The pro-metastatic effects of hemodynamic shear stress : breast cancer cells acquire aggressiveness during circulation

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2018

Ma, S. (2018). The pro-metastatic effects of hemodynamic shear stress : breast cancer cells acquire aggressiveness during circulation. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/73324>

<https://doi.org/10.32657/10356/73324>

**THE PRO-METASTATIC EFFECTS OF HEMODYNAMIC SHEAR
STRESS: BREAST CANCER CELLS ACQUIRE AGGRESSIVENESS
DURING CIRCULATION**

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2017

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BREAST CANCER CELLS ACQUIRE AGGRESSIVENESS DURING
CIRCULATION**

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A thesis submitted to the Nanyang Technological University

in fulfillment of the requirement for the degree of

Doctor of Philosophy

2017

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my two supervisors, Prof. Kathy Qian Luo and Prof. Sierin Lim. Prof. Luo's guidance and support have helped me a lot for the initiation and progression of my PhD project before and after she moved to the University of Macau. Prof. Lim's patience, continuous assistance and support allowed me to pursue my project freely; and her suggestions have inspired me to inquire the value of my work. Both of them are excellent scientists, and influence me a lot in my PhD career.

My sincere appreciation to Dr. Fu Afu, my mentor in the laboratory. Most of my experimental skills were learned from him. He also offered me many valuable suggestions and helped me overcome the tremendous problems in my project. His attitude to research and creative mind have widened my views from different perspectives. I would also like to thank him for designing the microfluidics system which I applied in my study. My deepest thanks to my lab mates who have helped me in my research, Sheraton Vivek Muniraj, Dr. Liu Xiaofeng, Dr. Padmaja Anand, Dr. Chen Yijun and Luo Man. My deepest appreciation to Dr. Yu Ting, Wei Na, Sagar Regmi, and Dr. Wang Qi for being great companions in the laboratory. I would also like to thank Tan Jiajun, Mridul Sarker, Bhaskar Sathya Moorthy, Dr. Chuah Yon Jin, Liu Shiyong and Fu Jiayin for providing laboratory materials and sharing research experience. My gratitude goes to my FYP students, Ong Shimin, T Rehka, Chai Mun Kit for assisting me in optimizing and performing experiments.

My many thanks to Chitra Devi D/O Subramaniam, our wonderful technician executive, as well as Dr Ong Teng teng and Dr. Yu Shucong for their assistance in

technical and administrative matters. To Chen Jie, Zheng Yangzi, Yu Hong, Boyce Law Hong Ping and Zhu Wenzhen for being great office friends.

Last but not least, I would like to thank my family for supporting my education, and for being the greatest family in the world. I want to thank my parents for the upbringing, and my sister for her care and help. A very special thanks to my wife, Geraldine Chiew Giap Ying, for helping me break off many bad habits, pushing me forward in my research, always keeping me company, and helping me with the imaging and data processing in the zebrafish experiments.

PUBLICATIONS

JOURNAL ARTICLES

1. **Ma S**, Fu A, Chiew GGY and Luo KQ. (2016). Hemodynamic shear stress stimulates migration and extravasation of tumor cells by elevating cellular oxidative level. *Cancer letters*. 388: 239-248. (Manuscript arising from this thesis)
2. **Ma S**, Fu A, Chiew GGY, Lim S and Luo KQ. (2017). The role of MnSOD in shear stress-promoted tumor cell migration and adhesion. (In preparation, manuscript arising from this thesis)
3. Fu A, **Ma S**, Wei N, Tan BX, Tan EY, and Luo KQ. (2016). High expression of MnSOD promotes survival of circulating breast cancer cells and increases their resistance to doxorubicin. *Oncotarget*. 31: 50239-50257
4. Chiew GGY, **Ma S**, Wee ML, Fu A, Lim S and Luo KQ. (2017). Function of oxidative stress-induced autophagy in breast cancer metastasis. (In preparation)

CONFERENCE PAPERS AND PRESENTATIONS

1. **Ma S**, Fu A, and Luo KQ. (2015) Study of effects of shear stress on tumor cells extravasation. *World Association for Chinese Biomedical Engineers (WACBE)*. July 6-8, 2015, Singapore (Poster Presentation)
2. **Ma S**, Fu A and Luo KQ. (2015) Fluidic shear stress stimulates invasiveness of tumor cells by elevating cellular ROS. *American Society for Cell Biology (ASCB) Annual Meeting*. December 12-16, 2015, San Diego, USA. (Poster Presentation)
3. **Ma S**, Fu A and Luo KQ. (2015) Hemodynamic shear stress stimulates invasiveness of circulating tumor cells. *The 5th International Conference on Optofluidics*, July 26-29, 2015, Taiwan. (Poster Presentation, **Best Poster Award**)
4. **Ma S**, Fu A and Luo KQ. (2016) Hemodynamic shear stress stimulates migration of tumor cells. *Biomedical Engineering Society 10th Scientific Meeting (BES10SM)*. May 14, 2016, Singapore. (Poster Presentation, **Best Poster Award**)
5. Fu A, **Ma S**, Lim S and Luo KQ. (2016) Studying the survival mechanisms of circulating tumor cells in bloodstream and identification of biomarkers for metastatic breast cancer. *16th International Biennial Congress of Metastasis Research Society (MRS)*. September 16-20, 2016, Chengdu, China. (Poster Presentation, **Poster Prize by Nature Reviews Cancer**)

ABSTRACT

Cancer metastasis is defined as the spread of cancer cells from the origin site to other parts of the body and is accountable for ninety percent of deaths from solid tumors. Circulation of cancer cells in blood stream is a vital step for distant metastasis, during which cancer cells are exposed to hemodynamic shear stress. It has been well studied that shear stress can damage circulating tumor cells (CTCs), however, the other actions of shear stress on tumor cells, and how shear stress modulates functions and properties of tumor cells are not understood. Based on the hypothesis that hemodynamic shear stress may enhance invasiveness of circulating tumor cells to assist them in exiting blood flow and invading secondary tissues, this thesis explores the pro-metastatic effects of shear stress.

By using a novel microfluidic circulatory system, I applied physiological fluidic shear stress on breast cancer cells and demonstrated that an arterial level of shear stress significantly enhanced tumor cell migration in transwell and wound healing assays. The results from an *in vitro* transendothelial assay indicated that extravasation of tumor cells was increased by shear stress treatment.

The mechanism study revealed that fluidic shear stress elevated the intracellular levels of reactive oxygen species (ROS), which is an early and indispensable event for increasing phosphorylation level of the extracellular signal-regulated kinases (ERK1/2). Subsequently, phosphorylated ERK1/2 promoted migration of tumor cells, which enhanced extravasation. I found that reducing cellular ROS production by antioxidants suppressed tumor cell extravasation in both *in vitro* transendothelial assay and *in vivo* zebrafish model.

I further explored the upstream signaling pathways and identified MnSOD in the mitochondria as an important regulator that mediates the shear stress-promoted tumor cell migration. Fluidic shear stress can induce tumor cells to generate mitochondrial superoxide, which is the major form of cellular ROS. MnSOD converts superoxide into hydrogen peroxide, which can diffuse into the cytosol and activate ERK1/2 to stimulate cell migration. Here, I verified that MnSOD played a vital role in shear stress-promoted tumor cell migration, by regulating the conversion and distribution of cellular ROS.

In addition to migration and extravasation, I investigated the other functions of circulated tumor cells and found that shear stress was able to enhance the adherence of tumor cells to extracellular matrix (ECM), as well as endothelial monolayer. The elevated cellular ROS level played important roles in shear stress-enhanced tumor cell adhesion, by promoting phosphorylation of focal adhesion kinase (FAK).

Overall, this study presents experimental evidence on the pro-metastatic effects of hemodynamic shear stress and reveals the underlying mechanism. Understanding how fluidic shear stress influences aggressiveness of tumor cells provides new insights into the origins and nature of cancer metastasis and have important implications in cancer treatment. Based on the study, I have identified different signaling molecules in the related pathway, including MnSOD, ROS, ERK1/2 and FAK, which can be used as the potential therapeutic targets for suppressing metastasis and tumor progression.

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ABBREVIATIONS

·OH	Hydroxyl radical
2D	Two-dimensional
3D	Three-dimensional
BSA	Bovine serum albumin
C3	Caspase-3
CCL21	Chemokine (C-C motif) ligand 21
CFP	Cyan fluorescence protein
CSF	Colony-stimulating factor
CTC	Circulating tumor cells
CuZnSOD	Copper and zinc-dependent superoxide dismutase
DCFDA	Dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
ECSOD	Extracellular superoxide dismutase
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescence protein
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal-regulated kinase mitogen
ETC	Electron transport chain
FAK	Focal adhesion kinase

FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green fluorescence protein
GSH	Glutathione
GPx	Glutathione peroxidases
H ₂ O ₂	Hydrogen peroxide
HIF	Hypoxia-inducible transcription factors
hpf	Hours post fertilization
hpi	Hours post injection
HUVEC	Human umbilical vein endothelial cells
ISV	Intersegmental vessels
l	Litre
LMW-PTP	Low molecular protein tyrosine phosphatase
M	Molar
MAPK	Mitogen-activated protein kinase
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinases
MnSOD	Manganese-dependent superoxide dismutase
MUC1	mucin 1
NAC	N-acetylcysteine
NF-κB	Nuclear factor kappa-light-chain-enhancer

NOX	NADPH oxidase
O ₂ ⁻	Superoxide
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PDGFR	Platelet derived growth factor receptor
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PG	Propyl gallate
PI3K	Phosphoinositide 3-Kinase
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
Rac	Ras-related C3 botulinum toxin substrate
RNS	Reactive nitrogen species
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
SDF	Stromal cell-derived factor
SOD	Superoxide dismutase
Src	Proton-oncogene tyrosine-protein kinase
SS	Shear stress
TAM	Tumor-associated macrophages
TEM	Transendothelial migration
VDAC	Voltage-dependent anion channels
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Y15	1,2,4,5-Benzenetetraamine tetrahydrochloride
YFP	Yellow fluorescence protein

CHAPTER 1 Introduction

1.1 Cancer Metastasis

Cancer metastasis is defined as the spread of cancer cells from the origin site to other parts of the body and is accountable for ninety percent of deaths from solid tumors [1, 2]. As one of the hallmarks of cancer, metastasis has been studied for more than a century [3]. The accumulation of discoveries in biomedical science provides insights into how metastasis starts and proceeds, however, the mechanism of metastasis is still not fully understood and there is currently no general and efficient strategy in the treatment of metastasis [2, 4, 5]. Therefore, further studies on metastasis are imperative and exploring pathogenesis of metastasis on the systemic, cellular and molecular levels is important for developing new therapeutic methods to suppress metastasis.

1.1.1 Distant Metastasis is a Multi-Step Process

The process of cancer metastasis consists of several discrete steps which include detachment of cells from primary tumors, migration and invasion of tumor cells into the local host tissue, entry into and survival within the circulation, followed by the exit of tumor cells and colonization at distal sites [1, 6] (Figure 1-1). Metastasis is usually initiated when the primary tumor cells gain specific aggressive traits as a response to alterations of tumor microenvironment. For example, tumor hypoxia occurs when a primary tumor outgrows the blood supply, which limits growth and survival of tumor cells [7, 8]. The hypoxic microenvironment will induce epithelial-mesenchymal transition (EMT) of tumor cells, which promotes detachment of cells from primary tumor and enhances cell migration and invasion [7, 9, 10]. Despite the environmental factors that drive the initiation and progression of metastasis, there are rate-limiting

barriers which make distant metastasis a very inefficient process [6, 11]. When tumor cells circulate in the blood circulatory system after intravasation, several factors are able to kill tumor cells, including detachment-induced anoikis, hemodynamic shear stress and immune cells in the blood [1, 12]. Only a part of tumor cells can survive circulation and extravasate, among which most undergo cell death due to the lack of appropriate interactions with the microenvironment of secondary organs [13, 14]. Only the tumor cells which successfully invade and colonize the secondary organs will form new metastases. In spite of the low efficiency of distant metastasis, many cancer patients face high risk of developing metastases [2].

Figure 1-1. Procedure of cancer metastasis. Tumor cells which detach from primary and vascularized tumors intravasate into blood vessels. These cells will circulate within the blood stream and are defined as circulating tumor cells (CTCs). Some of the CTCs are able to extravasate and invade a secondary organ. In a favorable microenvironment, these cells are able to grow and proliferate to develop distant metastases. Reprinted from reference [6] with permission from Nature Publishing Group.

1.1.2 Circulating Tumor Cells (CTCs)

Circulating tumor cells (CTCs) are defined as the cells that have shed into vasculature from a primary tumor and circulate in the blood stream [15]. In 1869, Thomas Ashworth observed and reported the existence of tumor cells in a cancer patient's blood for the first time. However, the importance of CTCs in cancer research had gained attention only since the mid-1990s [15, 16]. Nowadays, it is widely accepted that CTCs constitute seeds for subsequent development of metastases in distant organs [1, 17]. Many clinical studies revealed that the existence of CTCs was highly correlated with recurrence of cancer [16, 18, 19], which makes CTCs a potential prognostic marker. Various factors in the blood stream including immune-mediate killing, cell detachment and fluidic shear force, could kill CTCs [6, 20, 21]. However, once the CTCs survive the circulation and extravasate, they are able to invade secondary organs and form metastases (Figure 1-1).

1.1.3 Extravasation

In the case of malignant cancer metastasis, extravasation refers to the movement of circulating tumor cells from the capillaries to the tissues surrounding them [15, 22]. Extravasation is initiated when the CTCs stably attach to the endothelial monolayer of capillaries. The attachment leads to a series of complex interactions between the tumor cells and the endothelial cells, which involve various receptors, ligands and secreted factors [23, 24]. The adhesion of tumor cells to endothelial monolayer is stabilized and endothelial cell junction is disrupted [22]. The tumor cells then squeeze and transmigrate through the endothelial monolayer, which is defined as transendothelial migration [23-25].

Due to the importance of extravasation in cancer metastasis, different models have been developed to study cancer cell extravasation [23]. The most widely used *in vitro* model for studying extravasation is transendothelial assay. In transendothelial assay, endothelial cells are seeded in the upper side of transwell membrane to form an endothelial monolayer in Boyden Chamber. The number of cancer cells that migrate through the monolayer to the lower side of the transwell membrane can be quantified [25-27]. For *in vivo* study, the mouse model provides the most physiologically accurate presentation of conditions of extravasation, but the spatial resolution is very limited [23, 28, 29] (Figure 1-2). Compared to mouse model, the zebrafish model is highly preferred for studying extravasation [23, 30] (Figure 1-2). The zebrafish larvae are transparent and their immune systems are not fully developed. Moreover, the vascular system of a zebrafish larva is functional and precisely patterned. These features allow real-time observation and efficient detection of tumor cell extravasation in zebrafish [30-32].

Figure 1-2. *In vivo* models for studying extravasation. Left panel is the zebrafish model for studying extravasation. Tumor cells can be injected into the circulation system of a zebrafish larva at the pericardium site. The extravasation of tumor cells is monitored in the intersegmental vessels (ISV) of tail region. Right panel is the mouse model for studying extravasation. The

injection site of tumor cells is dependent on the cell type and observation site. Extravasation can be observed and monitored based on intravital imaging techniques. Reprinted from reference [23] with permission from Nature Publishing Group.

1.1.4 Metastasis of Breast Cancer

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide, with nearly 1.7 million new cases diagnosed in 2012 [33]. Breast cancer accounts for 14.7% of all female cancer death, and 90% deaths in breast cancer patients are due to the metastasis. Due to the urgent need to develop new prognostic markers and identify new therapeutic targets for treatment of breast cancer metastasis, the importance of understanding the mechanisms of breast cancer metastasis has been widely recognized.

To study breast cancer, many different breast cancer cell lines have been established since the mid-20th century [34, 35]. The breast cancer cell lines can be classified based on the derivations as well as the gene profiling of the cells [34, 36]. Both metastatic cell lines (e.g. MDA-MB-231) and non-metastatic cell lines (e.g. MCF7) are widely used by cancer biologists and play indispensable roles in advancing our understandings of breast cancer metastasis [37].

