THE REGULATION OF AUTOPHAGY IN YAP MECHANOTRANSDUCTION AND BREAST CANCER METASTASIS

THE REGULATION OF AUTOPHAGY IN YAP MECHANOTRANSDUCTION AND BREAST CANCER METASTASIS

By

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A Thesis Submitted to the Department of Mechanical Engineering and the School of Graduate Studies of McMaster University in Partial Fulfilment of the Requirements for the Degree of Master of Applied Science

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Master of Applied Science (2021) McMaster University

Department of Mechanical Engineering Hamilton, Ontario, Canada

TITLE: The Regulation of Autophagy in YAP Mechanotransduction and Breast Cancer Metastasis

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NUMBER OF PAGES: xvi, 93

Lay Abstract

Breast cancer is the most common cause of cancer-related death in women because it is challenging to develop effective treatment for breast cancer cells that often spread to another organ or tissue. Breakdown of cellular components and responses to microenvironment stiffness impact the migration of breast cancer cells. In addition, a critical molecule called YAP, controls cell degradation and can sense and transduce the microenvironmental signals. However, the interplay of the two factors on breast cancer cell migration is not well known. During this study, we examined the effect of the two factors on the migration of breast cancer cells individually and simultaneously. We concluded that the responses to microenvironment stiffness and cellular degradation together regulate YAP activity to affect cancer cell migration. Further investigation in this area will shed light on future therapeutics targeting the spreading of breast cancer.

Abstract

Breast cancer metastasis of a variety of vital organs is a major cause of breast cancer mortality. Autophagy has a crucial role in the metastatic breast cancer progression. As a critical mechanotransducer in the Hippo signalling pathway, YAP regulates cell proliferation and promotes autophagy. Previous publications also demonstrated extracellular matrix could regulate the nucleo-cytoplasmic transport of YAP. However, how YAP signalling connects to the interplay of autophagy and mechanotransduction in breast cancer metastasis remains entirely unknown. Through rapamycin-induced autophagy on the metastatic triple negative breast cancer (TNBC) cells, we observed upregulated YAP transcriptional activity and YAP nuclear localization in TNBC. Thus, we reported that YAP nuclear localization regulates autophagy to promote TNBC metastasis. Culturing TNBC cells on PDMS plates with various matrix stiffness demonstrated that stiff matrix promoted the migration of metastatic breast cancer cells in a YAP-dependent mechanism. Therefore, we proposed that YAP mechanotransduction promotes the migration of metastatic breast cancer cells. Then, we advance in these directions by reporting autophagy-mediated YAP nuclear localization is regulated by the response to stiff matrix when TNBC cells were cultured on different matrix stiffness upon autophagy. In conclusion, we suggest autophagy and mechanotransduction mediates YAP nuclear localization together. These findings expand the unknown gap in the convergence of YAP mechanotransduction and autophagy in metastatic breast cancer. They suggest that metastatic breast cancer cells have the potential to

exhibit different YAP signalling when they colonize on a secondary location with a distinct matrix stiffness from primary location. Our study further helped to understand YAP biology and the mechanism of breast cancer metastasis that will shed light on future YAP-targeting therapeutics for metastatic breast cancer.

Acknowledgements

First and foremost, I want to thank my supervisor, Dr. Fei Geng, for his mentorship. It has been an honour to be his master student. I am grateful for his understanding and willingness to listen to my concerns and opinions, although I know some have been very overwhelming. I appreciate his enthusiasm for his research, and all his contributions of time, ideas, funding, and the encouragement to attend conferences, to make my M.A.Sc. experience valuable and exciting and has shaped my personality as a researcher.

The members of the Geng group have contributed immensely to my personal and professional time at McMaster. The group has been a source of friendships as well as helpful advice and collaboration. I am incredibly grateful for Arjun, who worked on the cancer research project together. I very much appreciated his funniness and willingness to cover my "cell-sitting" when I am not available. I am also grateful for Sean Park, who has sat in the lab for hours with me to prepare PDMS substrate. I am also thankful for other past and present group members that I have had the pleasure to work with: Saeed Mohammadi, Yuxin Bai, and Chrishma Patel, and other capstone students who have come through the lab. I would also like to thank Alireza Shahin-Shamsabadi, for his kind advice and guidance on experiments.

The journey was made more delightful because of the many friends that became a part of my life. I am grateful for the time spent with my roommate Jennifer, and I loved the time we talk about daily tasks and items in scientific terms. Although my

study happened during the midst of the pandemic, I am grateful to my friends Kitty, Kelly, Carmen, and Josephine for their long-distance calls to make my journey cheerful.

Lastly, I would like to thank my family for all their love and encouragement throughout my life. For my parents who raised me with a passion for science and supported me in all my pursuits. For the curiosity of my sister Queenie and Brother Adrian constantly asking me for scientific proof. Most importantly, for the presence of my fiancé William, who supported me during every stage of this M.A.Sc. journey.

Thank you all.

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List of Tables and Figures

List of Abbreviations and Definitions

ANKRD1	Ankyrin repeat domain 1
ATCC	American Type Culture Collection
BC	Breast cancer
BSA	Bovine serum albumin
CNS	Central nervous system
CTC	Circulating tumour cell
CTCF	Corrected total cell fluorescence
CTGF	Connective tissue growth factor
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DCIS	Ductal carcinoma *in situ*
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FA	Focal adhesion
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HER2	Human epidermal growth factor receptor 2
LATS	Large tumor suppressor
LATS1/2	Large tumour suppressor kinases 1/2
LC3	Microtubule-associated protein 1A/1B-light chain 3
MBC	Metastatic breast cancer
MCF-7	Michigan Cancer Foundation-7, a breast cancer cell line
MRE	Magnetic resonance elastography
MST1/2	Mammalian Ste20-like kinases 1/2
mTOR	Mammalian target of rapamycin
N/C	Nuclear-cytoplasmic
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
PE	Phosphatidylethanolamine
PR	Progesterone receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SFM	Scanning force microscopy

SRE	Skeletal-related event
TAZ	Transcriptional coactivator with PDZ-binding motif
TEAD	Transcriptional enhanced associate domain
TNBC	Triple-negative breast cancer
YAP	Yes-associated protein

Declaration of Academic Achievement

Wei Chen contributed to the writing, experimental design, literature research, conducting of experiments, data analysis, interpretation of results, and figure generation for all chapters of this thesis.

Fei Geng contributed to experimental design, writing, interpretation of results, and revision of the manuscripts for all chapters of this thesis.

