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**STUDY OF THE DESTRUCTIVE EFFECTS
OF SHEAR STRESS AND ROS-GENERATING DRUGS
ON CIRCULATING TUMOR CELLS**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2018

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ON CIRCULATING TUMOR CELLS**

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School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University, Singapore

in fulfillment of the requirement for the degree of

Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

31-01-2019

A handwritten signature in blue ink, appearing to read 'Sagar', is written over a light blue rectangular stamp.

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SAGAR REGMI

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Assoc Prof Lim Sierin

Authorship Attribution Statement

This thesis contains material from 2 papers published in the following peer-reviewed journals where I was the first author.

Chapter 1 is “Introduction” where literature review is presented, and all the references are properly cited.

Chapter 2 is “Material and methods”. Mr. Sanam Pudasaini from School of Mechanical and Aerospace Engineering conducted the simulation of shear stress experienced by cancer cell in a microfluidic-based system using COMSOL multi-physics software. It is a pleasant part that I got to express my gratitude to Mr. Sanam Pudasaini in my humble acknowledgement. The simulations results are not published yet. I would include as co-authors if we use this results in my publication.

Chapter 3 is published as **Sagar Regmi**, Afu Fu, Kathy Qian Luo High Shear Stresses under Exercise Condition Destroy Circulating Tumor Cells in a Microfluidic System. *Scientific Reports*. 7, 39975; DOI: 10.1038/srep39975 (2017).

The contributions of the authors are as follows:

- Conceived and designed the experiments: Sagar Regmi, Afu Fu, and Kathy Qian Luo.
- Performed the experiments: Sagar Regmi.
- Analyzed the data: Sagar Regmi, Afu Fu, and Kathy Qian Luo.
- Contributed reagents/materials/analysis tools: Sagar Regmi, Afu Fu, and Kathy Qian Luo.
- Wrote the paper: Sagar Regmi, Afu Fu, and Kathy Qian Luo.

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The contributions of the authors are as follows:

- Sagar Regmi and Kathy Qian Luo conceived and designed the experiments.
- Sagar Regmi performed the experiments.
- Sagar Regmi and Kathy Qian Luo analyzed the data.
- Sagar Regmi, To Sing Fung, Sierin Lim, and Kathy Qian Luo contributed reagents/materials/analytical tools/project management.
- Sagar Regmi and Kathy Qian Luo wrote the paper.

Chapter 5 is conclusion and future directions of the work.

31-01-2019

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PUBLICATIONS

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5. Ju J, **Sagar Regmi**, Fu A, Lim S, Liu Q. (2018). Graphene quantum dot-based charge-reversal nanomaterials for nucleus-targeted drug delivery and control efficient photodynamic therapy. (Submitted to *Journal of Biophotonics* on 24th September 2018)

CONFERENCE PAPERS AND PRESENTATIONS

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6. Luo KQ, **Sagar Regmi**, and Fu AF. (2016) High Levels of Hemodynamic Shear Stress Achievable under Exercise Condition Destroy Circulating Tumor Cells in a Microfluidic System. 2016 American Society for Cell Biology (ASCB) Annual Meeting. December 3-7, 2016, Moscone Center, San Francisco, California, USA. (Poster presentation)
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9. Khairunisa Liyana Bte Abdul Malek, **Sagar Regmi**, Muniraj Vivek Sheraton, and Luo KQ. (2015) Effect of Hemodynamic Shear Stress on Circulating Breast Cancer Cells. SCBE FYP Symposium. May 14, 2015, Singapore. (Poster presentation)

ABSTRACT

Cancer metastasis is a complicated disease that causes around 90% of cancer deaths. It is one of the deadly diseases around the world killing almost 8.8 million people in 2015. Exercise, anti-cancer drugs, and anti-oxidants have attracted the interest in the rehabilitation of the cancer patients. Insights on the mechanism of prevention facilitate the treatment strategy of the disease. Hence, this thesis mainly focuses on the shear stress (SS)-based strategy for studying the metastatic properties of circulating tumor cells (CTCs) in a microfluidic system in the view of exercise, anti-cancer drugs, and antioxidants, to develop therapeutic strategies to prevent the cancer progression.

CTCs are the primary targets of cancer treatment as they cause distal metastasis. However, how CTCs response to exercise-induced high SS is largely unknown. To study the effects of hemodynamic microenvironment on CTCs, we designed a microfluidic circulatory system that produces exercise relevant SS. We explore the effects of SS on breast cancer cells with different metastatic abilities, cancer cells of the ovarian, lung and leukemic origin. High SS of 60 dynes/cm² achievable during intensive exercise killed more CTCs than low SS of 15 dynes/cm² present in human arteries at the resting state. High SS caused necrosis in over 90% of CTCs within the first 4 h of circulation. More importantly, the CTCs that survived the first 4 h-circulation, underwent apoptosis during 16–24 h of post-circulation incubation. Prolonged high SS treatment effectively reduced the viability of highly metastatic and drug-resistant breast cancer cells. As high SS had much less damaging effects on leukemic cells mimicking the white blood cells, we propose that intensive exercise may be a good strategy for generating high SS that can destroy CTCs.

To increase the relevance of the previous study and to develop a potential platform for the discovery of anti-cancer drugs with the intention of killing CTCs, we further tested the drug model in this microfluidic system. Many anti-cancer drugs are in practice in the clinic; however, little is known about their efficacy in CTCs. In this thesis, we investigated whether the pulsatile fluidic SS in human arteries can affect the efficacy of anti-cancer drugs. Cancer cells were circulated in our microfluidic circulatory system, and their responses to drug and SS treatments was observed. The results showed that fluidic SS significantly increased the potency of the reactive oxygen species (ROS)-generating drugs doxorubicin (DOX) and cisplatin but had an insignificant effect on the non-ROS-generating drugs Taxol and etoposide. Co-treatment with SS and ROS-generating drugs dramatically elevated ROS levels in CTCs, while adding antioxidants abolished the apoptotic effects of DOX and cisplatin. More importantly, the synergistic killing effects of SS and DOX or cisplatin were confirmed in the circulated lung, breast, and cervical cancer cells, some of which have a strong metastatic ability. These new findings suggest that ROS-generating drugs are more potent than non-ROS-generating drugs for destroying CTCs under pulsatile fluidic conditions. This latest information is highly valuable for developing novel therapies to abolish CTCs in the circulation and prevent metastasis.

Finally, the survival capabilities of lung metastasis-derived cell lines that were isolated from mice models in our lab were also studied in this thesis. The metastasis-derived breast cancer cells experienced increased basal levels of survival and underwent stronger resistance even during the high shear stress induced by exercise conditions for 4 h compared to the parental breast cancer cell line. However, the metastasis-derived cells could not resist the high shear stress for 8 h. This proves the fact that a high SS can also destroy highly metastatic and drug-resistant breast cancer cells. Furthermore,

metastasis-derived cells revealed differences in their survival capabilities. This contributes to increasing evidence of enhanced survival capabilities in cancer progression, metastasis and provides an insight into the role of killing the CTCs. As high SS had much less damaging effects on leukemic cells mimicking the white blood cells, we propose that intensive exercise may be a good strategy for generating high SS and use of ROS generating drugs that can destroy CTCs and prevent cancer metastasis.

Overall, I studied three important aspects in cancer metastasis: SS based exercise strategy, anti-cancer properties of drugs and the role of antioxidants during the circulation in a microfluidic system. I have revealed new insights correlating the exercise-induced SS to the survival of CTCs and provided the evidence of survival capabilities linked to reactive oxygen species (ROS) inducing drugs and non-reactive oxygen species (NROS) inducing drugs mechanism in cancer metastasis. Understanding how the fluidic SS with anti-cancer drugs influences the survival of tumor cells provides new insight into the cancer metastasis and have important application in the treatment of cancer. Discovering these new insights in the areas of cancer prevention may be an effective therapeutic target in the treatment of cancer.

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ABBREVIATIONS

APAF1	Apoptotic protease activating factor 1
ATCC	American type culture collection
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma
BMI	Body mass index
C3	Caspase-3
C8	Caspase-8
C9	Caspase-9
CCD	Charge-coupled device
CFP	Cyan fluorescent protein
CTCs	Circulating tumor cells
Cyto c	Cytochrome c
DCF	2', 7' Dichlorofluorescein
DCFDA	2',7' Dichlorofluorescein diacetate
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DTC	Disseminated tumor cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition

ER	Estrogen receptor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
H ₂ O ₂	Hydrogen peroxide
HPE	Human papillomavirus
IAPs	Inhibitor of apoptosis protein
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MTT	3-4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NK	Natural killer
p53	Tumor protein
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PI	propidium iodide

PG	Propyl gallate
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma
ROS	Reactive oxygen species
RNA	Ribonucleic acid
Sensor C3	Caspase-3 biosensor
SMACs	Second mitochondria-derived activator of caspases
SS	Shear stress
SS15	Shear stress of 15 dyne/cm ²
SS30	Shear stress of 30 dyne/cm ²
SS45	Shear stress of 45 dyne/cm ²
SS60	Shear stress of 60 dyne/cm ²
TNBC	Triple-negative breast cancer
TNF α	Tumor necrosis factor alpha
TNM	Tumor, lymph nodes, metastasis
TSCs	Tumor suppression genes
VEGF	Vascular endothelial growth factor
YFP	Yellow fluorescent protein

CHAPTER 1 Introduction

1.1 General understanding of cancer

Cancer is a group of complicated diseases that involves the growth of abnormal cells in an uncontrolled manner which has the potential to invade and spread in various parts of the body. Malignant neoplasms are also simply known as cancers. It starts when the normal cells lose the control and start to behave abnormally. It can start anywhere in the human body. This makes the diseases more complicated. It affects the people from many regions and countries of the globe. Cancer can be either a malignant or benign tumor. When cancer cells are malignant, they can spread to the different area of the body. But, benign tumor won't invade and spread to other areas of the body. However, only brain benign tumor may be life-threatening. But, malignant tumors are very dangerous and life-threatening as it can spread out even after the surgery.

1.2 Worldwide statistics of cancer

Cancer is the second leading cause of death around the globe which is caused by the mutation in the genome. It has been reported that there were 8.8 million deaths (2015), 8.2 million deaths (2012) [1] and 14.1 million new cancer patient identified in 2012 [2]. Worldwide, out of 6, 1 death is due to cancer. Mainly, cancer death was from lung (Around 1.6 million deaths), liver (Around 745,000 deaths), and stomach (Around 723,000 deaths). The most diagnosed cancer was lung (Around 1.82 million), breast (Around 1.67 million) and colorectal (Around 1.36 million) [2]. However, the most common cancer worldwide in case of the male is lung cancer and in the female is breast cancer. It is estimated that there will be around 23.6 million new cases of cancer by 2030 i.e. the number of cases will increase by 70% within the next 2 decades. Around 70% of

cancer deaths occur in low and middle-income countries. Around 33% of cancer death worldwide is due to five major dietary and behavioral cause 1) very high body mass index (BMI) 2) very low fruits and vegetable intake 3) lack of exercise 4) excessive use of tobacco and 5) alcohol use. Almost 22% of cancer death worldwide is due to using tobacco [3]. Around 25% of cancer cases are in low and middle countries due to hepatitis B and human papillomavirus (HPV) [4]. The cancer is diagnosed at the late stage. It is reported that only 26% of countries globally have the facility of pathology lab in 2017 making an inaccessible diagnosis in low-income countries. However, in high-income countries, almost 90% of the treatment facility is available compared with less than 30% of low-income countries. Worldwide, massive amounts are spent on cancer research for understanding the cause and treatment of cancer. Almost US\$ 1.16 trillion was estimated as the cost of cancer in 2010.

1.3 Hallmarks of cancer

There has been proposed the six acquired capabilities essential for normal cells to evolve progressively to neoplastic state [5]. These six hallmarks of cancer are: 1) Sustaining proliferative signals 2) Evading growth suppressor 3) Activating invasion and metastasis 4) Enabling replicative immortality 5) Inducing angiogenesis 6) Resisting cell death [5].

Figure 1-1. Six hallmarks of cancer. Reprinted from reference [5] with permission from Elsevier.

1.3.1 Sustaining proliferative signaling

Sustaining proliferative signaling is a very important hallmark of cancer. In the case of the normal cell, the cell number is carefully regulated through the Hippo signaling pathway and maintain normal tissue architecture and function [6, 7]. However, in the case of cancer cells, the cell number is not regulated. Cancer cells use the various mechanism to proliferate their number: 1) Elevated levels of the cell surface receptor-like epidermal growth factor receptor (EGFR) was discovered as a common factor in all cancer and thus promoting the growth of the solid tumor [8]. 2) HER 2 (human epidermal growth factor receptor 2) is an important member of the human epidermal growth factor receptor family which has a significant role in apoptosis, proliferation, migration, and differentiation. HER2 is amplified in many types of human cancer. The amplification of HER2 will lead to heterodimerization with EGFR and activation of MAPK and P13 signaling pathway which in turn promote cell growth and proliferation

[9, 10]. 3) Ras oncogene is among the most important oncogene which controls the process like proliferation, angiogenesis, and malignant transformation. Upon the activation of Ras oncogene, they initiate a signaling cascade resulting in the regulation of growth, development and subsequently, oncogenesis [11, 12].

1.3.2 Evading growth suppressor

Tumor suppression genes (TSGs) are well functioned in normal cells and prevent unnecessary cell growth and proliferation. However, cancer cells initiate the mutations in these TSGs and make them inactive which leads to unwanted cell growth and eventually tumorigenesis. These tumor suppressors' genes are also known as growth repressor and mainly there are three types of growth repressor namely: 1) tumor proteins (p53), 2) retinoblastoma proteins (RB) and 3) phosphate and tensin homolog (PTEN).

The Tumor protein p53 has mostly mutated the gene in human cancer. It is known by "the guardian of the genome" [13]. P53 plays a significant role in response to DNA damaged; whether a cell will repair the damaged DNA or will undergo the cell cycle arrest and apoptosis. In summary, p53 regulates the gene expression and prevent the mutation of the genome [14, 15]. The RB proteins receive the many signals from inside and outside of the cells and determine whether the cell to process for cell cycle and cell division. However, the deficiency in RB proteins lead the cells to uncontrolled cell proliferation and eventually cancer [16, 17]. P53 and RB are at the central of tumor suppressor pathway that prevents cells from potentially oncogenic stimuli. PTEN inactivates the phosphatidylinositol by dephosphorylating [18, 19] which plays a significant role in the initiation of the AKT pathway that is responsible for cell proliferation and survival [20, 21]. There is a report that the loss of PTEN function in the various type of cancer like breast, prostate etc. [22, 23].

1.3.3 Inducing angiogenesis

Angiogenesis is the physiological process of developing new blood vessels from the existing blood vessels [24]. It is very significant in the physiological process like reproduction, wound healing, and embryonic development [5]. The small tumor receives nutrition through the process of diffusion; however, for them to grow bigger than 1-2 mm [25], tumor requires an adequate blood supply. It is the blood circulatory system through which cancer cells spread from primary sites to other parts of the body and angiogenesis plays a significant role to support cancer cells to enter the blood vessels. The tumor cells release the pro-angiogenic factor like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factors (FGF) which activates the angiogenic pathways. The tumor vasculatures are leaky by their nature so that cancer cells enter the vessels to be disseminated to various parts of the body. There has been a report that angiogenesis deficient mice displayed an absence of metastasis [26]. Furthermore, the inhibition of angiogenesis shows the reduction in metastasis in mice models [5, 27]. In summary, angiogenesis which is essential for the expansion of tumor colony supports the tumor growth by providing oxygen and nutrients and removing the metabolic waste. Hence, anti-angiogenesis is identified as the potential therapeutic mechanism for the prevention as well as treatment of various cancer like hepatocellular carcinoma HCC [28, 29], late-stage cervical cancer [30] and non-small-cell lung cancer [31].

1.3.4 Enabling replicative immortality

Cancer cell requires the unlimited replicative potential to develop into the macroscopic tumors. However, the normal cell has two distinct barriers to proliferation. 1) Senescence that is the process where the cell will deteriorate with age. 2) The crisis

which is indeed a cell death [32]. In normal cells, the telomere is shortened in many cycles of cell division. It is the length of telomeric DNA in a cell that determines how many next generation of cells can pass before the telomeres lost their function entering the crisis [5, 33-37]. There is much evidence that shows in the tumor, the telomere protects the end of chromosomes by maintaining the length of telomere from end to end fusion and involved in the long-term proliferation [38-42]. Telomerase; the specialized DNA polymerase use reverse transcription to maintain the length of telomere by the addition of TTAGGG repeats at the end of telomere using the RNA molecule carried by the telomerase itself as a template [43]. The telomerase is normally absent in the normal cells but significantly expressed more in human cancer cells [44]. Hence, the capacity of cancer cells to evade the senescence or crisis by maintaining the length of telomere through activation of telomerase is an important hallmark of cancer.

1.3.5 Resisting cell death

Resisting cell death is an equally significant hallmark of cancer [5]. Apoptosis is a process of programmed cell death that has characteristics like nuclear fragmentation, mRNA decay, cell shrinkage, DNA fragmentation, and chromatin condensation. Apoptosis is a highly regulated and controlled process that is used to remove the unwanted cells during the organism's lifecycle.

Apoptosis serves as a natural barrier in the development of the tumor. The absence of apoptosis will lead to an increase in unwanted cell and thus cancer. There are two signaling pathways to regulate the process of apoptosis 1) Intrinsic pathway where the cell kills itself 2) Extrinsic pathway where the cells kill itself from the signal received from other cells [45, 46]. Both the pathway regulates the apoptosis through the activation of caspases.

Figure 1-2. The signaling pathway of apoptosis. Extrinsic pathway and the intrinsic pathway showing the activation of caspase-3. The figure is provided to me by my co-supervisor Prof Kathy Qian Luo.

The intrinsic pathway is triggered by irradiation and chemical reagents. It is related to stress-induced through the mitochondria. The decision to make whether cell to go for apoptosis or not is determined by the balance of pro and anti-apoptotic members of Bcl-2 family [47]. The anti-apoptotic member Bcl-2 binds with the pro-apoptotic member such as Bax which are embedded in the outer membrane of mitochondria to inhibit the cell apoptosis [48]. Upon triggering, the Bax is released which lead to their homo or hetero-dimerization and form the pores in the outer membrane of mitochondria or increase the permeability of the membrane which further helps to release cytochrome c (Cyto c) or SMACs (second mitochondria-derived activator of caspases) [49]. The presence of Cyto c in cytosol allow it to bind with

apoptotic protease activating factor – 1 (Apaf-1) and adenosine triphosphate (ATP) to form apoptosome which activates caspase-9 which helps in the activation of caspase-3 [49]. The SMACs binds with IAPs [50] (inhibitor of apoptosis proteins) to ensure the activation of caspase-9 and subsequently caspase-3.

The extrinsic pathway is activated by extracellular signals and regulated by cell surface receptors like TNF α (tumor necrosis factor alpha) and Fas ligand [51]. They initiate the activation of downstream effector caspase 8 and activates the down-stream key effector caspase-3. On the other side, caspase-8 cleave Bid into tBid [52] which supports to release of pro-apoptotic factors from mitochondria activating caspase 9 and subsequently caspase 3.

The cancer cell develops some new strategies to stop apoptosis. For instance, tumor cells overexpress anti-apoptotic like proteins Bcl-2 to protect the cell from apoptosis and makes a favorable condition for the survival of these tumor cells [53, 54]. In addition, p53 is tumor suppressor protein that recognizes the DNA damage and other abnormalities in cells. The p53 is responsible for the repair of the damaged DNA or initiate cell cycle arrest and apoptosis. However, the tumor cell develops some mutation in p53 leading to loss of apoptosis-inducing effect [13-15, 55, 56].

1.3.6 Activating invasion and metastasis

Normal tissue grows in the well-order manner to form tissue which eventually forms organs with a specific function. Two types of cells mainly form tissues namely epithelial and mesenchymal cells. Epithelial cells are lined one after another to form cell layers and protects the organs and body from the external environment whereas mesenchymal cells are alone and have the potential of migrating.

Malignant cells have the capability to invade the adjacent normal cell structure and metastasize to the distant organ at any point. Cells break off from the primary tumor and enter the blood vessels or lymphatic channel and travel to various parts of the body to form the new tumor. Their ability to invade eventually affects the function of normal tissue into which they are growing. Metastasis is a very complex interactions process among tumor cells, vessels of the lymph or vascular system, the basement membrane surrounding those vessels and extracellular matrix (ECM) which consists mainly of collagen. Tissue consists of a substantial proportion of extracellular space which is known as the extracellular matrix (ECM) [57]. Metastasis requires to break the bonds between the cells and ECM which allows tumor cells to travel freely. Activating invasion and metastasis which is the key hallmark of cancer is mostly associated with increased cellular motility and decreased cell adhesions [58, 59]. Integrins link cells to the ECM whereas the cadherins help in the cell to cell adhesions. One of the important protein in cell adhesion is E-cadherin which acts as a cement to bind them to other cells. The E-cadherin function is lost in cancer cells indicating the significance of E-cadherins in the deactivating invasion and metastasis [60, 61]. There has been the report that there is decreased E-cadherins expression and increased levels of another molecule known as N-cadherin. N-cadherins help cancer cell in migration to slip through the blood vessels. When some appropriate signals enter to the cells, a complex chain of events within the cytoplasm is set in motion, thus eventually lead into the cell nucleus where the transcription and gene regulating the cell cycle process progression and cell growth are stimulated. One protein produced through the activation process is matrix metalloproteinases (MMPs). When the tumor cells metastasize, it breaks off from the main tumor and enters the cellular space. Tumor cell secretes the MMPs, which degrades the collagen ECM [62, 63] or breaking through the basement membrane

around the tumor allowing the tumor cells to migrate along the lymph or blood vessels. When the MMPs reaches the vessels, they break down the basement membrane around the vessels through the enzymatic action. This opens the access to endothelial cells lining the vessels. Tumor cells then migrate into the blood and lymph by entering through the tight junction of the epithelial cells. Tumor cells are then transported through the lymph and blood to other tissues. It is known that metastasize tumor target some organs more than the other, although the reason is poorly understood. Initially, there is a weak adhesion of tumor cells to endothelial cells. This allows cells to roll with the vessels lining until stronger bond are formed. Once the metastatic cells are securely attached to endothelial lining, they leave the vessels and enter the tissue. They also leave the open pathway that allows less aggressive tumor cells to invade the tissue and grow [64, 65].

1.3.7 Emerging hallmarks of cancer

In addition to above-mentioned hallmarks, there are four additional hallmarks that have been realized recently: 1) Genome instability and mutation 2) Tumor-promoting inflammation 3) Reprogramming energy metabolism 4) Evading immune destruction [5].

A) Genome instability and mutation

There are various external factors like UV light, X-ray and chemical carcinogens that lead to mutations when exposed to cells [66, 67]. The reactive oxygen species (ROS) that is produced as a metabolic product during the normal functioning of cells alters the sequences of DNA and hence mutation [68]. But in case of normal cells, they have developed an effective mechanism such as nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR) to detect and fix the errors in

genomic level which make sure that level of mutation is very low [69]. The genomic stability is classified into two groups: 1) Nucleotide instability 2) Chromosome instability. The base insertions or deletions or substitutions constitutes nucleotide instability. The case where whole chromosomes are deleted or duplicated constitutes chromosome instability. In the case of cancer cells, it has been shown that genomic instability arises from mutations that affect mainly the DNA repair mechanism [70]. But there are some reports that show that chromosome instability leads to the mutation in tumor suppressor gene and hence genomic instability [71].

Figure 1-3. Emerging hallmarks of cancer. Reprinted from reference [5] with permission from Elsevier.

B) Tumor-promoting inflammation

There are reports that inflammatory responses contribute to the proliferation of cancer cells. It is clearly shown that the natural immune system supports in the promoting effects of tumors [72]. The macrophages and neutrophils, which are known by inflammatory cells are mainly involved in the cleaning of waste and wound healing [73]. These cells releases various signal to initiate growth factors to support wound healing

which further supports in the process of cell growth and proliferation of tumors [74]. Further, immune cells release certain chemicals like ROS which cause the mutation in DNA and thus supporting genetic instability [72].

C) Reprogramming energy metabolism

The tumor cells require a huge amount of energy in the process of cell growth and proliferation. To meet this requirement in the process of formation of a tumor, cancer cells reprogram the energy metabolism as an emerging hallmark of cancer [5]. In case of normal cells, with the help of oxygen, cells undergo aerobic respiration to convert glucose into pyruvate by the process of glycolysis in the cytosol and to pyruvate in mitochondria followed by the oxidation in Krebs cycle. But in the absence of oxygen, normal cells undergo anaerobic respiration to convert glucose into pyruvate by the process of glycolysis but not the oxidation of pyruvate in mitochondria. However, in the case of tumor cells, there is a production of lactate via pyruvate when there is oxygen supply known by term aerobic glycolysis [75]. This shows that cancer cells alter energy metabolism by restricting the production of pyruvate. In addition to this, the researcher has proposed that the increased glycolysis generates a huge production of glycolytic intermediated products. These products are used in the activation of various biosynthesis pathway leading to the synthesis of nucleotide, amino acids which is essential for the growth and proliferation of cancer cells [76].

D) Evading immune destruction

The immune system has a self-contradictory effect in the formation and progression of a tumor. On the one side, natural immune systems help in promoting the tumor [74]. On

the other side, in a recent year study in a mice model with immune deficient, it shows that carcinogen-induced cancer easily than in wild mice. This reflects that the immune system detects and eliminate the cancer cells. Hence, tumor cells have acquired the capability to survive from destruction by the immune system.