1.2 Hemodynamic Shear Stress

Hemodynamic is the dynamics of blood and explains the physical laws that govern the flow of blood. When blood moves along endothelial walls, the friction between blood flow and the surface of vessel wall causes the blood flow to shear, which generates hemodynamic shear stress [20, 38].

Figure 1-3. Calculation of hemodynamic shear stress. Fluidic shear stress can be calculated based on Poiseuille's equation, $\tau = 4Q\eta/\pi R^3$, where τ is shear stress in dyne/cm², Q is flow rate in cm³/s, η is the dynamic viscosity of the fluid, and R is the radius of the flow.

Hemodynamic shear stress can be calculated based on the inner radius of blood vessel, flow rate and viscosity of blood [39] (Figure 1-3). Studies have revealed the range of shear stress magnitudes in human circulation system and identified average level of human arterial shear stress (around 15 dynes/cm²), as well as the average human vein shear stress (around 5 dynes/cm²).

1.2.1 The Effects of Shear Stress on Endothelial Cells

Endothelial cells lining the cardiovascular system are constantly exposed to hemodynamic shear stress [39, 40]. Many studies have revealed that shear stress modulates the structure and functions of endothelial cells, which is highly associated with normal cardiovascular development and cardiovascular pathology [39, 40]. Traditional *in vitro* models to study the actions of shear stress on endothelial cells usually include a culture dish or microchannel in which endothelial monolayers are developed, a pump which can generate constant or pulsatile shear flow, and tubes for

connection [39, 41, 42]. Based on these models, how endothelial cells sense and respond to fluidic shear stress has been well studied [43].

The cytoskeleton is an important mechanotransducer for endothelial cells [44, 45]. Fluidic shear stress is able to modulate intracellular cytoskeleton network, which can change the distribution of cellular mechanical stress and transduce the mechanical signals throughout the cells [40, 46, 47]. Besides this, reorganization of cytoskeleton structure will activate certain biochemical signaling pathways[40]. For example, shear stress facilitates microtubule polymerization which subsequently activates Rac [48]. As an important GTPase of Rho family, Rac in turn influences cytoskeleton dynamics by promoting microtubule elongation and actin polymerization to induce lamellipodia formation [49].

The cellular membrane receptors also transduce mechanical signals. Fluidic shear stress can directly activate G protein-coupled receptors by modulating physical properties of cell membranes [50]. As mechanical forces can be transmitted through the whole cell, fluidic shear stress also influences cell-cell and cell-ECM interactions [47]. The receptors that mediate cell-ECM interaction (e.g. integrins) and cell-cell junctions (e.g. PECAM-1) play important roles in mechanosensing of cells [47, 51, 52]. By modulating cytoskeleton structures or activating membrane receptors, shear stress is able to trigger certain signaling pathways to regulate functions of endothelial cells, including migration, proliferation and differentiation, etc. [40, 41, 53]

1.2.2 The Effects of Shear Stress on Tumor Cells

In addition to the endothelial cells, the cells that circulate within the blood stream are also exposed to hemodynamic shear stress, including leukocytes and CTCs [6, 54]. The effects of shear stress on pseudopod formation and adhesion of leukocytes are well

defined [54, 55]. In recent years, the response of CTCs to hemodynamic shear stress has gained more and more attentions [6, 20].

Circulation of tumor cells within the blood stream is a rate-limiting step in cancer metastasis. Various factors that can eliminate tumor cells during circulation have been investigated [12]. Non-attachment status of CTCs will induce anoikis and lead to cytoskeleton disruption and cell death [56, 57]. Natural killer cells in the blood stream are able to kill tumor cells as a vital component of the innate immune system [58, 59]. The most important mechanism by which tumor cells are eliminated in the blood is the effects of hemodynamic shear stress, as the greatest portion of CTCs are destroyed by this mechanical force [6, 20]. Previous studies have found that fluidic shear stress can kill tumor cells by causing cell deformation. Dr. Fu Afu in our laboratory has further explored the effects of shear stress and found that shear stress is able to induce tumor cell apoptosis [12]. All of these studies have revealed the destructive actions of shear stress on tumor cells. However, how fluidic microenvironment influences other functions of tumor cells is poorly understood, and whether hemodynamic shear stress has pro-metastatic effects remains unknown.

To investigate the pro-metastatic effects of shear stress, we developed a microfluidic system to induce shear stress on tumor cells. Under physiological conditions, CTCs circulate in blood stream as non-adherent cells [17]. The models being used to study the response of endothelial cells to shear stress are not suitable for studying the response of tumor cells, as the cells are seeded in a dish or channel and the attachment may create transmission of mechanical force to cell-cell and cell-ECM (dish or channel *in vitro*) contacts [43, 53], which does not present the physiological conditions of CTCs. In 2012, J.M. Barnes developed a microfluidic system to study resistance of tumor cells to fluidic shear stress generated in a syringe needle [20]. In their study, they mimicked the

physiological conditions by using suspended tumor cells. However, only very high levels of transient shear stress can be applied using their system. In this thesis, I introduce a circulatory microfluidic system in which the levels of shear stress and circulation time can be well controlled. The system was originally designed by Dr. Fu Afu in our laboratory [12].

1.3 Tumor Cell Migration

Cell migration is a widely-studied process in cellular and medical biology [60]. It is generally defined as the orchestrated movement of cells to specific locations, which is required in many normal physiological process, including embryonic development, tissue repair and regeneration, immune responses, etc. In cancer biology, the migration capacity of tumor cells plays a vital role in distant metastasis [1, 61].

1.3.1 The Process of Cell Migration

Cellular movement across two-dimensional substrata is a well-studied process [60, 62] (Figure 1-4). For different migratory cell types, the spatial asymmetry which usually manifests as the polarization of cell morphology is required for cell migration [60]. The polarization leads to the extension of active membrane processes, including both lamellipodia and filopodia. The cellular membrane protrusions which are caused by actin filament polymerization and structural organization become the leading edge of cell migration [63, 64]. To provide the physical force which supports cell movement, the membrane extensions need to establish stable adhesions to the substrata. Different studies have revealed that the leading edge of cell migration is the preferential site where focal adhesions will form [65-67]. Afterwards, the contractile forces are generated through relative movement of actin filament to translocate the cell body

forward. The actin filament contraction is a well-documented process which is mediated by the motor protein myosin [68-70]. Finally, the disruption of cell adhesion to substrata at the tail region, together with the contractile force generated by cytoskeleton, lead to the rear retraction and movement of cells [60].

Figure 1-4. The process of cell migration in 2D. Migrating cells develop protrusive structures including lamellipodia and filopodia through actin filament polymerization. The membrane protrusions can rapidly form stable adhesions to the substrate. The detachment of tail region is caused by both disruption of focal adhesions and actin filament contraction. The actin filament contraction mediated by myosin is also responsible for the net force to support translocation of cell body. Reprinted from reference [60] with permission from Elsevier.

In recent years, cell migration in 3D has been widely discussed and investigated as 3D models better represent the microenvironment of living tissues [71]. Studies have revealed discrepancies between cell migration in 2D and 3D models, including the

secretion of MMPs to degrade ECM and the lamellipodia-independent mechanism of cell migration in 3D models, which are not observed in 2D models [71-73]. However, it is undeniable that the basic conceptions and principles of cell migration in 2D, including cytoskeleton rearrangement and dynamics of cell adhesion, are also essential for regulating cell migration in 3D or *in vivo* [45, 71].

1.3.2 ERK1/2 and Cell Migration

Extracellular signal-regulated kinases (ERKs) are members of the MAPK family, which is a type of protein kinase that is specific to the amino acids serine, tyrosine and threonine. MAPKs regulate different cell functions including proliferation, gene expression, survival and apoptosis, by directing cellular responses to a diverse array of stimuli, such as heat shock, mitogens and osmotic stress [74, 75]. ERKs are specific MAPKs that mediate cellular responses to extracellular signaling molecules by communicating signals from membrane receptors to downstream pathways [76, 77]. There are two similar (85% sequence identity) types of ERKs which are named as ERK1 and ERK2. Due to the high similarity, the two protein kinases are usually studied together in biological research and defined as ERK1/2 [76]. ERK1/2 play important roles in MAPK/ERK pathway, which involves a classical MAPK cascade (Figure 1-5). The MAPK/ERK pathway is initiated when an extracellular ligand binds to a tyrosine kinase receptor such as epidermal growth factor receptor (EGFR) [78]. EGFR then activates guanine nucleotide exchange factor SOS and SOS activates GTP binding protein Ras [79-81]. Ras subsequently activates Raf and the MAPK cascade begins from here [82]. As a MAP kinase kinase kinase (MAP3K), Raf can phosphorylate and activate MEKK, a MAP kinase kinase (MAP2K). MEK specifically phosphorylates ERK1/2, a MAP kinase (MAPK) [77, 83]. The phosphorylated ERK1/2 can

phosphorylate and activate several transcription factors and kinases, thus regulating gene expression and other important cell functions [76, 84].

Figure 1-5. Classical ERK/MAPK pathway. The ligands binding to EGFR triggers ERK/MAPK pathway. As the last section of the pathway, ERK1/2 have many different targets. By activating or inactivating several different transcription factors and kinases, ERK1/2 are able to regulate various cellular functions.

Previous studies have demonstrated that ERK1/2 play vital roles in regulating cytoskeleton dynamics and cell migration [85-87]. It is found that elevated phosphorylation level of ERK1/2 promotes cell migration and inhibiting ERK1/2

phosphorylation impairs cell migration [74, 85]. In 1997, Richard Klemke reported that ERK1/2 can directly phosphorylate myosin light chain kinase (MLCK) which then phosphorylates myosin light chain (MLC) [88]. Phosphorylation of MLC enables myosin to bind to actin filaments and mediate actin filament contraction which is important for cell migration [89, 90] (Figure 1-4). Recently, there were studies exploring other mechanisms by which ERK1/2 promote cell migration, such as regulation of cell adhesion dynamics [85, 91].

1.3.3 Tumor Cell Migration is Important for Metastasis

The migration capacity of tumor cells plays a vital role in metastasis, which makes cancer cell migration a widely studied field in cancer biology [61, 92, 93]. As mentioned above, distant metastasis is a multi-step process. Different stages of metastasis required effective migration of cancer cells, including invasion into surrounding tissues and penetration into the vasculature after detachment from primary tumors, extravasation, and invasion and colonization in the secondary organs [92, 94-96]. For example, the efficiency of extravasation is determined by how fast and efficiently tumor cells can transmigrate through the endothelium [23, 97, 98]. Although the disruption of endothelium during cancer cell extravasation has been reported by various studies, it is widely accepted that the size of the gap generated by disrupting endothelial junction is limited and breaching the endothelial barrier is difficult for cancer cells [23, 24]. The procedure of transendothelial migration was observed both *in vivo* and *in vitro*, which began with shape modulation of cells, followed by squeezing and transmigration through the endothelial monolayer [28, 30]. These facts imply that the migration capacity of cells plays a vital role during extravasation, which has been proven by various studies [26, 27, 99].

1.3.4 Tumor Cell Migration is Regulated by Extracellular Signals

Many different elements of the tumor microenvironment have been identified as important regulators of tumor cell migration, including chemokines, extracellular matrix (ECM) architecture, and resident host cells [1, 92, 100]. For example, macrophages within the tumor microenvironment have recently gained a lot of attentions as they are found to facilitate angiogenesis and promote tumor cell migration [101-103]. Normal macrophages function as part of immune system. However, tumor cells are able to recruit macrophages to assist them in growth, survival and migration, and these macrophages are generally defined as tumor-associated macrophages (TAM) [103, 104]. TAM are involved in an EGF/CSF-1 paracrine signaling loop with tumor cells to support migration and invasion of tumor cells [105]. The matrix remodeling capacity of TAM can also be exploited by tumor cells to penetrate basement membrane [101].

Despite these important findings, the complete molecular mechanism by which tumor cell migration occurs is not completely defined and is still under investigation [92]. Moreover, most of the studied factors that regulate tumor cell migration are biological or chemical factors. The role of physical factors in regulating tumor cell migration is poorly understood. During metastasis, there exist physical interactions between tumor cells and the microenvironment which are able to modulate certain properties and functions of tumor cells [6]. Hemodynamic shear stress, as a physical force in the microenvironment of tumor cells during circulation [20], could be able to modulate tumor cell migration.

1.3.5 Cell Migration Assays

Different *in vitro* models to study cell migration have been developed. The most widely used methods are the transwell assay and wound healing assay. In 1962, Stephen Boyden developed a two-chamber assay to study the chemotactic effect of antibody-antigen complexes on leukocytes, which was later named as Boyden Chamber, or the transwell assay [106]. Nowadays, the transwell assay has become one of the most used tools to access migration capacity of cells [107, 108]. In a classical transwell assay, two chambers are separated by a porous membrane which represents a physical barrier. In general, cells will be seeded in the upper chamber and the culture medium containing certain chemotactic agents will be placed in the lower chamber. As the size of pores in the membrane is much smaller than a cell, the cells in the upper chamber can only penetrate the membrane to the other side through active migration [106, 109]. After an appropriate incubation time, the membrane with tumor cells on both sides can be fixed and stained before removing the cells on the upper side. The cells on the lower side which have successfully migrated through the membrane can be detected and quantified [106, 110, 111].

Another commonly used method to study cell migration is wound healing assay [112-114]. To mimic cell migration during wound healing *in vivo*, a wound gap is created on an intact cell monolayer. The cells on both sides will migrate towards the center to close the gap. Images can be captured at different time points during wound healing and the migration rate of cells can be calculated. Wound healing assay is also used to study the dynamics of migration at cell-scale by tracking the migration of individual cells on the leading edge of the wound [115].

In recent years, various microfluidic-based cell migration assays have been developed [116-118]. Compared to the traditional wound healing and transwell assay,

microfluidic-based methods have several advantages, including simple operation steps and less cells and reagents consumption. However, as there is no decisive superiority of microfluidics-based methods over traditional assays and biologists are more comfortable with the existing well-established assays, none of the microfluidic-based methods is adopted widely for studying cell migration [108].

1.4 Tumor Cell Adhesion

Cell adhesion is the process by which cells interact and attach to a surface, substrate or another cell [119]. Cell adhesion is mediated by cell adhesion molecules, which are transmembrane glycoproteins, and include integrins, selectins, cadherins, etc. The attachment of cells to a substrate is not a simple physical process. The interactions of the adhesion molecules with the substrate will usually cause clustering or structural modulations of these molecules and induce signal transductions in the cells, which are important for cell survival and differentiation [119, 120]. Moreover, some of the adhesion molecules are directly or indirectly associated with cytoskeleton, which makes cell adhesion important for maintaining cell morphology and supporting cell migration [45, 65, 73].

1.4.1 Cell Adhesion to ECM

Extracellular matrix (ECM) is a collection of extracellular molecules that provides structural and biochemical support to the surrounding cells [121, 122]. The components of ECM include collagens, fibronectins, laminin, hyaluronic acid and different types of proteoglycans [123, 124]. The primary function of proteoglycans is to trap and store growth factors within ECM [125]. Hyaluronic acid functions to maintain the compressive strength of ECM through absorbing water and keeping the ECM hydrated

[126]. It also interacts with cell membrane receptor CD44 [127]. The proteins of ECM including collagens, fibronectins and laminins, are the main substrates that support cell adhesion. As the main components of ECM proteins, around 28 different types of collagens are identified and described, among which collagen type I comprises over 90% of the total collagens of human body [128]. These proteins act as ligands of integrins, the transmembrane receptors that mediate the interactions of cells to ECM [129, 130].

Integrins on the cell membrane are obligate heterodimers including two subunits, α and β . There are eighteen different types of integrin α and eight types of integrin β . The combination of different types of subunits can generate many different types of integrin heterodimers [131, 132]. However, there are only around 24 combinations of integrins commonly observed on mammalian cell membranes [133]. The integrins-ECM binding initiates the assembly of focal adhesions, the protein complexes which connect ECM-binding receptors on cell membrane with cytoskeleton (Figure 1-6). Focal adhesions generally contain over 100 different proteins, among which focal adhesion kinase (FAK), talin, vinculin, paxillin, p130CAS, and Src are considered the most important ones [134-136]. These proteins not only play indispensable roles in regulating focal adhesion assembly and disassembly, but also regulate intracellular signal transductions [119].