Lidan You contributed to experimental design and revision of the manuscript for chapter 3.

Chrishma Patel and Yuxin Bai contributed to performance of experiments in chapter 2 and chapter 3.

Arjun Raha and Saeed Mohammadi contributed to analysis and interpretation of the data for chapter 3.

Shihyun Park contributed to reagent and material making and conducting experiments in chapter 3.

Karim Henary contributed to performance of experiments in chapter 3.

Chapter 1

Introduction

1.1 Breast Cancer Metastasis

Breast cancer is the most prevalent female cancer globally and remains the leading cause of cancer-related deaths despite many potential preventive measures and new approaches to developing effective therapies (Jemal et al., 2009). Tumour forms when there is a build-up of cancer cells in the body, and metastasis happens when the malignant or cancerous tumours migrate and damage other areas of the body, such as the brain and the bone (Parsa et al., 2016). Breast cancer occurs when malignant tumours develop from the breast cells and can be further classified by their ability to metastasize to other tissues, the tumour's site of origin, the stroma surrounding the gland, and its ability to develop in the gland lumen (Kim & Myung, 2018). Therefore, in this thesis, we try to study the matrix stiffness effect on secondary metastasis site for breast cancer cells.

It is crucial to develop a deeper understanding of the breast cancer metastasis process that can potentially lead to the development of new therapeutic targets and biomarkers with the ultimate goal of preventing metastatic disease.

1.1.1 Triple Negative Breast Cancer

Clinically, breast cancer can be divided into distinct subtypes that have prognostic and therapeutic implications (Chavez, Garimella, & Lipkowitz, 2010). Breast cancer cells have receptors on their surface and in the cytoplasm and the nucleus. Thus, breast cancer patients routinely screen for the expression of three significant markers: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Chavez et al., 2010; Kim & Myung, 2018). Breast cancer tumours that do not express ER, PR, and HER2 markers are referred to as triple-negative breast cancer (TNBC) and are one of the clinically most aggressive subtypes (Chavez et al., 2010). TNBC represents approximately 10-15% of all breast cancers, and TNBC patients have an early relapse and poor outcome compared to other subtypes of breast cancer (Anders & Carey, 2009; Chavez et al., 2010). A high migratory behaviour associated with an increased metastatic spread is observed by many TNBC cell lines (Koedoot et al., 2019).

1.1.2 Metastasis Mechanism

Metastasis is a complex and multi-step process that can be divided into a series of stages: local invasion, intravasation, survival in the circulation, extravasation, survival at a second site, and outgrowth at a second site (E. E. Mowers, Sharifi, & Macleod, 2017). At the primary tumour site, metastasis begins with the invasion of tumour cells into the surrounding host tissue through the induction of epithelial-to-mesenchymal transition (EMT) (Lambert, Pattabiraman, & Weinberg, 2017; Erin E. Mowers, Sharifi, & Macleod, 2018). Next, the tumour cells continue to invade and intravasate into the blood or lymphatic vessels, where they spread through the

bloodstream or lymphatic vessels to distant sites (Scully, Bay, Yip, & Yu, 2012). This is followed by selective pressure on escaped tumour cells, where they undergo cell cycle arrest within the target site before extravasating into and proliferate within the secondary site (Erin E. Mowers et al., 2018). While undergoing the cascade, each step of the cascade involves the physical translocation of cancer cells to new microenvironments where the tumour cells must evade the host's immune responses and apoptotic signals and survive altered microenvironments in order to colonize successfully (E. E. Mowers et al., 2017). Besides locoregional relapse, breast cancer metastasizes preferentially to the bone, brain, liver, lung, and distant lymph nodes (W. Chen, Hoffmann, Liu, & Liu, 2018; Medeiros & Allan, 2019; Weigelt, Peterse, & van't Veer, 2005). Metastasis accounts for 90% of breast cancer mortality (Chaffer & Weinberg, 2011; Lambert et al., 2017); however, the specific mechanism in metastatic breast cancer migration remains largely unclear. Accordingly, it is critical to understand the cellular mechanism underlining metastatic breast cancer diseases.

1.2 Molecular Signalling of YAP/TAZ Pathway

The transcriptional coactivators, YAP (Yes-associated protein) and TAZ (Transcriptional coactivator with PDZ-binding motif) are the major downstream effectors of the Hippo signalling pathway. The Hippo signalling pathway is involved in controlling organ size and tumorigenesis, therefore, it is significant in studying human cancer (Moroishi, Hansen, & Guan, 2015; Piccolo, Dupont, & Cordenonsi, 2014; B. Zhao, Li, Lei, & Guan, 2010).

As the critical molecules of the Hippo pathway, YAP/TAZ shuttles between the cytoplasm and nucleus, where they regulate gene transcription through translocation (Piccolo et al., 2014). Upon the activation of the Hippo pathway, multiple upstream signals regulate the mammalian Ste20-like kinases 1/2 (MST1/2) where it phosphorylates and activates large tumour suppressor kinases 1/2 (LATS1/2) and result in the phosphorylation of YAP/TAZ (Meng, Moroishi, & Guan, 2016; Piccolo et al., 2014). Upon the inactivation of the Hippo pathway, the LATS1/2 kinases are inhibited and do not phosphorylate YAP/TAZ. Subsequently, YAP/TAZ translocate into the nucleus to bind the TEAD transcription factor family (TEAD1-4) and mediate target gene expressions such as connective tissue growth factor (CTGF) and Ankyrin Repeat Domain 1 (ANKRD1) that are involved in cell proliferation, survival, and migration (Boopathy & Hong, 2019; Meng et al., 2016). It is known that LATS-induced phosphorylation of YAP at Ser127 (pYAPSer127) is one of the primary inputs for YAP/TAZ subcellular localization as it promotes YAP nuclear exclusion, cytoplasmic retention, or proteasomal degradation (Meng et al., 2016; Piccolo et al., 2014; B. Zhao et al., 2010). Thus, the nuclear-to-cytoplasm (N/C) ratio is often used to study the nucleocytoplasmic transport for the diagnostic of YAP activity through immunofluorescent staining (Elosegui-Artola, Andreu, Beedle, Navajas, & Garcia-Manyes, 2017; Haka et al., 2005; Sero et al., 2015; Totaro et al., 2019).