1.4 Causes of cancer

Cancer has multiple causes; hence it is very difficult to predict what exactly has caused cancer to an individual. For example, a person who smokes heavy has a high risk of lung cancer; but there are reports of lung cancer to non-smoker too [77]. Cancer cause can be divided into two groups. They are 1) environmental factors that account for 90-95% of cancer 2) genetic mutation that lead to 5-10% of cancer [78].

Figure 1-4. The main cause of breast cancer. The environmental factors are associated with hereditary factors that lead to the major cause of breast cancer.

1.4.1 Chemicals

The certain chemical can damage DNA by altering the gene sequence. They are called mutagens. These mutagens which caused cancer are called carcinogens. Consider tobacco which contains more than 50 diverse types of carcinogens such as nitrosamines and polycyclic aromatic hydrocarbon. It is reported that smoking cause 25-30% of cancer deaths [78]. Smoking causes around 87.3% and 83.6% of lung cancer deaths to male and female respectively [79] in the UK. Smoking not only causes lung cancer but also increases the risk of other cancer like ovarian cancer, liver cancer etc. [79]. There has been a report regarding the alcohol consumptions increases the risk for the cancer of the oral cavity, pharynx, esophagus, larynx, colon, liver, rectum, breast etc. [80].

1.4.2 Diet and exercise

It is reported that approximately 30-35% of cancer death is related to diet, obesity, and physical inactivity. Lack of exercise increases the risk of cancer not only through obesity but also with the adverse effects on the immune system and metabolism of hormones [81]. The intake of red meat is risky for various cancers like prostate, breast, colorectal etc. It may be because of production of heterocyclic amines and pyrolysates in the process of cooking. But, having green vegetables in the diet reduces the risk of cancer [82]. Cancer can be prevented with diets which are rich in antioxidants like carotenoids (α -carotene, β -carotene, lycopene, lutein, cryptoxanthin), vitamin B12 as well as folic acid etc.

1.4.3 Infection

It is reported that around 18 % of cancer deaths are associated with infections [78]. The percentage varies with 10% in the developed world to 25% in African countries [83, 84]. Those viruses that cause cancer are called oncovirus. Viruses are most usual infective agents that lead to the formation of cancer whereas bacteria and parasites have minimal effects. Human papillomavirus, Epstein Barr virus, human T-lymphotropic virus 1, Hepatitis B virus (HBV), Hepatitis C virus (HCV), Human immunodeficiency virus (HIV) etc. can cause skin, cervical, nasopharyngeal, T-cell leukemia, liver cancer, and B-cell lymphoma. Human papillomavirus can directly cause the DNA mutation by changing the viral genes E6 and E7 [85] whereas HBV affects the DNA mutation by generating reactive oxygen species (ROS) through chronic inflammatory responses [86-88].

1.4.4 Radiation

It is reported that almost 10% of cancer is caused by radiation exposure [78]. There are basically two types of radiation. They are ionizing radiation which is from medical imaging, radon gas etc. and non-ionizing radiation which is from UV lights. Radiation exposure can cause skin cancer like basal cell carcinoma, melanoma etc. The ionizing radiation has a different strategy than other carcinogens. If ionizing radiation happens to strike a chromosome, it will lead to the DNA mutation hence inducing carcinogenesis.

1.4.5 Heredity

Most of the cancers are non-hereditary. It is reported that around 5-10% of cancer is due to inherited gene defects [78]. Mutation in BRCA1 AND BRCA2 is related to ovarian,

breast, prostate cancer etc. [89]. The people carrying a genetic mutation associated cancer is no more than 0.3% of the population worldwide which cause less than 3–10% of cancer [89].

1.4.6 Physical agents

It is reported that not only chemical agents but also physical agents can cause cancer. For example, the prolonged exposure of asbestos is very risky to develop lung cancer. It is possible to get skin cancer if there is a repeated burn of the same skin and the presence of carcinogenic chemicals. The scalding generated by repeated drinking of hot tea can cause esophageal cancer.

1.4.7 Hormones

There are many reports that hormones are associated to increase the risk of various cancer like breast, ovary, thyroid, bone, and prostate [90, 91]. For example, even if there is no breast cancer gene, the daughter of women who has breast cancer has more risk of having breast cancer than the daughter of women without breast cancer. This is because of an elevated level of estrogen and progesterone generated in the daughter of women with breast cancer.

1.5 Treatment of cancer

With the change of lifestyle, cancer is a preventable disease [78]. The way of reducing the risk of cancer is defined as cancer prevention. As 90-95% of cancer is caused by an environmental factor, cancer can be prevented. There was 8.8 million death (2015), 8.2 million deaths in 2012 caused due to cancer and approximately 14 million new cancer

patients were discovered [2]. It is expected that the number of a new cancer patient will increase by 70% within the next 2 decades and hence, the urgent need for cancer prevention. Even though there is no effective treatment for cancer, there are some methods which are used for the treatment of cancer. There is mainly five standard way of treatment used in the clinical practice namely surgery, radiation, chemotherapy, targeted and hormone therapy [92] in case of breast cancer. Chemotherapy with higher dose together with transplantation of stem cells are the new treatment strategies in clinical practice [93, 94].

1.5.1 Surgery

Surgery is the direct method for removal of the solid tumor and prolonged the survival of cancer patients. Surgery is done for cancer prevention, deciding the stage of cancer, debulking. Surgery is mostly combined with other treatment strategies like radiation, and chemotherapy to ensure that all the tumor cells are completely removed. In general, the whole tumor is removed and, in some cases, the lymph nodes or entire organs like breast which is close to tumor site is removed to increase the effectiveness of surgery. Majority of breast cancer patient remove the tumor [95] using surgery which includes the followings:

1. Lumpectomy: This is known as breast-conserving surgery where the tumor is removed along with some normal tissue but the whole breast is not removed.
2. Mastectomy: This is known as total mastectomy too where the entire breast that has a tumor is removed.
3. Modified radical mastectomy: This also includes the removal of the whole breast with the tumor, lymph nodes below the arms as well as some parts of the chest muscle.

1.5.2 Chemotherapy

Chemotherapy is the widely used method for treating cancer by killing the cancer cells using cytotoxic drugs. It uses one or many drugs to kill cells by disturbing the cell replication mechanism [96]. The major limitation of chemotherapy is that it cannot distinguish between tumor cells and normal cells. For example, these anti-cancer drugs not only kill the normal cells but also have side effects like alopecia, stomatitis, myelosuppression, etc. [97]. The chemotherapy is given to the breast cancer patients either by oral administration or intravenous injection etc. as per stage and type of breast cancer [98]. Many anti-cancer drugs are developed for treating breast cancer. However, chemotherapy that is used in combination of drugs works much better than single drugs via various action of mechanism [99, 100]. The current combination treatment of drugs for breast cancer approved by FDA are: 1) AC namely doxorubicin hydrochloride (Adriamycin) and cyclophosphamide 2) AC-T namely doxorubicin hydrochloride (Adriamycin) along with cyclophosphamide and Taxol 3) CAF namely cyclophosphamide along with doxorubicin hydrochloride (Adriamycin) and fluorouracil 4) CMF namely cyclophosphamide combined with methotrexate as well as fluorouracil 5) FEC namely fluorouracil with epirubicin hydrochloride and cyclophosphamide 6) TAC namely docetaxel with doxorubicin hydrochloride (Adriamycin) in presence of cyclophosphamide [99].

1.5.3 Radiation

Radiotherapy uses high energy X-rays as well as electrons to kill or reduce the symptoms of cancer cells [101]. There are mainly two kinds of radiation therapies I) external radiotherapy where the source of radiation is delivered outside the body II) internal radiation where the radiation source is delivered inside the body e.g. radiation

iodine in blood circulation. The clear objective of this technique is to provide a very specific dose of ionizing radiation for targeted cancer cells and have very minimal damage to normal cells located nearby. Radiation can kill the cancer cells directly by damaging DNA or by producing the reactive oxygen species (ROS) to damage DNA [102]. It is mostly used in treating solid tumors like lung, breast brain, prostate, stomach etc. [103].

1.5.4 Biological therapies

Biological therapies or immunotherapy use immune system for the treatment of cancer. It reduces the side effect generated by other cancer therapies [104]. The human body has its own immune power to fight with other diseases like cancer. The immune system generally detects and kill the foreign particle but there is a probability that cancer cell bypasses the immune system and became metastatic cancer. The main aim of biological therapies is to improve the immune system of the human body through biological modifications such as monoclonal antibodies, gene therapy, vaccination, interferon's, interleukins etc. [104, 105].

1.5.5 Targeted therapies

In targeted therapies, molecular inhibitors, as well as monoclonal antibodies, are used to inhibit the growth and differentiation of tumor cell where certain proteins or molecules involving in tumorigenesis [106] are targeted. It is done either by blocking the cell growth signaling pathway or by increasing the immune power for the elimination of cancer cells.

The targeted therapies are the current focus of the anti-cancer drug development program. The targeted therapies are different than the current chemotherapy like most of chemotherapy works by attacking the normal and cancer cells whereas targeted therapies work in a specific molecular target that is associated with cancer. Many chemotherapies kill the cells whereas targeted therapies are designed to target particularly. Chemotherapy is cytotoxic i.e. it works by killing the tumor cells whereas targeted therapies are cytostatic i.e. it works by blocking the tumor proliferation. For example, 1) Trastuzumab (Herceptin): Here a monoclonal antibody interferes HER2/neu receptors [107]. 2) Pertuzumab: Here a monoclonal antibody acts by binding and inhibiting with HER2 and inhibits HER2 dimerization [108]. 3) Lapatinib: It blocks the activation of EGFR pathways by blocking the phosphorylation of HER2 protein [109] for the breast cancer treatment. 4) Palbociclib (PD-0332991) is a cyclin-dependent kinase inhibitor used for ER-positive and HER2-negative breast cancer treatment [110].

1.5.6 Angiogenesis inhibitor

Angiogenesis involves the formation of new blood vessels leading to the progression of tumor growth and metastasis [111]. The Vascular endothelial growth factor (VEGF) stick together with VEGF receptors on the endothelial cell surface which in turn activates the downstream signaling pathway responsible to form new blood vessels. Angiogenesis inhibitors like Avastin can bind with VEGF and block the binding of VEGF to its receptors. Angiogenesis inhibitors stop the formation of blood vessels, however, it does not kill the tumor cell [112] which is a limitation of this method. However, using angiogenesis inhibitors with other cancer therapies may be an effective way for cancer treatment.

1.5.7 Vaccination and screening

The cervical cancer is increasing especially in the reproductive age group of women. There has been developed in the vaccines to reduce the risk of cancer caused by a virus. Cervical cancer can be reduced by human papillomavirus (HPV) vaccination programs. Similarly, hepatitis B virus is one of the major infections to cause liver cancer. However, the hepatitis B vaccine can reduce the risk of getting liver cancer [113]. Searching for cancer at the precancerous stage is helping for the cancer prevention. Majority of physicians believes that cancer like colorectal cancer is prevented via screening [114].

1.6 Fundamental of cancer metastasis

Cancer metastasis is basically a process in which cancer cells spread from the main site where they are formed to distal sites in various parts of the body. Majority of the current clinical research focuses on the treatment of primary tumors. However, metastasis causes almost 90% of cancer-related deaths [115]. The process of metastasis involves a series of complicated steps. They are i) depart from the primary sites ii) enter into the blood circulating system iii) travel in bloodstream iv) finally survived circulating tumor cells (CTCs) will extravasate to form secondary tumors in different organs of the body [116]. After which, cancer becomes life-threatening and very difficult to control and treat.

Figure 1-5. The diagram illustrating the process involved in cancer metastasis. The physical interactions of cancer cells with the microenvironment is essential in cancer metastasis. Cancer cells detaching from the primary sites undergoing the intravasation to form the circulating tumor cells (CTCs). These tumor cells extravasate and develop the secondary tumors in distant organs of the body. Reprinted from reference [115] with permission from Nature reviews cancer.

1.6.1 Breast cancer staging

The staging of breast cancer is done using TNM system which considers factors like size of the tumor (T), the involvement of the lymph node (N) as well as whether cancer has spread or metastasized (M) to different areas of the body.

The breast cancer staging is mainly expressed in five stages, a scale of 0 to IV where stage 0 means no cancer and stage IV represents the case where cancer has spread from the breast to different areas around the body [117].

Stage 0: This stage is also known by a non-invasive case where there are no abnormal cells or cancer cells starts to invade out of the parts of the breast. For example, Ductal

carcinoma in situ (DCIS) is the non-invasive case where the cancer cells start at the ducts of milk and haven't transferred out of the milk duct.

Stage 1: Stage 1 is divided into two groups as IA and IB.

Stage 1A is an invasive breast cancer where tumor size is 2 cm, as well as cancer, haven't moved out of breast with no involvement of the lymph node. (T1N0M0)

Stage 1B is an invasive breast cancer without a tumor in the breast but the tumor has moved to the lymph node with the size 0.2 mm to 2 mm. (T0N1M0) OR the tumor is present in the breast with size below 2 cm as well as the tumor spread to the lymph node with the size of 0.2 -2 mm (T1N1M0).

Stage 2: Stage 2 is divided into two groups as IIA and IIB.

Stage 2A is without tumor in the breast but tumor bigger than 2 mm is found in 1-3 axillary lymph node (T0N1M0) OR there is a tumor in the breast smaller than 2 cm and tumor spread to axillary lymph node (T1N1M0) OR there is a tumor in breast with the size of 2 to 5 cm but haven't move to lymph node (T2N0M0).

Stage 2B where there is a tumor in breast with a size of 2 to 5 cm as well as tumor has scattered to lymph node close to breastbone with size of 0.2 to 2 mm (T2N1M0) OR where there is tumor in breast with size bigger than 5 cm but haven't moved to the lymph node (T3N0M0)

Stage 3: Stage 3 is divided into three groups as IIIA, IIIB, and IIIC

Stage 3A where there is no cancer in breast but tumor has spread to 4 to 9 axillary lymph nodes or lymph nodes close to bone associated with breast (T0N2M0) OR tumor is bigger than 5 cm as well as tumor with size around 0.2 to 2 mm is observed in the lymph node (T3N2M0) OR there is cancer with size larger than 5cm in breast and tumor has spread to 4-9 axillary lymph nodes closer to the bone of the breast (T3N2M0).

Stage 3B where the size of cancer is any and found all around the wall of the chest or around breast skin (T4N0M0) OR cancer has found in all 9-axillary lymph node (T4N1M0) OR tumor move to the lymph nodes along with bone of breast (T4N2M0).

Stage 3C where the tumor may be of any size and moved to 10 or more axillary lymph nodes which might be below or above the collarbone and spread to breast skin (Any T, N3, M0).

Stage 4 is very invasive breast cancer with any size where cancer spread out of breast as well as lymph nodes to many parts of the body like skin, liver, brain or lung etc. (Any T, Any N, M1). It is an advanced and metastatic case [117].

1.6.1 Triple-negative breast cancer (TNBC)

Breast cancer cells which do not express estrogen receptors (ER-) along with progesterone receptors (PR-), as well as HER2 (HER2-), are called triple negative breast cancer (TNBC). It means that the growth of cancer is independent of hormones progesterone, estrogen as well as HER2 receptors. TNBC do not respond to targeted therapies or hormonal therapy. About 10-17 % of breast cancer is found to be TNBC [118, 119]. TNBC tends to be most aggressive than any kind of cancer associated with breast as they spread out of the breast and recur even after the treatment. TNBC has a higher grade than the rest of cancer. The more the grade means very less cancer resemble normal breast cells in terms of cell growth and morphology. TNBC is graded as 3 out of the scale of 1 to 3. TNBC is like basal-like meaning that the cells are close to basal cells. TNBC is more usual in a young woman and mostly diagnosed at a late stage [120]. Unlike other breast cancer, TNBC has a shorter survival [120] provided cancer starts to metastasize [121]. Due to an absence of a specific target [122] as well as limitation of hormonal and targeted therapy, the treatment of TNBC remains a challenge in cancer

research [119]. To understand the metastatic breast cancer, many breast cancer cell lines were used since the mid-20th century [123]. The widely used cancer cell lines by cancer biologist are both metastatic cell lines MDA-MB-231 as well as non-metastatic cell line MCF7 to advance our understanding in cancer metastasis [124].

1.7 Important steps in cancer metastasis

Breast cancer is very common cancer associated with the woman [125]. There were 1.7 million cases of breast cancer or about 25.2% new breast cancer cases diagnosed in a woman around the globe in 2012 [2]. It is second prevalent cause associated with cancer death in woman [126] with a report of 522,000 death in 2012 [127]. Regardless of such a high incidence rate, the mortality is relatively low with 14.7% of death in the female. Almost 90 % of female cancer death is due to metastasis via the lymphatic system to form secondary tumors mostly to bone and lung.

Metastatic cancer remains the biggest challenges in cancer treatment. The steps of cancer metastasis are thoroughly investigated. The process of metastasis is linked in a sequences as detachment of cancer cells from primary sites, invade into local tissue, entry to blood circulatory system known as intravasation, transit in the blood or lymph system, exit of cancer cells known as extravasation, followed by the colonization at various sites of the body [128]. Only a very few numbers of cancer cells can overcome these diverse challenges making metastasis as a very inefficient process [129]. The epithelial-mesenchymal transition (EMT) is associated with initial steps of metastasis where the cancer cells get the potential for invasion and migration to intravasate into the bloodstream by changing their genomic signaling [130]. Then these cancer cells are widely spread to various organs by lymphatic and blood circulatory system. The cancer cells stick to the wall of capillary and extravasate mainly to organ parenchyma where

suitable microenvironment initiate the growth, proliferation, and colonization of secondary tumors.

1.7.1 Cell motility and invasion

The cells having a property of invasion is the first requirement for cancer metastasis. There has been the development of various techniques like multiphoton confocal imaging expressing fluorescent proteins, optical coherence tomography to observe the cell motility with greater depth at a tissue level [131]. Majority of our understanding about the motility of normal as well as cancer cells come from the study in two dimensional (2D) substrate [132]. The characteristic of cell motility *in vivo* is significantly different than 2D *in vitro* substrate model [133]. Cancer cells can move at a higher speed of 15 μm per minute reflecting the potential of the invasion [129]. The separation of cancer cells from the primary sites followed by invasion on stroma at the cellular and molecular level is characterized by well-known epithelial to mesenchymal transition (EMT). EMT is a widely explored area associated with the process of cancer metastasis [134]. The E-cadherin loss and cytokeratin's change the cell morphology from cuboidal epithelial to mesenchymal [135]. This forces cells to release the matrix metalloproteinases (MMPs) on the surface of the basement membrane. It digests the lamin and collagen IV rich membrane of the basement to invade the blood vessels [136].

Figure 1-6. The diagram showing the process associated with invasion and intravasation during metastasis. The downregulation of E-cadherin in EMT associated with adhesion loss followed by the invasion of cancer cells as regulated by physiochemical properties of ECM. These cancer cells enter in blood vessels in this step of intravasation. Reprinted from reference [115] with permission from Nature reviews cancer.

1.7.2 Pro-metastatic microenvironment

The pro-metastatic microenvironment of a cell is responsible for determining the metastatic potential of cells. It has been proved that moving breast cancer cells in tumor areas have the number of macrophages associated with tumor [137]. These macrophages produce the epidermal growth factor (EGF) for promoting the invasion behavior of cancer cells [138]. The chemotactic gradient is also responsible to monitor the motility of cancer cells as shown by intravital imaging [129]. The cells that can express EGF receptor are more motile as compared with control keeping the same microenvironment. This highlights the epidermal growth factor receptor EGFR role in increasing metastatic potential of cells [139]. However, when the EGFR overexpressing cells were injected into the blood, it didn't promote the formation of metastases. This provides the hint that

EGFR support in cancer metastasis by helping the detachment of cells from primary sites [140].

1.7.3 Intravasation

The cancer cells must enter either via the blood or lymphatic system to spread into the distant organs. The previous study by *Li.et.al* showed that breast cancer cells show the orientation inclined to the blood vessels [141]. Even the non-metastatic MCF7 cells inclined towards the blood vessels [142]. However, the non-metastatic cells undergo fragmentation after experiencing the shear stress (SS). Majority of the study suggest that cancer cells with elongated morphology need to be rounded shape for making the intravasation efficiently. Initially, the carcinoma cells separate from epithelium and invade with well-known epithelial-to-mesenchymal transition (EMT) which involves the loss of E-cadherin and cytokeratin. This helps to change the morphology of the cells from epithelial to mesenchymal which results in the detachment of cells from the main sites of tumor. The cells then express matrix metalloproteinases (MMPs) on the surface for digestion of basement membrane with collagen IV rich as well as laminin. After the detachment from the primary tumor, these cells then encounter fibronectin and collagen I associated with very complex extracellular matrix (ECM). The physiochemical change that is occurred in matrix enhance the cell proliferation and invasion. Cancer cells need to withstand the SS generated due to flow in blood vessels.

1.7.4 Circulating tumor cells (CTCs)

In the process of metastasis through the circulatory system, tumor cells are exposed to hemodynamic shear force, collision with the host cells like endothelial cells lining the vessel wall or blood cells and immunological stress [115]. The shear flow affects the survivability and proliferation of circulating tumor cells (CTCs) and CTCs that survive the mechanical stress and immune will adhere to vascular endothelial of different organs [143]. Majority of CTCs will die in the process of circulation and few CTCs that survive generate the metastases.

The adjacent layer of blood flow with the viscosity (μ) which is moving at different velocities is responsible for the shear stress (SS). SS (τ) is influenced by factors like the diameter of blood vessels, patterns of flow of blood and collisions of cells in the blood vessels and viscosity of the blood etc. As per the no-slip condition, the viscosity is maximum at the center but zero at the periphery of the cylindrical tube. The shear rate is defined as relative velocities of an adjacent parallel layer in the cylindrical tube. Shear rate is denoted by $\frac{d\gamma}{dt}$, γ refers to the amplitude deformation in time t . The product of shear rate and viscosity of the fluid is called SS. Its unit is $\frac{\text{Newton}}{\text{meter}^2}$ in S.I unit and $\frac{\text{dynes}}{\text{centimeter}^2}$ in C.G.S system. The viscosity of blood (4 centipoise) is relatively higher than the viscosity of water (0.7 centipoise) because of the presence of erythrocytes. If the shear rate is higher than 100 S^{-1} then the blood is supposed as a Newtonian fluid as the correlation in between the SS and shear rate is linear. The SS is $1\text{-}4 \text{ dynes/cm}^2$ in venous circulation compared to $4\text{-}30 \text{ dynes/cm}^2$ in arterial circulation [144]. The SS is maximum at the wall of blood vessels.

Figure 1-7. The trajectory of CTCs in a blood vessel during cancer metastasis.

Cancer cells whose dimension is less than the blood vessels form a trajectory in the blood vessels. If the cancer cells encounter the small vessel dimension it will undergo mechanical trapping which is known as physical occlusion. Reprinted from reference [115] with permission from Nature reviews cancer.

Shear rate and Shear flow in human blood vessels

Consider an artery of diameter 4 mm, the average blood velocity is 0.45 m/sec and for a vein of a diameter of 5 mm, the average velocity is 0.1 m/sec. The shear rates $\frac{dy}{dt} = 8 \frac{V_{ave}}{d}$ is 900 S^{-1} for arteries whereas 160 S^{-1} for a vein. The renal epithelial cells experienced the SS of 0.5 dynes/cm^2 which increases relatively in the case of hypertension. The cell in gastrointestinal tract experienced SS up to 30 dynes/cm^2 and interstitial fluid velocity for consideration of cartilage and bone experienced up to 30 dynes/cm^2 at the time of mechanical loading for normal daily activity.

1.7.5 Extravasation

For metastatic cancer, extravasation means the movement of CTCs from the capillaries to the nearby tissue surrounding them [145, 146]. When the CTCs attaches to the endothelial monolayer of capillaries, then the process of extravasation is initiated. This

leads to the activation of various receptors and secreted factor [147, 148] resulting in the disruption of the endothelial cell junction [146] and hence extravasation. The CTCs need to bind with the wall of blood vessels before it exits the circulatory system. Based on the diameter of blood vessels, there are following mechanism of arrest [115]:-

1) Physical occlusion

If the diameter of vessels is smaller than the diameter of CTCs ($d_{\text{vessel}} < d_{\text{cell}}$), so arrest has proceeded with the mechanical trapping of cells. CTCs are usually greater than 10 μm for an epithelial origin and physical occlusion occurs in the capillaries whose diameter is less than 10 μm .

2) Cell adhesion.

If the diameter of a cell is less than a diameter of vessels, then tumor cells need the adhesion to the wall of blood vessels. When the tumor cells get attach to the wall of blood vessels, then leukocytes joined together and they are considered as first cells to extravasation together with tumor cells [149]. The process of extravasation usually takes a couple of hours after the attachment to the lining of blood vessels. Sometimes the metastatic tumor proliferates within the blood vessels which in turn may physically damage the endothelium allowing cancer cells to nearby tissue [150]. Majority of the non-metastatic cells are broken into fragments and do not metastases.

Figure 1-8. The recent development of various optical imaging technique to study the process of cancer metastasis. Reprinted from reference [129] with permission from Nature reviews cancer.