1.4.2 Tumor Cell Adhesion to ECM is Important for Metastasis

The dynamics of cell adhesion play a vital role in tumor progression, as metastasis proceeds along with the detachment and attachment of tumor cells [1, 137]. For example, during the initiation stage of metastasis, tumor cells which undergo epithelial-to-mesenchymal transition (EMT) will lose certain adhesion molecules and detach from the primary tumors [138], while during colonization in a secondary organ, tumor cells

need to interact with the ECM and form stable adhesions to support cell survival and growth [139, 140]. Interestingly, the low efficiency of colonization of disseminated tumor cells in distant organs has been reported [4, 14, 141, 142]. The survival and growth of tumor cells in the secondary organs are determined by interactions of tumor cells with constituents of the local microenvironment, including ECM components and host stromal cells [1, 140]. Several studies have revealed that disruptions of tumor cell adhesion to ECM, such as knocking down of integrins and decreasing FAK activities, significantly diminish metastatic colonization [13, 143].

Figure 1-6. Components of focal adhesion complex. Integrins interact with ECM. The integrin binding proteins talin and paxillin recruit FAK and vinculin, FAK subsequently binds

to Src and p130 Cas. α -Actinin is a cytoskeletal protein which is activated by FAK. α -Actinin binds to the actin stress fibres and tethers them to focal contacts. Reprinted from reference [134] with the permission from Nature Publishing Group.

1.4.3 Tumor Cell Adhesion to Endothelium

The adhesion of leukocytes to endothelial monolayer is a well characterized process which generally includes three steps: initial adhesion, rolling and stable adhesion [144]. Studies have proven that leukocyte adhesion and the interactions between leukocytes and endothelial cells are very important for leukocyte extravasation and immune response [144-146]. In contrast, the details of tumor cell adhesion to endothelial wall are poorly understood.

Studies of leukocyte adhesion to endothelium provide initial sight on the process of tumor cell adhesion, which leads to the hypothesis that the procedure of tumor cell adhesion to endothelium is same or similar to leukocytes [23, 24]. Although rolling of tumor cells on endothelium has been observed [147, 148], there is no conclusive evidence to prove that the rolling and initial adhesion is necessary for tumor cell extravasation [24]. In fact, there are two different theories of how tumor cells are captured by the endothelium before extravasation. It has been proven that tumor cells can be trapped physically by the capillaries. Studies based on different models have shown that the blood vessels with smaller diameter than tumor cells can mechanically arrest them [149, 150]. These trapped tumor cells will undergo either cell death or extravasation [22]. In comparison, the famous “seed and soil” theory indicates that the organ specificity of distant tumor metastasis cannot be explained by mechanical trapping of tumor cells. The theory speculates that the properties of the tumor cells (seeds) and the microenvironment of the secondary organs (soil) collectively determine

the sites of metastasis [3, 151]. One of the mechanisms that support organ specificity is the preferential adhesion of tumor cells to endothelium of specific tissues and organs without size restriction, which has been proven by many studies [152, 153]. Although the two theories seemed opposing, recent studies indicated that both theories are correct, which means tumor cells can either be trapped by narrow capillaries or preferentially attach to the endothelium depending on the types of tumor cells and the site of extravasation [3, 4, 141]. More importantly, despite the fact that the dynamics of tumor cell adhesion to endothelium is not fully understood, for both tumor cells trapped mechanically or captured by endothelial cells through receptor-ligand interactions, the development of appropriate tumor cell-endothelium interaction is an important and essential step before extravasation [23, 154].

Different membrane proteins on tumor cells including integrins, CD44 and mucin 1(MUC1) are found to mediate tumor cell-endothelium interactions [24, 155-157]. Several types of integrins are identified as the adhesion molecules that contribute to tumor cell attachment and extravasation [99, 158, 159]. As mentioned above, integrins are connected to cytoskeleton through focal adhesions, which presents the possibility that components of focal adhesion complex like FAK may also contribute to the interactions of tumor cells and endothelium. However, the roles of focal adhesion proteins in mediating tumor cell adhesion to endothelium are poorly explored.

1.4.4 FAK and Cell Adhesion

As one of the most important components of focal adhesions, the function of FAK in cell adhesion is very complicated and highly debated [134, 136]. In 1995, Dusko Ilic reported that the FAK-null (FAK^{-/-}) fibroblasts could still form focal adhesions which indicated that FAK is actually not essential for focal adhesion formation [160].

Interestingly, despite increased number of focal adhesions in FAK^{-/-} fibroblasts, these cells were rounder in shape and less spreading compared to normal fibroblasts, which implied inappropriate cell-ECM interactions and abnormal cytoskeleton organization. It was later found that the focal adhesions formed in FAK^{-/-} cells do not undergo maturation cycle and are unable to connect to actin stress fibres, which indicates the importance of FAK in formation of normal and functional focal adhesions [161-163]. Several studies have proven that inhibiting activation of FAK could impair cell adhesion, spreading and migration [164, 165]. On the other hand, the role of FAK in regulating the disassembly of focal adhesions was reported, which is highly associated with the tail region detachment and rear retraction of migrating cells [134, 166]. It is generally accepted that FAK play important roles in regulating the dynamics of cell contacts to ECM, including both assembly and disassembly of focal adhesions.

FAK contains several tyrosine phosphorylation sites including tyrosine 397 (Y397), 576 (Y576), 861 (Y861), etc. The phosphorylation site of Y397 is the most prominent site for activation and function of FAK. The interactions of integrins with ECM lead to autophosphorylation of FAK at Y397, as well as several additional sites within the kinase [167]. The activated FAK binds to Src and form a FAK-Src complex, an important part of focal adhesion complex which recruits and activates other adaptor proteins to stabilize cell-ECM contacts [134]. The FAK-Src complex also plays vital roles in signaling transduction and regulation of cytoskeleton rearrangement, as well as migration, proliferation and other cellular functions [168, 169].

In addition to the integrin-mediating cell attachment to ECM, FAK phosphorylation is regulated through many different mechanisms, among which the action of protein tyrosine phosphatase (PTPs) is the most studied [134]. The activities of many enzymes are regulated through phosphorylation and dephosphorylation of amino acid residues.

Kinases are the enzymes that catalyze phosphorylation by transferring phosphate groups to specific substrates. In contrast, phosphatases act to remove the phosphate group from their substrates through hydrolysis [170]. Proteins tyrosine phosphatase (PTPs) are specific phosphatases which target the phosphorylated tyrosine residues on proteins [171]. The activity of FAK is found to be regulated directly or indirectly by many different types of PTPs, including PTP-PEST, PTEN, PTP1B and LMW-PTP [172-175]. These PTPs are able to remove the phosphate group at tyrosine residues of FAK and inactivate this protein. For example, LMW-PTP can dephosphorylate the activated FAK at the sites of Y397, the prominent site for FAK function, thereby inactivating FAK [173].

1.5 Reactive Oxygen Species

Reactive oxygen species (ROS) are reactive chemical species with strong oxidation ability, which include superoxide, hydrogen peroxide, singlet oxygen, etc. Under normal conditions, ROS are produced in cells as byproducts of oxygen metabolism and are essential in regulating signal transductions and homeostasis [176]. Excessive ROS production which is a double-edge sword for the cells can be caused by different stimuli, especially environmental stresses [177, 178]. High level of cellular ROS causes severe damage to DNA, RNA and cellular macromolecules including lipids and proteins, which will impair normal cellular functions and induce cell death [179]. On the other hand, increased cellular ROS production rapidly triggers specific signaling pathways and enhances cell survival and proliferation, and endows cells resistance against external stresses [176, 180]. For example, unlimited growth of primary tumor leads to hypoxia, which is a hallmark of cancer development. It is well documented that hypoxia causes elevation of cellular ROS level, which is damaging to tumor cells [181]. The

hypoxia- induced ROS elevation can also activate MAPK/ERK pathway which promotes tumor cell survival, proliferation and migration [182]. Moreover, ROS stabilize hypoxia-inducible factor 1 α (HIF1 α), a transcription factor which mediates cellular response to hypoxia [183].

1.5.1 Cellular ROS Generation

Cells generate ROS through different mechanisms. NADPH oxidase (NOX) complexes and mitochondria are the two major sources of cellular ROS [184]. NOX generates superoxide by transporting electrons from NADPH across membranes and coupling the electrons to oxygen. NOX is present in the plasma membrane or the membranes of phagosomes and the superoxide anions generated by NOX are usually released to extracellular space or inside phagosomes. The ROS in mitochondrial is produced by the electron leakage of electron transport chain (ETC) [185]. In ETC, Complex I accepts electrons from NADH and passes them to coenzyme Q. Coenzyme Q then passes the electrons to Complex III, which passes them to cytochrome c. Finally, the electrons are passed to Complex IV, which donates the electrons to oxygen in the presence of hydrogen ions to form water molecules. However, during ETC, especially in the stages of Complex I and III, some electrons may leak from the chain and are passed prematurely and inappropriately to oxygen to generate mitochondrial superoxide [183, 185].

Studies have revealed that different stimuli including hyperglycemia, leptin and mitochondrial DNA damage, are able to enhance mitochondrial superoxide generation [176, 179]. Although the underlying mechanisms are not fully understood, it is well documented that stimulated superoxide production is highly associated with mitochondrial network dynamics [186]. Morphology, density and distribution of the

mitochondria are frequently modulated through mitochondrial fusion and fission, which play critical roles in maintaining normal mitochondrial functions, including ROS generation [187, 188]. It was reported in 2006 by Tianzheng Yu that mitochondrial fragmentation caused by abnormal fission increased superoxide generation [189].

1.5.2 Superoxide Dismutase

Superoxide dismutase (SOD) is an enzyme that specifically catalyzes the dismutation of superoxide into hydrogen peroxide. There are three different types of SOD in mammalian cells [190]. CuZnSOD, also named as SOD1, is a copper and zinc-containing homodimer located in cytosol [191]. MnSOD, or SOD2, is only found in the mitochondrial matrix as a manganese-containing enzyme [192]. ECSOD, or SOD3, a copper and zinc-containing tetramer, is secreted by the cells into extracellular spaces [193]. As the mitochondria is the major source of cellular superoxide, MnSOD is widely accepted as the main superoxide dismutase that protects cells from ROS damaging [194]. Accumulation of superoxide disrupts mitochondrial membrane potential and causes cell death. MnSOD converts mitochondrial superoxide to hydrogen peroxide, which is less toxic and damaging [195]. Unlike the superoxide anion, hydrogen peroxide is able to diffuse through mitochondrial membrane to cytosol, which avoids mitochondrial ROS accumulation [196].

1.5.3 Redox Signaling

As mentioned above, ROS play important roles in signal transduction in cells. ROS can directly modulate structures and functions of specific proteins in a cysteine oxidation-based manner [176]. The exposed cysteine residues in proteins contain thiol group (-

SH), which can be oxidized by ROS to form highly reactive sulphenic acid group (-SOH). The sulphenic acid can form a disulphide bond with a proximal thiol group or sulphonamide with a proximal nitrogen, which causes allosteric changes within the proteins and alters protein functions [180, 197]. A lot of proteins are found to be the direct targets of ROS, including low molecular weight protein tyrosine phosphatase (LMWPTP) [198], PTEN [199], protein kinase C (PKC) [179], etc. By regulating activities of the target proteins, ROS can trigger a number of signalling pathways and regulate different cellular functions including proliferation, migration and differentiation [182, 200, 201] (Figure 1-7).

Figure 1-7. Examples of the ROS targets in important signaling pathways. Transcription factors including hypoxia-inducible factors (HIFs) and NF-κB, as well as important kinases including MAPKs and PI3K are well studied targets of ROS. Reprinted from reference [202] with permission from Elsevier.

It is well documented that ROS can trigger the MAPK/ERK pathway and activate ERK1/2 [182, 203]. However, the underlying mechanism is not fully understood. Some studies suggested that one of the direct targets of ROS, PKC is involved in ROS-ERK signalling [182]. In 1996, Ueda Y reported that PKC interacts with and activates Raf, which is a MAP3K in ERK pathway. Raf can activate MEK which subsequently activates ERK1/2 [204]. Another potential mechanism is the inactivation of protein tyrosine phosphatase (PTPs) by ROS. PTPs are able to dephosphorylate and inactivate kinases including ERK1/2. After oxidized by ROS, the activities of PTPs are inhibited which increases the phosphorylation levels of ERK1/2 [205, 206]. However, the evidence that supports this view is limited and the types of PTPs involved in ERK dephosphorylation are not specified in related studies [202].

Another important signalling function of ROS is to regulate the dynamics of focal adhesion complex [207-209]. As a specific type of PTPs, LMWPTP which dephosphorylates FAK and down-regulates cell adherence to ECM can be oxidized and inactivated by ROS [172, 173]. During the process of cell adhesion, the integrin-ECM interaction induces intracellular ROS generation which then inhibits the activity of LMWPTP and promotes FAK activation and cell adhesion [207]. In addition, other components of focal adhesion complex including Src, paxillin and P130CAS are found to be activated by ROS [208]. Several studies have revealed that inhibiting cellular ROS generation by antioxidants suppressed cell adherence to ECM and increasing cellular ROS level by treating cells with exogenous ROS or ROS inducer enhanced cell adhesion [207].

1.5.4 ROS and Tumor Progression

Due to the DNA damaging effects, ROS was previously regarded as a carcinogen. Along with the increased knowledge on tumor progression as well as the nature and functions of ROS, it is realized that ROS plays much more important and complicated roles in tumor initiation, growth and metastasis. Studies have revealed that tumor cells function with higher levels of endogenous oxidative stress compared to normal cells [179, 210]. Elevated oxidative stress and increased levels of oxidative damage products in different cancer cell types were observed and reported [180, 211]. Although the mechanisms of excessive ROS production in cancer cells are not fully understood, various intrinsic factors including mitochondrial dysfunction, aberrant metabolism and mutations of p53 were identified to increase cellular ROS levels [176].

High level of ROS plays an important role in malignant transformation of normal cells, which subsequently leads to further increased ROS production [178, 212]. The increased ROS stress in cancer cells promotes tumor progression and metastasis by promoting proliferation, migration and invasion of cancer cells [177, 212]. Interestingly, if the level of ROS reaches a certain threshold level, the cytotoxic effects of ROS will lead cancer cell death. In fact, some anticancer agents such as paclitaxel and radiation therapy are found to kill tumor cell through, at least partly, increasing cellular ROS level [200]. It was later found that tumor cells are able to develop redox adaptation through enhanced cellular antioxidant system, including increased expression of SOD and catalase [211] (Figure 1-8). These adaptive mechanisms keep the cellular ROS levels within the safe range for cancer cells.

Figure 1-8. ROS generation and antioxidant system in cells. In conclusion, ROS is a double-edge sword for cancer cells. A moderate increase of ROS promotes tumor growth and metastasis. However, when the level of ROS reaches a certain level, it will cause tumor cell death. Compared to normal cells, cancer cells develop both increased cellular ROS production and enhanced antioxidant system, which produce an elevated ROS level, but not beyond the cytotoxic threshold. Reprinted from reference [202] with permission from Elsevier.

1.6 Rationale of Project

1.6.1 Hypothesis of Project

Exploring how tumor cells respond to environmental stresses has always been an interesting topic in cancer research. Hemodynamic shear stress has long been acknowledged as a restriction factor for survival of CTCs, which makes circulation of tumor cells within blood flow a rate-limiting stage for distant metastasis. However, the cancer studies focusing on hemodynamic shear stress are limited to investigating the damaging effects of shear stress. Whether hemodynamic shear stress have positive effects on tumor cells is not understood.

Here, I hypothesized that hemodynamic shear stress has pro-metastatic effects. Different traits of tumor cells that are important for distant metastasis might be

influenced by shear stress. I mainly focused on the migration capacity of tumor cells in this project, which is a hallmark of tumor metastasis. The adhesion capacity of tumor cells which plays important roles in extravasation, invasion and colonization would also be studied. I hypothesized that shear stress would promote migration, adhesion and extravasation of breast cancer cells.

1.6.2 Overall Aims of PhD Project

As the process of cancer metastasis is still not fully understood and there lacks efficient therapeutic methods targeting distant metastasis, further studies of metastasis are imperative. This thesis aims to explore the pro-metastatic effects of shear stress on tumor cells. How shear stress influences tumor cell migration, as well as tumor cell extravasation and adhesion would be studied. The new findings would not only advance our understandings of metastasis, but also provide new insights on tumor cell response to external stresses. I proposed that inhibiting the pro-metastatic effects of shear stress could be a new therapeutic strategy for metastasis. The thesis also aims to investigate the underlying mechanism and identify the key signalling molecules that mediate the effects of shear stress on tumor cells, which would lead to potential therapeutic strategies for suppressing metastasis.