1.2.1 YAP and Metastasis

It is previously known that knockdown of YAP/TAZ in MCF-7 and Hs 578T breast cancer cells reduces cell migration and invasion, indicating YAP/TAZ plays a critical role in the migration, invasion, and tumorigenesis of breast cancer cells (Chan et al., 2008). YAP/TAZ hyperactivation and nuclear localization are often reported in many human cancers, including breast cancers (Harvey, Zhang, & Thomas, 2013; Johnson & Halder, 2014; Kim & Myung, 2018). Thus, inhibition of YAP/TAZ at multiple levels may be a novel therapeutic target for breast cancer.

1.3 Autophagy

Autophagy (also known as macroautophagy) is a highly conserved self-degradative mechanism that has a key role in homeostasis by targeting cellular contents to the lysosomal compartment for degradation (Galluzzi, Bravo-San Pedro, Levine, Green, & Kroemer, 2017; Levine, 2007; E. E. Mowers et al., 2017). During this process, the formation of double-membrane-bound autophagosomes engulf the intracellular proteins and organelles, then the subsequent fusion of autophagosomes with lysosomes promotes the breakdown of autophagosomal contents (Klionsky et al., 2016; Liang et al., 2014; Sharifi, Mowers, Drake, & Macleod, 2015). To study autophagy, the autophagy marker, microtubule-associated protein 1A/1B-light chain 3 (LC3), is often focused for the experimental visualization of autophagosomes and measurement of autophagic flux (Klionsky et al., 2016; Sharifi et al., 2015). Upon the initiation of autophagy, the cytoplasmic LC3-I is conjugated to phosphatidylethanolamine (PE) to form LC3-II and then recruits to the autophagosome membranes. The recruitment of LC3-II helps with the

elongation and fusion of autophagosomes, where the degradation of the autophagic cargo also results in LC3-II degradation. Thus, fluorescently tagged exogenous LC3 or immunofluorescence for endogenous LC3 is a good marker to be examined for the identification of autophagosomes by fluorescence microscopy (Klionsky et al., 2016; Sharifi et al., 2015; D. Wang et al., 2020).

The mammalian target of rapamycin (mTOR) signalling is often upregulated in cancer and is a crucial player in controlling cell growth, proliferation, and survival (Ballou & Lin, 2008). There are two distinct signalling complexes, the mTOR complex 1 (mTORC1) and mTORC2. Research has shown that mTOR signalling is often highly active in breast cancer and mTOR complexes activation leads to increased tumour progression, increased protein synthesis, and increased inhibition of autophagy (Hare & Harvey, 2017). Specifically, the activation of mTORC1 has prominent autophagy-suppressing functions, thereby promotes cell growth (Galluzzi et al., 2017; Laplante & Sabatini, 2012). It is known that rapamycin can be used to pharmacologically induce autophagy through inhibition of negative regulators such as mTORC1 signalling (Levine & Kroemer, 2008; Rubinsztein, Gestwicki, Murphy, & Klionsky, 2007). Autophagy is strongly associated with cancer as it is involved in several tumour progression stages, including tumorigenesis, progression, and tumour maintenance, making autophagy an interesting target for therapeutics (Li, He, & Ma, 2020).

1.3.1 Autophagy and YAP Signaling

As a critical molecule in the Hippo signalling pathway, YAP promotes autophagic flux to regulate cell proliferation (Pavel et al., 2018). Double YAP/TAZ knockdown treatment inhibited autophagosome formation suggesting YAP/TAZ signalling regulates autophagy (Pavel et al., 2018). As autophagy acts downstream of YAP/TAZ, YAP/TAZ transcriptionally regulates autophagic flux by regulating the degradation of autophagosomes into autolysosomes (Pavel et al., 2018; Totaro, Panciera, & Piccolo, 2018). Furthermore, YAP knockdown inhibited autophagic flux in breast cancer cells. However, YAP increased autolysosome degradation to enhance cellular autophagic flux to protect breast cancer cells upon nutrient deprivation-induced apoptosis (Song et al., 2015).

Nevertheless, other studies have shown that autophagy is another process to affect YAP subcellular localization. Research has shown that YAP is an autophagy substrate, and YAP can be degraded by autophagy (Lee et al., 2018; P. Wang et al., 2019). It was shown that knockdown of the autophagy-related proteins Atg7 displayed autophagy inhibition and increased nuclear YAP localization and YAP protein level (Lee et al., 2018). Similarly, Wang et al. reported that the blockage of autophagy increases YAP expression and YAP target genes (P. Wang et al., 2019). This relationship between autophagy and YAP is further complicated due to YAP has been shown to function as a potential oncogene, and its activity enhances the metastatic potential of breast cancer (Lamar et al., 2012; T. Wang et al., 2018).

1.3.2 Autophagy and Cancer Metastasis

Autophagy is often upregulated in metastatic progression of multiple human cancers, including breast cancer, and the expression of autophagy markers is associated with poor prognosis (Erin E. Mowers et al., 2018). Various studies using surrogate markers have identified increased autophagy has an association with metastasis. For example, it has been shown that increased LC3-II was associated with proliferation and lymph node metastasis and was correlated with reduced survival in TNBC (Lazova et al., 2012; H. Zhao et al., 2013).

On the other hand, another study using human breast ductal carcinoma *in situ* (DCIS) reported elevated autophagy is associated with increased migration and invasion capabilities (E. E. Mowers et al., 2017). Autophagy increases as tumour cells progress to invasiveness, which is directly linked to cell migration, invasion, and EMT (E. E. Mowers et al., 2017). It has been demonstrated that autophagy promotes cell spreading, migration, and invasion of highly metastatic TNBC cells and that the focal adhesion (FA) protein paxillin is degraded by autophagy through interaction with LC3 to promote FA disassembly (Sharifi et al., 2016). Although autophagy has been identified as a critical mechanism in the progression of TNBC, the specific molecular mechanism underlying autophagy-regulated metastatic progression remains unclarified. Therefore, in Chapter 2, we want to understand the mechanism and function of autophagy regulation through YAP signalling in the context of cancer metastasis, and we discovered that autophagy promotes TNBC metastasis through YAP nuclear localization.

1.4 Mechanotransduction and Matrix Stiffness

Mechanotransduction is a process of how cells sense, interpret, and respond to mechanical stimulus and convert it into biochemical signals to elicit specific cellular responses (Hoffman, Grashoff, & Schwartz, 2011; Luis Alonso & Goldmann, 2016). The mechanotransduction pathway can be broken down into three stages: the transmission of applied load to specialized structures called mechanotransmission, the transduction of forces into biochemically detectable signal called mechanosensing, and the subsequent cellular response called mechanoresponse (Luis Alonso & Goldmann, 2016). Mechanotransduction triggers biochemical responses in the cells to allow cells to sense and adapt to external forces and physical constraints in the microenvironments, leading to gene expression, protein synthesis, and cellular phenotype change (C. S. Chen, 2008; Hoffman et al., 2011; Luis Alonso & Goldmann, 2016).