1.7.6 Establishment of metastases

The site of a secondary tumor based on primary tumor is investigated for a long time [151]. The tumor from primary sites can metastasize to any organs of the body to form a secondary tumor. But there is a certain probability being formed at a specific organ. For e.g., prostate cancer metastasizes to liver and bone marrow whereas breast cancer metastasizes to bone marrow and lungs. But pancreatic/colon cancer usually metastasize to lung and liver.

1.8 Clinical significance of CTCs

Circulating tumor cells (CTCs) are rare cells which cause distal metastasis. These cells have the potential to shed into the blood vessels from tumor developed at primary sites, circulate in the bloodstream and extravasation which forms secondary tumors in vital far organs. Almost a one and a half century ago by the Australian pathologist Thomas Ashworth in 1869 had found some cells in metastatic cancer patients which looks very different from the common blood cells. They resemble exactly to the patient's solid tumors. In the visionary words of Ashworth "cells identical with those of cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumors existing in the same person" [152]. Many studies have shown that CTCs as a prognostic marker [153] from the patients with metastatic breast, colorectal and prostate cancer however the low frequency 1 to 10 CTCs in 1 ml of blood in a cancer patients makes the diagnosis of cancer very challenging [154]. Even, it was reported earlier that CTCs is used as an alternative prognostic marker to estimate the life of metastatic breast cancer patients [145, 155]. The protein expression profile and gene characteristics of CTCs might provide a piece of important information regarding the mode and stages of cancer [156]. The cancer cells were observed in the blood from cancer patients during surgery [157]. However, the frequency of CTCs in a blood sample is very less. Currently, various methods are in the field for the isolation and detection of CTCs like cell search method [158], epic sciences method [159] etc. Regardless of the approach for detecting CTCs in the blood of cancer patients as well as with the advancement of various CTCs detection and isolation method, the clinical utility of CTCs is still unclear [160]. Therefore, measurement of CTCs is not included in the clinical guidelines. However, the ability of CTCs to remain to survive mainly depends upon its capacity to withstand hemodynamic shear stress in blood vessels.

1.9 Derivation of hemodynamic shear stress experienced by CTCs

Consider the length of an arterial segment as 'l'. The force resulting from the pressure difference $\Delta P = P_1 - P_2$ times the cross-sectional area πr_i^2 is equal to the opposing force generated by friction.

Figure 1-9. Calculation of hemodynamic shear stress

Frictional force on the wall = Shear stress times the lateral surface. Now we have, frictional force = $\tau \cdot 2\pi r_i \cdot l$. So, combining above two equations we get

$$\tau = \frac{\Delta P}{l} \frac{r_i}{2} \quad \dots\dots\dots (M)$$

Using the Poiseuille's law co-relating the pressure difference ΔP and steady flow Q through a uniform radius and stiff blood vessels, we have

$$Q = \frac{\Delta P \cdot \pi \cdot r_i^4}{8 \cdot \eta \cdot l} \quad \dots\dots\dots (N)$$

Rearranging the equations (M) and (N), we get

$$\tau = \frac{4 \cdot Q \cdot \eta}{\pi r_i^3}$$

This is the required formulae [161] for hemodynamic shear stress (τ) where Q is the flow rate in ml/sec; η is the viscosity of medium; r_i is the radius of the blood vessels. On an average, the hemodynamic shear stress in arteries is 15 dyne/cm² and 1-6 dynes/cm² in veins [161]. During arm cycle exercise, the blood flow and shear stress increased to 60 dynes/cm² in femoral artery [162]. The shear stress generated by the flow of blood changes the behavior of CTCs and makes the metastasis ineffective.

1.9.1 The measurement of shear stress received by CTCs during the physiological situation

The normal stress that occurs with the blood pressure is distributed in all the wall layer of vessels. But the shear stress is applied at the inner layer of the wall of blood vessel. The calculation of shear stress is done with the basic assumptions of fluid mechanics principle i.e. no-slip condition. This creates the velocity gradient i.e. velocity decreases from a maximum at the center to zero at the wall as shown in figure 1-10.

Figure 1-10. The velocity distribution profile in the tubes with various shear rates. Reproduced with permission by Papaioannou et al. from reference [163] published in Hellenic Journal of Cardiology.

In the physiological normal condition, shear stress maintains the values in a wide range. The venous and arterial shear stress is calculated by the characterization of flow velocity. The normal value of shear stress in veins is from 1-6 dynes/cm². However, the shear stress at arterial is in the range of 10 and 70 dynes/cm². The higher value of shear stress is found in the region of arteries with strong curvature like an aortic arch, bifurcations etc. The shear rate and shear stress are shown in figure 1-10 as published in [164, 165].

Figure 1-11. The correlation between shear stress with shear rates in the different region of blood vessels. Reproduced with permission by Papaioannou et al. from reference [163] published in Hellenic Journal of Cardiology.

The high shear stress increases the thickness of the wall leading to the increase of the diameter of a vessel so that normal shear stress is achievable. However, in the case of low shear stress decreases the diameter of a vessel so that normal shear stress is achievable. In any case, the mean achievable shear stress in arteries is 15 dynes/cm² [166-168].

1.9.2 Shear stress received by CTCs during exercise condition

Previously, there is a systematic review as well as meta-analyses of randomized controlled trials which suggest that physical exercise is benefited for the patients with HIV/AIDS [169], coronary heart disease [170] and cancer [171]. It is also suggested that the way to prevent plaque to be formed in the arteries i.e. atherosclerosis is by increasing the shear stress via exercise. Exercise mainly increase the heart rate which increases the

cardiac output resulting in the increase of blood flow followed by the increase of shear stress. There has been shown that the study that regular exercise can prevent atherosclerosis [172] and reduced the risk of cardiovascular disease (CVD). However, there is very little known about the effect of physical exercise on the viability of CTCs. In other words, it is still not clear how elevated level of shear stress generated during the exercise condition affect the viability of CTCs.

Exercise training increases the flow of blood and elevated the shear stress with the release of nitric oxide. The figure 1-11 shows that femoral blood flow increased during graded arm cycle exercise resulting in the increment of calculated femoral shear stress. The femoral blood flow increased by 3.5-fold from rest to 30 W as shown in figure 1-11. If the exercise is continuous, the shear stress is steady while shear stress will vary from low to high value in case of interval exercise. As there was no change in arterial diameter, this increment of blood flow in femoral was mainly due to an increment of the velocity of blood [173]. The work rate elevated in every 3 min by 5W during arm exercise and data shows the maximum work rate can be attained by individual i.e. 30 W for arm exercise [173].

The central message from this study [173] was that blood flow increased to non-working limbs directly proportional to work rate. This study further shows that increment in blood flow during exercise leads to the significant increment of shear stress during exercise. There was a similar finding in previous studies [174, 175] where dye dilution and thermos dilution techniques were used for the calculation of flow of blood in limb while performing an exercise. In those study too, it has been clearly shown that blood flow in inactive leg increased by many folds while performing arm cycle exercise. Interestingly, the femoral blood flow increased in linear proportion with the increasing intensity of exercise while performing the arm exercise. In short, blood flows as well as

shear stress increase while doing exercise [173]. During arm cycle exercise, the shear stress in the femoral artery can increase to 60 dynes/cm² [173].

Figure 1-12. The blood flows in the femoral artery and calculated shear stress in dynes/cm² during arm cycle exercise. Reprinted from reference [173] with permission from Medicine and Science in Sports and Exercise.

1.9.3 Effects of shear stress on the survivability of CTCs

It is known that fluid shear stress has a central role in cancer metastasis [176]. The shear stress changes the behavior of cancer cells in vascular as well as tumor microenvironment contributing to cancer metastasis. It changes the phenotypic

properties of nearby cancer cells probably enhancing the tumor invasion and cell migration. The understanding of these mechanical force with the change in biochemical properties of cancer cells might reveal new therapeutics. The response of shear stress on blood cells [177], smooth muscle cells (SMCs) [178], endothelial cells [179] are well studied. It is surprising that there is very little information about the effect of shear stress on the viability of CTCs [115]. In other words, even though it is clear that hemodynamic shear stress causes the destruction of CTCs leaving few CTCs to survive to generate metastasis [115, 180, 181], the shear stress effect on the survivability and proliferation of CTCs is largely unknown. There is certain progress with the development of cone-and-plate viscometer as well as stirring bath, to investigate the mechanism of shear stress in the regulation of CTCs [182-184]. But these previous studies [182, 185] focus on the viability of cell after the application of shear stress. But whether the cells that survived the shear stress will proliferate or not remains largely unknown[186]. These survived cells are mainly responsible for the formation of secondary tumors in many parts of the body. Understanding the mechanism of these survived cells is useful in the innovation of targeted drug.

1.10 Exercise and cancer

Cancer is a serious medical problem. The current treatment of chemotherapy and radiation therapy is very toxic and produces various side effects like pain, reduced fitness of cardio-respiratory, poor quality of life (QOL) as well as the suppression of immune system [187]. The physical activity helps to prevent cancer. The report from the International agency for research on cancer (IARC) shows that around 25% of cancer around the globe is caused by obesity and an unhealthy lifestyle. There has been documented that many studies showing the benefit of exercise in improving the quality

of life [188], composition and body weight [189], strength of muscle [190], cardio fitness [191, 192], as well as improvement of immune system [193] in case of cancer survivors. Physical exercise contributes a vital role in the rehabilitation of cancer patients. It has been clearly shown that breast, as well as colorectal cancer risk, is reduced proportionally with increasing physical exercise [194]. Exercise is beneficial in the prevention of cancer. Exercise helps cancer patient from the recovery of treatment as well as extend the survival.

1.10.1 The role of exercise in the prevention of cancer

Almost 150 studies have been done to study the correlation between the exercise and cancer for a specific site [194]. Regardless of the limitation of each study, the result shows that higher physical activity is related to the reduced risk of the breast as well as colon cancer. Many of these studies [194] conclude that exercise helps to reduce the risk of formation of cancer either by improving the immune system [195], reducing the weight and obesity [196] in changing metabolic and sexual hormone level [197] etc. In case of breast cancer, the reduction of risk is around 30-40% probably by I) reduction in menarche i.e. first time occurrence of menstruation, II) reduced time of exposure to estrogen [197]. In the case of prostate cancer, the physical activity reduced the risk around 10-30% by decreasing the production of testosterone [198]. In case of colon cancer, the risk is reduced by I) 40-50% by reducing the time of gastrointestinal transit (i.e. decreased mucosal exposure if any carcinogens), II) reducing the secretion of bile acid [199].

1.10.2 The benefits of exercise in cancer survivors

Many studies show the consistent finding that exercise is beneficial in cancer survivors by I) strengthening muscle II) composition and body weight III) reducing pain, tiredness, anxiety, depression and IV) improving the overall well-being of life. American cancer society recommends exercise to cancer survivors as a therapy. A systematic review by Kirshbaum *et al.* shows that exercise is safe for breast cancer patient [200]. Markes *et al.* conclude that exercise improves the overall activity of normal daily life [201]. Schmitz *et al.* showed that exercise improves the fitness of cardiorespiratory while taking or after the treatment of cancer [202]. These all studies show that the exercise helps to extend the life of cancer survivors by slowing the progression of cancer or reducing the chance of reoccurrence of the disease. The cancer of breast and colon has a lower rate of reoccurrence with the intense regular exercise.

1.10.3 The exercise program prescribed in cancer

The American college of sports and medicine (ACSM) recommends the following exercise which consists of five components namely the FITT principle (Frequency, intensity, time and type) and progression.

FITT principal components	Frequency	Intensity	Time	Type
Cardiorespiratory (Aerobic training)	3 to 5 days per week	40% or 50-85% HRR 40% or 50-85% V _{O2} R 55% or 65-90% HR _{max}	20 to 60 minutes	Dynamic use of large muscle groups

Muscular strength and endurance (Resistance training)	2 to 3 days per week	12-16 RPE	1 set of 3 to 20 repetitions (e.g. 3 to 5, 8 to 10, 12 to 15)	8 to 10 exercises (All major muscles)
Flexibility	2 to 7 days per week	Stretch to tightness at the end of the range of motion but not to pain	15 to 30 seconds 2 to 4 times/ stretch	Static stretches (All major muscles)

FITT = frequency, intensity, time, type; HRR = heart rate reserve; HR max = maximum heart rate; RPE = rate of perceived exertion; VO₂R = maximum oxygen consumption reserve. Data adapted from ACSM.

Table 1-1. The prescription of FITT exercise by the American college of sports and medicine (ACSM) for healthy individuals. Reprinted from reference [194] with permission from Indian Journal of Medical and Paediatric Oncology.

1.10.4 Types of exercise

Physical activity (PA) is defined as the motion of the body from skeleton muscle which helps in the expenditure of energy in comparison with resting state. Mainly three types of exercise are recommended namely aerobic, resistance and flexibility. However, all mode of exercise is beneficial for cancer, in fact, no exercise has been reported so far which is harmful to the cancer patient. Aerobic is related to improve the cardio-respiratory system like cycling, running and walking etc. Resistance type of exercise is

related to the improvement of muscle strength with intention of increasing muscle mass. Flexibility type of exercise is related to improve the length of the muscle. Chemotherapy drugs have side effects like the formation of a scar in the joint and obstacle in the motion and can be benefited by the resistance training [194].

1.10.5 Frequency, intensity, and duration of exercise

ACSM recommends three to five days in a week of aerobic exercise, two to three days in a week of resistance training (non-consecutive days per week) and two to seven days per week of flexibility training. The aerobic exercise intensity is 65-90% of maximum heart rate (MHR) which is given by age-predicted maximum heart rate (APMHR) as calculated like $220 - \text{Age}$. The intensity recommended for resistance training is a 12-16 rate of perceived exertion (RPE). The intensity level for the flexibility training is in the range of motion which stretches to tightness at the end without pain. As per ACSM, aerobic is performed for 20-60 min, resistance not more than an hour and flexibility training for 15-30 seconds for 2-4 times per stretch [194].

1.10.6 Physical exercise reduces breast cancer risk

The epidemiology study has clearly shown that there exists an indirect proportional between exercise and postmenopausal breast cancer i.e. inverse relation between the exercise with incident breast cancer [203]. Almost 70 studies show that breast cancer risk reduces by 25% in physically active compared to inactive women [204]. As breast cancer is a common problem in the case of women, more than 7 hours of walking per week reduces the breast cancer [203]. In the absence of other physical activity, even 1 hour of walking per day reduces breast cancer risk. In a separate study conducted in

Japanese women concludes that exercise twice a week reduced the risk of breast cancer [205]. Any activity like playing sports, swimming, cycling that is associated with the enhancement of breath and heart rate done on a regular basis reduces the risk of breast cancer. The risk is further decreased by performing an intense activity like running, walking on the hill, aerobics etc. for more than 150 min per week on a regular basis. The current guidelines are 75 min per week of high intensity or 150 min per week of the medium intensity of aerobic exercise recommended for the overall health of adult people [203]. Exercise helps to maintain the healthy weight which helps to reduce the risk after menopause. Exercise helps to improve the immune system as well as change a different kind of hormone in the body. Exercise reduces the level of estrogen level and low level of estrogen reduces the risk of breast cancer in women, but the exact mechanism behind how exercise decreases the risk of breast cancer is not clear yet.

1.11 Reactive oxygen species (ROS) and cell death

Those chemical species that contain oxygen are called reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot\text{OH}$) and superoxide anion (O_2^-). ROS plays a significant role in cell signaling for maintaining homeostasis [206], however, a high concentration of ROS induces the cell death via damaging DNA and degrading the protein [207, 208].

1.11.1 The generation of cellular ROS

ROS is produced through various mechanism depending upon cell and tissue. The ROS production takes place in mitochondria, NADPH oxidase (NOX) complexes, peroxisomes and endoplasmic reticulum (ER). In mitochondria, where adenosine

triphosphate (ATP) is produced, involves the electron escape in the electron transport chain (ETC) producing O_2^- which are converted to H_2O_2 by the superoxide dismutase. A second important source for generation of ROS is NADPH oxidase (NOX) which is in the plasma membrane or phagosomes. NOX generate the superoxide O_2^- and other ROS [209] by transporting the electron across the plasma membrane. These superoxides are then released into extracellular space or phagosomes.

Formation of superoxide followed by hydrogen peroxide as well as hydroxyl radical

Reduction of oxygen forms the superoxide: $O_2 + e^- \rightarrow \cdot O_2^-$

Hydrogen peroxide is formed by the dismutation of superoxide

This H_2O_2 is later converted to hydroxyl radical (partly) and water (fully)

Figure 1-13. Understanding the fundamental of ROS. Figure reprinted from reference [210] with permission from Elsevier.

1.11.2 ROS and redox signaling

Redox signaling refers to the activation of the various signaling pathway that helps to maintain the normal physiological function by regulating biological process [211]. ROS is a signaling molecule that contributes a significant role in signal transduction in the cell. ROS regulates the activity of certain protein via oxidation of cysteine (Cys) residues of proteins i.e. oxidized thiol group (-SH) to sulphenic acid group (-SOH). This sulphenic acid changes the structure of the target protein which ultimately alters the function of the protein [212].

Figure 1-14. Understanding the fundamental of ROS. Figure reprinted from reference [210] with permission from Elsevier.

ROS helps in cell proliferation with the redox reaction in cancer cells [210] via redox-signaling reactions by involving pathway like mitogen-activated protein kinases (MAPKs). Another important ROS target is NF-κB that is responsible to control the survival of cancer cells which is regulated by ROS [213]. The NF-κB transcription factor gets activated by H₂O₂. But, N-acetyl-L-cysteine (NAC) suppress ROS and block the activation of NF-κB by H₂O₂ [213]. Similarly, the hypoxia condition increases the production of ROS which helps in stabilization of transcription factors called hypoxia-inducible factors (HIFs) [214]. Hypoxia-inducible factor-1 (HIF-1) helps to regulate

various aspects related to metabolism and angiogenesis [215]. Another signaling pathway that regulates the cell growth is by phosphoinositide 3-kinase (PI3K) pathway. ROS can directly interact with various signaling molecules like PI3 kinase to start the signaling process of cell growth [216]. ROS helps in oxidation of protein phosphatases which helps in the activation of the PI3K pathway. In short, tumor cells generate an elevated level of ROS which activates a various signaling pathway that helps in metabolism, angiogenesis, growth, survival, and proliferation. Cancer cells also maintain a prominent level of antioxidants to prevent the cell death from oxidative stress generated by ROS. This provides a thoughtful approach to ROS therapy in cancer. The question arises whether the ROS level is reduced to prevent the signaling pathway or increase the ROS level to kill the cancer cells? The level of ROS is regulated by the presence of the antioxidant defense mechanism in the regulation of redox signaling.

1.11.3 Oxidative stress-induced cell death

ROS is generated at a constant level to control the signaling pathway [217]. The cell controls the level of ROS under the normal physiological condition by balancing the production and elimination of ROS. But, oxidative stress which means the huge amount of production of ROS damages DNA, RNA, protein, and lipids. On one side, ROS helps in the survival of cancer cells as ROS activation facilitates the cell cycle progression [218]. ROS acts as a signaling molecule that helps to maintain the normal physiological function by regulating the biological process. But, on the other side, ROS suppress the growth of cancer cells by activating cell death. ROS can cause the cell death either by necrosis or apoptosis. ROS regulates the apoptosis by activating caspase, proteins of Bcl-2 family and dysfunction of mitochondria. Majority of chemo-drugs, as well as radiotherapy, kills tumor cell via the action of ROS. Thus, the medium level of ROS

helps tumor cells to survive but higher production of ROS kills the cancer cells. Understanding how ROS production induces cell death would allow for developing better therapeutics for cancer.

1.11.4 Relation between ROS and cell death

ROS has a destructive effect on lipids and protein molecules. It has been already shown in 1991 that hydrogen peroxide induces the apoptosis that is prevented by catalase [219]. ROS induces the apoptosis in various cell system. For example, ROS induced by hydrogen peroxide is prevented by catalase in neutrophils [220]. In the case of eosinophil, the ROS generated by cause sodium arsenite induces the apoptosis which is prevented by N-acetylcysteine (NAC) and glutathione. The mitochondria regulate the induction of apoptosis. Bcl-2 proteins can detect the damage of mitochondria and activates the protein named Bax. Bax knocks a hole in the membrane of mitochondria leaking the cytochrome C. This cytochrome C bind with apoptotic protease activating factor-1 (Apaf-1). This cytochrome c and Apaf-1 binds with apoptosomes for activating caspase-9. The caspase-9 then activates caspase-3 which eventually leads to the apoptosis [221].

Figure 1-15. The relation between ROS and cell death. Figure reprinted from reference [221] with permission from Apoptosis.

1.11.5 Types of cell deaths: apoptosis and necrosis

Resisting to cell death is one of the important hallmarks of cancer [5] and hence understanding cell death in cancer cells is important to understand the metastatic disease. There are several types of cell death based on morphological criteria without distinction in an exact biochemical mechanism. Cell death is mainly classified into apoptotic, necrotic, autophagic or anoikis. The cell is dead if any one of the following morphology is observed (1) Cell lost the plasma membrane integrity (2) Cell, as well as the nucleus, fragments to apoptotic bodies (3) Engulfment by neighboring cells.

The word “apoptosis” was first used in 1972 by Kerr, Wyllie, and Currie in a scientific publication to explain an identical kind of cell death [222]. Apoptosis is defined as a programmed cell death that takes place in the multicellular organism in a

natural way. It involves the cell rounding, decrease of cellular volume, chromatin condensation, breakage of the nucleus, followed by the formation of apoptotic bodies [45]. Necrosis is sudden cell death or premature death of a cell that happens with an increase in cellular volume, loss of intracellular contents by the rupture in the plasma membrane. Necrosis is un-programmed cell death that occurs with various external factors like toxins, trauma or infection etc. Apoptosis is usually beneficial which keeps a body in balance and maintain homeostasis whereas necrosis is mostly harmful. There are various stimuli condition that can induce apoptosis in a cell. Many anti-cancer drugs used in chemotherapy damages the DNA and induce the apoptosis by p53 dependent pathway [45]. Apoptosis happens during the normal condition where an adult human loses around 50-70 billion cells every day whereas children lose around 20-30 billion cells every day. The special type of apoptosis that occurs with the loss of attachment to the substrate or neighboring cells known as anoikis. The molecular mechanism associated with anoikis is identical with that of classical apoptosis [223].

Figure 1-16. The relation between apoptotic and necrotic cell death. Figure reprinted from Wikipedia

1.11.6 Signaling pathway of apoptosis

Apoptosis is mainly regulated by two different pathways: namely intrinsic pathway (known by mitochondrial pathway also) and the extrinsic pathway. The intrinsic pathway is regulated by various internal stimuli like irradiation i.e. UV, X-ray, γ -ray, toxic chemicals. Upon triggering, Bcl-2 proteins involve in the binding on the outer membrane associated with mitochondria and create the pores which cause to release cytochrome C. The cytochrome c combines along APAF1 (apoptotic protease activating factor 1) in presence of ATP resulting in the complex formation known by apoptosome.

This apoptosome activates the procaspase-9 which then activates the caspase-3 as well as caspase-7. This caspase regulates the cell death [224].

Figure 1-17. Intrinsic and extrinsic pathway of apoptosis. Figure reprinted from reference [224] with permission from Springer Nature.

The extrinsic pathway is regulated by death-receptor ligation by death ligands. For instance, tumor necrosis factor (TNF) binds with its death ligands TNFR1. TNFR1 is a death receptor that initiates the process of apoptosis. The TNFR1 binds with two different cytoplasmic adaptor proteins namely TRADD (TNR receptor-associated death domain) as well as FADD (Fas-associated death receptor) in presence of activated procaspase-8 molecules. Then caspase-8 further activates the caspase-3 which induces cell death [224]. But, to those cells which do not respond with extrinsic pathway requires to target BH3 protein, Bid (BH3 interacting-domain death agonist). This helps

to activate the intrinsic pathway by the activation of pro-apoptotic proteins. This further induces mitochondrial outer membrane permeabilization (MOMP).

1.11.7 Fluorescence resonance energy transfer (FRET)-based caspase sensor for detecting cell apoptosis

The fluorescence resonance energy-based caspase sensor is used for detecting the cell apoptosis. This was discovered in 2001 by my co-supervisor Prof Kathy Qian Luo [225]. Activation of caspase-3 is a required step in the process of apoptosis. Caspase-3 activity in cells is measured using methods like cyan fluorescent protein (CFP) fused with a yellow fluorescent protein (YFP) by DEVD i.e. 16 amino acid-based peptide linker [225]. This DEVD sequence connecting CFP and YFP is within close proximity to bring FRET [226]. The idea of FRET is to use two colored green fluorescent protein (GFP) and assess the activity of caspase-3 [227]. When these two colored proteins are linked with each other, then energy transfer takes place from donor to acceptor making the FRET effective [228]. However, upon the induction of apoptosis, the effect of FRET is eliminated [225] due to breakage of linker by the activated caspase-3.

In sensor C3, CFP protein and YFP protein are linked by a specialized linker of 16 amino acids known as DEVD. Both CFP-YFP and sensor C3 have a single band gap at 63 kDa (lane 1 & 3). When caspase-3 is added, the control CFP-YFP does not change (lane 2). But with the addition of caspase-3, sensor C3 gets split into 2 bands YFP and CFP (lane 4). When the caspase-3 inhibitor namely Ac-DEVD-CHO is put together, the sensor C3 doesn't cleaved into YFP and CFP (lane 5) indicating that biosensor C3 is cleaved by caspase-3 [225].