CHAPTER 2 Materials and Methods

2.1 Antibodies and Reagents

N-acetylcysteine (NAC), propyl gallate (PG), pluronic F-127, and paraformaldehyde (PFA) were purchased from Sigma Aldrich (Saint Louis, USA). FAK inhibitor 1,2,4,5-Benzenetetraamine tetrahydrochloride (Y15), MEK inhibitor U0126, dichlorofluorescein diacetate (DCFDA) and were purchased from Thermo Fisher Scientific (Waltham, USA). Collagen I solution was purchased from BD Biosciences (San Jose, USA). Hydrogen peroxide (H₂O₂) was purchased from Calbiochem (San Diego, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, USA). MnSOD antibody was purchased from Abcam (Cambridge, UK). Phospho-ERK1/2, ERK1/2, phosphor-FAK (tyrosine 397), FAK, GAPDH antibodies were purchased from Cell Signaling (Boston, USA).

2.2 Cell Culture

The breast cancer cell line MDA-MB-231 was provided by Professor Xiaofeng Le from the Department of Experimental Therapeutics at The M.D. Anderson Cancer Center, University of Texas. MCF7, MDA-MB-157 and BT-549 cells were purchased from American Type Culture Collection (ATCC, Manassas, USA), and cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% FBS (Hyclone, Logan, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, USA). The 231-C3 and MCF7-C3 cells are stable cell lines that express the CFP-DEVD-YFP fusion protein. They were generated by transfecting a fluorescence resonance energy transfer (FRET)-based C3 biosensor into MDA-MB-231 and MCF7 cells, respectively [213, 214]. HUVEC-dt cells expressing the red fluorescent dTomato

protein were kindly given to us by Dr. Chong Seow Khoon (Nanyang Technological University, Singapore).

2.3 Cellular Functional Assays

2.3.1 Circulatory Microfluidic System

A circulatory microfluidic system was assembled using a peristaltic pump (Ismatec, Germany) which can produce a circulatory pulsatile flow in a micro-tubing of 500 μm in diameter and 1.5 m in length. Shear stress was calculated using Poiseuille's equation [30], $\tau = 4Q\eta/\pi R^3$, where τ is shear stress in dyne/cm^2 , Q is flow rate in cm^3/s , η is the dynamic viscosity of the fluid, and R is the radius of the tubing (250 μm). Control cells were seeded in 96-well plates coated with a solution of pluronic f127 (Invitrogen, Carlsbad, USA).

2.3.2 Transwell Migration Assay

Cells were collected and re-suspended in DMEM without serum at a density of 1×10^5 cells/ml. Next, 100 μl of cell suspension was added to the upper side of a transwell chamber with a pore size of 8 μm (Corning, Corning, USA). Next, 600 μl of DMEM with 10% FBS was added to the lower chamber. The cells were allowed to migrate from the upper to the lower chamber for 18 h at 37°C . The membranes of the chambers were fixed with 4% paraformaldehyde. The cells that remained on the upper side of the membrane were gently removed using a cotton swab and cells left in the transwell chamber were stained with H&E staining solutions using the standard protocol (Table 2-1). The purple dye haematoxylin stains the nuclear and the pink-color dye eosin stains cytoplasm of cells, which makes the migrated cells appear pink and purple in color.

Afterwards, the membrane was cut out from the transwell chamber and mounted on a glass slide using Clarion Mounting Medium (Sigma-Aldrich, Saint Louis, USA). Migrated cells were directly counted in each slide on a microscope with a 10X objective using a bright field filter (Axio Observer Z1, Carl Zeiss, Germany). The quantification of migrated cells was based on three independent repeats.

Table 2-1. Steps for H&E staining of migrated cells

Station	Duration (min unless otherwise stated)
Distilled water	1
Haematoxylin	1
Water	2
Distilled water	1
Differentiation solution	10 sec
Tap water	5
Distilled water	1
95% Ethanol	1
Eosin	30 sec
90% Ethanol	30 sec

2.3.3 Transendothelial Migration Assay

HUVEC-dt cells (2×10^4 cells/well) were seeded into the upper chamber of the transwell insert to form a monolayer. After 24 h incubation, the integrity of the endothelial monolayer was examined based on the red fluorescence of the HUVEC-dt cells. 231-

C3 cells (1×10^4 cells/well) were re-suspended in DMEM without serum and were added to the upper chamber of a transwell insert. Next, 600 μ l DMEM medium with 10% FBS was added to the lower chamber, and the cells were allowed to migrate for 24 h at 37°C. The transwell membrane were then fixed with 4% paraformaldehyde (PFA). The cells on the upper side of the membrane were gently removed with a cotton swab. The migrated 231-C3 cells were imaged using an inverted fluorescence microscope with YFP filters. At least 6 pictures were captured from various observation fields in each group. The numbers of migrated cells were counted in each image, and the average number was calculated for each group.

2.3.4 Wound Healing Assay

Silicone culture-inserts (Ibidi, Germany) were attached to a 35-mm dish. A volume of 100 μ l cell suspension at the cell density of 4×10^5 cells/ml was added into each compartment of the insert. After 2 h of incubation, the insert was gently removed and a wound gap of 500 ± 50 μ m was generated. Images were taken at 0 h and 18 h during the process of wound healing. The area of the wound gap for each group was measured using software ZEN. The area of wound closure was calculated using wound area of 0 h to subtract wound area of 18 h. The presented final results were all normalized to the control group.

2.3.5 Cell Adhesion Assay (Adhesion to ECM)

Prepare collagen I solution 50 μ g/ml with 0.02 M acetic acid. Add 100 μ l of the solution to the wells of 96-well plates, incubate under 37°C for 2 h. Remove the collagen solution and let the wells dry for 1 h. Use fresh DMEM to wash the wells twice before

use. Add 100 μ l suspension of breast cancer cells at the concentration of 1×10^5 cells/ml to wells of the collagen I-coated plates. Incubate the plates under 37°C for 20 min. After taking images using an inverted fluorescence microscope with YFP filters, aspirate the medium and wash the wells with PBS 2 $\times$ 10 min. Add fresh medium to the plates and take images again. At least 6 pictures were captured from various observation fields in each group. The images were processed and the cell numbers were counted for each group. The cell adhesion rate of each group is calculated as the ratio of cell numbers after wash to cell numbers before wash.

2.3.6 Cell Adhesion Assay (Adhesion to Endothelial Monolayer)

HUVEC-dt cells were seeded in 96-well plates at the density of 2×10^4 cells/well. After incubation for overnight, HUVEC monolayers were developed. The integrity of the monolayers could be examined based on both phase contrast imaging and the red fluorescence of HUVEC-dt cells (Figure 2-1). Add 100 μ l suspension of breast cancer cells at the concentration of 1×10^5 cells/ml to the plates. The washing and imaging steps are same with the ECM adhesion assay.

Figure 2-1. Observation of HUVEC-dt monolayer. Left panel is the red fluorescence image of HUVEC monolayer. Center panel is the phase contrast image of the monolayer. Right panel the merged image. Scale bar, 100 μ m.

2.3.7 Observation of Cell Spreading

Breast cancer cells were collected and re-suspended in fresh medium. The cells were seeded in collagen I-coated 96-well plates or plates with established HUVEC-dt monolayers at the density of 1×10^4 cells/well and incubated under 37 °C. Both phase contrast and YFP fluorescence images were taken at different time points (20, 40, 60 min).

2.4 Molecular Analysis Assay

2.4.1 Detection of Cellular ROS Level

MDA-MB-231 cells were circulated and collected at different time points. The cells were then immediately stained with 10 μ M DCFDA (Thermo Fisher Scientific, Waltham, USA) in DMEM. After incubation for 30 min, the cells were collected, re-suspended in Fluoro-brite medium (Thermo Fisher Scientific, Waltham, USA), and seeded in 96-well plates. The cells were immediately imaged using an inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany). At least 5 pictures were captured for each group. The fluorescence intensity of each cell was measured using the ZEN software, and the average values were calculated.

2.4.2 MTT Assay

100 μ l of cell suspension of 231-C3 (1×10^5 cells/ml) were seeded in each well of a 96-well plate. After incubation of 24 h, hydrogen peroxide was added into each well at the concentration of 50 μ M. After treating the cells with H₂O₂ for 6, 12, 24, and 48h, 10 μ l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution

(5mg/ml) was added. Three hours later, the formazan was solubilized by the addition of 100 μ l 10% SDS, 0.01 M HCl solution. After overnight incubation, the optical density at 595nm of the mix was determined using a plate reader.

2.4.3 Western Blot Analysis

The cells were lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors (Sigma-Aldrich, Saint Louis, USA). The protein concentrations were determined using a Bradford assay kit (Bio-Rad, Berkeley, USA). SDS-PAGE gels were loaded with the same amount of proteins and samples underwent electrophoresis at the same time. After the transfer process, the PVDF membranes were probed with primary antibodies overnight at 4°C. The next day, the blots were probed with HRP-conjugated secondary antibodies for 1 h at room temperature. The blots were developed in ECL solution, and the intensities of the bands on the blots were measured using Image Pro Plus.

2.5 Chemical Reagents Treatment

NAC was applied at the concentration of 5 mM, PG at 20 μ M, hydrogen peroxide at 50 mM, MEK inhibitor U0126 at 10 μ M, FAK inhibitor Y15 at 10 μ M. For the treatment of all the different reagents, breast cancer cells were pre-treated with the reagents for 1 h before collection. The collected cells were re-suspended in DMEM with the same reagents at the same concentration before circulation or being kept under static condition. NAC and PG were purchased as powder, both of which were directly dissolved in medium for using. Hydrogen peroxide was also directly dissolved in medium. Y15 was firstly dissolved in deionized water as stock solution. U0126 was

firstly dissolved in DMSO at the concentration of 10 mM. To exclude the effects of DMSO on tumor cells, a vehicle control group was added in the U0126 experiment, in which same concentration of DMSO was dissolved in medium. As the final working concentration for U0126 is 10 μ M, DMSO was dissolved at the concentration of 0.1% in vehicle control group. For NAC treatments, NAC was firstly dissolved in DMEM at the concentration of 500 mM. The pH was adjusted using NaOH as solution of NAC was acidic. Then NAC was diluted with DMEM to the final concentration of 5 mM.

2.6 Transfection of siRNA

Two Silencer Select siRNAs against MnSOD (SiRNA No.1 ID: s13268, and No.2 ID: s13269) and Silencer Select negative control siRNA were purchased from Ambion (Life Technologies, USA). The siRNA targeting FAK and siRNA targeting ERK1/2 were purchased from Cell Signaling Technology. Breast cancer cells were seeded on 60 mm petri dishes and allowed to attach overnight. Cells were washed with OptiMEM (Life Technologies) once before transfected with 20 nM of siRNAs using Lipofectamine RNA/iMAX (Life Technologies), according to the manufacturer's instructions. After incubation for 8 h, the transfection medium was replaced with fresh culture medium. Cells were collected for circulation at 72 h after transfection.

2.7 Microinjection of Tumor Cells into Zebrafish and Quantification of Extravasation

All zebrafish experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University (NTU). *Tg(fli1:EGFP)* zebrafish was kindly given to us by Professor Christoph Wolfram Winkler at National

University of Singapore. Zebrafish were maintained according to the standard operational guidelines. Fish embryos were obtained using standard mating conditions and incubated in zebrafish embryo medium (E3 medium). The details of the injection method have been described previously [215]. Briefly, the zebrafish larvae were collected and anesthetized with 0.01% tricaine at 72 hours post fertilization (hpf). The 231-C3 cells were collected and re-suspended in PBS at a concentration of 2×10^4 cells/ μ l. Cell suspensions were then injected into the larvae at the pericardium site. At 24 hours post injection (hpi), the larvae were placed in imaging chambers. A GFP filter set was used to observe the distribution of both the sensor cells and the vascular endothelial cells in the tail region. The cell numbers in and out of the blood vessel in the ISV were directly counted [215].

2.8 Statistical Analysis

All data are presented as the mean $\pm$ SEM from three independent experiments. The significance between two groups were assessed by the Student's *t*-test (two-tailed) in all experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant.

CHAPTER 3 The Effects of Shear Stress on Tumor Cell Migration and Extravasation

3.1 Introduction

The migration capacity of tumor cells plays a vital role in metastasis [1, 3, 216]. The effects of biological and chemical factors on tumor cell migration, including immune cells, fibroblasts and cytokines, have been studied extensively [1, 100, 101, 217]. However, the role of physical factors is poorly understood, especially the mechanism by which hemodynamic shear stress affects the migration of tumor cells during metastasis [6, 17]. Circulating tumor cells (CTCs) are tumor cells that have detached from primary tumors and have penetrated into blood vessels during metastasis [23, 218]. When CTCs travel in the circulatory system, they are exposed fluid shear stress (SS) [6, 18, 219], which could damage tumor cells during circulation [6, 20, 24].

The recent work in our lab reported that physiological levels of shear stress at 15 or 30 dyne/cm² could kill non-metastatic breast cancer cells (e.g. MCF7 cells) mainly by triggering apoptosis, while aggressive cell lines (e.g. MDA-MB-231) are much more resistant to SS [215]. Only aggressive tumor cells which survive circulation can extravasate and invade secondary organs to develop new tumors. There were other studies exploring effects of SS on tumor cells which revealed that stress could damage circulating tumor cells in different ways. However, how tumor cells response to hemodynamic SS did not draw enough attention. The external stresses in tumor microenvironment usually has dual effects on tumor cells, such as hypoxia and immune system. These stresses are damaging to tumor cells, but on the other hand, the stresses are able to modulate certain properties and functions of tumor cells which enhance the cell resistance to the environmental stresses or increase the invasiveness of tumor cells.

I suggested that hemodynamic SS has the similar dual effects on tumor cells. For the surviving tumor cells in circulation, shear forces may promote their migration capacity, which is crucial for extravasation [23, 24, 26, 27], and inhibition of the positive effect of SS on tumor cell migration might significantly suppress metastasis.

To test this hypothesis, I applied a microfluidic system which was originally designed by Dr. Afu Fu to recreate physiological pulsatile shear flows and mimic the physical environment of CTCs [215]. I found that an arterial level of SS significantly enhanced tumor cell migration in transwell and wound healing assays, and enhanced extravasation in a transendothelial assay. Circulatory treatment elevated the intracellular levels of reactive oxygen species (ROS), which is an early and indispensable event for activation of the extracellular signal-regulated kinases (ERK1/2). Subsequently, ERK1/2 activation promoted the migration of tumor cells and enhanced their extravasation. Interestingly, the original type of cellular ROS generated by shear stress is mitochondrial superoxide. Superoxide is not able to penetrate mitochondrial membrane freely and cannot be released into cytosol to activate kinases like ERK1/2. As an important component of cellular antioxidant system, manganese-dependent superoxide dismutase (MnSOD) functions to convert mitochondrial superoxide into hydrogen peroxide. Unlike superoxide, hydrogen peroxide can diffuse through mitochondrial membrane and enter cytosol, which then oxidize different kinases and transcription factors and regulate their activities. Therefore, I explored and identified the role of (MnSOD) in mediating shear stress-promoted cell migration. Finally, I found that reducing cellular ROS production suppressed tumor cell extravasation in both a transendothelial assay and a zebrafish model. This new understanding of how fluid shear stress promotes tumor cell migration has important

implications in cancer treatment and can help us to identify potential therapeutic targets for inhibiting tumor progression.

3.2 Results

3.2.1 Shear Stress Promotes Tumor Cell Migration

A microfluidic circulatory system was previously developed by Dr. Fu Afu to mimic physiological fluid shear forces. This system uses a peristaltic pump to generate pulsatile shear flow in which tumor cells circulate and experience SS [215] (Figure 3-1). Engineered breast cancer MDA-MB-231 (231-C3) cells which express a fluorescent apoptotic sensor were introduced into the system through the reservoir, and circulated under SS of 5, 15, and 30 dynes/cm². The SS of 5 and 15 dynes/cm² represent the average SS values in a human vein and artery, respectively, while the higher SS of 30 dynes/cm² can be achieved when the heart rate reaches 140 beats per minute [220]. Meanwhile, a control group was set up with uncirculated (static) suspending cells, which aims to exclude the interference of non-adherent condition by culturing 231-C3 cells in non-adhesive 96-well plates pre-coated with 1% pluronic f127.