1.4.1 Matrix Stiffness and Metastasis

Cells within tissues are surrounded by a complex microenvironment that includes the composition of extracellular matrix (ECM). Cells are mechanically integrated structures in which the ECM and actin cytoskeleton are connected by integrins and mechanosensitive FA proteins where mechanical stimuli can be applied directly through the ECM or transmitted through the cytoskeleton to mediate cellular response (Hoffman et al., 2011).

Tissue matrix stiffness regulates many biological and cellular processes, including cell motility, proliferation, and differentiation (Discher, Janmey, & Wang, 2005; Jansen, Atherton, & Ballestrem, 2017). In particular, the mechanical stiffness

of the ECM profoundly influences cell behaviour (Dupont, 2016). Similarly, it has been reported that tumour progression and invasion are promoted by changes in the ECM stiffening (Levental et al., 2009). ECM stiffening is not only associated with breast tumour development; it is also essential to sustain cancer proliferation and malignant behaviours such as local invasiveness and migration, which ultimately promotes metastasis (Acerbi et al., 2015). ECM stiffness is considered as one of the major factors regulating cancer progression and cancer malignancy (McConnell et al., 2016; Provenzano et al., 2008).

The knowledge of the mechanical properties of breast tissue is critical for primary breast cancer diagnosis. A review of the biomechanical properties of breast tissue reported a wide variation in stiffness properties within the tissue type (Ramião et al., 2016). Using the method of mechanical testing of *ex vivo* breast tissue, different studies showed a variation in moduli in which the normal glandular tissue is 0.73 ± 0.18 kPa to 66 ± 17 kPa and the ductal carcinoma *in situ* (DCIS) tumour tissue is 3.5 ± 0.5 kPa to 2162 kPa (Ramião et al., 2016). On the other hand, the *in vivo* magnetic resonance elastography method for breast tissue showed the modulus of normal glandular tissue is 0.90 ± 0.18 kPa to 45 kPa and 3.1 ± 0.7 kPa to 75 kPa for tumour tissue (Ramião et al., 2016).

As mentioned previously, breast cancer metastasis to the secondary site often occurs at the bone, brain, liver, lung and distant lymph nodes, with the bone and the brain being the most common sites (W. Chen et al., 2018; Medeiros & Allan, 2019). However, breast cancer metastasis is highly inefficient because less than 0.01% of

circulating tumour cells (CTCs) will eventually form secondary tumours (Langley & Fidler, 2011). The metastatic process is determined by a complex series of interactions between breast cancer cells and metastatic sites, and thus, it is essential to understand the mechanisms involved in this complicated dialogue.

1.4.2 Breast Cancer Bone Metastasis

Bone metastasis accounts for 70% of breast cancer metastasis (W. Chen et al., 2018) and contributes to significant morbidity due to the development of pain and skeletal-related events (SREs) (Tahara, Brewer, Theriault, & Ueno, 2019). Breast cancer metastasis to bone promotes bone degradation and deregulates normal bone remodelling, leading to bone loss (Brook, Brook, Dharmarajan, Dass, & Chan, 2018; W. Chen et al., 2018). Metastatic breast cancer cells extravasate from capillaries to the bone marrow and produce bone cell-like factors, which promote their adhesion, proliferation, and survival in the bone microenvironment (W. Chen et al., 2018). The ability of breast cancer cells to produce bone cell-like properties facilitates breast cancer cell survival and colonization within the bone microenvironment, thus, promoting bone metastasis (Brook et al., 2018). In addition, the medial overall survival rate from bone metastasis diagnosis is 40 months in breast cancer patients (Kuchuk et al., 2013).

Since bone metastasis is very common in metastatic breast cancer patients, it is important to know the physiological stiffness of the tissue for *in vitro* model. A study examined the specimen between 35 to 92 years of age reported the elastic

moduli of human femoral bone was 15.2 GPa at the age of 35 and fell by 2.3% every ten years (Zioupos & Currey, 1998).

1.4.3 Breast Cancer Brain Metastasis

There are 10 – 30% of metastatic breast cancer patients develop brain metastases (W. Chen et al., 2018), where the prognosis of TNBC patients was poorest with a median survival rate of six months (Martin et al., 2017). Brain metastasis forms by the CTCs breaching the blood-brain barrier to invade the local microenvironment for survival and colonization (W. Chen et al., 2018).

In studies using magnetic resonance elastography (MRE) to visualize and quantify mechanical properties of human brain tissues, the shear moduli were measured to be in the range of 1-3 kPa (Green, Bilston, & Sinkus, 2008; Sack, Beierbach, Hamhaber, Klatt, & Braun, 2008), which translate to effective elastic moduli of 400 – 1200 Pa (Christ et al., 2010). A more recent study used MRE to identify the shear stiffness of different brain tissue regions *in vivo* in adults and adolescents. It was reported that the shear moduli range from 0.188 ± 0.032 kPa in the hippocampus to 0.286 ± 0.050 kPa cerebellum in adolescents, and 0.187 ± 0.012 kPa ± in the hippocampus to 0.271 ± 0.039 kPa occipital lobe in adults (McIlvain, Schwarb, Cohen, Telzer, & Johnson, 2018).

1.4.4 Mechanotransduction and YAP

In mammalian cell systems, the YAP/TAZ activity and their nucleo-cytoplasmic shuttling are found to be regulated by the ECM elasticity and cell geometry (Dupont et al., 2011). Monitoring YAP/TAZ transcriptional activity in human Mammary Epithelial Cells (MEC) and MDA-MB-231 cells grown on

fibronectin-coated acrylamide hydrogels of various stiffness showed YAP/TAZ activity and its subcellular localization was regulated by ECM stiffness (Dupont et al., 2011). When cells grow on stiff ECM substrate, they develop cytoskeletal tension by pulling on ECM, allowing the maturation of cell-substrate adhesions and the development of stress-fibres. Subsequently, YAP/TAZ transcriptional coactivators accumulate in the nucleus to bind to the TEAD family to regulate gene transcription, promote cell proliferation, and inhibit differentiation, enabling cells to behave according to the mechanical microenvironment. In conditions where cells are cultured on soft ECM, there was no development of cytoskeletal tension and have reduced or no stress-fibres. Thus, YAP/TAZ delocalize into the cytoplasm, and the activity is inhibited with proteasomal degradation (Dupont et al., 2011; Piccolo et al., 2014). Moreover, cells stretched over a large ECM adhesive area induced YAP translocation from the nucleus to the cytoplasm (Dupont et al., 2011; Piccolo et al., 2014). Furthermore, experiments comparing HEK293A cells gene expression profiles on stiff versus soft substrates revealed that Hippo signalling and YAP/TAZ play a central role in transcriptional regulation in response to different substrate stiffness (Meng et al., 2018).