Figure 1-18. (A) Basic design of fusion constructs for CFP-YFP and sensor C3, the latter contains the caspase-3 cleavage sequence DEVD in the linker, the length of which is given by the number of amino acid (aa). (B) Western blot results showing the cleavage of the purified sensor C3 protein by caspase-3. The positions of YFP and polyhistidine-tagged CFP are indicated. CHO (Ac-DEVD-CHO) is a caspase-3 specific inhibitor. This was discovered by my co-supervisor Prof Kathy Qian Luo. Figure reprinted from reference [225] with permission from Elsevier.

The apoptosis rate was determined using fluorescence resonance energy transfer (FRET) microscopy. An inverted Zeiss Axiovert S100 fluorescence microscope (Carl Zeiss, Germany) and a computer-controlled charge coupled device (CCD) camera (AxioCam MRm, Carl Zeiss, Germany) were used to capture the FRET images [143, 225, 229, 230]. The cells were excited with a wavelength of 436 ± 10 nm, and two emission wavelengths, one from cyan fluorescent protein (CFP) (480 ± 20 nm) and

another from yellow fluorescent protein (YFP) (535 ± 15 nm), were individually captured. The Image-Pro Plus software (Media Cybernetics, Inc., USA) was used to merge the digital fluorescence images of CFP and YFP. In the merged FRET images, live cells are in green color while cells undergoing apoptosis is in blue color. This difference occurs because, in the live cells, the fusion protein CFP-DEVD-YFP is intact and emits more green fluorescence. When sensor cells enter apoptosis, caspase-3 is activated, resulting in the cleavage of the peptide linker at the DEVD sequence between CFP and YFP. Because of the separation of CFP from YFP, the energy cannot be transferred from CFP to YFP. Consequently, the color in merged FRET image alters from green to blue, indicating apoptotic cells [143, 225, 229, 230].

Figure 1-19. Phase (a & l) and FRET images (b-k) of HeLa-C3 cells undergoing apoptosis. UV irradiation was done for 3 min to activate apoptosis. The time after the UV irradiation is shown at the top of each image. Scale Bar: 10 μ m. Figure reprinted from reference [225] with permission from Elsevier.

1.11.8 Application of FRET sensor for the understanding of CTCs survival during circulation

This sensor C3 has many advantages and application which can be investigated:

1. The sensor C3 is very sensitive to caspase-3 and can detect the apoptosis of cell in real time.
2. The efficiency of FRET is very high and change of color from green (living) to blue (dead) while undergoing apoptosis is clearly seen.
3. The gene of the sensor C3 is encoded in DNA plasmid and can be incorporated in a cell to produce it automatically without changing the morphology of cells [225, 229, 230].

Our group extensively utilized this sensor C3 for detecting apoptotic cells in various application. After this sensor C3 is incorporated into Hela-C3 cells [225], it is used for identifying various anti-cancer compounds as a tool for drug discovery [231]. Our group has utilized this sensor C3 for drug screening of herbal compounds [232-236]. Our group has utilized this sensor C3 to understand how these compounds regulate the action in cancer cells. For example, compounds that were modified from tanshinone hinder the growth of tumor growth by activating intrinsic pathway of apoptosis [237] whereas compound, oblongifolin C hinder tumor growth and induces apoptosis by the activation of Bax [238] using Hela-C3 cells. On the other sides, our group has transfected this sensor C3 to generate human umbilical vein endothelial cells (HUVEC-C3) [239] to study angiogenesis [240] and develop this platform to screen anti-angiogenesis drugs for liver cancer [241]. Our group has utilized this sensor cells to understand the generation of reactive oxygen species (ROS) under various shear stress (SS) condition as well as glucose concentration in endothelial cells [242]. Our group has

further utilized this sensor C3 to study endothelial cell apoptosis in a microfluidic chip system [243] for diabetes mellitus patients [244]. Further, our group has generated the highly metastatic breast cancer cells [230] out of which 231-M1 and 231-M1 were utilized in this thesis to understand the survival of CTCs during circulation [143]. In the process of metastasis, cancer cells find the way to enter in blood vessels to form secondary tumors in the different part of the body. However, very little is understood about shear stress effect on the survivability as well as the proliferation of CTCs. We have extensively utilized this sensor C3 for the understanding the effect of shear stress (SS) on CTCs survival during circulation. Furthermore, we have facilitated the combined study of SS and drugs and develop a noble platform for drug screening targeting the CTCs utilizing this sensor C3 in this thesis.

1.12 Anti-cancer drugs and anti-oxidants

Many major classes of anti-cancer drugs like alkylating agents, antimetabolites, natural products, and hormones which are known by the antineoplastic drug are used on the treatment of cancer. The first drug used in cancer treatment in modern medicine was alkylating agent mechlorethamine. It was discovered in the 1940s for the treatment of lymphomas. Later, in 1956s, antimetabolite methotrexate was discovered for the first time for the treatment of a solid tumor. Five years later, 5-fluorouracil was discovered for the first time as a new class of cancer-killing compounds known by pyrimidine analogs.

Many anti-cancer drugs were successfully tested after this which can be administered intravenously or given orally or injected intramuscularly or intrathecally (administered into the spinal cord). Anti-cancer drugs mainly destruct the tumor cells by interfering in the cellular function like damage to DNA or replication. This induces

the cell death activating apoptosis. Some cancer drugs generate free radicals to destroy the cancer cells [245]. Some of the drugs that work on the mechanism of free radical are anthracyclines like doxorubicin, platinum coordination complexes like carboplatin, cisplatin, an alkylating agent like alkylsulfonates, hydrazines, podophyllin derivatives like etoposides as well as camptothecins like irinotecan, topotecan etc. [246].

The use of anti-cancer drugs depends upon several factors like type of cancer, stage of cancer whether the tumor can be removed by surgery or radiation therapy. The specificity of anti-cancer drugs plays a vital role in reducing the side effect like hair loss, sores on the mucous membrane, nausea, vomiting, bone marrow toxicity etc. related to the used drug. Anti-cancer drugs are very harmful as well as toxic to normal cells. They cause all these mentioned side effects which may require reducing the dosage of the drug or changing to new drug as per the tolerable limit of the cancer patient. In a very rare case, long use of these anti-cancer drugs like etoposide leads to the formation of secondary tumors like myelodysplastic syndrome or acute leukemia which is caused by alkylating agents or topoisomerase inhibitors. Anti-cancer drugs interfere with the different part of cell cycle like growth phase, cell division or resting phase etc. One cancer drug may be used to pause the growth of tumor cells whereas another drug may be working to control the different phase. In addition to these multiple drugs, the treatment is often combined with surgery for removal of tumor cells together with radiation therapy to destroy leftover tumor cells.

Antioxidants eliminate the free radicals and prevent the damage of cell and thus finding significance in chemotherapy. The use of antioxidants has attracted interest in cancer therapy. Antioxidants may protect from oxidative stress produced by chemotherapy [247]. But the principle of action of few chemo drugs involves the formation of free radical to induce death in cancer cells. This may result in a fear to use

the anti-oxidants in chemotherapy as the antioxidants may interfere with the drugs. It is debatable whether the antioxidants whether increases or decreases the efficacy of anti-cancer drugs. It is still unclear whether the antioxidants protect the normal cells from the side effects of chemo drugs. Thus, the use of antioxidants is a controversial issue in chemotherapy [246].

1.12.1 The current anti-cancer drugs in the clinics

Conventional anti-cancer drugs, like taxanes, platinum compounds, anthracyclines, and topoisomerase I inhibitors are used in clinics for treating cancer. The platinum-based anticancer drugs cisplatin was approved in 1979 for the treatment of various cancer like ovarian, lung, testicular as well as bladder cancer. Since then only 23 platinum-based drugs are developed which enter into clinics where carboplatin and oxaliplatin are worldwide approved and other three are approved in individual country i.e. nedaplatin approved in Japan, lobaplatin approved in China and heptaplatin approved in Korea. In addition to these, four additional platinum-based anticancer drugs i.e. satraplatin, picoplatin, lipoplatinTM, and proLindacTM are in clinical trials [248]. Many monoclonal antibodies are also developed to improve the efficacy of anti-cancer drugs in the last few decades due to their targeting specificity.

Many anticancer drugs are approved by the food and drug administration (FDA) for various cancers. The drugs used for the prevention of breast cancer are Evista (Raloxifene Hydrochloride), Keoxifene (Raloxifene Hydrochloride), Nolvadex (Tamoxifen Citrate), Raloxifene Hydrochloride and Tamoxifen Citrate. The individual chemo drugs given in combination for treating breast cancer as approved by FDA are AC where A stands for dox hydrochloride widely known by adriamycin and C stands for cyclophosphamide, AC-T where additional T refers to paclitaxel, CAF where

additional F refers to fluorouracil, CMF where M stands for methotrexate and F for fluorouracil, FEC where E is epirubicin hydrochloride and TAC where T stands for docetaxel known by Taxotere, etc. These drugs are given in a combination of chemotherapy as the combination is usually better than single drugs because the mechanism of treating cancer cells works in a unique way. Anti-angiogenic agents in combination with chemotherapy or combined anti-angiogenic drugs are under development as they work by different mechanisms. Even though there are no clinic drugs developed so far with this concept, there are some promising results with bevacizumab, which works targeting vascular endothelial growth factor (VEGF). Few small molecules like ZD6474, AZD2171, and PTK/ZK are in clinical trials which works inhibiting VEGF-tyrosine kinase.

1.12.2 Understanding of oxidative stress generated by anti-cancer drugs

Anti-cancer chemotherapy drugs damage the normal as well as cancer cells through the generation of oxidative stress [246]. Cardiac damage [249], liver damage, a complication in pulmonary, kidney damage (as elimination pathway for drugs) [250] etc. are due to the generation of ROS by drugs. The oxidative stress induced by drugs attacks various cellular targets slowing cell cycle and ultimately inhibiting and killing tumors [251]. The anticancer produces oxidative stress in cancer patients. It is characterized by the increment of lipid peroxidation, decrease of radical trapping potential by blood plasma, the decrease of antioxidants like Vitamin c, vitamin E in plasma. The highest level of ROS is generated by anthracyclines i.e. doxorubicin, epirubicin, and daunorubicin, platinum-based anticancer drugs like cisplatin, oxaliplatin & carboplatin, epipodophyllotoxins that includes teniposide, etoposide & camptothecins like irinotecan & topotecan etc. [247]. The anthracycline produces oxidative stress by

converting electron of ETS of mitochondria into superoxide. The taxanes group of anti-cancer drugs like paclitaxel and docetaxel, vinca alkaloids like vincristine, vinblastine generate a very low level of oxidative stress [247]. The side effects that are caused by the generation of oxidative stress from anti-cancer drugs is prevented by antioxidants as a supplement during the treatment [247].

1.12.3 Role of an anti-oxidant system in cancer

The use of antioxidants in chemotherapy is a very controversial topic. For a low dose of antioxidants, the data indicate that antioxidants protect the normal as well as cancer cells from being killed. However, the high dose of antioxidants inhibits the tumor cell growth but not for normal cells [246]. It is interesting to understand whether the antioxidants taken as a supplement during the chemotherapy protects the normal cell from side effects without influencing the drug efficacy on a tumor. The review on antioxidants and other nutrients taken during the chemotherapy suggest that 1) Antioxidants do not interfere with chemotherapy drugs 2) Antioxidants improves the efficacy of chemotherapy by enhancing the cytotoxicity of drugs 3) Antioxidants protects the normal cells as well as tissue 4) Antioxidants had increased the survival of cancer patients [252].

There is certain evidence that in taking antioxidants as a supplement has additional benefits taken together with certain kinds of chemotherapy. In a study to understand the effect of antioxidants supplement on survival and recurrence of breast cancer, almost 81% of women were using the antioxidants supplement [253]. The key finding of this research is that vitamin C and vitamin E reduces breast cancer recurrence risk whereas vitamin E reduces the mortality risk [253]. But, surprisingly the using carotenoids increase the death risk with breast cancer as well as for all-cause getting

mortality [253]. This shows that the choice of using antioxidants supplement together with chemotherapy is very important in increasing or decreasing the efficacy of anti-cancer drugs. However, the use of chemotherapy reduces the level of antioxidants in cancer patients due to lipid peroxidation [246] which increase the production of oxidative stress. Thus, the additional supplement of antioxidants helps to maintain the level of oxidative stress and hence improve the health condition of cancer patients receiving the chemotherapy [254].

1.12.4 Strategies of anti-oxidants in the modulation of ROS generation

The regulation of ROS is a significant factor in both cancer development as well as responses with respect to chemotherapy drugs. Many signaling pathways are associated with cancer regulation through the generation of reactive oxygen species (ROS). We know that ROS is a signaling molecule which modulates cell growth, differentiation, proliferation, cell death as well as gene regulation by activation of various transcription factors like NF- κ B, HIF α etc. An elevated level of ROS is harmful to cells and is balanced by antioxidants. As ROS contributes to the proliferation of cancer cells, the idea of treating cancer with antioxidants has already been in cancer therapy [255]. Various antioxidants like red wine and green tea have long been studied in chemoprevention, chemotherapy [256, 257] and promising anti-angiogenic agents [258]. The negative effects of antioxidants supplement for vitamin C, β -carotene, and vitamin E is currently receiving much attention [259]. The major concern of antioxidants is in the modulation of ROS levels which allows the proper functioning of normal cells as well as induced apoptosis of cancer cells [259]. The antioxidants used in cancer therapy is a very controversial topic. Few researchers suggest that antioxidants eliminate the side effects of chemo drugs whereas other researchers suggest that antioxidants

interfere with the efficacy of chemo drugs [260] as the cause is mainly dependent on the level of ROS. ROS regulates the death receptors as well as mitochondria-mediated apoptosis [260] by helping in opening the permeability transition pore complex so that cytochrome c be released with the apoptosome formation and ultimately activating caspases-3 [260]. Thus, intake of antioxidants alter the cell apoptosis pathways [261] which is to be considered very carefully while prescribing anticancer drugs in treating the cancer patients.

1.13 Rationale of project

1.13.1 Hypothesis of project

Delving the effect of hemodynamic shear stress (SS) to understand the mechanism of metastasis may help to develop new treatment strategies [115]. CTCs are exposed to hemodynamic SS but very little is known about how the high SS-induced in blood vessels during the exercise condition affects the biology of CTCs. In addition to this how the addition of various clinical anti-cancer drugs with different reactive oxygen species (ROS) level together with anti-oxidants alters the survivability of these CTCs is not understood.

Prior to my work, it is understood that regular exercise changes the level of certain hormone-like estrogen or insulin etc. For example, physical exercise reduces the level of insulin or estrogen which reduces the probability of developing breast cancer. But here, I have a new hypothesis that intensive exercise may be a good strategy for generating high SS that can destroy CTCs and prevent cancer metastasis. The survival of CTCs is a crucial step in the process of metastasis which is regulated by SS. Here, I mainly focused on the effects of physiologically relevant SS as well as high exercise-

induced SS on the two-different way of cell death: necrosis and apoptosis, which is an important hallmark of cancer. I hypothesized that ROS-generating drugs are more potent for destroying CTCs than are non-ROS-generating drugs. In other words, I hypothesized that fluidic shear stress increases the anti-cancer effects of ROS-generating drugs in CTCs. Further, I hypothesized that antioxidants abolished the apoptotic effect of ROS-generating drugs in CTCs.

1.13.2 Overall aims of the Ph.D. project

The current focus of cancer treatment targets on primary tumors whereas almost ninety percent of deaths are due to metastasis [181]. Once cancer cells spread to distant organs, cancer becomes life-threatening and very challenging to treat using modern medicine. This thesis mainly focuses on understanding the survivability of tumor cells during circulation in cancer metastasis: role of high SS generated in blood vessels under exercise condition for survivability of CTCs, efficacy of anti-cancer drugs in destroying CTCs together with physiological relevant pulsatile fluidic SS that exists in the bloodstream of a human artery and finally the interaction of antioxidant together with anti-cancer drugs targeting CTCs in a bio-mimicking circulatory system.

In this thesis, I aim to study the survivability of CTCs on a broad range of hemodynamic SS that can be generated in a range of physiologically and exercise relevant SS. Therefore, I aim to study SS-induced cell death either by apoptosis or necrosis in this thesis. How SS influences the survival of CTCs of various cancer would be studied. Our research group had isolated various cell lines with higher metastatic potential with respect to parental cell lines from lung metastases in two rounds of such experiments. These newly derived breast cancer cell lines with stronger metastatic

potential as well as leukemia as white blood cell model would be studied to find the difference in their survivability capabilities with respect to high SS-induced during the exercise condition.

In this thesis, I aim to address a very important question: can the efficacy of anti-cancer drugs in destroying CTCs be affected by the pulsatile fluidic SS that exists in the bloodstream of a human artery? The thesis aims to investigate whether the ROS generated in CTCs in the circulation affect their sensitivity to anti-cancer drugs. The thesis also aims to gain a clear understanding of how the level of ROS production in clinical drugs treated cancer cells together with physiological relevant SS using a microfluidics system. Further, I aim to investigate the role of antioxidants to see whether they can counteract the anti-cancer effects of ROS-generating drugs. I think the novel findings of this thesis will have significant clinical implications to design more effective regimens for cancer treatment. In addition, the findings of this thesis will also provide valuable insights for developing new strategies to attack CTCs and prevent cancer metastasis.

CHAPTER 2 Materials and Methods

2.1 Antibodies and reagents

The human breast cancer cells MDA-MB-231 was provided by Prof. Xiao- Feng Le from the Department of Experimental Therapeutics at the University of Texas M.D. Anderson Cancer Center, Houston, TX, USA. The human breast cancer MCF-7, lung cancer A549, ovarian cancer 2008, breast cancer UACC-893 and leukemia K562 cells were purchased from American type culture collection (ATCC, Manassas, VA, USA). The FRET sensor cell lines 231-C3, MCF-7-C3, Hela-C3, 231-M1 and 231-M1A used in this thesis were previously generated by our laboratory [225, 229, 230]. We purchased the Dulbecco's modified eagle's medium (DMEM) from Thermo Fisher Scientific, Waltham, MA, USA. The Roswell park memorial institute (RPMI) 1640 medium was bought from life technologies, gibco, Grand Island, NY, USA. The fetal bovine serum (FBS) and penicillin/streptomycin were purchased from hyClone, Buckinghamshire, the UK and Thermo Fisher Scientific, Waltham, MA, USA respectively. The phosphate buffered saline (PBS) was purchased from Lonza, Walkersville, MD, USA. The anti-cancer drugs DOX, cisplatin, etoposide, and Taxol were purchased from Selleck Chemicals, Houston, TX, USA. The antioxidants N-acetylcysteine (NAC) and propyl gallate (PG) were purchased from Sigma Aldrich, USA. The pluronic F-127 was purchased from (Invitrogen, USA). Further, Methylthiazolyldiphenyl-tetrazolium bromide (MTT), Lactate dehydrogenase (LDH or LD), propidium iodide (PI), paraformaldehyde (for fixation) and Triton X-100 were purchased from Sigma Aldrich, USA. The flow rate controlling tubing (PharMed®) and a circulatory tube (silicone tubing) were purchased from Cole-Parmer, USA. Fluoro-brite medium, 2', 7' –

dichlorofluorescein diacetate (DCFDA), Dihydroethidium (DHE) and phalloidin (for staining actin) were purchased from Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Cell line and cell culture

Breast cancer MDA-MB-231 was provided by Prof. Xiao- Feng Le from the Department of Experimental Therapeutics at the University of Texas M.D. Anderson Cancer Center, Houston, TX, USA. The fluorescence resonance energy transfer (FRET)-based caspase sensor C3 [225] was transfected into MDA-MB- 231 cells to generate a stable cell line named 231-C3 [229]. The 231-C3 cells can express a CFP-DEVD-YFP fusion protein, which can be cleaved by caspase-3 or caspase-7, thus serving as a live indicator of apoptosis. A family of 231-C3 cells with increased metastatic ability were generated by injecting 231-C3 cells into the tail vein of nude mice. Afterward, metastatic sensor cells were isolated from lung metastases in two rounds of such experiments [230]. Other cell lines including lung cancer A549, ovarian cancer 2008, breast cancer UACC-893, breast cancer MCF7, cervical cancer HeLa and leukemia K562 cells were obtained from ATCC. MDA-MB-231, 231-C3, 231-M1, 231-M1A, MCF7-C3, HeLa-C3 and UACC - 893 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA). A549, 2008, and K562 cells were cultured in Roswell Park Memorial Institute (RPMI 1640, (Invitrogen, USA). All the culture media were supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, USA), 100 units/ml penicillin, and 100 mg/ml of streptomycin (Gibco, USA). Cells were cultured in a 37 °C incubator with 5% CO₂.

2.3 Microfluidic circulatory system

A microfluidic circulatory system with a peristaltic pump (Ismatec, Germany) was constructed to control the flow rate of the system. Our microfluidic system is based on 6 rollers (Fig. 3-2a) which pump the fluid in a pulsatile manner mimicking the flow of bloodstream in the human body [262]. DMEM or RPMI 1640 containing 10% FBS, 1% penicillin and streptomycin, pH 7.4 (PS, Gibco, USA) was used as a fluid composition in the circulating system. The SS that the cells encounter in the circulation can be calculated using Poiseuille's equation [263]: $\tau = \frac{4 \cdot Q \cdot \eta}{\pi R^3}$, where Q is the flow rate in ml/sec, η is the dynamic viscosity of the fluid, which equals to 0.012 dynes.sec/cm², and R is the inner radius of the circulatory tube (R₂). We have tested various cell densities ranging from 1 × 10⁴ to 1 × 10⁶ cells/ml and found that 2 × 10⁵ cells/ml is the best condition at which enough cells can be collected for analysis, and they can circulate in the system without conjugation or precipitation. The length of the silicone-based circulatory tube (Cole Parmer, USA) is 1.5 m; the size of R₁ is either 0.25 mm or 0.5 mm, and the size of R₂ is fixed at 0.25 mm (Fig. 3-2a). The tube was sterilized by washing with 70% ethanol (4 ml), followed by rinsing with autoclaved distilled water (4 ml). To reduce the adhesion of the circulating cells to the tube, the inner surface of the tube was treated with 1% Pluronic F-127 (Invitrogen, USA) for 30 min. Then, the Pluronic F-127 was removed, and the tube was air dried for at least 30 min. The morphology of cells is examined every time before the cells were introduced into the microfluidic system to make sure that only healthy cells were used. The cells were trypsinized and suspended in fresh DMEM at a density of 2 × 10⁵/ml. One milliliter of the cell suspension (2 × 10⁵ cells) was injected into the circulatory system and subjected to SS treatment for

various durations at 37 °C in a 5% CO₂ incubator. The same volume (100 µl) of cell suspension were then collected at the designated time points.

Figure 2-1. The peristaltic pump

2.4 Calculation of hemodynamic SS

To determine the shear stress generated by the flow in a peristaltic pump (Fig. 2-1), we have used the Poiseuille's equation: $\tau = \frac{4Q\eta}{\pi R_2^3}$ Where Q is the flow rate in ml/sec, η is the dynamic viscosity of the fluid, which is $0.012 \frac{\text{dynes} \cdot \text{sec}}{\text{cm}^2}$, and R_2 is the inner radius of the tube in cm.

Condition: 1

Radius of flow rate controlling tube (R_1) = 0.25 mm = 0.025 cm

Radius of circulatory tubing (R_2) = 0.25 mm = 0.025 cm

Case 1: Speed setting = 27

Volume (ml)		Time (sec)	Q value ($\mu\text{l/sec}$)	Q Average ($\mu\text{l/sec}$)	SS (dynes/cm ²)
Tube A	2	125	16.00	16.03	15.68
	3	187	16.04		
	4	249	16.06		
Tube B	2	127	15.74	15.94	15.59
	3	187	16.04		
	4	249	16.06		

Case 2: Speed setting = 58

Volume (ml)		Time (sec)	Q value ($\mu\text{l/sec}$)	Q Average ($\mu\text{l/sec}$)	SS (dynes/cm ²)
Tube A	2	63	31.74	31.58	30.88
	3	96	31.25		
	4	126	31.74		
Tube B	2	63	31.74	31.75	31.04
	3	96	31.25		
	4	124	32.25		

Case 3: Speed setting = 87

Volume (ml)		Time (sec)	Q value ($\mu\text{l/sec}$)	Q Average ($\mu\text{l/sec}$)	SS (dynes/cm ²)
Tube A	2	41	48.78	46.29	45.26
	3	68	44.11		
	4	87	45.97		
Tube B	2	42	47.61	46.58	45.55
	3	65	46.15		
	4	87	45.97		

Case 4: Speed setting = 99 i.e. (maximum)

Volume (ml)		Time (sec)	Q value (μl/sec)	Q Average (μl/sec)	SS (dynes/cm ²)
Tube A	2	39	51.28	52.26	51.10
	3	56	53.57		
	4	77	51.94		
Tube B	2	41	48.78	51.27	50.14
	3	58	51.72		
	4	75	53.33		

At the fastest speed setting of 99, the highest shear stress of this microfluidic circulatory system was 50 dynes/cm². To increase the shear stress, we doubled the radius of the flow rate-controlling tube to $R_1 = 0.50$ mm; this tube was connected to the circulatory tube with the same radius of $R_2 = 0.25$ mm used in the previous experiment.