Figure 3-1. The circulatory microfluidic system. (A) The schematic diagram of the system. Tumor cell suspension was injected into the system through a reservoir, which allows for gas exchange. The cells can be collected from the tubing end after circulation. Cotton filter is used to prevent contamination. (B) The peristaltic pump that generates pulsatile shear flow. (C) The circulatory system with cell suspension in reservoir and silicone tubing. The system was originally designed by Dr. Fu Afu.

After 6 h, both the non-circulated and circulated 231-C3 cells were collected separately and subjected to an 18-h transwell migration assay. More 231-C3 cells migrated to the lower side of the transwell chamber after SS treatment, compared to cells which were kept under static conditions. Furthermore, the number of migrated cells increased along with the increment of SS (Figure 3-2). In the static group, around 320 cells migrated to the lower side of membrane in each transwell insert. SS15 significantly increased migrated cell number to 940, by approximately 3-fold compared to the static group. Raising SS level from 15 to 30 dynes/cm² further enhanced cell migration very slightly (1140 migrated cells, 3.5-fold compared to the static group) (Figure 3-2). As SS15 represents the average SS in a human artery, I decided to continue using SS15 for subsequent experiments in this study.

To verify the effects of SS, two other metastatic breast cancer cell lines, MDA-MB-157 and BT-549, were circulated under SS15 for 6 h and subjected to the transwell assay. The two cell lines exhibited a 2.7 to 3.9-fold increased level of migration after SS15 treatment compared to the static conditions, which was similar to the 3.0-fold increase in cell migration observed in 231-C3 cells under SS15 treatment (Figure 3-2). I further validated this finding by performing a wound healing assay using 231-C3 cells

and the results confirmed that a physiological arterial level of SS15 enhanced the migration of the tumor cells by approximately 50% (Figure 3-3).

Figure 3-2. SS stimulates the migration of breast cancer cells in transwell assay. (A) The 231-C3 cells were first circulated under SS of 5, 15, and 30 dynes/cm² (labelled as SS5, SS15, and SS30, respectively) for 6 h before subjected to the transwell assay. In the static (control) group, cells were incubated in a non-adhesive culture plate for 6 h. Upper panel shows the images of 231-C3 cells that had migrated to the lower side of a porous membrane. The dark arrows indicate the migrated cells. The cytoplasm was pink and the nucleus was purple in migrated cells because of H&E staining. The white dots with black borders are the pores of transwell membrane. Scale bar, 100 μ m. Lower panel shows the quantification of migrated cells. (B) SS enhanced migration of MDA-MB-157 and BT-549 cells. Both MDA-MB-157 and BT-549 cells were circulated under SS15 for 6 h and subjected to the 18-h transwell assay. Upper

panel shows the images of cells that had migrated through the porous membrane. Scale bar, 100 μm . Lower panel shows the quantification of migrated cells.

Figure 3-3. Shear stress promotes migration of breast cancer cells in a wound healing assay. Left panel shows representative images of the 231-C3 cells in a wound healing assay. Scale bar, 100 μm . Right panel shows the quantification of wound closure. The results were normalized to the static group.

The same experiment was then conducted on non-metastatic MCF7-C3 cells. In the static group, around 100 MCF7-C3 cells migrated to the lower side of membrane in each transwell insert. Migration of the MCF7-C3 cells circulated under SS15 showed no significant difference compared to the static group, with 95 migrated cells in each transwell insert (Figure 3-4). Taken together, the data indicated that SS stimulates the migration of metastatic breast cancer cells, but not the non-metastatic MCF7 cells.

Figure 3-4. MCF7-C3 cells circulated under SS15 had similar migration capacity as cells maintained under static conditions. Left panel shows images of MCF7-C3 cells that had migrated to the lower side of the porous membrane. The cytoplasm was pink and the nucleus was purple in migrated MCF7-C3 cells because of H&E staining. The white dots with black borders are the pores of transwell membrane. Scale bar, 100 μ m. Right panel shows the quantification of the migrated cells.

3.2.2 Shear Stress Stimulates Generation of Cellular ROS

In the previous studies of our group, Dr. Fu Afu pre-stained MDA-MB-231 cells with a ROS dye CM-H₂DCFDA (DCFDA) before circulation and observed that the intracellular level of ROS was increased within the first 2 h of circulation [215]. DCFDA is a widely-used ROS indicator, which mainly reacts with cellular hydrogen peroxide. Cellular hydrogen peroxide has long been recognized as an important signaling molecule which regulates different cellular functions including cell migration [179, 182, 221]. I hypothesized that ROS generation was stimulated in cells circulated under SS, thus enhancing tumor cell migration.

Here, I optimized the experimental conditions to determine the dynamics of cellular ROS during the 6-h circulation under SS15. MDA-MB-231 breast cancer cells were

circulated under SS15, collected at different time points and stained with DCFDA. The green fluorescence of DCF in MDA-MB-231 cells were imaged and fluorescence intensities were quantified using the ZEN software. The fluorescent micrographs indicated that ROS levels increased after 0.5 h of SS15 treatment and peaked at 3 h, which was approximately 4.5-fold higher than the initial ROS level at 0 h. Furthermore, this increase in ROS levels persisted to the end of the circulatory experiment at 6 h (Figure 3-5).

Figure 3-5. SS increases ROS production in breast cancer cells. Upper panel shows the fluorescent images of MDA-MB-231 cells that were circulated under SS15 for the indicated durations and stained with DCFDA. Scale bar, 100 μm . Lower panel is the quantified fluorescence intensity of DCF from the cells that had been circulated under SS15 for the indicated durations. The results were normalized to 0 h.

To investigate the correlation between SS and ROS levels, MDA-MB-231 cells were circulated under SS of 5, 15, or 30 dynes/cm² for 6 h and stained with DCFDA (Figure 3-6). When SS was increased from 5 to 15 dynes/cm², the intensity of DCF elevated from 2.7-fold to 4.2-fold over the basal ROS level at 0 h. However, further increasing SS from 15 to 30 dynes/cm² did not significantly change the ROS levels. The ROS level of MDA-MB-231 cells under static conditions also increased very slightly, which is much lower compared to the cells subjected to SS.

Figure 3-6. Shear stress stimulates generation of cellular ROS. Upper panel shows the representative fluorescent images of MDA-MB-231 cells that were circulated under SS of 5, 15, and 30 dynes/cm² for 6 h and stained with DCFDA. Scale bar, 100 μ m. Lower panel is the quantified fluorescence intensity of DCF from the cells that had been circulated under different levels of SS for 6 h. The results were normalized to 0 h.

3.2.3 Shear Stress-promoted Cell Migration is Mediated by ROS

In order to find connections between ROS elevation and cell migration, I used two antioxidant agents, N-acetyl-cysteine (NAC) [222] and propyl gallate (PG) [223], to effectively prevent SS-induced ROS accumulation (Figure 3-7).

Figure 3-7. Application of antioxidants NAC and PG successfully reduced ROS levels of the cells circulated under SS15 for 6 h. Upper panel shows the images of MDA-MB-231 cells stained with DCFDA. Scale bar, 100 μ m. Lower panel shows the quantification of DCF intensity in the cells. The results were normalized to the static group.

231-C3 cells were pre-treated with 5 mM NAC or 20 μ M PG for 1 h, circulated under SS15 for 6 h, and subjected to the transwell migration assay for 18 h. SS15 increased migration of 231-C3 cells by 3.5-fold; however, this increase was almost completely lost following pre-treatment with either NAC or PG (Figure 3-8). These results suggest that ROS acts as an intermediary signaling molecule in regulating SS-promoted tumor cell migration. Treating cells with either NAC or PG did not affect cell migration under static conditions (Figure 3-8), suggesting that ROS elevation is not essential for migration of non-circulating cells.

Figure 3-8. ROS is indispensable for shear stress-induced cell migration. Antioxidants suppressed the migration of 231-C3 cells circulated under SS15. Upper panel shows representative images of migrated 231-C3 cells in the 18-h transwell assay. The cytoplasm was pink and the nucleus was purple in migrated 231-C3 cells because of H&E staining. The white dots with black borders are the pores of transwell membrane. Scale bar, 100 μ m. Lower panel shows the quantification of migrated cells.

To explore the importance of high levels of ROS in promoting cell migration, I applied H_2O_2 as the external ROS source to elevate cellular oxidative level. The cytotoxic effects of H_2O_2 on tumor cells were examined by Dr. Fu Afu. 231-C3 cells were treated with 50 μ M H_2O_2 for 6, 12, 24, 48h, and the viabilities of 231-C3 cells were examined using MTT assay. The viabilities were calculated as the ratio of MTT readings of the H_2O_2 treated cells to MTT readings of the control cells without H_2O_2 treatment at each time point, respectively. The results showed that 231-C3 cells were highly resistant to H_2O_2 (Figure 3-9). 231-C3 cells were then treated with 50 μ M H_2O_2 for 6 h under static conditions. The results of an 18-h transwell assay demonstrate that H_2O_2 treatment significantly increased tumor cell migration by approximately 3.5-fold compared to the control group (Figure 3-10). This result is consistent with the hypothesis that excessive ROS generation mediates shear stress-enhanced cell migration.

Figure 3-9. 231-C3 cells are highly resistant to cytotoxic effects of H₂O₂. 231-C3 cells were treated with or without 50 μ M H₂O₂ for 6, 12, 24 and 48 h before subjected to MTT assay. The viabilities of cells were calculated as the ratio of MTT reading of H₂O₂ group (H₂O₂ treatment) to MTT reading of control group (no H₂O₂ treatment). For both the treatment of 6 and 12 h, the viability of 231-C3 cells is around 95%. Extending the treatment time to 24 h decrease the cell viability slightly to 88%. Treating 231-C3 cells with 50 μ M H₂O₂ decrease cell viability to 81%.

Figure 3-10. ROS promotes tumor cell migration. H₂O₂ enhanced the migration of 231-C3 cells under static conditions. The cells were seeded in pluronic-coated 96-well plates and treated with or without 50 μ M H₂O₂ before subjected to transwell migration assay. Left panel shows representative images of migrated 231-C3 cells in the transwell assay. The cytoplasm was pink and the nucleus was purple in migrated cells because of H&E staining. The white dots with

black borders are the pores of transwell membrane. Scale bar, 100 μ m. Right panel is the quantification of migrated cells.

3.2.4 Shear Stress Activated ERK1/2 in Tumor Cells

Extracellular signal-regulated kinases (ERK1/2) are important kinases in the mitogen-activated protein kinases (MAPK) signaling pathway and they play an important role during tumorigenesis and metastasis [182, 224]. I hypothesized that shear stress could promote cell migration through activating ERK1/2. 231-C3 cells were circulated under SS15 and collected at different time points for Western blot analysis. The results indicated that SS exposure caused an increase in the level of phosphorylated ERK1/2 (pERK1/2) (Figure 3-11). In particular, there was a 2.1-fold increase in pERK1/2 levels after a 4-h treatment, and the levels peaked with a 3.0-fold increase after 6 h. In contrast, no significant change of pERK1/2 levels was detected in static cells after 6 h of incubation. The level of total ERK1/2 did not change significantly in both the static and circulated 231-C3 cells (Figure 3-11).

I further examined the effects of a range of SS conditions on ERK1/2 phosphorylation. 231-C3 cells were circulated under SS5, 15 or 30 for 6 h before being subjected to Western blot analysis. The results showed that the pERK1/2 levels significantly increased as SS increased. A 1.9-fold increase compared to the static group was observed in the SS5 treatment group. A 3.1-fold increase was observed at SS15, which was further enhanced by SS30 exposure very slightly (Figure 3-11). This is in agreement with our previous results, which showed that SS levels greater than the arterial level of SS15 did not cause a further increase in cell migration (Figure 3-2), or ROS production (Figure 3-6).

Figure 3-11. Shear stress promotes ERK1/2 activation in 231-C3 cells. (A) Western blot analysis of ERK1/2 phosphorylation levels in 231-C3 cells that were circulated under SS15 or seeded in a pluronic-coated 96-well plate for 0, 2, 4 and 6 h. Upper panel is the representative blot image. Lower panel shows the quantification of phosphorylation level of ERK1/2, which is calculated as the ratio of the band intensities of pERK1/2 to the band intensities of total ERK1/2. The results were normalized to 0 h. (B) Western blot analysis of the levels of ERK1/2 phosphorylation in 231-C3 cells circulated under SS5, SS15, and SS30 for 6 h. Upper panel is the representative blot image. Lower panel shows the quantification of phosphorylation level of ERK1/2, which is calculated as the ratio of the band intensities of pERK1/2 to the band intensities of total ERK1/2. The results were normalized to static group.

The role of SS in regulating ERK1/2 activity in metastatic breast cancer cells was further examined using MDA-MB-157 and BT-549 cells. Both cell lines were circulated under SS15 for 6 h. The Western blot analysis indicated that treatment with SS15 increased phosphorylation levels of ERK1/2 by 2.4-fold and 2.7-fold, respectively, in MDA-MB-157 and BT-549 cells (Figure 3-12).

Figure 3-12. Shear stress promotes the ERK1/2 activation in MDA-MB-157 and BT-549.

Western blot analysis of ERK1/2 phosphorylation in MDA-MB-157 and BT-549 cells which were circulated under SS15 for 6 h. Left panel is the representative blot image. Right panel shows the quantification of phosphorylation level of ERK1/2, which is calculated as the ratio of the band intensities of pERK1/2 to the band intensities of total ERK1/2. The results were normalized to static group.

Next, I studied the effects of ERK1/2 phosphorylation on cell migration. I used the inhibitor U0126 to inhibit the activity of MAPK kinase (MEK), thereby blocking ERK1/2 phosphorylation. 231-C3 cells were pre-treated with 10 μ M U0126 for 1 h, circulated under SS15 for 6 h. The cells were then collected for Western blot analysis and the results indicated that pre-treating 231-C3 cells with U0126 completely prevented SS-induced phosphorylation of ERK1/2 (Figure 3-13). Transwell migration assay was applied to examine the effects of U0126 on cell migration. As the U0126 was dissolved in DMSO as stock solution, a vehicle control group was included in transwell migration experiment to exclude the influence of DMSO, as described in Chapter 2.5. Cells in the blank control group did not receive any treatments. I found that the vehicle DMSO did not influence cell migration and U0126 significantly reduced SS-stimulated transmembrane migration by approximately 6-fold (Figure 3-13). A much smaller 2-

fold reduction in cell migration was detected in the non-attached cells in the static group following U0126 treatment (Figure 3-13). These results demonstrate that ERK1/2 activity is important for cell migration with or without SS, and is indispensable for regulating SS-enhanced cell migration.

A

B

Figure 3-13. Shear stress-promoted tumor cell migration is mediated by ERK1/2. (A) The MEK inhibitor U0126 inhibited SS-induced ERK1/2 phosphorylation in 231-C3 cells. Left panel is the representative blot image. Right panel shows the quantification of phosphorylation level of ERK1/2, which is calculated as the ratio of the band intensities of pERK1/2 to the band intensities of total ERK1/2. The results were normalized to static group. (B) The MEK inhibitor U0126 suppressed migration of 231-C3 cells that were circulated under SS15 and the cells under static conditions, compared to vehicle control group. No obvious difference between vehicle control group and blank control group was detected. Upper panel shows the images of migrated 231-C3 cells in an 18-h transwell assay. The cytoplasm was pink and the nucleus was purple in migrated cells because of H&E staining. The white dots with black borders were the pores of transwell membrane. Scale bar, 100 μ m. Lower panel shows the quantification of migrated cells.