1.4.5 Modelling Matrix Stiffness to Observe YAP Mechanotransduction

Breast cancer cells seeded on hydrogel or polydimethylsiloxane (PDMS) are often used as an *in vitro* model system to study the connection between ECM stiffness and YAP/TAZ. YAP/TAZ are nuclear and active when cells proliferate and display invasive phenotypes on a stiff ECM substrate; however, when cells cannot grow and be stretched on soft ECM substrate, the cells take on a rounded

shape and YAP/TAZ is cytoplasmic and inhibited (Dupont, 2016). Therefore, ECM stiffness induces phenotypic changes in breast cancer, cell proliferation, and migration through the modulation of YAP signalling, which then enhances cancer metastasis (Haga, Irahara, Kobayashi, Nakagaki, & Kawabata, 2005; Ishihara et al., 2013; Paszek et al., 2005; Umesh, Rape, Ulrich, & Kumar, 2014; Zanconato, Cordenonsi, & Piccolo, 2016).

A previous study reported metastatic process is determined by the interactions between breast cancer cells and metastatic sites (Langley & Fidler, 2011). However, the role of YAP mechanotransduction in the interplay between breast cancer and matrix stiffness on secondary metastatic site is not well known. Chapter 3 examined different matrix stiffness to mimic different tissue stiffness and demonstrated that the stiff matrix promotes the migration of metastatic breast cancer cells in a YAP-dependent mechanism.

1.5 Unknown Interplay between Autophagy and Mechanotransduction in Cancer Metastasis

1.5.1 Connection between Autophagy and YAP

Numerous studies have concluded that YAP/TAZ signalling regulates autophagic flux to control cell proliferation, differentiation, and transformation (Pavel et al., 2018; Song et al., 2015; Totaro et al., 2018). Concurrently, autophagy blockage can also increase the expression of YAP and YAP target genes (P. Wang et al., 2019). The relationship between autophagy and YAP is further eluded due to YAP is an oncogene and can enhance the metastatic potential of breast cancer (Lamar et al., 2012; T. Wang et al., 2018).

1.5.2 Connection between YAP and Matrix Stiffness

The Piccolo group first demonstrated that increased ECM stiffness promotes YAP/TAZ nuclear localization and upregulates YAP/TAZ target genes (Dupont et al., 2011). Emerging evidence showed mechanoregulation of YAP/TAZ plays a pivotal role in various human disease progressions (Cai, Wang, & Meng, 2021). To date, various studies have observed that stiff matrix substrates activate YAP/TAZ to increase primary cancer cell growth, migration, and chemotherapy resistance (Levental et al., 2009; Meng et al., 2018; Qin et al., 2020). It is hypothesized that the hyperactivation of YAP/TAZ in cancer cells is required to overdrive the mechanical checkpoints for growth (Aragona et al., 2013). However, our mechanistic understanding of how ECM stiffness regulates YAP/TAZ remain fragmentary as emerging players have been reported in various context (Cai et al., 2021).

1.5.3 Connection between Autophagy and Mechanotransduction

Nonetheless, current literature remains inadequate to understand how microenvironmental signals control autophagy in different cell types. Totaro et al. reported YAP/TAZ transcriptionally controls autophagy by regulating the fusion of autophagosomes with lysosomes (Totaro et al., 2019). Then, they seeded MII-GFP-LC3 cells on soft (2.0 kPa) and stiff (40 kPa) fibronectin-coated acrylamide hydrogels and observed increased GFP-LC3 puncta in the cells on soft hydrogels, which is similar to the effect of YAP/TAZ knockdown, suggesting autophagy is mechanically regulated. Subsequently, they seeded MII-GFP-LC3 cells infected with a doxycycline-inducible lentiviral vector expressing an activated YAP

(YAP5SA) on soft hydrogels and observed no GFP-LC3 puncta accumulation in the cells. Taken together, their research indicated that cell mechanics regulate autophagic flux through the regulation of YAP/TAZ transcriptional activity (Totaro et al., 2019).

Indeed, many evidence demonstrates the association between YAP signalling and autophagy and between YAP signalling and mechanotransduction. However, there is limited research investigating the interacting effect of autophagy and mechanotransduction through YAP signalling in cancer metastasis. The regulatory mechanism of the combined effect remains largely unknown; therefore, in Chapter 4, we aim to study the intermixing effect of autophagy and mechanotransduction in breast cancer metastasis.

1.6 Summary

The knowledge on the interplay between autophagy and YAP mechanotransduction in breast cancer metastasis remains entirely unknown. Piccolo et al. were among the first groups to demonstrate the YAP/TAZ nucleo-cytoplasmic transport regulates gene transcription. It is known that LATS-induced phosphorylation of YAP leads to YAP nuclear exclusion, cytoplasmic retention, or proteasomal degradation and is one of the primary inputs for YAP/TAZ subcellular localization (Figure 1A). Since autophagy is strongly associated with cancer, the role of autophagy in association with YAP signalling and cancer metastasis was previously demonstrated. Various studies have demonstrated the positive association between autophagy and metastasis, and simultaneously, YAP plays a

critical role in cancer progression and metastasis (Figure 1B). However, the upstream signal of YAP activation and the downstream effects of YAP activation on TNBC remained ambiguous. In addition, numerous studies reported that ECM stiffness enhances cancer metastasis through the modulation of YAP signalling (Figure 1C). Despite the emerging efforts that have been made over the years to investigate how YAP mechanotransduction and autophagy are involved in cancer metastasis, the combined effect of the stimuli was incompletely understood. Here, we propose that autophagy (Figure 2A) or stiff matrix substrate (Figure 2B) promotes breast cancer metastasis through YAP nuclear localization, and that both autophagy and matrix stiffness play a synergic role to enhance metastasis through YAP nuclear localization (Figure 2C).