Condition: 2

Radius of flow rate controlling tube (R_1) = 0.50 mm = 0.050 cm

Radius of circulatory tubing (R_2) = 0.25 mm = 0.025 cm

Case 1: Speed setting = 10

Volume (ml)		Time (sec)	Q value (μl/sec)	Q Average (μl/sec)	SS (dynes/cm ²)
Tube A	2	118	16.94	16.66	16.30
	3	180	16.66		
	4	244	16.39		
Tube B	2	114	17.54	16.78	16.41
	3	182	16.48		
	4	245	16.32		

Case 2: Speed setting = 36

Volume (ml)		Time (sec)	Q value (μl/sec)	Q Average (μl/sec)	SS (dynes/cm ²)
Tube A	2	33	60.60	60.51	59.17
	3	49	61.22		
	4	67	59.70		
Tube B	2	32	62.5	61.4	60.08
	3	49	61.2		
	4	66	60.60		

Case 3: Speed setting = 99 i.e. (maximum)

Volume (ml)		Time (sec)	Q value (μl/sec)	Q Average (μl/sec)	SS (dynes/cm ²)
Tube A	2	12	166.6	161.52	157.94
	3	19	157.89		
	4	25	160		
Tube B	2	12	166.6	164.44	160.75
	3	18	166.6		
	4	25	160		

As a result, the flow rate was increased, which in turn significantly elevated the maximum shear stress of the system from 50 to 160 dynes/cm². Therefore, we have developed a microfluidic circulatory system that can generate a range of physiologically and exercise relevant shear stresses, including 15, 30, 45 and 60 dynes/cm².

2.5 Numerical simulation

Computational modeling (COMSOL Multiphysics Inc., Burlington, MA) of a straight channel as per our microfluidics system was done in three dimensions to understand the correlation among the laminar fluid flow, velocity distribution and shear stress experienced by cancer cells. The Navier-Stokes equation for incompressible, laminar flow is expressed as following [264]:

$$\nabla \cdot \vec{u} = 0 \quad \dots\dots\dots(1)$$

$$\rho \left[\frac{\partial \vec{u}}{\partial t} + (\vec{u} \cdot \nabla) \vec{u} \right] = -\nabla p + \nabla \cdot [\mu \nabla \vec{u}] \quad \dots\dots\dots(2)$$

Where $\vec{u}$: is the velocity vector for the medium under flow, ρ is the density of the medium, t is the time, p is the pressure and μ is the dynamic viscosity of DMEM. The Newtonian fluid dynamics module was used to solve the Navier-Stokes equation at a steady state conditions [265]. The density and viscosity values were assumed constant throughout the simulation and were specified for a medium at the room temperature.

Table 2-1. Governing equations used during numerical simulation.

Name	Equation
Equation of Continuity	$\nabla \cdot \vec{u} = 0$
Equation of Momentum	$\rho[(\vec{u} \cdot \nabla) \vec{u}] = -\nabla p + \mu \nabla^2 \vec{u}$

$\vec{u}$: Velocity, ρ : density, p : pressure, μ : dynamic viscosity.

Table 2-2. Physical parameters used in a numerical simulation.

Parameter	Value	Description
ρ	997 kg/m ³	Density of DMEM
μ	0.0012 Pa*s	Dynamic viscosity of DMEM

2.6 Simulation of shear stress experienced by cancer cell in a microfluidic-based system using COMSOL multi-physics software

We have developed a computational model using COMSOL multi-physics software to determine the laminar fluid flow, velocity distribution and shear stress experienced by cancer cells in three dimensions of a straight channel as per our microfluidics system. Shear stress was experimentally calculated using the Poiseuille's equation as mentioned in our earlier publication [143, 230, 266, 267]. The goal is to validate the shear stress experienced by cancer cells in the experimental work of our microfluidic system with a numerical simulation using a COMSOL multi-physics software. The geometric model was developed using solid works to mimic the tubes with the same dimension used in the experiments. This model was imported in COMSOL to perform single phase flow simulation to understand how various parameters like flow rate, velocity distributions, and shear stress are inter-related. We present the velocity and shear stress distributions using the channel dimensions and layout designed in solid works based on our circulatory microfluidics device using the Navier-Stokes equation. We have considered three boundary conditions [268]. I) Mass flow boundary conditions were applied at an inlet to provide a prescribed mass flow rate, II) Fluid at zero absolute pressure at the outlet, and (III) A no-slip fluid-wall interface.

These boundary conditions were imposed to solve the velocity distribution for the fluid flow using the Navier-Stokes equation assuming the laminar, incompressible,

Newtonian flow and steady-state condition [265]. Solving the Navier-Stokes assuming the above conditions is a reasonable approximation [264]. The mesh was refined to minimize the flux of the flow between the inlet and outlet. After the successive refinement, for the total 41150 mesh element with the minimum error of 0.5%, the velocity and shear stress distribution were calculated. To calculate the total shear stress experienced by the cancer cells, we constructed plots for the average velocity distributions for the cancer cell perimeter for various flow rates based on our experimental model. The velocity distributions show the maximum velocity at the center of the tubing and the zero at the outer periphery of the tubing. The resulting velocities distributions for the flow rate were used to validate the COMSOL simulations to determine their accuracies for shear stress of the fluids experienced by the cancer cells. The simulation results showed that the maximum shear stress at the outer periphery of the tubing and minimum at the center of the tubing.

Case 1: - Speed setting = 27

For the case of flow rate 16.03 $\mu\text{l/s}$ corresponding to 15.68 dynes/cm^2 for our experimental model, the velocity at the center was simulated to be 16.5 cm/s at the center and zero at the periphery and the simulated value of shear stress experienced by the cancer cells is 15.4 dynes/cm^2 (Fig. 2-2). The analytically calculated values were very close to the simulated values and hence the model based on our system can be considered accurate for determining the shear stress experienced by the cancer cells. Comparing the analytical and simulated shear stress, we found that both values were in very close agreement, with a $\approx 2\%$ error between them, showing that the computational model is also a valid approximation to calculate shear stress based on a flow of fluid in the microfluidic device.

Figure 2-2. Velocity and shear stress distribution for the flow rate of 16.03 $\mu\text{l}/\text{sec}$

Case 2: - Speed setting = 58

For the case of flow rate 31.58 $\mu\text{l}/\text{s}$ corresponding to 30.88 dynes/cm² for our experimental model, the velocity at the center was simulated to be 32.5 cm/s at the center and zero at the periphery and the simulated value of shear stress experienced by the cancer cells is 32.5 dynes/cm² (Fig. 2-3).

Figure 2-3. Velocity and shear stress distribution for the flow rate of 31.58 $\mu\text{l}/\text{sec}$

As expected the experimentally calculated SS equal to 30.88 dynes/cm², the simulated value of shear stress experienced by the cancer cells is 30.3 dynes/cm². The simulated values are very close to the experimental values.

Case 3: - Speed setting = 87

As expected the velocity distribution was maximum at the center and zero at the periphery of the tubing. The velocity at the center was simulated to be 47.5 cm/s at the center and zero at the periphery.

Figure 2-4. Velocity and SS distribution for the flow rate of 46.16 $\mu\text{l}/\text{sec}$

As expected the experimentally calculated SS equal to 45.26 dynes/cm², the simulated value of shear stress experienced by the cancer cells is 44.3 dynes/cm².

Case 4: - Speed setting = 36

This corresponds to condition 2 where the radius of flow rate controlling tube (R_1) = 0.50 mm = 0.050 cm and radius of circulatory tubing (R_2) = 0.25 mm = 0.025 cm.

Figure 2-5. Velocity and shear stress distribution corresponding for (R_1) = 0.50 mm and radius of circulatory tubing (R_2) = 0.25 mm.

The experimental value of shear stress is 59.62 dynes/cm² and the simulated value is 57.4 dynes/cm² which is very close in agreement. As close to the case 1,2 and 3 the experimentally values are very close to the simulated values and hence the model based on our system is very accurate.

2.7 Fluorescence resonance energy transfer (FRET) imaging microscopy

The FRET images were captured using an inverted Zeiss Axiovert S100 fluorescence microscope (Carl Zeiss, Germany). The cells were excited with a light at the wavelength of 436 ± 10 nm. The emission fluorescent images of cyan fluorescent protein (CFP) (480 ± 20 nm) and a yellow fluorescent protein (YFP) (535 ± 15 nm) were captured using a computer-controlled charge-coupled device (CCD) camera (AxioCam MRm, Carl Zeiss, Germany). The digital fluorescence images of CFP and YFP were merged using Image-Pro Plus software (Media Cybernetics, Inc. USA). The merged FRET images were further analyzed to determine the percentage of apoptotic cells. The live cells appeared in green in the merged FRET image. When cells undergo apoptosis, caspase-3 is activated, which leads to the cleavage of the peptide linker at the DEVD sequence between CFP and YFP. This cleavage disrupts the energy transfer from CFP to YFP. As a result, the FRET image changes from green to blue, indicating the occurrence of apoptosis. The rate of apoptosis is calculated using the following formula:

$$\% \text{ of apoptosis} = \frac{\text{Total number of blue cells}}{\text{Total number of green and blue cells}}$$

2.8 Analysis of cell apoptosis with FRET apoptotic sensor

The activation of caspase-3 is a key step in apoptosis. In order to determine the caspase-3 activation, my co-supervisor Prof Kathy Qian Luo invented this sensor in 2001 [225]. As explained earlier, this sensor contains the cyan fluorescent protein (CFP) fused with a yellow fluorescent protein (YFP) connected with a specialized linker DEVD (caspase-3 cleavage sequence). As illustrated in the figure, in case of live cells, when the FRET sensor is excited with excitation wavelength for CFP (433 nm), the CFP acts as a donor, and transfer the energy via FRET effect to YFP which acts as an acceptor. The cells appear green due to the light emitted from YFP (526 nm). However, in the case of apoptotic cells, caspase-3 is activated; this will cleave the DEVD sequence linking between CFP and YFP. When the FRET sensor is excited with an excitation wavelength of CFP (433 nm), CFP is not able to transfer energy to the YFP making FRET ineffective. Hence, the light is emitted from CFP at 480 nm and the apoptotic cells appear to be blue. The real-time detection of cell apoptosis was measured using the cell lines transfected with this FRET sensor.

Figure 2-6. Illustration for FRET principle for the determination of cell apoptosis.

Figure reprinted from reference [244] with permission from The Royal Society of Chemistry.

The rate of apoptosis was determined using fluorescence resonance energy transfer (FRET) microscopy. An inverted Zeiss Axiovert S100 fluorescence microscope (Carl Zeiss, Germany) and a computer-controlled charge-coupled device (CCD) camera (AxioCam MRm, Carl Zeiss, Germany) were used to capture the FRET images [143, 225, 230]. The cells were excited with a wavelength of 436 ± 10 nm, and two emission wavelengths, one from cyan fluorescent protein (CFP) (480 ± 20 nm) and another from yellow fluorescent protein (YFP) (535 ± 15 nm), were individually captured. The Image-Pro Plus software (Media Cybernetics, Inc., USA) was used to merge the digital fluorescence images of CFP and YFP.

In the merged FRET images, live cells appeared in green color while apoptotic cells appeared in blue color. This difference occurs because, in the live cells, the fusion protein CFP-DEVD-YFP is intact and emits more green fluorescence. When sensor cells enter apoptosis, caspase-3 is activated, resulting in the cleavage of the peptide linker at the DEVD sequence between CFP and YFP. As a result of the separation of CFP from YFP, the energy cannot be transferred from the CFP donor to the YFP acceptor. Consequently, the color of the merged FRET image changes from green to blue, indicating apoptosis [143, 225, 230].

Figure 2-7. Detection of apoptosis using the FRET-based caspase sensor in breast cancer cell lines. UV for the various time as indicated at the top of each panel and FRET imaging after 24 h.

2.9 MTT assay

MTT assay can measure the level of reductase in mitochondria which reflects the level of cell metabolic activity. In this assay, the activity of NAD (P) H-dependent cellular oxidoreductase represents the total number of viable cells. This enzyme basically reduces the MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide into formazan (insoluble). After various SS treatments, the cells were collected from the circulatory system and 100 μ l of suspended cells were added to each well of a 96-well plate. After various incubation times, 10 μ l of sterilized MTT solution (5 mg/ml) was added to the cells. After 3 h incubation, 100 μ l of solubilization solution containing 10% SDS and 0.1% HCl was added to each well. 12 h later, the optical density was measured at 595 nm using a plate reader (PerkinElmer Victor³).

Figure 2-8. (A) Mechanism of cell viability assay (B) Formation of formazan crystals

2.10 Detection of necrosis by propidium iodide (PI) staining.

Propidium iodide (PI) can penetrate damaged cell membranes and stain the nucleus red; thus, PI staining has been used as the conventional assay for determining necrotic cell death. One milliliter of 2×10^5 cells was injected into the microfluidic system. In this study, after different SS treatment for 1–4 h, the same volume (100 μ l) of cell suspension were stained with 10 μ M PI (Sigma, USA) for 30 min in the dark. The images were

captured with an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany) at an excitation wavelength of 590 nm and an emission wavelength of 630 nm.

2.11 Lactate dehydrogenase (LDH) assay.

The lactate dehydrogenase assay was performed using a commercial test kit (Sigma-Aldrich). One milliliter of 2×10^5 cells was injected into the microfluidic system. After various SS treatments, the same volume (100 μ l) of cell suspension was collected from the circulatory system and centrifuged at 1500 RPM for 3 min. The cell pellet was discarded and 100 μ l of supernatant was transferred to each well of a 96-well plate. The LDH mixture (200 μ l/well) was added to the supernatant, immediately after which, the plate was covered with aluminum foil. After 30 min of incubation at room temperature, 30 μ l of 1 N HCl was added to each well to terminate the reaction. Then, the primary absorbance and background absorbance was measured at 490 nm and 690 nm respectively using a plate reader (Spectramax M5). The background absorbance at 690 nm was subtracted from the primary absorbance at 490 nm which was then used to calculate the LDH release.

2.12 Anti-cancer drugs

The anti-cancer drugs DOX, cisplatin, etoposide, and Taxol were bought from Selleck Chemicals (Houston, TX, USA). The working concentrations of all four drugs were freshly prepared from their own stock solutions. The drugs were uniformly mixed with the cells in DMEM containing 10% FBS and 1% penicillin and streptomycin, pH 7.4 (PS, Gibco, USA), before being applied to the microfluidic circulatory system or added to each well of a 96-well culture plate. These cells that were treated with drugs were

maintained at 37°C in a 5% CO₂ incubator and were then collected at the designated time points for various analyses. All drug testing experiments were conducted in triplicate.

2.13 Antioxidants

The antioxidants N-acetylcysteine (NAC) and propyl gallate (PG) were purchased from Sigma, USA. The working concentrations of both antioxidants were freshly prepared from their stock solutions. The antioxidants NAC or PG and the ROS-generating anti-cancer drugs DOX or cisplatin were uniformly mixed with cells in DMEM containing 10% FBS and 1% penicillin and streptomycin, pH 7.4 (PS, Gibco, USA), before being applied to the microfluidic circulatory system. The cells that were treated with drugs and antioxidants were maintained at 37°C in a 5% CO₂ incubator and were then collected at the designated time points for various analyses. All experiments testing the combined effects of antioxidants and drugs were conducted in triplicate.

2.14 Measurement of cell death by annexin V and propidium iodide (PI) staining

To detect apoptotic cell death in our A549 lung cancer cells that do not express our caspase sensor C3, annexin V and PI staining were used. Phosphatidylserine (PS) is in the inner layer of a cell membrane in normal cells. However, in the case of apoptotic cells, PS translocate from the inner to an outer layer of a cell membrane, and this change can be used to indicate apoptosis. Annexin V is a 35–36 kDa calcium-dependent phospholipid-binding protein that can bind PS exposed to the outer layer of the cell membrane. After various circulatory and drug treatments, the cells were collected by centrifugation at 1,500 RPM for 3 min. Then cells were washed twice with cold PBS

and resuspended in 1X annexin-binding buffer. For every 100 μ L of cell suspension, we added 10 μ L of annexin V solution (Thermo Fisher Scientific, Waltham, MA, USA) and 2 μ L of PI at 100 μ g/mL (Sigma, USA) and incubated for 30 min at a normal temperature in the dark. Then, cells were washed with 1X annexin-binding buffer. Annexin V and PI fluorescence were immediately observed under an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany). Those cells that had an annexin V+/PI- staining pattern were in early apoptosis, while cells with an annexin V+/PI+ pattern are in late apoptosis and necrosis.

2.15 Detection of intracellular ROS levels using 2',7'-dichlorofluorescein diacetate (DCFDA)

MDA-MB-231 cells were treated with or without different drugs and circulated or left under static conditions in Fluoro-brite medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (HyClone, Buckinghamshire, UK) for 3 h. Then, the cells were collected and centrifuged to remove various drugs. The cells were immediately stained with 10 μ M CM-H₂DCFDA (DCFDA) (Thermo Fisher Scientific, Waltham, MA, USA) prepared in Fluoro-brite medium. After incubation for 30 min, the medium with DCFDA was removed, and the cells were resuspended in fresh Fluoro-brite medium. Then, 100 μ L of Fluoro-brite medium containing cells were added to 96-well plates. Green fluorescence images of DCFDA were immediately captured using an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany) at an excitation wavelength of 495 nm and an emission wavelength of 529 nm. Five images were collected for each condition, and the average value of fluorescence intensity was calculated from these images using ZEN software.

2.16 Analysis of the formation of ROS by dihydroethidium (DHE) staining

MDA-MB-231 cells were treated with or without different drugs, circulated or left under a static condition in Fluoro-brite medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Hyclone, Buckinghamshire, UK) for 3 h. After which the cells were collected and centrifuged to remove various drugs. The cells were immediately stained with 10 μ M dihydroethidium (DHE) (Thermo Fisher Scientific, Waltham, MA, USA) prepared in Fluoro-brite medium. After incubation for 30 min, the medium with DHE was removed and cells were re-suspended in fresh Fluoro-brite medium. Then, 100 μ l of Fluoro-brite medium containing cells were added in 96-well plates. The red fluorescent images of DHE were captured using an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany) at an excitation wavelength of 535 nm and an emission wavelength of 610 nm. Five images were taken for each condition and an average value of fluorescence intensity was calculated from these images using ZEN software.

2.17 Immunofluorescent staining of the cytoskeleton and Hoechst staining

After collecting the cells as mentioned time, cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were then washed with 1X PBS three times followed by permeabilized with 0.2% Triton-X 100 for 15 min. Cells were again washed with 1X PBS three times. Then, actin was stained using 1X phalloidin-rhodamine (Invitrogen) at room temperature which was dissolved in methanol for 30 min. Cells were again washed with 1X PBS three times. The nucleus was stained using 5 μ g/ml Hoechst 33342 prepared in 1X PBS for 15 min at room temperature. Cells were again washed with 1X PBS three times. Then, cells were mounted onto a coverslip using 30 μ l Mowiol® 4-88 (Calbiochem) which was kept for overnight. Images were captured

next day using LSM 710 META laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA).

2.18 Statistical analysis

All the data are presented as the means $\pm$ standard deviations from at least three independent experiments. Student's t-test for two-tailed distributions and two-sample unequal variances were used to calculate the p -values between two groups. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ were considered significant. The FRET images were quantified using micrographs obtained from three independent experiments. A total of 300 cells were counted.

CHAPTER 3 Effects of Hemodynamic Shear Stress on the Behavior and Survival of Circulating Tumor Cells

3.1 Introduction

Cancer metastasis is a major medical problem because it causes 90% of human cancer deaths [269], thus the most effective way to save the life of cancer patients is to prevent metastasis. Metastasis occurs through a series of complicated steps including 1) tumor cells depart from the primary tumor sites; 2) the cells undergo intravasation to enter the circulatory system [64, 181]; 3) the cells travel in the bloodstream known as circulating tumor cells (CTCs); and 4) finally, the survived CTCs extravasate and form secondary tumors in different parts of the body [270]. As only the survived CTCs can become the initial metastatic tumor cells, destroying these CTCs represents a promising strategy to prevent metastasis [271]. Many studies have shown that CTCs can serve as a prognostic marker [153] for patients with prostate, metastatic breast and colorectal cancer [154]. However, how to eliminate CTCs without damaging the blood cells remains a big challenge. Previously, systematic reviews and meta-analyses of randomized controlled trials suggested that physical exercise can benefit patients with HIV/AIDS [169], coronary heart disease [170] and cancer [171]. However, little is known about the effect of physical exercise on the viability of CTCs.

CTCs can potentially be destroyed in the bloodstream by several mechanisms including hemodynamic shear stress (SS), anoikis due to the detachment of the CTCs from the extracellular matrix, and immune-elimination [272]. Among them, hemodynamic SS is the main focus of this study because it has been reported that SS generated by the bloodstream can destroy cancer cells, rendering the metastatic process ineffective [64, 163]. Previously, several studies have investigated the effects of SS on

endothelial cells [242, 243, 273], cardiovascular disease [274], atherosclerosis [275], etc. Recently, we also reported that physiological levels of SS could induce apoptosis in circulating breast cancer cells [230]. However, it is not well understood how elevated levels of SS achievable under intensive exercise conditions can affect CTCs, especially the ones with increased levels of malignancy.

To address this question, we have developed a bio-mimicking circulatory system that can produce a broader range of SS than the one reported in our previous study [230]. On average, hemodynamic SS is 15 dynes/cm² in human arteries and 1–6 dynes/cm² in veins at resting state [163, 263]. During arm cycle exercise, the SS can increase to 60 dynes/cm² in the femoral artery [173]. In a human body, the blood flows in a pulsatile manner [262], hence we also mimicked this pulsatile mode in our microfluidic system [230]. We then compared the effects of low and high SS on a series of breast cancer cells with different metastatic abilities [230], lung and ovarian cancer cells. The in vitro microfluidic circulatory system developed in this study circumvents a major obstacle in studying clinically isolated CTCs, i.e. the extremely low level of CTCs (1–5 cells/ml of patient's blood sample [154]). Some of the breast cancer cells used in this study also stably expressed apoptotic sensor proteins which allow real-time detection of apoptosis [225, 229, 230]. By combining the three technologies including the microfluidic circulatory system, metastatic cell lines, and apoptotic sensor, we were able to closely examine how high SS generated during intensive exercise destroys CTCs.

3.2 Objectives and Significance of Research

3.2.1 Objective 1: Establish a microfluidic system for generating a range of physiologically and exercise relevant shear stress

The main aim of the current study focuses on the cancer cell metastasis. In the process of metastasis, tumor cells enter in blood vessels to gain access to the far organ but how this kind of fluid micro-environment affects cancer cells is still a puzzle. As the cancer cell experience different shear stress in different part of the blood vessels, our first specific objective is to develop a novel peristaltic pump system to mimic the shear stress generated in human circulatory system at resting and exercise condition. We aim to generate the physiological resting and exercise condition relevant value of shear stress by mimicking the flow rate of the blood and diameter of the blood vessels. The objective is to design a microfluidic system to mimic the shear stress generated in the human circulatory system, which can be used to study cancer metastasis.

3.2.2 Objective 2: Explore the effects of the shear stress on apoptosis of metastatic cells

Circulating tumor cells (CTCs) obtained from primary tumors undergo apoptosis from a hemodynamic shear force which makes metastasis ineffective. However, we still do not fully understand how cell sense and response to mechanical force. It is experimentally observed that non-metastatic cancer cells (MCF7-C3) undergo more apoptosis than metastatic cancer cells (231-C3) under hemodynamic stress force [230]. Here, we aim to distinguish for survivability of metastatic cancer cells having different metastatic potentials under mechanical force. We aim to figure out how shear stress variations kill tumor cells during the process of circulation. Our objective is to figure

out the suitable value of physiologically relevant shear stress either in normal or exercise conditions where more than 90% of cell death is achievable. The next objective would be to identify the suitable conditions where we can differentiate between the metastatic cell lines with different metastatic potential based on shear stress. We aim to figure out the cell-type dependent survival against high shear stress.

3.2.3 Objective 3: Explore the effect of shear stress in post-circulation periods for metastatic cells

We further aim to demonstrate the cell morphology; behavior after post-circulation. We aim to understand how tumor cells respond to various shear stress conditions in the post-circulation period. This could present new directions to cancer metastasis and provide novel therapeutic targets in cancer. In short, we want to answer how shear stress influences the survival as well as the behavior of CTCs in circulation throughout metastasis. To demonstrate the morphological structural and behavioral change of cells after post-circulation, we aimed to explore the actin, nucleus and mitochondria changes in the application of hemodynamic shear stress. Based on the preliminary results we will investigate the underlying mechanism. We aim to correlate the dynamic interrelationship of these components in response to hemodynamic shear stress.

3.2.4 Objective 4: Identify the differences between the survivability of metastatic cancer cells

Our objective is to explore the effect of shear stress on different metastatic cells with various potential using an established microfluidic system. Many researchers have highlighted that the effects of hemodynamic shear stress on the survivability of the

cancer cells is unknown [115]. By identifying the case of survivability of cancer cells that directly trigger the metastasis during the circulation in a blood vessel, new directions to the destructions of CTCs would be discovered. We aim to test whether high shear stress treatment can be used as a strategy to eliminate highly metastatic CTCs. We aim to utilize a family of 231- C3 cells with increased metastatic ability and resistance to an anticancer drug, doxorubicin [230]. Fluorescence resonance energy transfer (FRET)-based caspase sensor C3 was transfected into MDA-MB-231 cells to generate a stable cell line of 231-C3 [229]. These 231-C3 cells express a fusion protein of CFP-DEVD-YFP which can be cleaved by caspase-3 or caspase-7, thus serving as a live indicator of apoptosis. To generate more metastatic sensor cells, 5×10^5 of 231-C3 cells were injected into the tail vein of nude mice; after 45 days, it caused the formation of micro-metastases on the surface of the lung. Some sensor cells were isolated from a lung micro-metastasis and grow into a stable cell line which was named as 231-M1 (Fig 1). To obtain more metastatic sensor cells, 5×10^5 of 231-M1 cells were injected into the tail vein of nude mice, and after 90 days, more and bigger metastases were formed on the surface of the lung. Some sensor cells were isolated and grow into another stable cell line named as 231-M1A (Fig 1). Thus, the metastatic capacities of these cell lines should be in the order of 231-M1A > 231-M1 > 231-C3.