To further verify the role of ERK1/2 in SS-mediated cell migration, an siRNA targeting both ERK1 and ERK2 (named as siERK1/2) was transfected into 231-C3 cells. The cells in negative group were transfected with non-targeting siRNA purchased from Thermo Scientific. The blank control is normal 231-C3 cells without any treatments. Cells were collected at 72 h after transfection for Western blot analysis and the results indicated that the cellular levels of ERK1/2 were significantly decreased by siERK1/2 (Figure 3-14). The transfected 231-C3 cells were then collected and circulated under SS15 or kept under static conditions for 6 h, before subjected to an 18-h transwell migration assay. Knockdown of ERK1/2 suppressed migration of 231-C3 cells both under static condition and SS15. The effect of SS on migration of 231-C3 cells was totally blocked by decreasing ERK1/2 level (Figure 3-14). This result is consistent with the previous experiment using ERK1/2 inhibitor (Figure 3-13).

Figure 3-14. Knockdown of ERK1/2 inhibits the SS-enhanced tumor cell migration. (A)

231-C3 cells were collected at 72 h after transfection of siRNAs targeting ERK1/2 (named as siERK1/2) and lysed for Western blot analysis. The levels of ERK1/2 were detected and GAPDH was used as the internal control for equal loading. The siERK1/2 significantly

decreased total ERK1/2 levels in 231-C3 cells. (B) 231-C3 cells were collected at 72 h after transfection of siERK1/2. The cells were circulated under SS15 for 6 h before subjected to transwell migration assay. Upper panel shows representative images of migrated cells. The cytoplasm was pink and the nucleus was purple in migrated cells because of H&E staining. The white dots with black borders were the pores of transwell membrane. Scale bar, 100 μ m. Lower panel is the quantification of migrated cells.

Because ROS elevation occurred 3 h prior to the SS-induced ERK1/2 phosphorylation and inhibiting either event reduced SS-stimulated cell migration, I investigated the relationship between ROS elevation and ERK1/2 phosphorylation. 231-C3 cells were pre-treated with 5 mM NAC or 20 μ M PG for 1 h, circulated under SS15 for 6 h, and analyzed by Western blot. The results showed that inhibiting ROS generation with antioxidants significantly reduced ERK1/2 phosphorylation in the circulated 231-C3 cells (Figure 3-15).

To confirm the role of ROS in activating ERK1/2, I added 50 μ M H₂O₂ to 231-C3 cells and incubated cells under non-adhesive conditions for 6 h. Results from the Western blot analysis showed that elevating the intracellular level of ROS significantly increased ERK1/2 phosphorylation (Figure 3-15). Taken together, these results suggest that ROS generation occurs upstream of ERK1/2 activation and both events play essential supportive roles in promoting the migration of circulated cancer cells.

Figure 3-15. Cellular ROS generated by shear stress activates ERK1/2. (A) Western blot analysis of the ERK1/2 phosphorylation levels in the 231-C3 cells. The cells were treated with or without 5 mM NAC or 20 μ M PG and circulated under SS15. Upper panel is the representative blot image. Lower panel shows the quantification of phosphorylation level of ERK1/2, which is calculated as the ratio of the band intensities of pERK1/2 to the band intensities of total ERK1/2. The quantification is based on three independent experiments. The results were normalized to static group. (B) Western blot analysis of the ERK1/2 phosphorylation levels in the 231-C3 cells that were kept under static conditions and treated with or without 50 μ M H₂O₂. Upper panel is the representative blot image. Lower panel shows the quantification of phosphorylation level of ERK1/2, which is calculated as the ratio of the band intensities of pERK1/2 to the band intensities of total ERK1/2. The results were normalized to control group.

3.2.5 The Function of MnSOD in Shear Stress-promoted Cell Migration

Previous studies conducted in our laboratory have identified MnSOD as a key antioxidant enzyme that protects tumor cells during circulation [12]. Dr. Fu revealed

that shear stress stimulates generation of mitochondrial superoxide in tumor cells which is highly damaging to the mitochondria. MnSOD converts superoxide to less toxic hydrogen peroxide and protects mitochondria and tumor cells. Interestingly, another difference between superoxide and hydrogen peroxide is that hydrogen peroxide can diffuse through mitochondrial membrane easily while superoxide is unable to penetrate mitochondrial membrane without ionic channels. Most of the targets of ROS locate inside the cytosol, including ERK1/2. Thus, I proposed that MnSOD plays important roles in SS-promoted cell migration by converting superoxide to hydrogen peroxide to activate ERK1/2 in the cytosol.

To verify the function of MnSOD, two functionally validated siRNAs targeting MnSOD were purchased from Thermo Scientific, which were named as siMnSOD-1 and siMnSOD-2. The 231-C3 cells were separately transfected with the two siRNAs. The cells in negative group were transfected with non-targeting siRNA purchased from Thermo Scientific. The blank control is normal 231-C3 cells without any treatment. The effects of siRNAs on cellular MnSOD levels were examined 72 h after transfection by Western blot analysis. The results showed that MnSOD levels were significantly decreased by both siMnSOD-1 and siMnSOD-2 (Figure 3-16).

Figure 3-16. Silencing of MnSOD using two different siRNAs. 231-C3 cells were collected at 72 h after transfection of siRNAs and lysed for Western blot analysis. The levels of MnSOD were detected and GAPDH was used as the internal control for equal loading. Both siMnSOD-

1 and siMnSOD-2 showed significant reduction of MnSOD levels compared to the control and negative groups.

The transfected 231-C3 cells were then collected and circulated under SS15 or kept under static conditions for 6 h, which were then subjected to an 18-h transwell migration assay. For the cells kept under static conditions, although the reduction in MnSOD level suppressed the migration of 231-C3 cells to some extent, the difference was not significant when comparing groups of siMnSOD-1 and siMnSOD-2 with the control and negative groups (Figure 3-17). In contrast, for the 231-C3 cells circulated under SS15, both siMnSOD-1 and siMnSOD-2 groups displayed substantially decreased cell migration compared with the negative group (Figure 3-17). This is in good agreement with the previous results of suppressing SS-promoted cell migration by using antioxidants, which indicates that ROS and MnSOD are not the main factors regulating cell migration under normal conditions, but are the key signaling molecule and enzyme mediating enhanced cell migration and invasiveness as cellular responses to external stresses like shear force.

Figure 3-17. Silencing of MnSOD impaired shear stress-promoted tumor cell migration.

231-C3 cells were collected at 72 h after transfection of siRNAs and circulated under SS15 or kept under static conditions. The cells were then subjected to a 18-h transwell migration assay. Upper panel shows the representative images of migrated cells on transwell membranes in different groups. The cytoplasm was pink and the nucleus was purple in migrated cells because of H&E staining. The white dots with black borders were the pores of transwell membrane. Scale bar, 100 μ m. Right panel shows the quantification of migrated cells.

To further explore the functions of MnSOD, the cells circulated under SS15 or kept under static conditions were collected for Western blot analysis. I detected the levels of phosphorylated ERK1/2 (pERK1/2), and total ERK1/2. Apparently, reducing MnSOD

levels suppressed phosphorylation levels but not the total protein levels of ERK1/2, for the cells circulated under SS15 (Figure 3-18). In harmony with the transwell migration results, silencing of MnSOD only suppressed ERK1/2 phosphorylation very slightly for the cells in static group.

Figure 3-18. Silencing of MnSOD suppressed shear stress-promoted ERK1/2 phosphorylation. 231-C3 cells were collected at 72 h after transfection of siRNAs and circulated under SS15 or kept under static conditions. The cells were then collected and lysed for Western blot analysis. Levels of phosphorylated ERK1/2, total ERK1/2 were detected and analyzed. GAPDH was used as the internal control for equal loading. Phosphorylation levels of ERK1/2 were significantly decreased by knocking down of MnSOD, while total levels of ERK1/2 are not influenced. Upper panel is the representative blot image. Lower panel shows the quantification of phosphorylation level of ERK1/2, which is calculated as the ratio of the

band intensities of pERK1/2 to the band intensities of total ERK1/2. The results were normalized to control group under static condition.

3.2.6 Antioxidants Reduced Shear Stress-stimulated Tumor Cell Extravasation

The migration capacity of tumor cells plays a vital role in extravasation during metastasis. The transendothelial migration assay was conducted to determine if SS-enhanced cell migration can assist tumor cells in extravasation. This assay has been widely used to assess cell migration capacity across an endothelial monolayer. HUVEC-dt cells were seeded on the upper side of the transwell chamber to establish an endothelial monolayer. Subsequently, static 231-C3 cells or cells exposed to SS15 for 6 h were added to the top of the endothelial monolayer. After 24 h, all of the cells that remained on the upper side of the transwell chamber were gently removed, and 231-C3 cells that migrated to the lower side of the chamber were detected based on their green fluorescence. I found that circulating 231-C3 cells at SS15 for 6 h significantly enhanced their transendothelial migration ability by 2.6-fold (Figure 3-19), which suggests that SS stimulates tumor cells to pass through the blood vessel during extravasation.

My previous results indicated that the effects of SS on cell migration are mediated by ROS generation, therefore, I tested the effect of suppressing ROS-generation on SS-enhanced extravasation. 231-C3 cells were pre-treated with 5 mM NAC or 20 μ M PG for 1 h, circulated under SS15 for 6 h, and subjected to a 24-h transendothelial migration assay. In agreement with the results of our 18-h transwell assay, antioxidant treatment suppressed transendothelial migration of circulated 231-C3 cells (Figure 3-19).

Figure 3-19. Shear stress enhances transendothelial migration of tumor cells. A greater number of 231-C3 cells subjected to SS15 migrated through endothelial monolayer compared to cells maintained under static conditions. Antioxidants significantly reduced the SS-promoted transendothelial migration. Upper panel shows fluorescent images of 231-C3 cells that had migrated to the lower side of a porous membrane in a 24-h transendothelial assay. Scale bar, 100 μ m. Lower panel shows the quantification of migrated cells in the transendothelial assay. The results were normalized to the static group.

Thus far, our *in vitro* data demonstrate that SS can elevate the intracellular levels of ROS in tumor cells, thereby activating MEK/ERK-mediated signaling pathway to promote cell migration and extravasation. To validate our findings *in vivo*, I employed transgenic zebrafish *Tg(fli1:EGFP)* in my study. The *Tg(fli1:EGFP)* zebrafish uniformly express GFP throughout the vasculature. Since the body of a zebrafish larva is transparent, we can visualize the circulation system of a *Tg(fli1:EGFP)* larva under a fluorescence microscope (Figure 3-20). Intersegmental vessels (ISVs) in zebrafish

vascular system have a highly patterned structure, which allows for efficient detection of extravasated tumor cells. These features make the *Tg(fli1:EGFP)* zebrafish a widely used *in vivo* model for studying extravasation [23, 30, 215].

Figure 3-20. Using a zebrafish model to study extravasation. (A) *Tg(fli1:EGFP)* zebrafish larvae at 72 hpf were imaged under a fluorescence microscope. The arrow indicates the injection site of tumor cells. (B) Sample images of extravasated 231-C3 cells (Out) and cells located inside the ISVs (In). Images were taken at 24 hpi.

I injected 231-C3 cells into the vascular system of larvae through the pericardium site (Figure 3-20). The tumor cells circulate in the vascular system and experience hemodynamic SS. Previously, Dr. Fu reported that the ROS levels of cancer cells within the vascular system of zebrafish rise during circulation [12]. I suspected that the excessive cellular ROS generated by SS would facilitate extravasation of 231-C3 cells in our zebrafish model. To test this hypothesis, I pre-treated both 231-C3 cells and zebrafish larvae at 72 hpf with 5 mM NAC or 20 μ M PG for 3 h. The 231-C3 cells were then injected into zebrafish larvae and the larvae were incubated in zebrafish embryo medium with or without 5 mM NAC or 20 μ M PG. At 24 hpi, both the tumor cells and the ISVs could be visualized based on their fluorescence. The number of extravasated cells (cells outside the ISVs) and total cell number (cells located inside and outside the

ISVs) were determined by counting the cells directly under microscope. These numbers were then used to calculate the percentage of extravasation. The quantification results showed that the extravasation occurred in 35% of control tumor cells, which was significantly reduced to 16-17% when cells were treated with NAC or PG (Figure 3-21). Interestingly, I observed that the extravasated cells in the larvae of control group had altered morphological features with elongated shapes and protrusions that might facilitate aggressive invasion of the surrounding ISV tissues. In contrast, most of the 231-C3 cells treated with NAC or PG, including the extravasated cells, appeared more rounded and less invasive (Figure 3-21). These results indicated that antioxidants were able to suppress extravasation and might further reduce the invasiveness of extravasated tumor cells *in vivo*.

Figure 3-21. Antioxidants suppressed *in vivo* tumor cell extravasation in zebrafish.

Percentage of extravasation of 231-C3 cells treated with NAC or PG were significantly lower than the control group. Left panel shows representative images of larvae in each group at 24

hpi. The arrows indicate the extravasated 231-C3 cells. The extravasated cells in the control group were more elongated and had protrusions, characteristics that indicated invasion into the larval tissue. Most of the cells, including the extravasated cells treated with NAC or PG had less aggressive morphological characteristics. Right panel shows the quantification of percentage of extravasation based on three independent experiments. At least ten fish were analyzed in each experiment, and each fish had at least five cells located in their ISVs.

3.3 Discussion

It has been reported that hemodynamic SS modulates different phenotypes and functions of endothelial cells. However, little is known about the effects of SS on tumor cells. In this study, I demonstrate that hemodynamic SS could up-regulate migration and extravasation of surviving tumor cells in circulation, and facilitate metastasis.

Traditional methods to study cellular effects of fluidic forces are usually performed by applying SS to attached cells [41, 43]. However, it was overlooked that the tumor cells which experience SS are the circulating tumor cells (CTCs) in the blood flow, all of which are non-adhesion cells. In order to simulate how CTCs encounter SS in circulation, I directly injected the suspended cancer cells into an in-house designed microfluidic circulatory system [215]. Using this system, I was able to circulate breast cancer cells under physiological SS and study the effects of SS on the invasiveness of tumor cells.

In conclusion, I produced the following important findings. First, SS could promote migration of metastatic tumor cells *in vitro*. All three metastatic cell lines, including 231-C3, MDA-MB-157 and BT-549, displayed a higher migration capacity after exposure to SS. The migration of 231-C3 cells was increased in both transwell assay and wound healing assay, which indicated that SS enhanced general motility of tumor cells, but not chemotaxis. SS did not increase the migration of non-metastatic MCF7-C3 cells, suggesting the specificity of the SS-conferred metastatic effect. Second, I elucidated the underlying mechanism: SS induced tumor cells to generate excessive ROS, which activates ERK1/2 to stimulate tumor cell migration (Figure 3-22). Finally, I found that SS enhances extravasation of breast cancer cells and identified the importance of ROS elevation in mediating the SS-stimulated extravasation. Application

of antioxidants that prevent cellular ROS elevation reduces SS-promoted cell migration *in vitro*, and suppresses extravasation in a zebrafish model.

Figure 3-22. Summary of the signaling pathway the regulate SS-enhanced tumor cell migration and extravasation

The use of antioxidants in cancer therapy has long been a heavily debated issue [225, 226]. The controversy over antioxidant application was due to the complicated and volatile roles of ROS during cancer progression [177, 226]. In this respect, understanding the function of ROS in tumor cells during metastasis is enormously important for developing new anti-cancer strategies. Based on my results, I found that ROS plays a positive role in SS-promoted extravasation and that reducing ROS by antioxidants decreases the extravasation of tumor cells. It should be noted that although DCFDA can be oxidized by different types of ROS, it is widely accepted that it mainly

reacts with hydrogen peroxide in cells [227, 228]. Thus, the cellular ROS I detected during cell circulation which functions to regulate cell migration, should be hydrogen peroxide. Moreover, I identified the importance of MnSOD in SS-promoted tumor cell migration by regulating existing forms and distributions of cellular ROS. MnSOD converts the mitochondrial superoxide to hydrogen peroxide, which diffuses to cytosol and activates ERK1/2. Interestingly, mitochondrial superoxide is responsible for killing tumor cells. By scavenging the superoxide, MnSOD not only promotes ERK1/2 activation but also protects the tumor cells. In this view, the application of general antioxidants might inhibit tumor cell migration and extravasation, but at the same time protect the circulating tumor cells. Based on my study, I propose using the antioxidants which specifically target hydrogen peroxide but do not eliminate superoxide (e.g. ebselen) to inhibit metastasis. More importantly, targeting MnSOD, an important component of cellular antioxidant system has multiple actions, including facilitating SS-induced tumor cell death and decreasing the aggressiveness of tumor cells. The other molecules regulating pro-metastatic effects of shear stress could also function as anti-metastasis targets, including ERK1/2 or other ROS targets.