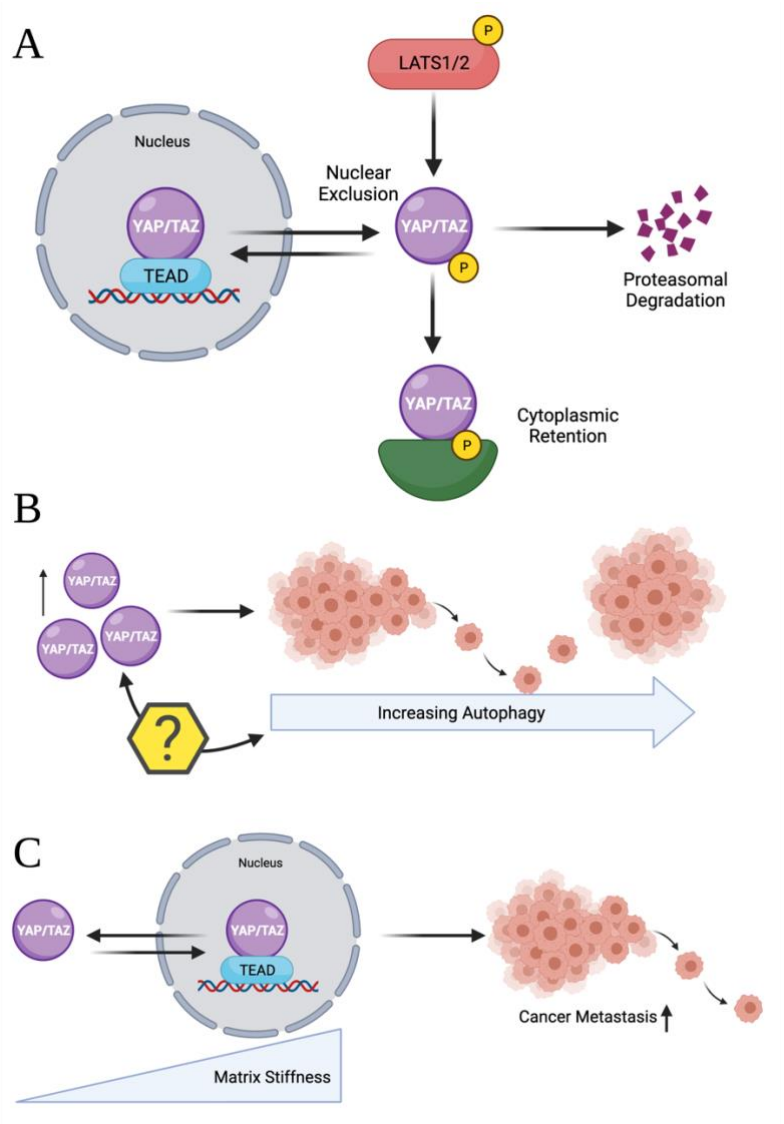

Figure 1.1 Previous research demonstrating the relationship between autophagy, metastasis, and YAP mechanotransduction. (A) YAP subcellular localization is determined by LATS-induced phosphorylation of YAP, leading to YAP nuclear exclusion, cytoplasmic retention, or proteasomal degradation. (B) An increase in autophagy is associated with an increase in cancer metastasis. (C) ECM stiffness promotes YAP nuclear localization and enhances cancer metastasis. Figure created with BioRender.com.

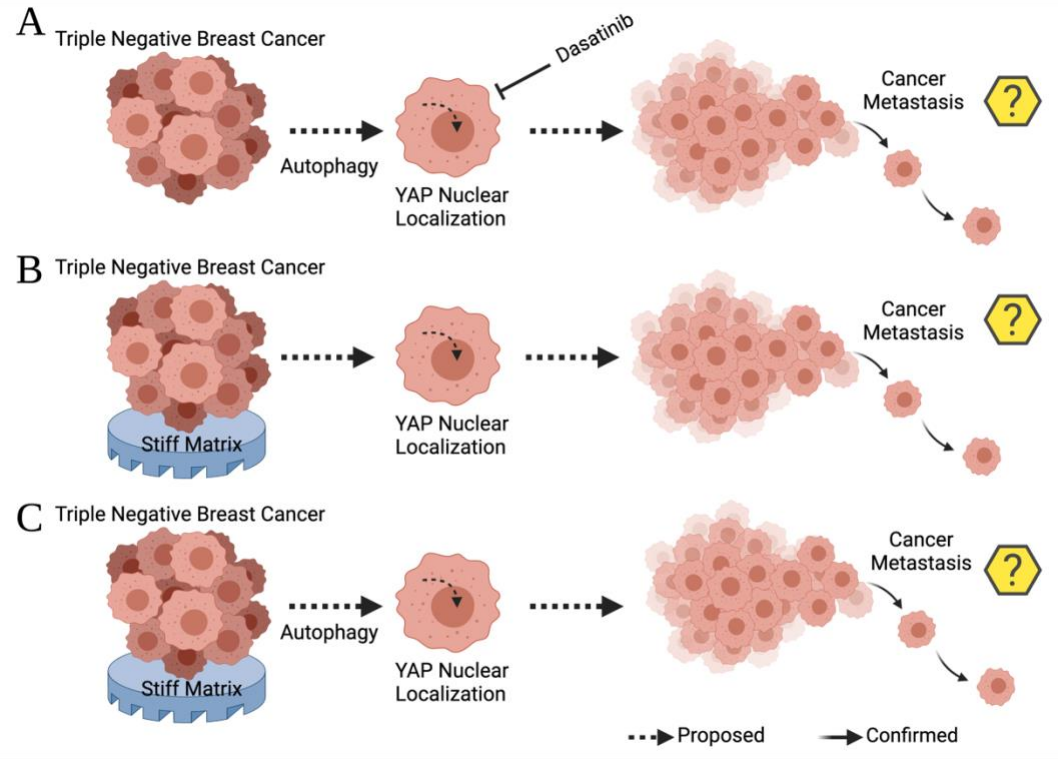

Figure 1.2 Proposed mechanism based on previous literature. (A) Autophagy and (B) Stiff matrix substrate will promote breast cancer metastasis through YAP nuclear localization. (C) The intermixing effect of autophagy and stiff matrix will promote YAP nuclear localization synergically to enhance breast cancer metastasis. Figure created with BioRender.com.