Figure 3-1. Generation of a family of 231-C3 cell lines including 231-M1 and 231-M1A from lung metastases in mice [230].

3.2.5 Objective 5: Identify the differences between the survivability of different types of cancer cells and leukemic cells

To explore the possibility of using high shear stress as a strategy to kill CTCs in circulation, we aim to evaluate its destructive effect on three other cancer cell lines including: 1) Lung cancer cell line (A549), 2) Ovarian cancer cell line (2008), and 3) another cell line of breast cancer origin (UACC-893). We want to address one major concern that whether the results exist same outside the range of metastatic breast cancer cell line (231-C3) family. If different types of cancer cell lines could be tested in this microfluidic system, the conclusion of the research would be more persuasive. Thus, with our established novel microfluidic system, we aim to study shear stress effect on different types of cancer cells. To address the concern that high shear stress might damage white blood cells while it destroys the CTCs in the bloodstream, we aim to study the leukemia K562 cells as model cells to check their survivability after the circulatory treatments.

3.3 Results

3.3.1 Design of a microfluidic system for generating a broad range of hemodynamic shear stress.

A microfluidic circulatory system was developed based on our previous work [230] to study the effects of hemodynamic SS on CTCs (Fig. 3-2a). This system can generate various levels of SS that CTCs may encounter in the human vascular system under both resting and intensive exercise conditions. This circulatory system consists of four parts: 1) a reservoir for loading the cell suspension into the system that also allows oxygen and carbon dioxide to get into the tubing system. To ensure the culturing condition of the circulatory system is similar to that of the incubator, we have put the whole system

including the pump into the CO₂ incubator and maintained the whole system within the incubator during the entire circulation time; 2) a cotton filter for preventing both airborne contamination and evaporation of the culture medium in the reservoir; 3) a durable controlling tube (PharMed®) that contacts the six rollers of the peristaltic pump to control the flow rate (shown in yellow color in Fig. 3-2a); and 4) a circulatory tube (silicone tubing) that allows cell suspension to flow unidirectionally and return to the reservoir. The rotation of the rollers that contact the durable tube can drive the fluid to flow in a pulsatile manner with an adjustable flow rate. The durable tube has a radius R_1 of either 0.25 mm or 0.5 mm and a fixed length of 30 cm, whereas the circulatory tube has a radius R_2 of 0.25 mm and a length of 1.5 m.

The highest SS used in our previous study was 30 dynes/cm² which is two times higher than the average artery SS [163, 230, 263]. In order to produce a higher level of SS (60 dynes/cm²) that can be generated in the femoral artery during intensive arm cycle exercise [173], we first determined the correlations between the pump speed and SS by using the same radius ($R = 0.25$ mm) for both controlling (R_1) and circulatory (R_2) tubes. The flow rate (Q) in ml/sec was calculated by measuring the volume of culture medium that exited the circulatory tube in 30 secs at each tested speed (Fig. 3-2b). With the measured flow rate, the fluid SS can be calculated using Poiseuille's equation: $\tau = \frac{4Q\eta}{\pi R^3}$ where η is the fluidic dynamic viscosity which is 0.012 dynes•sec/cm². At the fastest speed setting of 99, the highest SS of this microfluidic circulatory system was 50 dynes/cm² (Fig. 3-2c). To increase the SS, we doubled the radius of the flow rate-controlling tube to $R_1 = 0.50$ mm; this tube was connected to the circulatory tube with the same radius of $R_2 = 0.25$ mm used in the previous experiment. As a result, the flow rate was increased (Fig. 3-2b), which in turn significantly elevated the maximum SS of

the system from 50 to 160 dynes/cm² (Fig. 3-2c). Therefore, we have developed a microfluidic circulatory system that can generate a range of physiologically and exercise relevant shear stresses, including 15, 30, 45 and 60 dynes/cm².

Figure 3-2. Design of the microfluidic circulatory system. (a) Schematic diagram of the microfluidic circulatory system that can generate various levels of SS. Correlations between the speed settings of the peristaltic pump with the flow rate (b) and levels of SS (c).

3.3.2 High shear force can kill CTCs.

To investigate the effects of SS on inducing apoptosis in CTCs, we utilized engineered human breast cancer MDA-MB-231 cells (231-C3) that express apoptotic sensor for real-time detection of caspase activation [229]. Four SS conditions were selected in this experiment: 15, 30, 45, and 60 dynes/cm² covering the range of arterial SS from physiologically resting state to heavy exercise condition [173]. After 231-C3 cells were circulated in the microfluidic system for 0, 2, 4, 9, and 18 h, the cells were collected from the end of the tube and subjected to FRET imaging analysis. The fluorescent micrographs showed that many 231-C3 cells were still alive after being circulated under lower SS conditions of SS15 and SS30 for 18 h, while very fewer cells survived the higher SS treatment of SS45 for 18 h (Fig. 3-3a). No viable cells were detected under the highest SS60 treatment for 9 and 18 h, whereas some cells survived this harsh circulatory condition at 2 and 4 h (Fig. 3-3a). These results indicate that high SS can kill more CTCs than low SS within the same duration of circulatory treatment.

We further validated this finding by measuring the cell viability using the 3 - (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The MTT results showed that 52% and 36% of cells were viable after being circulated under lower levels of SS of 15 and 30 dynes/cm² for 18 h (Fig. 3-3b). But much lower cell viability of 18% and 1% were detected from the cells treated with higher SS of 45 and 60 dynes/cm² (Fig. 3-3b). These results confirm the finding that high SS kills more CTCs than low SS within the same duration.

Figure 3-3. Shear force- and time-dependent death of 231-C3 cells. (a) Merged FRET images of 231-C3 cells immediately after being treated with No SS, SS15, SS30, SS45, and SS60 for 0, 2, 4, 9 and 18 h. The white arrows indicate the blue apoptotic cells. Scale bar: 100 μ m. (b) The viability of 231-C3 cells was measured by the MTT

assay under the same conditions indicated in panel (a). The results were normalized to 0 h. All the data are presented as the means $\pm$ standard deviations from at least three independent experiments. Student's t-test for two-tailed distributions and two-sample unequal variances were used to calculate the p-values between SS15 and higher SS groups. *p <0.05, **p <0.01, ***p <0.001 were considered significant.

To more closely examine the time frame of high SS-induced cell death, the 231-C3 cells were circulated in our microfluidic system under SS15 and SS60 for 2, 3 and 4 h and analyzed by FRET/light imaging microscopy and MTT assay. Both the FRET and phase images showed that high SS60 treatment significantly reduced the number of live cells at 2–3 h and abolished most of the cells at 4 h (Fig.3-4a), meanwhile, more cell debris could be seen in the phase images of 3–4 h. The results of MTT assay (Fig. 3-4b) also showed that the cell viability was decreased to 45.6%, 29.3% and 8.5% after high SS treatment of 60 dynes/cm² for 2, 3 and 4 h, respectively. While the cell viability of SS15 -treated cells was found to be 86% after 4 h of circulation. These results indicate that 4 h of high SS60 circulation is enough to destroy most of the CTCs, while low SS15 treatment is not effective to kill the CTCs within 4 h.

Figure 3-4. Examination of the SS effects. (a) Phase and merged FRET images of the 231-C3 cells after 0, 2, 3 and 4 h of circulation under the treatment of SS15 and SS60. Scale bar: 100 μ m. (b) The viability of 231-C3 cells was measured by the MTT assay under the same treatment. The results were normalized to 0 h. *** $p < 0.001$.

3.3.3 High shear stress can induce necrosis in CTCs.

Because the cell viability was drastically reduced from 100% to 8.5% within the first 4 h of high SS treatment, we hypothesized that most of the cells might be killed by necrosis (unregulated cell death) rather than apoptosis (programmed cell death). We tested this hypothesis by using a previously described fluorescence staining method [276]. 231-C3 cells were circulated in our microfluidic system under SS15 or SS60 for 4 h. Then, the cells were collected from the ends of the tubing and added to a 96-well plate. Afterward, cells were stained with 10 μ M propidium iodide (PI) for 30 min. Cells are considered necrotic if their cell membranes are damaged which allows a membrane impermeable dye, such as PI, to enter the cells. The fluorescence images of the PI staining and FRET microscopy were obtained separately and merged to produce Fig. 3-5a. The fluorescent micrographs showed that high SS of SS60 ruptured the plasma membrane of 231-C3 cells showing positive for PI staining.

To validate the necrotic effect of high SS on CTCs, we performed a lactate dehydrogenase (LDH) assay. LDH is a cytoplasmic enzyme which can be released from the cells into an extracellular environment when the cell membrane is damaged [277, 278]. Thus, LDH assay can be used to detect necrotic cell death. The assay is based on the reduction of nicotinamide adenine dinucleotide (NAD) by LDH which is then utilized in the stoichiometric conversion of a tetrazolium dye. After circulated at SS15 or SS60 for 1–4 h, the cell suspension was centrifuged, and the supernatant was used to perform the LDH assay. The experimental results showed that the level of LDH was significantly increased by ~12 fold after 1 h of high SS60 treatment compared with the uncirculated control cells, and the LDH level was further increased to ~17 fold after 4 h of SS60 treatment. In contrast, much lower levels of LDH release were detected from

the cells circulated under low SS15 treatment for 1–4 h (Fig. 3-5b). This result verifies that high SS can induce necrosis in CTCs.

Figure 3-5. Examination of the necrotic effects of high SS on circulated 231-C3 cells. (a) Optical micrograph of 231-C3 cells that were circulated under SS15 and SS60 for 4 h. Necrotic cells were visualized using propidium iodide (PI) staining, while apoptotic cells were indicated by FRET imaging. Necrotic cells emit a red color, whereas the live cells emit a green color. The white arrows indicate the red necrotic cells. Scale bar: 100 μ m. (b) Relation of LDH release with the time (1–4 h) of SS treatment. The results were normalized to 0 h.

3.3.4 High shear stress prevents CTCs to attach and induces apoptosis after circulation.

We next compared the impacts of low SS vs. high SS on the cell recovery during the post-circulation time. 231-C3 cells were exposed to SS15 or SS60 for 4 h and allowed to recover for 12, 16, and 24 h in 96-well plates. The FRET images show that after 4 h of SS15 treatment, many live green cells are still visible. Cells started to attach to a normal 96-well plastic culture plate at 12 h and most of them became fully attached at

24 h. Importantly, very few blue apoptotic cells appeared in the FRET images of SS15-treated cells, and quantitative analysis of the FRET images also showed that apoptotic rate of SS15-treated cells was 11% at 24 h of post-circulation (Fig. 3-6 a, c). This result indicates that exposure to low SS of 15 dynes/cm² did not affect cell attachment or induce apoptosis. In contrast, the cells that were treated with high SS of 60 dynes/cm² for 4 h could not attach to the plate after 12 h of incubation. More significantly, most of these cells changed their color from green to blue at 16 h, and 92% of them underwent apoptosis at 24 h (Fig. 3-6 a, c).

The MTT results confirm the findings of the FRET imaging microscopy. The graph in Fig. 3-6 b shows that after circulated at SS15 for 4 h, about 90% of the cells were alive at 12 h post-circulation and even higher viability of 96.7% was registered at 24 h post-circulation, probably due to the growth of cells after the recovery (Fig. 3-6 b). However, circulating cells at high SS60 for 4 h reduced cell viability to 8.5%, which was further reduced to 4% at 24 h post-circulation (Fig. 3-6 b).

Figure 3-6. Cell fate after exposure to low and high SS. The cells were exposed to SS15 and SS60 for 4 h and allowed to recover under a static condition. The images were captured during the post-circulation period at the indicated times. (a) Merged FRET images of the 231-C3 cells at 12, 16 and 24 h post-circulation under SS15 and SS60 for 4 h. The white arrows indicate the blue apoptotic cells. Scale bar: 100 μ m. (b) The viability of the 231-C3 cells was measured by the MTT assay under the same treatment conditions as in (a). The results were normalized to before circulation. (c) The

percentage of apoptotic cells was quantified from the FRET images (n =300 cells, *** $p < 0.001$).

3.3.5 High shear stress can effectively kill other types of cancer cells.

To explore the possibility of using high SS as a strategy to kill CTCs in circulation, we evaluated its destructive effect on three other cancer cell lines including: 1) Lung cancer cell line (A549), 2) Ovarian cancer cell line (2008), and 3) another cell line of breast cancer origin (UACC-893). All three types of cells were circulated in our microfluidic system under the SS15 and SS60 conditions for 4 h. The physical presence of the cells after the circulatory treatment was examined by phase-contrast light microscopy. The micrographs in Fig. 3-7 a show that very similar to the case of 231-C3 cells, very few numbers of cells remained intact after the treatment of SS60 for 4 h. As these cells do not have a FRET sensor, we used the MTT assay to quantify the cell viability. The MTT results show that less than 8% of the cells survived the high SS treatment, while much higher levels of viability were detected from all three types of cells under a low level of SS15 treatment. These results show that high SS is an effective approach to kill diverse types of CTCs.

Figure 3-7. Examining the cytotoxic effects of SS on multiple types of cancer cells.

(a) Optical micrographs showing the effects of the low and high SS conditions on three cell lines of A549, 2008 and UACC-893. (b) The cell viability was measured by the MTT assay after 4 h of circulation under SS15 and SS60. *** $p < 0.001$. Investigation of signaling molecules important for tubule formation in the co-culture model.

3.3.6 High shear stress can also destroy highly metastatic and drug-resistant breast cancer cells.

To test whether high SS treatment can be used as a strategy to eliminate highly metastatic CTCs, we utilized a family of 231-C3 cells with increased metastatic ability and resistance to an anticancer drug, doxorubicin [230]. The 231-M1 cells were isolated from a tiny lung tumor after 231-C3 cells were injected into the tail vein of a nude mouse for 45 days, and 231-M1A cells were isolated from a much bigger metastatic lung tumor after 231-M1 cells were intravenously injected into the mice for 90 days. Thus, the metastatic capacities of these cell lines should be in the order of 231-M1A > 231-M1 > 231-C3. All three types of cells were circulated in the microfluidic system under the high SS of 60 dynes/cm² for 4 or 8 h. Afterward, the cells were collected from the tube and analyzed by FRET imaging microscopy. The FRET images showed that although some highly metastatic 231-M1A and 231-M1 cells survived the high SS treatment at 4 h; much less number of these strongly metastatic cells stayed alive after a prolonged high SS treatment for 8 h (Fig. 3-8 a).

We further validated this finding using the MTT assay. The results show that although highly metastatic 231-M1 and 231-M1A cells exhibited stronger resistant than the original 231-C3 cells against the cytotoxic effect of SS60 treatment for 4 h. The viability of these metastatic cells reduced to 22% for 231-M1 cells and 26% for 231-M1A cells after the treatment of SS60 was prolonged to 8 h (Fig. 3-8 b). This result suggests that this physical method may be an effective approach to destroy metastatic and drug-resistant cancer cells.

Figure 3-8. Effects of high SS treatments on cancer cells with different metastatic and drug-resistant abilities. (a) Merged FRET images of 231-C3, 231-M1 and 231-M1A cells after 4 and 8 h of SS60 treatment. Scale bar: 100 μ m. (b) The viability of 231-series cells was measured by the MTT assay at 4 and 8 h after SS60 treatment. The results were normalized to 0 h. *** $p < 0.001$ vs. 231-C3 cells.

3.3.7 Leukemic cells are more resistant to high shear stress than other types of cancer cells.

To address the concern that high SS might damage white blood cells while it destroys the CTCs in the bloodstream, we used leukemia K562 as model cells to check their survivability after the circulatory treatments. This is because K562 cells are of the erythroleukemia type. They are non-adherent, rounded and bear some proteomic resemblance to both undifferentiated granulocytes [279] and erythrocytes [280]. Cells were circulated in our microfluidic system under the treatments of SS15 and SS60 for 1–4 h, respectively, and analyzed by imaging microscopy and MTT assay. Unlike the breast, lung and ovarian cancer cells, where more than 92% of cells died after circulation under SS60 for 4 h (Fig. 3-9 b); many K562 cells remained intact after 4 h of SS60 treatment (Fig. 3-9 a). We also performed the MTT assay and the results showed that 68.3% of cells were viable under SS60 for 4 h which is slightly lower than the viability (80.4%) of SS15 at 4 h (Fig. 3-9 b). As SS15 is the average arterial SS, while SS60 represent a higher SS achievable during arm cycle exercise, we predict that applying a high SS60 for 4 h can selectively damage CTCs without significantly affecting the white blood cells in the circulatory system.

Figure 3-9. Leukemic cells are highly resistant against strong shear forces. (a) Optical micrographs showing the effects of the low and high SS conditions on K562 cells. Scale bar: 200 μm . (b) The viability of K562 cells was measured by the MTT assay after 1–4 h of SS15 and SS60 treatment. The results were normalized to 0 h. * $p < 0.05$, ** $p < 0.01$.

3.3.8 High shear stress destroys the actin dynamics in breast cancer cells

We know that actin filament provides the mechanical support to cells. Hemodynamic shear stress during the circulation of cancer cells regulates the structure and function of the cytoskeleton [281]. However, the effects of shear stress on actin dynamics have not been previously characterized nor the mechanism of cytoskeleton rearrangement after shear stress is understood [282]. Further, we hypothesized that high shear stress destroys the cytoskeleton structure. We choose the actin cytoskeleton to test this hypothesis as it is mainly responsible to provide mechanical strength to a cell. In this study, we exposed the human breast cancer cells under SS15 and SS60 for 1 h and allow it to grow for the next 24 h. We observed that after SS15 for 1 h, the actin filament spread to reshape to same as no-SS conditions and rearrange back to the original morphology after 24 h. However, SS60 for 1 h treated actin couldn't rearrange back to its normal position (Fig. 3-10). This observation indicates the role of shear stress to maintain the architecture and shape of the cells.

Figure 3-10. Actin and nucleus staining in human breast cancer cells. (a) Optical micrographs showing the effects of the low and high SS conditions on MDA-MB-231

cells immediately after 1 h as observed under 10 x and 100 x magnification (b) Optical micrographs showing the effects of no SS, low and high SS conditions for 1 h on MDA-MB-231 cells captured after 24 h as observed under 10 x and 100 x magnification. Phalloidin (red) was used to stain the reorganization of the actin filaments while Hoechst 33342 was used to stain the nuclei. Arrows indicate the actin fiber arrangement.

To understand the role of shear stress on the remodeling of actin rearrangement, we applied SS15 and SS60 for 2 h and allow it to grow for the next 24 h. We observed that even after SS15 for 2 h, the actin filament spread to reshape back to the original morphology after 24 h (Fig. 3-11). However, SS60 for 2 h treated actin resembles with SS60 for 1 h treated and as expected couldn't rearrange back to its normal morphology (Fig. 3-10). Our result suggests the significant role of shear stress for the actin remodeling of cancer cells. Hence, the mechanics of cytoskeleton dynamic is a major focus in understanding the response of mechanical force and the signaling pathway associated with it as a therapeutic target.

Figure 3-11. Actin and nucleus staining in human breast cancer cells. (a) Optical micrographs showing the effects of the low and high SS conditions on MDA-MB-231 cells immediately after 2 h as observed under 10 x and 100 x magnification (b) Optical micrographs showing the effects of low and high SS conditions for 2 h on MDA-MB-231 cells captured after 24 h as observed under 10 x and 100 x magnification. Phalloidin (red) was used to stain the reorganization of the actin filaments while nuclei were stained using Hoechst 33342. Arrows indicate the actin fiber arrangement.

3.3.9 Both the high shear stress from vigorous exercise as well as the duration of exposure are equally important for causing cell death

From a practical application of this study in the cancer population, it is important to address a key factor: Do high shear stress from a vigorous exercise or the duration of exposure contribute for the elimination of circulating tumor cells (CTCs)? The trained endurance athlete can maintain sufficient intensity for a duration of a few hours, however, it can be very challenging to perform such intensive exercise by cancer patients. But, it is important to understand from fundamental science approach whether the high shear stress from vigorous exercise as well as the duration of exposure is equally important.

We hypothesized that high shear stress might kill most of the cells by necrosis (unregulated cell death) rather than apoptosis (programmed cell death). We tested this hypothesis by using a previously described fluorescence staining method [276]. To address this curiosity, 231-C3 cells were circulated in our microfluidic system under SS0, SS15, SS60, and SS160 for 15 min and 30 min. Then, the cells were collected from the ends of the tubing and added into a 96-well plate to monitor its response for the next 24 h. Afterward, cells were stained with 10 μ M propidium iodide (PI) for 30 min. Cells are considered necrotic if their cell membranes are damaged which allows a membrane impermeable dye, such as PI, to enter the cells. The fluorescence images of the PI staining and FRET microscopy were obtained separately and merged to produce Fig. 3-11. The fluorescent micrographs showed that No SS and SS15 for 15 min and 30 min followed by 24 h shows negative for PI staining of 231-C3 cells. However, high SS60 and SS160 for 15 min and 30 min showing positive for PI staining of 231-C3 cells. However, more necrotic cells were observed under SS60 for 15 min and 30 min followed

by 24 h. But, in the case with cells under SS160 for 15 min and 30 min followed by next 24 h showed apoptosis as well as necrosis.

We further validated this finding by measuring the cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT results showed that 92.29% and 92.85% of cells were viable after being circulated under lower levels of SS15 for 15 min and 30 min respectively (Fig. 3-12). The cell viability is 98.23% and 94.83% after 24 h under exposure of low SS15 for 15 min and 30 min respectively (Fig. 3-12). But with SS60 for 15 min, the cell viability is 87.72% which is reduced to 77.52% after 24 h. With increasing the time to 30 min under SS60, the cell viability is 79.58% which is then reduced to 59.28% after 24 h. However, with SS160 for 15 min and 30 min, the cell viability is 80.25% and 55.91% which is further reduced to 36.26% and 25.09% after 24 h respectively (Fig. 3-12). These results confirm the finding that both high shear stress from vigorous exercise as well as the duration of exposure is equally important.

Figure 3-12. Effects of high SS treatments on metastatic cancer cells under the lower duration of exposure. (a) Merged FRET images of 231-C3 cells after 15 and 30 min of No SS, SS15, SS60 and SS160 treatment followed by next 24 h. Scale bar: 100 μ m. (b) The viability of 231 cells was measured by the MTT assay under the same conditions as (a). * $p < 0.05$, ** $p < 0.01$ were considered significant between SS group compared with

No SS. The error bars represent standard deviations. The results were normalized to No SS.

3.4 Discussion

Previous studies on hemodynamic SS mainly focused on its role in the structure and function of endothelial cells and its involvement in cardiovascular diseases [274, 283, 284]. Separate studies also focused on the isolation of CTCs [285]. However, few studies investigated the destructive effects of SS on CTCs, whose survival is a prerequisite for metastasis. Many tumor cells can enter the circulatory system during metastasis; however, only the survived CTCs can reach the metastatic sites and grow into secondary tumors in distinct organs of the body [64, 181, 270]. If we can understand how CTCs withstand the fluid shear force, we can develop a new strategy to specifically destroy CTCs, thus effectively preventing metastasis.

Hemodynamic SS exists everywhere in the circulatory system and every CTC will experience this mechanical force during circulation [115, 263]. Previously, other studies have used various devices including a cone-plate viscometer and a syringe needle to generate fluid SS to study its damaging effect on cancer cells [182, 263, 284, 286]. Recently, our lab has developed a microfluidic circulatory system that can generate physiologically relevant SS. Using this system, we showed that low SS of 15 dynes/cm² was effective to induce apoptosis in non-metastatic breast cancer MCF7-C3 cells, but did not cause significant cell death against metastatic breast cancer 231-C3 cells [230]. In this study, we modified this microfluidic system to generate higher levels of SS that can be produced in the femoral artery during intensive exercise [173, 263].

Using this system, we found that circulation under high SS of 60 dynes/cm² for 4 h could kill 90% of the CTCs derived from multiple types of cancer including breast

cancer MDA-MB-231, UACC-893, lung cancer A549, and ovarian cancer 2008. High SS killed CTCs through two mechanisms: first via necrosis within the 4 h of circulatory treatment, and second via apoptosis during the 24 h of post-circulatory incubation. Furthermore, circulation under SS60 for 8 h could destroy 74% of metastatic breast cancer cells (231-M1A) that were found to be highly malignant and strongly resistant to doxorubicin in our previous study [230]. Interestingly, leukemia K562 cells representing the white blood cells showed strong resistance to the high SS treatment.

Based on these findings, we speculate that cancer patients may use exercise as a mean to produce high SS which in turn can kill CTCs in their vascular system. This physical approach shall have at least three advantages over chemo- and radio-therapies: (1) it has higher specificity on CTCs than the surrounding blood cells; (2) it has stronger efficacy against metastatic and drug-resistant CTCs; and (3) it should have much fewer side effects to the cancer patients.