The functions of MnSOD in shear stress-promoted tumor cell migration and extravasation need to be further verified. Moreover, further studies using cancer cells of different origins and deeper investigations on the specific mechanisms at play are required to fully understand the involvement of ROS in tumor progression and metastasis.

CHAPTER 4 Shear Stress Stimulates Tumor Cell Adhesion

4.1 Introduction

The adhesion capacity of cells to ECM, which indicates how efficiently cells can form stable interactions with ECM, is very important in determining cell survival and growth [167, 168]. During cancer metastasis, some of the CTCs are able to extravaste and invade a secondary organ. Appropriate and effective adhesions of the extravasated tumor cells to the ECM of the secondary organ have a great influence on the invasion and colonization of the tumor cells in the new microenvironment [13, 143]. The seed and soil theory proposed that the nature of ECM in the secondary organ, especially the stiffness, regulates tumor cell adhesion to ECM, which has been proven by many studies [140]. On the other hand, the adhesive capacity of tumor cells is also important for the interactions between tumor cells and ECM.

The adhesion of tumor cells to ECM is mainly mediated by integrins. After binding to the ligands in ECM, integrins will cluster and the cytoplasmic domain will activate kinases including FAK and Src, which induce the formation of focal adhesion complex [136, 229]. Focal adhesion complex not only transduces signals and triggers downstream pathways for cell survival and growth, but also connects ECM-binding integrins with cytoskeleton to support stable tumor cell adherence to ECM [129, 158]. It was discovered that expression level and translocation of integrins, as well as the activities of kinases involved in focal adhesion formation determine the adhesive capacity of tumor cells to ECM [162, 230, 231]. Among the kinases in focal adhesion complex, FAK is one of the most widely studied. The emphasis placed on FAK is owing to its importance in focal adhesion formation and the fact that FAK activities are regulated by various factors, including ROS [229].

In cancer metastasis, the adherence of tumor cells to endothelium is an indispensable step before extravasation. Although the dynamics of tumor cell adhesion to endothelium is not fully understood and controversial, it is widely accepted that stable attachment of tumor cells to endothelial monolayer is essential for disruption of endothelial cell junction and transendothelial migration of tumor cells [23, 24]. Previous studies have revealed different receptors on tumor cell membrane including CD44, MUC1 and integrins, as well as the receptors on endothelial cell membrane including selectins, that mediate the interaction of tumor cells and endothelial cells [155, 157, 232]. However, understanding of the basic mechanism of the adherence, including how the tumor cell-endothelium binding sites are linked to cytoskeleton of tumor cells, is still limited.

As both tumor cell extravasation and invasion to secondary organs happen after circulation of tumor cells in blood stream, I would like to investigate the effects of shear stress (SS) on the adhesion of tumor cells to both ECM and endothelium. Based on the microfluidic system that we developed, different levels of SS were applied on the breast cancer cells. The circulated cells were then collected and subjected to cell adhesion assays. Type I collagen was coated or an intact HUVEC monolayer was established in 96-well plates before cancer cells were seeded. After washing twice with PBS, the tumor cells which still remained on the plates, indicating stable adhesions to the coated ECM or HUVEC monolayer, were imaged and quantified. I found that SS promotes tumor cell adhesion to both ECM and endothelial monolayer. I further explored the underlying mechanism and found that SS-promoted tumor cell adhesion is mediated by SS-generated cellular ROS. The activities of FAK are increased after shear stress treatment, which can be suppressed by the application of antioxidants. Although the function of FAK in cell-ECM interaction has been well documented [166, 229], whether

FAK influences tumor cell-endothelium interaction remains unknown. I investigated the roles of FAK in mediating SS-promoted tumor cell adhesion and found that FAK not only determines the adherence of circulated tumor cells to ECM but also influences tumor cell adhesion to endothelium.

In conclusion, I disclosed the effects of SS in up-regulating tumor cell adherence to both endothelium and ECM, which is important for tumor cell extravasation and metastases formation. This finding will advance the understanding of the distant metastasis and provides new insights on how tumor cells response to external stresses. The mechanism study revealed potential therapeutic targets including ROS and FAK for inhibiting tumor metastasis.

4.2 Results

4.2.1 Shear Stress Promotes Tumor Cell Adherence to ECM and Endothelium

Engineered breast cancer MDA-MB-231 (231-C3) cells were introduced into the microfluidic system and circulated under SS of 5, 15 and 30 dynes/cm² for 6 h. After circulation, the cells were immediately collected and re-suspended in fresh DMEM medium. The circulated 231-C3 cells were then seeded in plates coated with either type I collagen or HUVEC-dt monolayer as substrates to support tumor cell adhesion. After 20 min of incubation under 37°C, the 231-C3 cells which failed to form stable adhesion contacts with collagen or HUVEC-dt monolayer would be washed away. The 231-C3 cells can be detected based on their green fluorescence. Images were taken before and after wash, and the numbers of cells were counted and the cell adhesion rates were calculated as the ratio of cell number after wash to cell number before wash.

The results showed that after SS treatment, more 231-C3 cells remained on both the plates coated with collagen and the plates with HUVEC monolayer. For the cells kept under static conditions, there were around 26% of cells remaining in collagen I-coated plates and 39% of cells on HUVEC monolayer. SS5 did not change the percentage of adherent cells on collagen I-coated plates significantly, which was around 24%, and slightly increased the adhesion rate of tumor cells on HUVEC monolayer to 48%. SS15 significantly increased the percentage of adherent cells to 66% for collagen I-coated plates and 82% for HUVEC monolayer (Figure 4-1). Yet, raising SS level from 15 to 30 dynes/cm² increased cell adhesion rates very slightly for both substrates. The numbers of adherent cells are positively correlated with SS levels, which indicates that SS promotes adhesion capacity of 231-C3 cells. Interestingly, for both the static group and SS groups, there were always more cells remaining on HUVEC monolayer

compared to collagen-coated plates after PBS wash, which implies that the adhesion of tumor cells to endothelium may be more stable than adhesion to ECM.

Figure 4-1. Shear stress promotes tumor cell adhesion. (A) Shear stress promotes tumor cell adherence to ECM. 231-C3 cells were circulated under SS5, 15 and 30 for 6 h before seeded in collagen I-coated plates. The cells were washed with PBS after incubation for 20 min. Upper

panel shows representative fluorescent images of cells remaining in plates after PBS wash. Scale bar, 100 μ m. Lower panel is the quantification result of cell adhesion rates. (B) Shear stress promotes tumor cell adherence to endothelial monolayer. HUVEC-dt cells were seeded in 96-well plates and incubated for 24 h to establish intact HUVEC monolayers. 231-C3 cells were circulated under SS5, 15 and 30 for 6 h before seeded on HUVEC monolayers. The cells were washed with PBS after incubation for 20 min. Upper panel shows representative fluorescent images of cells remaining on HUVEC monolayer after PBS wash. Scale bar, 100 μ m. Lower panel is the quantification result of cell adhesion rates.

4.2.2 Shear Stress Promotes Tumor Cell Spreading on ECM

Moreover, I observed that circulated 231-C3 cells exhibited more cell spreading on ECM compared to cells of static group after 20 min of incubation (Figure 4-2), indicating efficient development of cell adhesions to ECM, which is in agreement with the results of cell adhesion assay (Figure 4-1). In contrast, 231-C3 cells of both SS groups and static group remained round-shaped on HUVEC monolayer (Figure 4-3).

Figure 4-2. Shear stress promotes tumor cell spreading on ECM. 231-C3 cells were circulated under SS5, 15 and 30 for 6 h before seeded in collagen I-coated plates. After 20 min of incubation, both phase contrast and fluorescence images were taken to evaluate cell spreading. Scale bar, 100 μ m.

Figure 4-3. Shear stress does not influence tumor cell spreading on endothelial monolayer. HUVEC-dt cells were seeded in 96-well plates and incubated for 24 h to establish intact HUVEC monolayers. 231-C3 cells were circulated under SS5, 15 and 30 for 6 h before seeded on HUVEC monolayers. After 20 min of incubation, both phase contrast and fluorescence images were taken to evaluate cell spreading. HUVEC-dt cells were detected based on red fluorescence and 231-C3 were detected based on green fluorescence. Scale bar, 100 μ m.

I extended the incubation time to 1 h and monitored cell spreading at different time points. For the cells seeded on collagen-coated plates, SS15 group always demonstrated greater cell spreading than the static group (Figure 4-4). However, most of the cells still did not show apparent cell spreading on HUVEC monolayer, in either SS15 group or static group (Figure 4-5). Other studies have also shown similar observations, where

tumor cells spread very slowly on endothelial monolayer [233-235]. The more stable adhesions and less spreading of tumor cells may be caused by involvement of other adhesion molecules, and different mechanisms regulating cytoskeleton organization when tumor cells attach to endothelial monolayer compared to ECM.

Figure 4-4. Observation of tumor cell spreading on ECM at different time points after circulation. 231-C3 cells were circulated under SS15 for 6 h before seeded into collagen I-coated plates. During the incubation, both phase contrast and fluorescence images were taken to evaluate spreading of cells at 20, 40, 60 min. Scale bar, 100 μ m.

Figure 4-5. Observation of tumor cell spreading on endothelial monolayer at different time points after circulation. HUVEC-dt cells were seeded in 96-well plates and incubated for 24 h to establish intact HUVEC monolayers. 231-C3 cells were circulated under SS15 for 6 h before seeded on HUVEC monolayers. During the incubation, both phase contrast and fluorescence images were taken to evaluate spreading of cells at 20, 40, 60 min. HUVEC-dt cells were detected based on red fluorescence and 231-C3 were detected based on green fluorescence. Scale bar, 100 μ m.

4.2.3 The Shear Stress-Promoted Tumor Cell Adhesion is Mediated by ROS

In chapter 3.2.2, I revealed that SS was able to simulate generation of cellular ROS, which worked as the signaling molecule mediating enhanced cell migration [32]. To

explore the role of SS-generated ROS in tumor cell adhesion, I pre-treated the 231-C3 cells with 5 mM NAC or 20 μ M PG for 1 h before circulating the cells under SS15. The results of cell adhesion assay showed that PG decreased adhesion rates of the circulated 231-C3 cells from 69% to 45% on ECM and from 82% to 41% on HUVEC monolayer, while NAC decreased cell adhesion rate to 34% and 39% respectively (Figure 4-6).

Figure 4-6. ROS is indispensable for shear stress-enhanced tumor cell adhesion. (A)

Antioxidants impaired the effects of shear stress on tumor cell adhesion to ECM. 231-C3 cells were circulated under SS15 with or without pre-treatment of antioxidants. The cells were then collected and seeded in collagen I-coated plates. The cells were washed with PBS after 20 min incubation. Upper panel shows representative fluorescent images of cells that remained in plates after PBS wash. Scale bar, 100 μ m. Lower panel is the quantification results of cell adhesion rates. (B) Antioxidants impaired the effects of shear stress on tumor cell adhesion to endothelial monolayer. 231-C3 cells were circulated under SS15 with or without pre-treatment of antioxidants. The cells were then collected and seeded on HUVEC monolayer. The cells were washed with PBS after 20-min incubation. Upper panel shows representative fluorescent images of cells that remained in plates after PBS wash. Scale bar, 100 μ m. Lower panel is the quantification results of cell adhesion rates.

To explore the importance of high levels of ROS in promoting cell adherence, 231-C3 cells were treated with 50 μ M H₂O₂ for 6 h under static conditions. The cells were then collected and subjected to cell adhesion assay. The exogenous ROS significantly increased cell adhesion rates from 21% to 58% on ECM and from 30% to 70% on endothelial monolayer (Figure 4-7). This result is consistent with the hypothesis that excessive ROS generation mediates shear stress-enhanced cell adhesion.

Figure 4-7. Exogenous ROS promotes tumor cell adhesion. (A) Hydrogen peroxide stimulates tumor cell adherence to ECM. 231-C3 cells were seeded in non-attachment 96-well plates with or without 50 μM H_2O_2 for 6 h. The cells were collected and seeded into collagen-I coated plates and washed with PBS. Left panel shows representative images of 231-C3 cells remaining in the plates after PBS wash. Scale bar, 100 μm . Right panel is the quantification result of cell adhesion rates. (B) Hydrogen peroxide stimulates tumor cell adherence to endothelial monolayer. 231-C3 cells were seeded in non-attachment 96-well plates with or without 50 μM H_2O_2 for 6 h. The cells were collected and seeded onto a well-established HUVEC-dt monolayer and washed with PBS. Left panel shows representative images of 231-C3 cells that remained on HUVEC-dt monolayer after PBS wash. Scale bar, 100 μm . Right panel is the quantification result of cell adhesion rates.

4.2.4 Shear Stress Enhances Phosphorylation of FAK

Different components of focal adhesion complex including FAK, Src and paxillin are known targets of ROS, among which FAK is the most studied one. FAK plays important roles in both attachment and detachment of cells by regulating assembly and disassembly of focal adhesions [134, 166]. Several studies have proved that phosphorylation level of FAK is highly associated with adhesion and spreading of cells on ECM [173, 207]. I hypothesized that the SS-generated cellular ROS promotes tumor cell adhesion by regulating the activation of FAK. To determine the activity of FAK, I examined the phosphorylation level of Tyrosine 397 residue in FAK, which is the key phosphorylation site for FAK activation.

231-C3 cells were pre-treated with 5 mM NAC or 20 μ M PG for 1 h before circulation under SS15 for 6 h. The circulated cells were allowed to adhere to collagen I-coated dish for 20 min and the cells were collected for Western blot analysis. Results showed that phosphorylation at Tyrosine 397 of FAK was significantly increased in cells circulated under SS15 compared to cells under static conditions, which was suppressed by the application of PG and NAC (Figure 4-8). Moreover, I treated the 231-C3 cells under static conditions with 50 μ M H₂O₂ for 6 h. The cells were then allowed to attach to collagen I coated dish for 20 min and collected for Western blot analysis. H₂O₂ treatment drastically elevated phosphorylation level of FAK in the cells under static condition (Figure 4-8). Taken together, these results indicated that SS is able to promote FAK activation by elevating cellular ROS level.

Figure 4-8. Shear stress promotes FAK activation through stimulating cellular ROS generation. (A) 231-C3 cells were circulated under SS15 for 6 h with or without pre-treatment of antioxidants. Cells were seeded on ECM for 20 min before being collected and subjected to Western blot analysis. Levels of phosphorylated and total FAK were examined. Upper panel is the representative blot image. Lower panel shows the quantification of phosphorylation level, which is calculated as the ratio of the band intensities of pFAK to the band intensities of total FAK. The results were normalized to static group. GAPDH was used as internal control for equal loading. (B) 231-C3 cells were seeded in non-adhesive 96-well plates with or without H₂O₂. Cells were seeded on ECM for 20 min before being collected and subjected to Western blot analysis. Levels of phosphorylated and total FAK were examined. Upper panel is the representative blot image. Lower panel shows the quantification of phosphorylation level of FAK, which is calculated as the ratio of the band intensities of pFAK to the band intensities of total FAK. The results were normalized to static group. GAPDH was used as internal control for equal loading.

4.2.5 Shear Stress-enhanced Tumor Cell Adhesion is mediated by FAK

To further explore the role of FAK in SS-enhanced cell adhesion to ECM, a well-established inhibitor of FAK, 1,2,4,5-Benzenetetraamine tetrahydrochloride (Y15) was used to suppress the activities of FAK [165, 236]. 231-C3 cells were pre-treated with Y15 at 10 μ M for 1 h before circulation for 6 h. The cells were collected and seeded into 96-well plates coated with collagen I to assess the adhesion capacity. The results of adhesion assay showed that for cells both circulated under SS15 and kept under static conditions, the inhibitor Y15 notably impaired cell attachment to ECM. Specifically, for the cells under static conditions, treatment with Y15 decreased cell adhesion on collagen I-coated plates from 27% to 9%. On the other hand, for the cells circulated under SS15, Y15 decreased cell adhesion on collagen I-coated plates from 68% to 12% (Figure 4-9). For both the circulated under SS15 and the cells kept under static condition, the FAK inhibitor Y15 almost totally blocked cell attachment to ECM, implying the indispensable function of FAK in mediating cell-ECM interaction, as well as the important role of FAK in SS-enhanced cell adhesion. These results verified the hypothesis that SS-promoted tumor cell adhesion to ECM is mediated by, at least partly, the ROS-enhanced FAK activation.