1.7 Objectives of the Thesis

The objective of this master's thesis is to investigate the interplay between autophagy, YAP signalling, and mechanotransduction in association with cancer metastasis with the hope to define a potential mechanism that can contribute to the application of YAP-targeting therapeutics for metastatic breast cancer. This objective is met by elucidating smaller objectives in each chapter to come to a conclusion. Chapter 2 intends to understand the mechanism and function of autophagy regulation through YAP signalling in the context of cancer metastasis. Chapter 3 addresses the role of YAP signalling in the interplay between matrix

stiffness and cancer metastasis on the secondary site. Finally, in chapter 4, we aim to examine the intermixing effect of autophagy and YAP mechanotransduction in breast cancer metastasis between the primary and secondary location.

1.8 Note to Reader

Chapters 2 and 3 are published with permission under the author's rights from their respective journal. As the published articles follow a coherent workflow, there are overlapping contents in the introduction and the methods section of chapter 2, chapter 3, and chapter 4.

Chapter 2

Autophagy, YAP Signaling and Cancer Metastasis

Many TNBC cell lines show high migratory behaviour in association with increases metastatic spread. Concurrently, numerous studies using surrogate markers such as LC3-II demonstrated an association between autophagy and metastasis. However, the underlying mechanism of how autophagy regulates metastatic progression remains unclarified. In addition, the Yes-associated protein (YAP) regulates cell proliferation and autophagy through nuclear localization and has been shown to function as a potential oncogene to enhance breast cancer metastasis. This chapter aims to elucidate the mechanism and function of autophagy regulation in YAP signalling in the context of cancer metastasis. As we elucidate the relationship between autophagy, YAP, and cancer metastasis and their potential role in TNBC, the results will shed light on future investigations for YAP-targeting therapeutics for TNBC patients.

Autophagy Promotes Triple Negative Breast Cancer Metastasis via YAP Nuclear Localization

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Status: Published

Full Citation:

Chen, W., Bai, Y., Patel, C., & Geng, F. (2019). Autophagy promotes triple negative breast cancer metastasis via YAP nuclear localization. *Biochemical and Biophysical Research Communications*, *520*(2), 263–268. https://doi.org/10.1016/j.bbrc.2019.09.133

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Abstract

The triple-negative breast cancer (TNBC) subtype is the most aggressive form of invasive breast cancer. Although autophagy is critical to the progression of TNBC, the mechanism of autophagy in regulating the metastatic potential of TNBC still remains unclear. Recently, the effector of the Hippo signaling pathway yes-associated protein (YAP) was shown to promote autophagy. To investigate autophagy regulation in YAP signaling in the context of cancer metastasis, we performed profiling analysis of YAP signaling, YAP subcellular localization, autophagosome formation and cell invasiveness in TNBC cell lines (MDA-MB-231 and Hs 578T) versus estrogen receptor (ER) positive breast cancer cell line MCF7. Our results showed that YAP transcriptional and protein expression was significantly upregulated in TNBC. When we triggered autophagy response in TNBC, YAP translocated into the nucleus and the expression of YAP target gene ankyrin repeat domain 1 *(ANKRD1)* increased remarkably. The correlation between autophagy response and YAP expression in TNBC was confirmed at the single-cell level. Furthermore, the inhibition of YAP nuclear entry greatly impeded the migration and invasion of TNBC cells while it did not affect the mobility of ER positive breast cancer cells. Therefore, this research established the autophagy-YAP-metastasis axis in TNBC and sheds light on the application of targeting YAP for TNBC therapeutics.

Keywords: YAP; cancer metastasis; autophagy; breast cancer; TNBC; autophagy-YAP-metastasis axis

2.1 Introduction

Breast cancer (BC) is the most prevalent form of cancer in women and remains the main cause of cancer-related deaths despite intensive attempts to develop effective therapies (Jemal et al., 2009). Triple-negative breast cancer (TNBC) represents approximately 10–15% of all breast cancers and it lacks the molecular markers of estrogen receptor (ER), progesterone receptor (PR), and amplification of HER-2/Neu (Brenton, Carey, Ahmed, & Caldas, 2005; Chavez, Garimella, & Lipkowitz, 2010; Maishman et al., 2017). TNBC relapses more frequently than ER-positive subtypes and patients with TNBC have a worse outcome compared to the other subtypes of breast cancer (Tseng et al., 2013). Many TNBC cell lines demonstrate a high migratory behavior in association with an increased metastatic spread (Koedoot et al., 2019).

Macro-autophagy (hereafter autophagy) is a highly conserved catabolic process that targets cellular contents to the lysosomal compartment for degradation (Mowers, Sharifi, & Macleod, 2017). At present various studies using surrogate markers have identified an association between increased autophagy and metastasis. For example, increased punctate staining for microtubule-associated protein 1A/1B light chain 3-II (LC3-II) was associated with lymph node metastasis and reduced survival in TNBC (Kabeya, 2000; Lefort et al., 2014). Although autophagy has been identified as a key mechanism in the progression of TNBC (Lefort et al., 2014), the molecular mechanisms underlying autophagy-regulated metastatic progression remain poorly understood.

As the key molecule in Hippo signaling pathway, yes-associated protein (YAP) regulates cell proliferation and promotes autophagy (Pavel et al., 2018; Zhao, Tumaneng, & Guan, 2011). YAP shuttles between the cytoplasm and nucleus, where it activates the TEA domain (TEAD) family of growth-promoting transcription factors to regulate the expression of genes such as *Ankyrin Repeat Domain 1* (*ANKRD1*) (Sayedyahossein, Li, Hedman, Morgan, & Sacks, 2016). Phosphorylation of YAP at Ser127 (pYAPSer127) by large tumor suppressor (LATS) is known to promote its nuclear exclusion and cytoplasmic accumulation, leading to YAP inactivation (Basu, Totty, Irwin, Sudol, & Downward, 2003; Sayedyahossein et al., 2016). It has also been shown that YAP functions as a potential oncogene and its activity enhances the metastatic potential of breast cancer (Lamar et al., 2012; Wang et al., 2018). Thus, in this research we sought to understand the mechanism and function of autophagy regulation in YAP signaling in the context of cancer metastasis.

2.2 Materials and Methods

2.2.1 Cell Culture and Reagents

Human breast cancer cell line MCF7 was purchased from American Type Culture Collection (ATCC). Hs 578T cells and MDA-MB-231 cells were kindly provided by Dr. Juliet Daniel at McMaster University. MCF7, Hs 578T and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific). All cultures were incubated at 37°C with 5% CO2. All

three cell lines were grown on 35 mm glass-bottom culture dishes (Thermo Fisher Scientific) for immunofluorescence staining.