We are currently investigating the mechanisms by which high SS induces cell death. There are two possibilities. First, we have observed in this study that the cells that were circulated under SS60 for 4 h could not attach to the surface after 12 h of incubation and underwent apoptosis 16–24 h later (Fig. 3-6a). It is possible that the circulatory treatment at high SS damaged the cytoskeleton, prevented cell adhesion, and induced apoptosis via anoikis. However, anoikis is unlikely to be the major cause of SS-induced cell death because we observed that circulating CTCs under low SS15 for 18 h caused only 48% of cell death (Fig. 3-3b). The second possible cause of cell death is related to our previous finding that SS could elevate the level of reactive oxygen species (ROS) in endothelial cells [242] and CTCs [230]. ROS may also be elevated in CTCs in response to high SS, which can thus trigger oxidative stress-induced cell death.

3.5 Conclusion

Circulating tumor cells (CTCs) are the primary targets of cancer treatment as they cause distal metastasis. However, how CTCs response to exercise-induced high shear stress is largely unknown. To study the effects of hemodynamic microenvironment on CTCs, we designed a microfluidic circulatory system that produces exercise relevant shear stresses. We explore the effects of shear stresses on breast cancer cells with different metastatic abilities, cancer cells of the ovarian, lung and leukemic origin. Three major findings were obtained. 1) High shear stress of 60 dynes/cm² achievable during intensive exercise killed more CTCs than low shear stress of 15 dynes/cm² present in human arteries at the resting state. 2) High shear stress caused necrosis in over 90% of CTCs within the first 4 h of circulation. More importantly, the CTCs that survived the first 4 h-circulation, underwent apoptosis during 16–24 h of post-circulation incubation. 3) Prolonged high shear stress treatment effectively reduced the viability of highly metastatic and drug-resistant breast cancer cells. As high shear stress had much less damaging effects on leukemic cells mimicking the white blood cells, we propose that intensive exercise may be a good strategy for generating high shear stress that can destroy CTCs and prevent cancer metastasis.

CHAPTER 4 Fluidic Shear Stress Increases the Anti-Cancer Effects of ROS-Generating Drugs in Circulating Tumor Cells

4.1 Introduction

Despite advancements in anti-cancer drug development in recent decades, metastatic cancer remains an incurable disease that takes the lives of more than 90% of cancer patients [287, 288]. Resistance to chemotherapy is believed to be a significant problem that causes treatment failure in most metastatic cancer patients [289]. Metastasis is a multistep process in which tumor cells travel through the circulatory system to form metastatic tumors at distance sites. Thus, circulating tumor cells (CTCs) are considered to be the origin of metastasis [290]. Previously, CTCs were identified in the blood of cancer patients with solid tumors, which demonstrated the presence of CTCs in the vascular system [291, 292]. Current research on CTCs mainly focuses on detecting and diagnosing CTCs [293]. For example, several studies have shown that the number of CTCs present in the blood determines the severity of the disease [294-297]. Many oncologists consider CTCs isolated from the blood to be a ‘fluid biopsy’ [298]. As CTCs are the cause of metastasis, more efforts should be made to develop new strategies to destroy these cells in the circulation, which will be an effective way to prevent metastasis.

To study CTCs, we have developed a microfluidic circulatory system that can generate physiological levels of fluidic shear stress (SS) similar to those present in human arteries and veins [230]. More importantly, this SS can be applied to CTCs in a pulsatile manner, mimicking the periodic movement of the blood flow in human arteries. With this system, we found that elevated levels of SS, such as the levels present in arteries (15-30 dyne/cm²), could elevate the levels of reactive oxygen species (ROS) in

CTCs, which damaged their mitochondria and induced them to undergo apoptosis. In contrast, low levels of SS, such as the levels present in veins (5 dynes/cm^2), devoid of such an effect [230]. These findings raised a question whether the ROS generated in CTCs in the circulation affect their sensitivity to anti-cancer drugs.

To address this question, we chose four anti-cancer drugs: doxorubicin (DOX), cisplatin, Taxol, and etoposide. These drugs were applied to cancer cells either under static conditions or in a circulatory environment. Their efficacy in inducing apoptosis and reducing cell viability was determined in three types of cancer cells, including breast, lung and cervical cancer. The results show that the ROS-generating drugs DOX, and cisplatin produced significantly stronger anti-cancer effects in CTCs from all three types of cancer, while the non-ROS-generating drugs Taxol and etoposide did not have such an effect. These findings suggest that ROS-generating drugs may be more effective in destroying CTCs in the vascular system. Upon validation in clinical studies, the results of this study may cause a paradigm shift in the use and dosing of these anti-cancer drugs to destroy CTCs during cancer therapy.

4.2 Objectives and Significance of Research

4.2.1 Objective 1: Establish a microfluidic model for drug screening

The circulating tumor cells (CTCs) are mainly responsible for the formation of secondary tumors by hematogenous dissemination. The previous studies in the last decade have suggested that the elimination of CTCs in circulation could be a way to prevent cancer metastasis. However, there is no previous report on the effects of a drug targeting the cancer cells during the process of circulation in blood vessels. Screening drugs targeting the CTCs has immense potential, but it is quite challenging due to the limitation of CTCs in the cancer patients. However, we have modified our microfluidics

system to mimic the study of drug response on cancer cells during circulation in blood vessels.

4.2.2 Objective 2: Distinguish between the anti-cancer efficacy of DOX in estrogen receptor (ER) + and metastatic triple-negative breast cancer cells

We would like to demonstrate the anti-cancer efficacy of DOX in estrogen receptor (ER) + and metastatic triple-negative breast cancer cells. Our objective is to resolve whether survival level differs in breast cancer cells with estrogen receptor (ER)+ and metastatic triple-negative breast cancer cells against the DOX-treated together with SS15.

4.2.3 Objective 3: Examine the level of reactive oxygen species (ROS) in drug-treated breast cancer cells under the application of shear stress

Our first objective is to measure the level of ROS in breast cancer cells treated with drugs under static condition. Our second objective is to understand the role of SS in ROS production in breast cancer cells. Then, compared the synergistic effect of SS and drugs on ROS production in MDA-MB-231 cells.

4.2.4 Objective 4: Examine the effects of shear stress and drugs in circulated lung and cervical cancer cells

Our objectives are to examine our results developed using our engineered breast cancer cells 231-C3 and MCF7-C3 in the circulated lung as well as cervical cancer cells. The examination of shear stress and drugs responses on multiple cancer cells would address a major concern whether the results remain same outside the range of metastatic breast cancer cell line (231-C3) and non-metastatic cancer cell line (MCF7-C3).

4.2.5 Objective 5: To test the effects of shear stress and drug in presence of an antioxidant in breast cancer cells.

We want to address one major concern that whether the results of shear stress and drugs treated cells gets influenced by the addition of the antioxidants. We would like to demonstrate the effects of 1) SS15 + drugs 2) SS + drugs + antioxidants on the apoptosis of metastatic breast cancer cells. In other words, does the intake of antioxidants during chemotherapy affects the efficacy of anticancer drugs? Majority of preclinical studies performed in vitro and animal model shows the benefits of antioxidants [299]. Our main objective is to elucidate the role of antioxidants for the impact of oxidative stress induced by anti-cancer drugs for the induction of apoptosis.

4.3 Results

4.3.1 Physiological fluidic shear stress significantly enhanced the anti-cancer efficacy of DOX in estrogen receptor (ER) + breast cancer cells

DOX is widely used as the first-line chemotherapeutic agent to treat numerous types of cancers, including breast, ovarian, bladder, lung, thyroid, and stomach cancers, neuroblastoma, Wilms' tumor, lymphoma, some acute leukemia, and Kaposi's sarcoma [300-303]. It is well documented that DOX uses two mechanisms to achieve its anti-cancer effect. The first mechanism is to damage DNA by inhibiting topoisomerase, which arrests the cells in S phase and further induces apoptosis [304, 305]. The second mechanism is to produce superoxide, which can damage mitochondria and induce apoptosis [230, 306]. In our previous study, we showed that pulsatile SS elevated the levels of ROS, including superoxide and hydrogen peroxide, in circulated breast cancer

cells [230, 267]. We thus hypothesized that these SS-elevated ROS can further increase the apoptotic effect of DOX.

To test this hypothesis, we compared the cytotoxic effects of DOX on ER+ MCF7 breast cancer cells under static or circulatory conditions. To detect apoptotic cell death immediately after the circulatory process, we used engineered MCF7-C3 sensor cells that can detect apoptosis in real time [229, 230]. The sensor cells were circulated in our microfluidic circulatory system under a pulsatile SS of 15 dyne/cm² for 12 h. We used SS15, as this value represents the average level of SS in a human artery in the resting state [263, 275]. Four experimental conditions were designed for this study: 1) Circulation under SS15 for 12 h; 2) No SS, but treatment with 2.5 μ M DOX for 12 h on an adhesive surface; our adhesive surface does not release any chemicals. 3) No SS, but treatment with 2.5 μ M DOX for 12 h on a non-adhesive surface, which was generated by treating a 96-well plate with 1% Pluronic F-127 for 30 min, to reveal the potential damaging effects of detachment that can occur during circulation; and 4) Circulating cells under SS15 with 2.5 μ M DOX for 12 h.

After various treatments, the MCF7-C3 cells were collected and subjected to phase microscopy and FRET imaging analysis. The fluorescence micrographs in Fig. 4-1A show that DOX significantly reduced the number of green live cells and increased the number of blue apoptotic cells when it was applied to MCF7-C3 cells under circulatory conditions at SS15 for 12 h. The quantified FRET images revealed that co-treatment with SS15 + 2.5 μ M DOX for 12 h resulted in 78.2% apoptotic cell death, while single treatment produced much lower apoptotic rates ranging from 14.2% for SS15-12 h and 16.2% for 2.5 μ M DOX on an adhesive surface to 20.3% for 2.5 μ M DOX on a non-adhesive surface (Fig. 4-1B).

To confirm the above results, we used an MTT assay to measure the viability of MCF7-C3 cells under the same conditions. The graph in Fig. 4-1C shows that single treatment resulted in high cell viabilities under all three conditions: 64.0% for SS15-12 h; 73.6% for 2.5 μ M DOX-12 h on an adhesive surface; and 60.0% for 2.5 μ M DOX-12 h on a non-adhesive surface. However, co-treatment with SS15 + 2.5 μ M DOX for 12 h reduced the cell viability to 17.3%. These results confirm that fluidic SS can significantly improve the cytotoxic effect of DOX on ER+ breast cancer cells during circulation.

Figure 4-1. Shear force improved the anti-cancer efficacy of DOX in ER+ MCF7-C3 breast cancer cells. (A) Optical micrographs of phase and FRET images showing the effects of single or co-treatment with SS15 and 2.5 μ M DOX on MCF7-C3 cells for 12 h

12 h. Scale bar: 100 μm . (B) The apoptotic rate was quantified based on FRET imaging analysis. $N \geq 300$ cells for each group. (C) Cell viability was determined by an MTT assay. The results were normalized to the condition before circulation. *** $p < 0.001$ by Student's t-test.

4.3.2 Fluidic shear stress increased the anti-cancer efficacy of DOX in metastatic triple-negative breast cancer cells

To determine whether fluidic SS can also enhance the anti-cancer effect of DOX in metastatic breast cancer cells, we used engineered MDA-MB-231-C3 (231-C3) sensor cells [229]. MDA-MB-231 is a commonly used metastatic breast cancer cell line that does not express therapeutic receptors, including ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). As such, triple-negative breast cancer is not sensitive to hormone or HER2 therapy and is routinely treated with traditional chemotherapeutic agents, such as DOX and Taxol. More importantly, triple-negative breast cancer has the highest rate of metastasis and the lowest rate of survival compared to ER+ and HER2+ breast cancer. Furthermore, we confirmed that these 231-C3 sensor cells retained their metastatic ability, as they could form metastatic tumors in the lungs of nude mice after being administered via intravenous injection [230].

In this experiment, 231-C3 cells were treated with the same four experimental conditions used for MCF7-C3 cells and subjected to phase microscopy and FRET imaging analysis. The FRET images in Fig. 4-2A show that for the cells treated with SS15 or 2.5 μM DOX alone, most cells appeared in green color; while for the cells treated with 2.5 μM DOX for 12 h under circulatory conditions, many cells appeared in blue color. The quantified FRET images revealed a low apoptosis rate of 4.8% for the

SS15-12 h group; rates of 10.0% and 8.3% were obtained for the 2.5 μ M DOX-12 h groups under adhesive and non-adhesive conditions, respectively (Fig. 4-2B). In contrast, the apoptotic rate of the co-treatment group (SS15 + 2.5 μ M DOX-12 h) was 66.7%, which was 8-fold higher than the apoptotic rate of cells treated with 2.5 μ M DOX alone (Fig. 4-2B).

We further validated these findings by measuring the viability of 231-C3 cells using an MTT assay. The quantified results showed that single treatment with 2.5 μ M DOX for 12 h generated high viabilities of 73.3% under adhesive conditions and 70.3% under non-adhesive conditions. In contrast, co-treatment with SS15 + 2.5 μ M DOX for 12 h produced much lower cell viability of 23.0% (Fig. 4-2C). Taken together, these results indicate that physiological fluidic SS can increase the efficacy of DOX in metastatic breast cancer cells.

Figure 4-2. Shear stress significantly enhanced the anti-cancer efficacy of DOX in metastatic 231-C3 cells. (A) Optical micrographs of phase and FRET images showing the effects of single or co-treatment with SS15 and 2.5 μM DOX on 231-C3 cells for 12 h. Scale bar: 100 μm. (B) The apoptotic rate was quantified based on FRET imaging analysis. $N \geq 300$ cells for each group. (C) Cell viability was determined by an MTT assay. The results were normalized to the condition before circulation. ** $p < 0.01$, *** $p < 0.001$ by Student's t-test.

4.3.3 Shear stress elevated the levels of ROS in DOX- and cisplatin-treated breast cancer cells

The observation that DOX exhibited a much stronger cytotoxic effect under circulatory conditions led us to hypothesize that co-treatment with DOX and circulation may produce more ROS in circulated cells. To test this hypothesis, we measured the level of hydrogen peroxide using the ROS indicator DCFDA. DCFDA is a fluorescent dye that emits a very low level of fluorescence inside of cells; the presence of hydrogen peroxide can convert DCFDA into DCF, which emits much stronger green fluorescence. Thus, the intensity of green fluorescence can indicate the level of ROS [242]. Using this dye, we previously showed that circulating MDA-MB-231 cells under SS15 for 1-6 h significantly elevated the intracellular level of ROS [230, 267].

In this study, MDA-MB-231 cells were treated with or without 2.5 μ M DOX under static or circulatory conditions at SS15 for 3 h. The cells were collected after various treatments and immediately stained with 10 μ M CM-H2DCFDA (DCFDA) prepared in Fluoro-brite medium for 30 min. The green fluorescence of DCF was captured using an inverted fluorescence microscope at an excitation wavelength of 495 nm and an emission wavelength of 529 nm. The fluorescence micrographs showed that more cells emitted green fluorescence after they were treated with either 2.5 μ M DOX or circulated under SS15 for 3 h than after they were left untreated, demonstrating that both conditions increased ROS (Fig. 4-3A). Significantly, many more cells emitted green fluorescence when they were treated with 2.5 μ M DOX under circulatory conditions (Fig. 4-3A). The fluorescence intensities were measured using the ZEN software. The quantified results showed that single treatment with SS15 or 2.5 μ M DOX increased the level of ROS by 5.27- or 4.55-fold, respectively, while co-treatment with SS15 + DOX produced a much higher level of ROS elevation (15.16-fold increase)

(Fig.4-3B and 4-3D). These data may explain why DOX exhibited much stronger anti-cancer potency when it was applied to cancer cells under circulatory conditions.

To validate the synergistic effect of SS and ROS-generating drugs on ROS production, we compared the levels of ROS in MDA-MB-231 cells treated with three additional anti-cancer drugs (cisplatin, Taxol, and etoposide) under static or circulatory conditions at SS15 for 3 h. The fluorescence images show that under static conditions, cisplatin increased the number of green fluorescent cells, while Taxol and etoposide did not; this finding indicates that cisplatin is a ROS-generating agent, while the other two anti-cancer drugs are non-ROS-generating agents (Fig. 4-3A). More importantly, among the three anti-cancer drugs tested, only co-treatment with cisplatin and SS15 for 3 h resulted in more cells that emitted green fluorescence corresponding to a 13.56-fold increase in ROS level (Fig. 4-3A). In contrast, cells treated with Taxol or etoposide under circulatory conditions compared to the single treatment of SS15 for 3 h showed no increase in ROS level (Fig. 4-3C). These results suggest that fluidic SS and ROS-generating drugs, such as DOX and cisplatin, can synergistically increase the intracellular level of ROS in CTCs.

Figure 4-3. Shear stress only elevated the ROS levels in DOX- and cisplatin-treated cells. (A) Fluorescent images of MDA-MB-231 cells treated with various anti-cancer drugs under static or circulatory conditions at SS15 for 3 h. Scale bar: 100 μ m. (B and C) Quantified fluorescence intensity of DCF from the cells in each group. $N \geq 300$ cells for each group. (D) The results were normalized to no drug treatment before circulation,

and the fold changes in fluorescence intensity are presented in this panel. $**p < 0.01$, $***p < 0.001$ by Student's t-test.

We have utilized another widely used ROS detecting dye, dihydroethidium (DHE) [307], to measure ROS levels. We measured the level of superoxide using the ROS indicator DHE. The presence of superoxide can convert DHE into 2-hydroxyethidium (2-OH-E⁺), which emits much stronger red fluorescence. Thus, the intensity of red fluorescence can indicate the level of ROS. Our group has used this DHE dye previously to show that MDA-MB-231 cells under SS5, SS15, and SS60 for 6 h enhanced the intracellular level of ROS [267].

In this experiment, MDA-MB-231 cells were treated with no drugs as a control, 2.5 μ M DOX, 100 μ M cisplatin, 100 nM Taxol and 100 μ M etoposide for 3 h. Cells were stained with 10 μ M DHE prepared in Fluoro-brite medium for 30 min and images were captured using inverted Zeiss Axiovert S100 fluorescence microscope (Carl Zeiss, Germany). The results showed that DOX and cisplatin are ROS generating agent whereas Taxol and etoposide are the non-ROS generating agent. (Fig. 4-4A). In another experiment, cells were circulated under SS15 treated with 2.5 μ M DOX, 100 μ M cisplatin, 100 nM Taxol and 100 μ M etoposide for 3 hours. Then cells were collected and stained with 10 μ M DHE for 30 min. The results obtained are consistent with the DCFDA results (Fig. 4-4A and 4-4B). The fluorescence micrographs showed that more cells emitted red fluorescence after they were treated with either 2.5 μ M DOX or circulated under SS15 for 3 h than after they were left untreated, demonstrating that both conditions increased ROS (Fig. 4-4A). Significantly, many more cells emitted red fluorescence when they were treated with 2.5 μ M DOX under circulatory conditions (Fig. 4-4A). While the red fluorescent images of DHE were captured using the same

microscope at an excitation wavelength of 535 nm and an emission wavelength of 610 nm. Five images were taken for each condition and the fluorescence intensities of DHE were measured using the ZEN software. The quantified results showed that single treatment with SS15 or 2.5 μ M DOX increased the level of ROS by 3.85- or 4.56-fold, respectively, while co-treatment with SS15 + DOX produced a much higher level of ROS elevation (13.50-fold increase) (Fig. 4-4B and 4-4D). These data remain consistent with the DCFDA results (Fig. 4-3B and 4-3D).

We compared the levels of ROS in MDA-MB-231 cells treated with SS15 together with three additional anti-cancer drugs (cisplatin, Taxol, and etoposide) for 3 h. The fluorescence images show that among the three anti-cancer drugs tested, only co-treatment with cisplatin and SS15 for 3 h resulted in more cells that emitted red fluorescence corresponding to a 9.50-fold increase in ROS level (Fig. 4-4A). However, cells treated with Taxol or etoposide under circulatory conditions compared to the single treatment of SS15 for 3 h showed no increase in ROS level (Fig. 4-4C and Fig. 4-4D). These results remain consistent with an elevation of ROS as detected using DCFDA.

Figure 4-4. Shear stress increases the production of ROS in DOX- and cisplatin-treated cells. (A) Fluorescent images of MDA-MB-231 cells treated with various anti-cancer drugs under static or circulatory conditions at SS15 for 3 h stained with DHE. Scale bar: 100 μ m. (B and C) Quantified fluorescence intensity of DHE from the cells

in each group. $N \geq 300$ cells for each group. (D) The fold changes in fluorescence intensity are presented in this panel and the results were normalized to no drug treatment and before circulation, *** $p < 0.001$ by Student's t-test.

4.3.4 Cisplatin displayed stronger destructive effect against cancer cells in circulation

We then compared the killing effect of cisplatin in 231-C3 cells under static and circulatory conditions. For the static condition, 100 μ M cisplatin was added to 231-C3 cells grown in either an adhesive or a non-adhesive 96-well plate and incubated for 12 h. For the circulatory condition, 100 μ M cisplatin was added to 231-C3 cells, and the drug-cell suspension was circulated in our microfluidic system under SS15 for 12 h. The treated cells were then collected and processed for phase and FRET imaging analyses. The FRET images indicated that many cells were alive after being treated with 100 μ M cisplatin for 12 h under static conditions. However, many blue apoptotic cells were observed after treatment with 100 μ M cisplatin for 12 h under circulatory conditions with SS15 (Fig. 4-5A). We calculated the rate of apoptotic cells from the FRET images and found that treating cells with 100 μ M cisplatin for 12 h resulted in 6.4% and 6.2% apoptosis for cells grown on adhesive or non- adhesive surfaces, respectively (Fig. 4-5B). However, the combined 12 h treatment with 100 μ M cisplatin and SS15 enhanced the apoptotic rate by seven-fold to 44.6% in 231-C3 cells (Fig. 4-5B).

To validate the findings obtained from FRET-imaging-based apoptotic analysis, MTT assay was performed. The graph in Fig. 4-5C shows that 231-C3 cells treated with 100 μ M cisplatin for 12 h under static conditions retain high cell viability values of 86.5% and 94.0% on adhesive and non-adhesive surfaces, respectively (Fig. 4-5C). In contrast,

co-treatment of 231-C3 cells with 100 μ M cisplatin for 12 h under circulation at SS15 significantly reduced the cell viability to 45.8% (Fig. 4-5C). These results demonstrate that SS can also effectively increase the anti-cancer effect of another ROS-generating drug, cisplatin, in circulation.

Figure 4-5. Cisplatin displayed a much stronger killing effect against circulated 231-C3 cells. (A) Optical micrographs of phase and FRET images from 231-C3 cells treated with 100 μ M cisplatin for 12 h under static conditions on an adhesive or non-adhesive surface or in circulation at SS15. Scale bar: 100 μ m. (B) The apoptotic rate was quantified based on FRET imaging analysis. $N \geq 300$ cells for each group. (C) Cell

viability was determined by an MTT assay. The results were normalized to the condition before circulation. $**p < 0.01$, $***p < 0.001$ by Student's t-test.

4.3.5 Fluidic SS did not enhance the anti-cancer effect of Taxol and etoposide in circulation

In Fig. 4-3, we showed that neither Taxol nor etoposide increased ROS in MDA-MB-231 cells under circulatory conditions. We thus predicted that these two anti-cancer drugs might not be more effective in CTCs than in attached cancer cells. To test this prediction, we treated 231-C3 cells with either 100 nM Taxol or 100 μ M etoposide for 12 h under static or circulatory conditions. We then used FRET-based imaging analysis to determine their apoptotic rates and an MTT assay to measure their viabilities. The results presented in Fig. 4-6 demonstrate that neither Taxol nor etoposide caused significant cell damage either alone or when applied in circulation for 12. The apoptotic rate obtained after 100 nM Taxol treatment under static conditions on a non-adhesive surface was 6.6%, and the apoptotic rate obtained with 100 nM Taxol in circulation at SS15 was 6.7% (Fig. 4-6A and 4-6C). Similarly, low apoptotic rates were obtained after single treatment with 100 μ M etoposide for 12 h (4.1%) and after co-treatment with etoposide and SS15 (5.5%) (Fig. 4-6A and 4-6C).

We further validated the results of the apoptotic analysis with those obtained in a cell viability assay. High viabilities were detected in 231-C3 cells treated with 100 nM Taxol for 12 h under both static conditions on a non-adhesive surface (74.3%) and circulatory conditions at SS15 (71.5%) (Fig. 4-6D). Similar high viabilities were obtained from 231-C3 cells treated with 100 μ M etoposide for 12 h under static conditions (85.3%) and in circulation (75.6%) (Fig. 4-6D). Taken together, these results

showed that physiological levels of SS did not enhance the anti-cancer effect of non-ROS-generating drugs, such as Taxol and etoposide.

Figure 4-6. Comparison of the anti-cancer effects of Taxol and etoposide under static and circulatory conditions. (A) Phase and merged FRET images of 231-C3 cells

before circulation and after 12 h of treatment with 100 nM Taxol or 100 μ M etoposide on a non- adhesive surface or in circulation under SS15. Scale bar: 100 μ m. (B) The percentage of apoptotic cells was quantified from at least 300 cells for each condition. (C) The cell viability was measured using an MTT assay for the same conditions. The results were normalized to the condition before circulation.