Figure 4-9. Inhibiting FAK activities impaired SS-enhanced tumor cell adhesion on ECM. 231-C3 cells were circulated under SS15 for 6 h with or without pre-treatment of FAK inhibitor Y15 before seeded in plates coated with collagen I. Left panel shows representative fluorescent images of cells remained on ECM after PBS wash. Scale bar, 100 μ m. Right panel is the quantification result of cell adhesion rates.

To further verify the role of FAK in SS-promoted cell adhesion, an siRNA targeting FAK (named as siFAK) was transfected into 231-C3 cells. The cells in negative group were transfected with non-targeting siRNA purchased from Thermo Scientific. The blank control is normal 231-C3 cells without any treatments. Cells were collected at 72 h after transfection for Western blot analysis and the results indicated that the cellular level of FAK was significantly decreased by siFAK (Figure 4-10). The transfected 231-C3 cells were then collected and circulated under SS15 or kept under static conditions for 6 h, before subjected to an adhesion assay. Knockdown of FAK suppressed attachment of 231-C3 cells to ECM in both static and SS15 groups. Specifically, for the cells under static conditions, knockdown of FAK decreased cell adhesion on collagen I-coated plates from 23% to 4%. On the other hand, for the cells circulated under SS15, knockdown of FAK decreased cell adhesion on ECM from 63% to 11%. The effect of SS on adhesion of 231-C3 cells was totally blocked by decreasing FAK level (Figure 4-10). This result is consistent with the previous experiment using FAK inhibitor (Figure 4-9).

A

B

Figure 4-10. Knockdown of FAK inhibits the SS-enhanced tumor cell adhesion on ECM.

(A) 231-C3 cells were collected at 72 h after transfection of siRNAs targeting FAK and lysed for Western blot analysis. The levels of FAK were detected and GAPDH was used as the internal control for equal loading. The siRNA (named as siFAK) significantly decreased total FAK level in 231-C3 cells. (B) 231-C3 cells were collected at 72 h after transfection of siFAK.

The cells were circulated under SS15 for 6 h before seeded in plates coated with collagen I. Upper panel shows representative fluorescent images of cells remained on ECM after PBS wash. Scale bar, 100 μ m. Lower panel is the quantification result of cell adhesion rates.

Although many different types of integrins are found to play vital roles in tumor cell-endothelium interactions [24, 158], the function of focal adhesions in tumor cell adhesion to endothelium is highly neglected. All integrins are associated with cytoskeleton through focal adhesions and the connections of membrane molecules with cytoskeleton are very important for establishing stable cell adhesion to the substrates, including the endothelium [167]. However, there is no direct evidence to support the preposition that focal adhesions are important for tumor cell adhesion to endothelium, in spite of the well-studied functions of integrins in tumor cell-endothelium interaction. As I had previously demonstrated that SS promotes the attachment of breast cancer cells to endothelial monolayer and the activation of FAK, I proposed that the SS-enhanced adhesion to endothelial monolayer was caused by the increased activities of FAK in cells.

To test the hypothesis, I pre-treated 231-C3 cells with Y15 for 1 h before circulation for 6 h. The cell adhesion assay showed that Y15 decreased adhesion to HUVEC-dt monolayer from 34% to 18% for cells under static conditions, and decreased adhesion of circulated cells to HUVEC-dt monolayer from 79% to 25% (Figure 4-11). To verify the finding, the siFAK was transfected into 231-C3 cells to decrease FAK level. The results of adhesion assay indicated that knockdown of FAK suppressed attachment of 231-C3 cells to HUVEC monolayer in both static and SS15 groups (Figure 4-12), which is consistent with the results in Figure 4-11.

These results indicated the importance of FAK in mediating tumor cell adhesion to endothelium, which has never been reported before. Most importantly, I demonstrated that the SS-enhanced tumor cell adhesion to endothelium is highly associated with the SS-induced FAK activation

Figure 4-11. Inhibiting FAK activities impaired SS-enhanced tumor cell adhesion on endothelial monolayer. HUVEC-dt cells were seeded in 96-well plates and incubated for 24 h to establish intact HUVEC monolayers. 231-C3 cells were circulated under SS15 for 6 h with or without pre-treatment of FAK inhibitor Y15 before seeded on HUVEC monolayers. Left panel shows the representative fluorescent images of cells remained on HUVEC monolayer after PBS wash. Scale bar, 100 μ m. Right panel is the quantification results of cell adhesion rate.

Figure 4-12. Knockdown of FAK inhibits the SS-enhanced tumor cell adhesion on HUVEC monolayer. 231-C3 cells were collected at 72 h after transfection of siFAK. The cells were circulated under SS15 for 6 h before seeded in plates with HUVEC monolayer. Upper panel shows representative fluorescent images of cells remained after PBS wash. Scale bar, 100 μ m. Lower panel is the quantification result of cell adhesion rates.

4.3 Discussion

The interaction of tumor cells with ECM in the surrounding microenvironment is of vital importance for survival and growth of tumor cells, especially in the stage of development of metastases [13, 130]. Recent studies reported that the initiation of metastases is a very inefficient process, where most tumor cells are unable to colonize and proliferate in secondary organs, which is determined by the interactions of tumor cells with constituents of the local microenvironment, including ECM components and host stromal cells [14, 94]. Therefore, the efficiency of tumor cell adhesion and spreading on ECM indicates how tumor cells adapted to the local microenvironment in distant organs after extravasation.

This study revealed that shear stress is able to promote tumor cell adhesion and spreading on ECM, which means circulation of tumor cells within the blood stream will assist in their survival and growth at the stage of colonization. I explored the underlying mechanism and identified the shear stress-ROS-FAK-cell adhesion pathway that regulated this effect of shear stress. Moreover, I found that shear stress was able to promote tumor cell adhesion to endothelial monolayer through the same mechanism. Recent studies exploring the interactions between tumor cells and the endothelium mainly focused on the membrane molecules [23, 24], while the importance of membrane protein-cytoskeleton connections is neglected. Integrins of tumor cells have been found to play important roles in the adherence to endothelium. Here, for the first time, I reported that the activities of FAK which is one of the key components of focal adhesion complex are highly associated with the stability of tumor cell-endothelium interactions. The signaling pathway is summarized as in Figure 4-13.

Figure 4-13. Summary of the signaling pathway that regulates SS-enhanced tumor cell adhesion.

The details on how SS-generated ROS enhanced FAK phosphorylation remains unclear, which needs further study. I hypothesize that the action of ROS on FAK activation is mediated by PTPs. The PTPs in tumor cells suppress the activities of FAK through dephosphorylation. PTPs are direct targets of ROS, and their activities are inhibited after oxidization. During circulation in shear flow, the cellular ROS could oxidize PTPs and suppress their activities, and the tumor cells are in a ready-to-adhere status. After the tumor cells attach to ECM, the integrin-ligands interaction triggers the phosphorylation of FAK, the lower levels of PTP activities in circulated tumor cells lead to higher levels of FAK phosphorylation compared to cells without SS treatment.

I previously investigated the functions of MnSOD in the SS-enhanced tumor cell migration. Interestingly, cellular ROS also plays important roles in SS-enhanced tumor

cell adhesion, which implies that MnSOD might regulate adhesion of tumor cells as well. This hypothesis can be verified by knocking down MnSOD in metastatic breast cancer cells. The roles of FAK in SS-mediated tumor cell adherence to endothelial monolayer also need to be further verified.

CHAPTER 5 Conclusions and Future Directions

Exploring the mechanisms that regulate and influence distant cancer metastasis is of vital importance because of the complexity of the process of metastasis and the lack of efficient therapeutic methods targeting metastasis [2, 94]. In this thesis, I studied the effects of hemodynamic shear stress on the progression of metastasis. Circulation of tumor cells within the bloodstream is an indispensable step of distant metastasis, during which tumor cells are exposed to fluidic shear stress. Shear stress has long been regarded as one of the environmental factors which restrict survival of tumor cells and limit the efficiency of tumor progression [6, 20]. Here, my study revealed the pro-metastatic effects of shear stress which had never been reported before.

I mainly focused on the influence of shear stress on cell migration as the migration capacity is an important hallmark for tumor progression. By utilizing an *in vitro* transwell migration assay and a wound healing assay, I disclosed that shear stress was able to promote migration of breast cancer cells. Staining of the circulated cells with different ROS dyes revealed that shear stress stimulates excessive cellular ROS generation, which was later identified as a vital event in regulating the migration of circulated cells. The cellular ROS activated ERK1/2, which then subsequently enhanced cell migration. More importantly, the function of MnSOD in mediating shear stress-promoted cell migration was identified. As an important component of the cellular antioxidant system, MnSOD converts mitochondrial superoxide into hydrogen peroxide, which is released into the cytosol to activate targets including ERK1/2. I also investigated the extravasation of the circulated breast cancer cells. The evaluations of extravasation based on both an *in vitro* transendothelial migration assay and an *in vivo*

zebrafish model showed that shear stress facilitated extravasation by elevating cellular ROS level.

In addition to migration and extravasation, the effects of shear stress on tumor cell adhesion were also explored. The results of *in vitro* cell adhesion assays showed that shear stress was able to promote tumor cell adhesion to ECM, as well as the endothelium. Adherence of tumor cells to ECM represents how efficiently and stably can the tumor cells interact with the surrounding ECM, which is important for tumor cell invasion and colonization in distant organs [13, 141]. On the other hand, establishing stable interactions with endothelial monolayer is essential for tumor cells extravasation [23]. The findings indicate that shear stress not only facilitates extravasation of tumor cells, but also influences the subsequent invasion and colonization. The mechanism study identified the shear stress-ROS-FAK-cell adhesion pathway that regulates SS-promoted cell adhesion to both ECM and endothelial monolayer.

In conclusion, I discovered the promoting-actions of shear stress on migration, extravasation and adhesion of tumor cells and revealed the underlying mechanisms, as summarized in Figure 5-1. The findings identified cellular ROS as important signaling molecules that mediate the effects of shear stress on cell migration and adhesion. The roles of ROS in cancer metastasis are very complicated and still under investigation, which makes the application of antioxidants in cancer therapy a heavily debated issue [179, 221]. There are several studies reporting that ROS limits metastasis [237, 238], but there are also studies providing solid evidences that support the pro-metastasis actions of ROS [239-241]. The controversy over application of antioxidants is due to the fact that ROS may have dual effects on tumor cells [179, 211]. In the studies performed by our group, Dr. Afu Fu had previously shown that SS-stimulated ROS

generation was damaging to tumor cells and induced cell apoptosis [12], while in this thesis, I investigated and examined how the elevated cellular ROS level could up-regulate invasiveness of tumor cells. Taken together, these findings revealed that ROS may function as a double-edge sword for the circulating tumor cells. Interestingly, however, the type of ROS identified as the main culprit for cell death in Dr. Fu's study is different from the type of ROS identified as the key signaling molecule in my study. Shear stress stimulates tumor cells to generate high level of mitochondrial superoxide, which then severely damage mitochondria and induce tumor cell apoptosis. The enzyme MnSOD protects the tumor cells by converting superoxide into hydrogen peroxide, which is much less damaging to cells. Moreover, superoxide is not able to penetrate mitochondrial membranes while hydrogen peroxide can diffuse through membranes freely. This means the hydrogen peroxide, instead of mitochondrial superoxide is responsible for activating kinases in the cytosol including ERK1/2 and FAK.

Figure 5-1. Summary of the signaling pathways that regulate SS-enhanced tumor cell migration, adhesion and extravasation.

The above findings provide new insights on the therapeutic strategies for suppressing metastasis. First, different types of ROS may have different functions on tumor cells which are determined by the nature of the properties and distributions of ROS. Taking Dr. Fu's and my studies as examples, at the stage of tumor cell circulation during metastasis, mitochondrial superoxide kills tumor cells by inducing apoptosis; whereas the less toxic hydrogen peroxide functions to activate different kinases to increase tumor cell invasiveness and assist the cells to exit blood flow. As a result, application of antioxidants that specifically target hydrogen peroxide but do not have much influence on mitochondrial superoxide (e.g. ebselen) can suppress shear stress-promoted tumor cell invasiveness without reducing tumor cell death. On the other hand, the blind use of antioxidants without considering the ROS targets may protect the tumor cells and facilitate metastasis. In other cases when ROS levels in tumor cells are increased, the similar mechanisms may regulate and determine the death or the enhanced aggressiveness of tumor cells. Thus, a better understanding of the existing forms and distributions of cellular ROS in tumor cells has significant meanings.

Second, instead of targeting a specific type of ROS, modulating the cellular antioxidant system could be another efficient way to suppress metastasis. By converting the damaging molecule superoxide into signaling molecule hydrogen peroxide, MnSOD not only protects the tumor cells under shear stress but also regulates the pro-metastatic effects of shear stress. Interestingly, compared to non-metastatic cancer cell lines, the metastatic cancer cell lines usually have higher levels of MnSOD [12], which makes them more resistant to shear stress and guarantees them enhanced aggressiveness

after surviving the circulation. MnSOD, as a component of cellular antioxidant system can function both as a prognosis marker and a therapeutic target. Decreasing the expression or inhibiting the function of MnSOD could facilitate SS-mediated cell death, as well as inhibit SS-promoted cell migration and extravasation.

Finally, a comprehensive understanding of the mechanisms regulating the enhanced aggressiveness of circulating tumor cells would provide different potential therapeutic targets. In addition to ROS and MnSOD, the kinases ERK1/2 and FAK can also be applied as the targets for suppressing metastasis. As the seeds for distant metastases, elimination of CTCs during circulation and inhibiting the extravasation of CTCs can be two efficient ways to suppress metastasis.

There are still limitations of this study, which requires future investigations. The role of MnSOD in regulating SS-promoted tumor cell migration was examined by knocking down MnSOD. Although two different siRNAs were used to exclude the off-target effect, overexpression of MnSOD in breast cancer cells, especially non-metastatic cells, is still needed to further validate the function of MnSOD. What's more, as both migration and adhesion of circulated tumor cells were regulated by ROS and distribution of ROS was regulated MnSOD, I suspected that MnSOD may also influence SS-promoted tumor cell adhesion. This was not examined in the current study and needs further investigation to give more comprehensive understandings of the mechanism regulating cellular responses to SS. For the *in vivo* studies, although zebrafish is a preferred animal model for studying extravasation, mammalian models are more widely used for verifying findings *in vitro*. As a result, I will consider using mouse models in the future.

In addition to the future work for completeness and perfection of the project, my current study raised some interesting topics which can be further explored.

Chemokines play important roles in cancer metastasis by interacting with chemokine receptors of tumor cells and triggering downstream pathways [242]. Different types of chemokines, including SDF-1 α and CCL21, are found to regulate tumor cell adhesion, migration, extravasation and survival. Some studies exploring responses of tumor cells to chemokines under flow conditions generated interesting findings which is highly associated with and supportive of the results in this thesis [243, 244]. For example, Amanda P. Struckhoff found that SDF-1 α -induced adhesion of MCF7 cells is more pronounced under flow conditions compared with static conditions [244]. The effects of shear stress on chemokine response of tumor cells were not studied in this thesis. Investigation of how shear stress influences chemokine actions, such as chemokine-directed cell migration, or chemotaxis, would be essential for a full understanding of pro-metastasis action of hemodynamic shear stress.

Cellular ROS is important in signaling transduction. Many kinases including Src, Akt, and JNK can be activated by ROS. The activities of these kinases can be easily detected through Western blot analysis and are important in different cellular functions. As the shear stress is able to stimulate cellular ROS generation, it is possible that shear stress could promote activations of other different types of kinases, in addition to ERK1/2 and FAK. Exploring the effects of shear stress on these kinases and the related downstream pathways would be interesting and important.

There are other potential future studies based on this thesis. The SS-enhanced phosphorylation of ERK1/2 and FAK could possibly assist tumor cells in surviving circulation, as both the kinases are found to regulate tumor cell survival; further investigations on the role of FAK in tumor cell attachment to endothelium may advance our understandings of the dynamics of tumor cell-endothelium interactions; the cellular

antioxidant system may play functions in tumor cell response to other external stresses, which could also be studied.

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