4.3.6 Antioxidants reduced the SS-stimulated anti-cancer effect of DOX and cisplatin

We have shown that the anti-cancer effects of DOX and cisplatin were significantly enhanced when they were applied to circulated cancer cells, and the elevation of ROS levels in CTCs was revealed as the mechanism of action. To validate the role of ROS in DOX- and cisplatin-mediated apoptosis in CTCs, we used two antioxidants, NAC and PG, to suppress ROS generation. Metastatic 231-C3 breast cancer cells were treated with 2.5 μ M DOX or 100 μ M cisplatin in the presence of either 5 mM NAC or 20 μ M PG and circulated under SS15 for 12 h. The cells were collected and examined by phase and FRET microscopy. The results in Fig. 4-7 show that co-treatment with SS15 and 2.5 μ M DOX resulted in a high level of apoptosis (66.7%) in 231-C3 cells, which was significantly reduced to 12.1% and 12.9% by the addition of the antioxidants 5 mM NAC and 20 μ M PG, respectively. Similarly, co-treatment with SS15 and 100 μ M cisplatin produced 44.6% apoptosis, which was significantly reduced to 7.5% and 5.7% after incubation with 5 mM NAC and 20 μ M PG, respectively. These results show that repressing ROS elevation can attenuate the synergistic effect of SS15 and DOX or cisplatin, which indicates the crucial role of ROS elevation in DOX- and cisplatin-induced apoptosis in CTCs.

Figure 4-7. Antioxidants significantly reduced the apoptotic effect of SS with DOX or cisplatin in 231-C3 cells. (A and B) Phase and FRET images of 231-C3 breast cancer cells after treatment with the following reagents alone or in combination: 2.5 μ M DOX or 100 μ M cisplatin with 5 mM NAC or 20 μ M PG under circulatory treatment conditions conducted under SS15 for 12 h. Scale bar: 100 μ m. (C) The percentage of

apoptotic cells was quantified from FRET images of more than 300 cells for each group, *** $p < 0.001$ by Student's t-test.

4.3.7 DOX and cisplatin also displayed stronger apoptotic effects in circulated lung and cervical cancer cells

To test the diverse application of the use of the ROS-generating drugs DOX and cisplatin to kill CTCs in circulation, we evaluated the destructive effects of these drugs on two more cancer cell lines: a lung cancer cell line (A549) and a cervical cancer cell line (HeLa-C3). A549 lung cancer cells were circulated in our microfluidic system under SS15 for 12 h with or without various anti-cancer drugs: 2.5 μM DOX, 100 μM cisplatin, 100 nM Taxol, and 100 μM etoposide. The cytotoxic effects of various treatments were determined by annexin V/PI staining, as described previously [308] since these A549 cells do not express the apoptotic sensor C3. Annexin V can bind to phosphatidylserine and thus detect apoptotic cells, whereas PI can stain apoptotic or necrotic cells with damaged plasma membranes [309]. The results presented in Fig. 4-8A show that annexin V and PI were barely detectable in A549 cells before circulation, while weak signals from annexin V and PI were visible after SS15 treatment for 12 h. Very high green and red fluorescence intensities appeared in the cells after being circulated under SS15 for 12 h with either 2.5 μM DOX or 100 μM cisplatin, while minimal levels of fluorescence were observed in A549 cells treated with 100 nM Taxol or 100 μM etoposide in circulation for 12 h.

To further investigate the effects of DOX and cisplatin on apoptosis induction in CTCs under circulatory conditions, we utilized another engineered sensor cell line to express our apoptotic sensor in human cervical cancer cells (HeLa-C3) [225]. HeLa-C3 cells were circulated in our microfluidic system under SS15 for 12 h with or without 2.5

μM DOX or 100 μM cisplatin and analyzed by phase and FRET microscopy. The results presented in panels B and C show that the apoptotic rate obtained after treatment with 2.5 μM DOX was quite low at 19.8% under static conditions and was significantly increased to 80.4% under the circulatory condition of SS15 for 12 h (Fig. 4-8C). This value is close to the high apoptotic rate of 78.2% produced by co-treatment with SS15 + DOX in MCF7-C3 breast cancer cells (Fig.3-2B). The circulatory condition also significantly increased the apoptotic rate of 100 μM cisplatin from 6.5% under static conditions to 55.0% in a circulatory environment in HeLa- C3 cells (Fig. 4-8C). These results confirm that DOX and cisplatin can also be used to effectively destroy CTCs derived from lung and cervical cancer.

Figure 4-8. Examining the effects of SS on drug efficacy in other types of cancer cell lines. (A) Annexin V and PI staining for detecting apoptosis in A549 cells. Overview of apoptotic status in the specified conditions: before circulation or after

circulation under SS15 for 12 h with four anti-cancer drugs (2.5 μ M DOX, 100 μ M cisplatin, 100 μ M etoposide or 100 nM Taxol). Scale bar: 100 μ m. (B) Phase and FRET images of HeLa-C3 cells before circulation or after circulation under SS15 for 12 h with 2.5 μ M DOX or 100 μ M cisplatin. Scale bar: 100 μ m. (C) The apoptotic rate of HeLa-C3 cells was quantified from FRET images under the same treatment conditions described in panel B. $N \geq 300$ cells for each group, $**p < 0.01$, $***p < 0.001$ by Student's t-test.

4.4 Discussion

This study shows, for the first time, that ROS-generating drugs, such as DOX and cisplatin, exhibit much higher cytotoxicity in cancer cells circulated under fluidic conditions than do non-ROS-generating drugs, such as Taxol and etoposide. This discovery was made based on two important developments. The first is the development of a microfluidic circulatory system that can generate a pulsatile fluidic SS of 15 dyne/cm², which is the average SS in human arteries [143, 230]. The second is the finding that in this circulatory system, pulsatile SS elevated the levels of ROS in CTCs [230]. By combining this latest information and technology, we were able to address the following important question: can SS affect the efficacy of anti-cancer drugs in CTCs? The results showed that DOX and cisplatin displayed much stronger potency than Taxol and etoposide under circulatory conditions, but such differences were not observed under static treatment conditions.

Based on these findings, we suggest that cancer patients may benefit more from ROS- generating drugs, such as DOX and cisplatin, than non-ROS-generating drugs, such as Taxol and etoposide. ROS-generating drugs can produce much higher levels of

ROS in the circulation, which allows them to kill more CTCs in the vascular system. On the other hand, cancer patients should be careful when they consume antioxidants, as they may counteract the anti-cancer effects of ROS-generating drugs.

This latest information may also help oncologists to design treatment strategies. For example, although both Taxol and DOX are first-line chemotherapeutic agents for treating breast cancer, there is no clear guideline concerning which drug should be used first in chemotherapy. If DOX is proven in clinical studies to produce much stronger anti-cancer efficacy in CTCs, DOX should be given to breast cancer patients before surgery, during which many cancer cells may be detached from the primary tumor and enter the vascular system to become CTCs.

In addition to the two ROS-generating drugs tested in this study, there are 20 more therapeutic agents available in the clinic or under clinical trials plus radiotherapy that can directly or indirectly generate ROS (Table 4-1). As some of these agents may also produce higher efficacy under fluidic SS conditions, their therapeutic effects on CTCs should be carefully examined.

Furthermore, our microfluidic circulatory system can be used to study how CTCs behave under the combined effects of fluidic SS and anti-cancer drugs. These studies will provide new insights for developing novel strategies to specifically enhance the destruction of CTCs, thus effectively preventing metastasis.

Table 4-1. Information about therapeutics that can directly or indirectly generate ROS

Mechanism	Name	Targeted cancer types	Stage
Directly generate ROS	5-fluorouracil	Colorectal cancer, head and neck cancer	FDA-approved
	Cisplatin	Multiple types of cancer	
	Daunorubicin		
	Doxorubicin		
	Ionizing radiation		
Mitochondrial dysfunction	2-methoxyestradiol	Prostate cancer and leukemia	
	Arsenic trioxide	Leukemia and myeloma	
	Lanperasone	KRAS ^{G12D} -expressing tumor cells	
	N-(4-hydroxyphenyl) retinamide	Prostate cancer, breast cancer, and neuroblastoma	
	N-benzyloxy-carbonyl-Ile-Glu (O-t-butyl)-Ala-leucinal	Leukemia	
	Erastin	RAS ^{V12} -expressing tumor cells	Phase I/II
Perturb the ER stress pathway	Bortezomib	Multiple myelomas	FDA-approved
	Celecoxib	Colorectal and prostate cancer	
	Nelfinavir	Cervical and pancreatic cancer	
	G202	Liver and prostate cancer	Phase II
Alter GSSG/GSH levels	6-aminonicotinamide	Colon cancer	Phase I
	Buthionine sulphoximine	Ovarian and breast cancer, melanoma	Phase I
	Dibenzophenanthridine	Lymphoma and breast cancer	Preclinical
	L-aspariginase	Leukemia and pancreatic cancer	Phase II
	NOV-002	Lung, breast and ovarian cancer	Phase III
	Sulphasalazine	Pancreatic and lung cancer	Phase I/II
Alter NADP/ NADPH levels	AG-22	Advanced hematological malignancies	Phase II
	AGX-891	Glioma and leukemia	Preclinical

4.5 Conclusion

Many anti-cancer drugs are used in the clinic; however, little is known about their efficacy in circulating tumor cells (CTCs). In this study, we investigated whether the pulsatile fluidic shear stress (SS) in human arteries can affect the efficacy of anti-cancer drugs. Cancer cells were circulated in our microfluidic circulatory system, and their responses to drug and SS treatments were determined. The results showed that fluidic SS significantly increased the potency of the reactive oxygen species (ROS)-generating drugs doxorubicin (DOX) and cisplatin but had an insignificant effect on the non-ROS-generating drugs Taxol and etoposide. Co-treatment with SS and ROS-generating drugs dramatically elevated ROS levels in CTCs, while adding antioxidants abolished the apoptotic effects of DOX and cisplatin. More importantly, the synergistic killing effects of SS and DOX or cisplatin were confirmed in the circulated lung, breast, and cervical cancer cells, some of which have a strong metastatic ability. These new findings suggest that ROS-generating drugs are more potent than non-ROS-generating drugs for destroying CTCs under pulsatile fluidic conditions. This latest information is highly valuable for developing novel therapies to abolish CTCs in the circulation and prevent metastasis.

CHAPTER 5 Conclusion and Future Directions

Understanding the response of circulating tumor cells (CTCs) that influence the cancer metastasis is very important [180]. The current medicine requires effective therapeutic methods for the treatment of cancer metastasis. Metastasis is the deadly diseases that occur when cancer starts to spread to different regions of the body. In this thesis, a vital component of cancer metastasis was investigated. Here, I studied the effect of hemodynamic shear stress on the survival and proliferation of CTCs. In the process of metastasis, cancer cells undergo the blood circulation to form secondary tumors in distinct parts of the body. During the process of circulation, cancer cells are exposed with the hemodynamic shear stress. Shear stress is a very important parameter that controls to restrict the survival of cancer cells making metastasis ineffective. Here, my studies reveal the effect of hemodynamic shear stress on the elimination of CTCs with an intention to eliminate cancer metastasis which was not reported earlier [115].

I mainly looked into the influence of fluidic shear stress on the survivability and proliferation of cancer cells as activating invasion in metastasis is a very notable hallmark of cancer [5]. Our group has previously established a microfluidics system [230] and I have demonstrated the flow rate and shear stress generated underflow using both experimental [143] and simulation studies. By utilizing the engineered metastatic breast cancer MDA-MB-231 cells that can express the C3 sensor (231-C3), I disclosed that high shear stress induced at exercise was able to kill the breast cancer cells undergoing circulation in a microfluidic system. Further staining of these circulated cells with propidium iodide (PI) dyes revealed that high shear stress induces necrosis which was later discovered as a key type of cell death using lactate dehydrogenase (LDH) assay. High shear stress further activated the apoptosis subsequently enhancing the cell

death. As vital information, high shear stress kills other types of circulated lung, ovarian and another cell line of breast cancer origin.

Our group has injected the metastatic breast cancer 231-C3 cells into the tail vein of mice and isolated the 231-M1 cells from lung micro-metastasis. Later, these 231-M1 cells were again injected into the tail vein of mice and 231-M1A was isolated from lung micro-metastasis. All these cell lines 231-C3, 231-M1 and 231-M1A which have different metastatic potential were developed in our lab from triple negative breast cancer MDA-MB-231 cells [229, 230]. 231-M1A is more aggressive as well as metastatic than 231-M1 and subsequently followed by 231-C3 cells [230].

Figure 5-1. Diagram showing the generation of highly metastatic cell lines in nude mice. The figure is taken and modified from reference [230] with permission from the first author, Dr. Afu Fu.

Here, in my thesis, I studied these three newly derived highly metastatic cell lines to distinguish the survivability against fluidic shear stress. In other words, a clear distinction between the metastatic cancer cells in a family of 231-C3 cells was identified based on fluidic shear stress. The association between the survivability capabilities of among these three cell lines were explored in depth. 231-C3 was found to be more apoptotic and less resistant to fluidic shear stress than 231-M1 and 231-M1A cell lines.

High survivability was observed in metastatic 231-M1A followed by 231-M1 than parental 231-C3 cell line under SS60 for 4 h. However, 231-M1 and 231-M1A cell lines were destroyed under SS60 within 8 h of circulation. High shear stress is enough to destroy the drug-resistant as well as highly metastatic breast cancer cells. I further investigated to understand the survivable capabilities of leukemic cells under high shear stress. Interestingly, both the phase and cell viability assay showed that high shear stress had little effects on the survivability of leukemic cells.

Cancer metastasis is a devastating disease. The main aim of my thesis is to understand the mechanism of elimination of CTCs. Despite advancements in anti-cancer drug development in recent decades, metastatic cancer remains an incurable disease that takes the lives of more than 90% of cancer patients [115]. The survival rate for cancer patients with benign tumors is significantly higher than malignant tumors. The effective measures for the elimination of CTCs are achieved by 1) Destroying CTCs under exercise-induced shear stress 2) Increasing the anti-cancer effects of ROS generating drugs like DOX. The use of suitable drugs targeting the CTCs is the least understood topic in metastasis. The mechanism of the drugs to induce the apoptosis of CTCs is not addressed before.

To understand the efficacy of drugs involved in the treatment of cancer, I focused on the drug targeting on CTCs and antioxidants role for the elimination of cancer cells. In other words, in addition to the effects of hemodynamic shear stress, the synergetic effects of shear stress and drugs were also explored. I investigated the effects of four anti-cancer drugs together with the application of shear stress targeting the cancer cells during circulation. The results *in vitro* showed that fluidic shear stress can significantly improve the cytotoxic effect of DOX on ER+ breast cancer cells during circulation. Furthermore, the effect of shear stress together with DOX was investigated in triple

negative breast cancer cell line. Shear stress together with DOX-induced the higher apoptotic rate in triple negative breast cancer cells. The higher apoptotic rate of cancer cells indicates how effectively the DOX can interact with tumor cells to eliminate the CTCs. On the other hand, the normal interaction of DOX on cancer cells has a very less apoptotic rate. These findings suggest that the shear stress facilitates in targeting the tumor cells by increasing the efficacy of anti-cancer drugs. The results indicate that physiological fluidic shear stress can increase the efficacy of DOX in metastatic breast cancer cells. The mechanism studies revealed that DOX and cisplatin are ROS generating agent whereas Taxol and etoposide are non-ROS generating drugs.

Furthermore, the mechanism studied showed that the shear stress induced the ROS production in breast cancer cells [267]. Interestingly the combined effect of shear stress enhances the ROS production in DOX and cisplatin-treated cells with no effects in Taxol and etoposide-treated cells. Finally, these results of ROS production using DCFDA remains same as verified by another popularly used ROS dye DHE. Due to the heterogeneity of cancer cells, I employed shear stress and ROS producing drugs to study the effect on circulated lung as well as ovarian cancer cells. These finding developed using our engineered breast cancer 231-C3 cells remains consistent even in circulated lungs and ovarian cancer cells.

The role of antioxidants in the prevention of cancer is a heavily debated topic [310]. I revealed the importance of antioxidants in cancer therapy in this thesis. Here I focus on the approaches in which antioxidants suppressing ROS are used together with anti-cancer drugs DOX and cisplatin. The debated application of antioxidants arises from the dual nature of ROS on cancer cells [311]. Shear stress stimulates ROS which damages cancer cells and induces cell death via apoptosis and necrosis. Furthermore, our group had previously demonstrated that shear stress elevates ROS which further

increases the invasiveness of a cancer cell [267]. These findings have produced an insight to eliminate the CTCs and ultimately provides one of the way to reduce metastasis. This thesis has opened a new area for those who blindly use the antioxidants while taking chemo drugs. Further studies must be done to understand the role of various anti-oxidants taken from our food while undergoing chemotherapy both *in-vitro* as well as an *in-vivo* model.

Finally, this thesis has developed a potential future study for the destruction of CTCs *in vivo* model. It has been thought that the change in hormonal level might be responsible for the prevention of cancer. However, this thesis has opened a new area that it is the shear stress generated while performing the exercise is responsible for the prevention of cancer. However, future work must be conducted to understand in terms of exercise and cancer *in vivo* model. The correlation between cancer cells survival with respect to exercise condition in mice model still needs to be investigated. One new direction in the field for future studies is to talk about stents used for heart patients to increase the local shear stress which might be useful for preventing metastasis. What is the probability of developing cancer in heart patients who have installed the stent? This thesis has opened a new area in further medical science. There is some limitation in this current study which needs more detail investigation. The immediate further interesting future investigation is on epidemiology studies to see the impact of exercise with higher shear stress on the human population itself.

Many research has shown the significant role of platelets in breast cancer metastasis. Platelets is a major component of blood in a human being and their interactions with CTCs is largely unknown. When the blood vessels get some damage or bleeding, these platelets form an aggregate together with other agglutinating factors known by the blood clot. How this formation of blood clot affects the CTCs is unknown.

It is known that less metastatic MCF7 cells activate the platelet by ADP release. The tumor cells interact with many external factors like immune cells, platelets and natural killer (NK) cells etc. in blood vessels. In my Ph.D. study, the role of external factors is excluded which might be interesting to look in future. But, it is important to investigate a single component at a time in such complicated microenvironment. In other words, it is unknown about the survival of tumor cells in the presence of external factors of blood.

However, in addition to the above mentioned future works, my current study has raised some interesting questions which need further investigation. There are potential directions based on this thesis to make this project perfect and complete. The shear stress interactions with mitochondria require further investigations. We still do not understand the mechanism of production of ROS during the shear stress. It is interesting to investigate the role of shear stress on the cell membrane and cell organelles. Further investigations on the role of shear stress in the EMT induction will help to make this project complete. The role of re-modeling in cytoskeleton dynamics (Intermediate filaments, microtubules, actin filaments) with the shear stress at a real-time is unknown. The interactions of anti-cancer drugs with the cytoskeleton in the absence or presence of shear stress is an interesting future potential project.

In conclusion, this thesis has provided new knowledge to understand the metastatic cancer research and treatment. I explored innovative ideas which are important for cancer metastasis and understood the areas that have been neglected. However, these results developed based on cultured breast cancer cells in microfluidic is much preliminary to be tested in clinics. Many efforts are required to understand these concepts developed *in vitro* model to move forward in clinical application.

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Appendix I

1. Actin and nucleus staining

1. Cells were collected as mentioned time.
2. Remove the DMEM and wash the cells thrice with prewarmed PBS (pH =7.4, 200 µl)
3. Fix cells with 4% PFA for 15 min (200 µl) at room temperature.
4. Remove 4% PFA and wash cells with PBS thrice, 5 min each.
5. Incubate with 0.2 % Triton X 100 for 15 min at room temperature.
6. Remove 0.2 % Triton X 100 and wash with PBS thrice, 5 min each.
7. Prepare 1 X phalloidin in PBS and add to slides for 30 min at room temperature.
8. Remove 1 X phalloidin and wash cells with PBS thrice, 5 min each.
9. Add 5 µg/ml Hoescht 33342 prepared in 1 X PBS for 15 min to stain cell nucleus.
10. Remove Hoescht and wash again with PBS thrice, 5 min each.
11. Then rinse the glass slide in Milli-Q water to remove any salts from PBS.
12. Then put 30 µl of mowiol and mount the coverslip on the glass slide.
13. Remove excess mowiol and clean coverslip and glass slide.
14. Keep the glass slide on for dry in the dark overnight.
15. Images were captured next day using LSM 710 META laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Appendix II

2. Detection of intracellular ROS levels using 2',7'-dichlorofluorescein diacetate

1. MDA-MB-231 cells were treated with or without different drugs and circulated or left under static conditions in Fluoro-brite medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (HyClone, Buckinghamshire, UK) for 3 h.
2. Then, the cells were collected and centrifuged to remove various drugs.
3. The cells were immediately stained with 10 μ M CM-H₂DCFDA (DCFDA) (Thermo Fisher Scientific, Waltham, MA, USA) prepared in Fluoro-brite medium.
4. After incubation for 30 min, the medium with DCFDA was removed, and the cells were resuspended in fresh Fluoro-brite medium.
5. Then, 100 μ L of Fluoro-brite medium containing cells were added to 96-well plates.
6. Green fluorescence images of DCFDA were immediately captured using an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany) at an excitation wavelength of 495 nm and an emission wavelength of 529 nm.
7. Five images were collected for each condition, and the average value of fluorescence intensity was calculated from these images using ZEN software.

Appendix III

3. Analysis of the formation of ROS by dihydroethidium (DHE) staining

1. MDA-MB-231 cells were treated with or without different drugs, circulated or left under a static condition in Fluoro-brite medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Hyclone, Buckinghamshire, UK) for 3 h.
2. After which the cells were collected and centrifuged to remove various drugs.
3. The cells were immediately stained with 10 μ M dihydroethidium (DHE) (Thermo Fisher Scientific, Waltham, MA, USA) prepared in Fluoro-brite medium.
4. After incubation for 30 min, the medium with DHE was removed and cells were re-suspended in fresh Fluoro-brite medium.
5. Then, 100 μ l of Fluoro-brite medium containing cells were added in 96-well plates.
6. The red fluorescent images of DHE were captured using an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany) at an excitation wavelength of 535 nm and an emission wavelength of 610 nm.
7. Five images were taken for each condition and an average value of fluorescence intensity was calculated from these images using ZEN software.

Appendix IV

4. Measurement of cell death by annexin V and propidium iodide (PI) staining

1. To detect apoptotic cell death in our A549 lung cancer cells that do not express our caspase sensor C3, annexin V and PI staining were used.
2. Phosphatidylserine (PS) is in the inner layer of a cell membrane in normal cells.
3. However, in the case of apoptotic cells, PS translocate from the inner to an outer layer of a cell membrane, and this change can be used to indicate apoptosis.
4. Annexin V is a 35–36 kDa calcium-dependent phospholipid-binding protein that can bind PS exposed to the outer layer of the cell membrane.
5. After various circulatory and drug treatments, the cells were collected by centrifugation at 1,500 RPM for 3 min.
6. Then cells were washed twice with cold PBS and resuspended in 1X annexin-binding buffer.
7. For every 100 μL of cell suspension, we added 10 μL of annexin V solution (Thermo Fisher Scientific, Waltham, MA, USA) and 2 μL of PI at 100 $\mu\text{g/mL}$ (Sigma, USA) and incubated for 30 min at a normal temperature in the dark.
8. Then, cells were washed with 1X annexin-binding buffer. Annexin V and PI fluorescence were immediately observed under an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany).
9. Those cells that had an annexin V+/PI[−] staining pattern were in early apoptosis, while cells with an annexin V+/PI⁺ pattern are in late apoptosis and necrosis.

Appendix V

5. Lactate dehydrogenase (LDH) assay

1. The lactate dehydrogenase assay was performed using a commercial test kit (Sigma-Aldrich).
2. One milliliter of 2×10^5 cells was injected into the microfluidic system.
3. After various SS treatments, the same volume (100 μ l) of cell suspension was collected from the circulatory system and centrifuged at 1500 RPM for 3 min.
4. The cell pellet was discarded and 100 μ l of supernatant was transferred to each well of a 96-well plate.
5. The LDH mixture (200 μ l/well) was added to the supernatant, immediately after which, the plate was covered with aluminum foil.
6. After 30 min of incubation at room temperature, 30 μ l of 1 N HCl was added to each well to terminate the reaction.
7. Then, the primary absorbance and background absorbance was measured at 490 nm and 690 nm respectively using a plate reader (Spectramax M5).
8. The background absorbance at 690 nm was subtracted from the primary absorbance at 490 nm which was then used to calculate the LDH release.

Appendix VI

6. Detection of necrosis by propidium iodide (PI) staining

1. Propidium iodide (PI) can penetrate damaged cell membranes and stain the nucleus red; thus, PI staining has been used as the conventional assay for determining necrotic cell death.
2. One milliliter of 2×10^5 cells was injected into the microfluidic system.
3. In this study, after different SS treatment for 1–4 h, the same volume (100 μ l) of cell suspension were stained with 10 μ M PI (Sigma, USA) for 30 min in the dark.
4. The images were captured with an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany) at an excitation wavelength of 590 nm and an emission wavelength of 630 nm.

Appendix VII

7. MTT assay

1. MTT assay can measure the level of reductase in mitochondria which reflects the level of cell metabolic activity.
2. In this assay, the activity of NAD (P) H-dependent cellular oxidoreductase represents the total number of viable cells.
3. This enzyme basically reduces the MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide into formazan (insoluble).
4. After various SS treatments, the cells were collected from the circulatory system and 100 µl of suspended cells were added to each well of a 96-well plate.
5. After various incubation times, 10 µl of sterilized MTT solution (5 mg/ml) was added to the cells.
6. After 3 h incubation, 100 µl of solubilization solution containing 10% SDS and 0.1% HCl was added to each well.
7. Then, 12 h later, the optical density was measured at 595 nm using a plate reader (PerkinElmer Victor3).