2.2.2 RNA Extraction

DMSO control and rapamycin-treated MCF7, Hs 578T and MDA-MB-231 cells were harvested and total RNAs were extracted using PureLink RNA Mini kit (Thermo Fisher Scientific) according to the manufacturer's directions. RNA concentrations were then measured using Qubit Fluorometer and Qubit RNA BR (Broad-Range) Assay Kit (Thermo Fisher Scientific).

2.2.3 qRT-PCR

YAP and *ANKRD1* gene expression were analyzed using qRT-PCR with *GAPDH* as the internal reference gene. SYBR Green Quantitative RT-qPCR Kit was obtained from Sigma-Aldrich.

YAP gene was amplified using: YAP forward primer: 5′-GCACCTCTGTGTTTTAAGGGTCT-3′; YAP reverse primer: 5′-CAACTTTTGCCCTCCTCCAA-3′. *GAPDAH* gene was amplified using: GAPDH forward primer: 5′-CTCCTGCACCACCAACTGCT-3′; GAPDH reverse primer: 5′- GGGCCATCCACAGTCTCCTG-3′.

As per suppliers instructions, a master mix for each sample was made of: 25 μL of 2x SYBR Green Quantitative RT-PCR Buffer, 0.5 μL reference dye, 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 17.25 μL of PCR grade water, and 0.25 μL of MMLV RT enzyme per reaction. Following distribution of the master mix, 5 uL of RNA (2 ng/uL) was added to each sample. The negative

controls were comprised of the same mixture, but PCR grade water was added instead of RNA.

qPCR was performed on C1000 Thermal Cycler (Bio-Rad) using the following conditions: 30 minutes of first strand synthesis at 43 °C, 30 seconds of denaturation/RT inactivation, followed by 40 cycles of step 1 with 95 °C for 5 seconds, step 2 with 55 °C for 15 seconds, and step 3 with 72 °C for 10 seconds. Raw data was then analyzed with CFX Maestro Software (Bio-Rad) and gene expression levels were normalized against *GAPDH*.

2.2.4 Immunoblotting

Cells were scraped from the culture flask and washed three times with phosphate-buffered saline (PBS, Thermo Fisher Scientific) before the lysis in Triton X-100 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 and, 5 mM EDTA). The protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Then 10 ug of cell lysates were mixed with 2x laemmli sample buffer (Bio-Rad) and boiled at 100°C for 10 minutes prior to the immunoblotting. Proteins were separated on Bio-Rad Mini-PROTEAN TGX Gels and transferred to nitrocellulose membrane (Thermo Fisher Scientific). The membrane was incubated overnight with antibodies against YAP (Cell Signaling Technology), Phospho-YAP (Ser127) (Cell Signaling Technology) or β-actin (Cell Signaling Technology) at the dilution of 1:1000. Then the membrane was incubated with the corresponding secondary antibody coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories) at the dilution of 1:10,000 for 1 hour at

room temperature. Labeled proteins were visualized with Enhanced Chemiluminescence (Perkin Elmer) using Bio-Rad ChemiDoc Imager.

2.2.5 Immunofluorescence Staining

Cells were seeded on glass-bottom culture dishes and grown as described (Geng, Zhu, Anderson, Leber, & Andrews, 2012). Following rapamycin treatment, control cells and treated cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with lysis buffer (0.1% Triton X-100 in PBS). Then the cells were incubated with blocking buffer (3% BSA in PBS) before the subsequent overnight incubation with mouse anti-YAP (Santa Cruz Biotechnology) at a dilution of 1:2000 and rabbit anti-LC3-II antibody (Cell Signaling Technology). Then samples were washed and incubated with secondary antibodies fluorescein isothiocyanate (FITC) conjugated goat anti-Mouse antibody (Sigma-Aldrich) and Rhodamine (TRITC)-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) at room temperature for one hour. Cell nuclei of each sample were then stained with DAPI (Thermo Fisher Scientific) solution at room temperature for 10 minutes prior to the subsequent fluorescent microscopy (IX51S1F-3, Olympus).

2.2.6 YAP and LC3-II Co-Staining Image Analysis

Images were scored for YAP expression and for LC3-II using ImageJ software (Wayne Rasband, National Institute of Health). Comparison of the green fluorescent intensity (YAP) in each cell line (15 images per sample) was used to set an arbitrary threshold intensity to identify YAP expressing cells in the population. Corrected total cell fluorescence (CTCF) of red fluorescent intensity (LC3-II) was

then analyzed in those cells with green intensity above the threshold (expressing YAP protein) using the following equation:

CTCF = Integrated Density – (Area of selected cell × Mean fluorescence of background readings).

2.2.7 Boyden Chamber Assay

MCF7, Hs 578T and MDA-MB-231 cells were seeded and treated with DMSO control or 5 uM Dasatinib (Cell Signaling Technology) for 24 hours prior to the assay. 5×10^4 cells of each sample were suspended in 200 μL serum-free medium and seeded into the upper chamber and 600 μL complete medium into the lower chamber. After 24 hours, the upper surface of the insert was wiped gently with a cotton swab to remove non-migrating cells. Cells that migrated and invaded through the membrane were stained with crystal violet solution (0.5 % crystal violet in 25 % methanol/PBS) and photographed by a microscope with a camera in five random fields at a magnification of 40 ×. Quantification was expressed as the percentage of area covered with migrated cells by using ImageJ software (Wayne Rasband, National Institute of Health).

2.2.8 Wound Healing Assay

1×10^5 MCF7, Hs 578T and MDA-MB-231 cells were seeded on 24 well plates. Cells were grown to form a confluent monolayer in the wells before wounding. A sterilized pipette tip was used to generate wounding across the cell monolayer, and the debris was washed with PBS. Then cells were treated with DMSO control or 5 uM Dasatinib for 24 h. Then cells migrating into the wounded area were visualized and photographed under an inverted microscope (IX51S1F-3,

Olympus) at varying intervals. A total of six areas were selected randomly in each condition and photographed at a magnification of 10×.

2.2.9 Statistical Analysis

Gene expression analysis was reported as mean ± SEM. For evaluation of differences in gene expression, unpaired 2-tailed Student's t test was performed assuming equal variance. Differences were considered significant at $P < 0.05$.

2.3 Results

2.3.1 YAP signaling was greatly activated in MDA-MB-231 compared to MCF7 cells

To understand the role of YAP signaling in cancer metastasis, we utilized highly metastatic TNBC cell lines MDA-MB-231 and Hs 578T for YAP profiling analysis (Chavez et al., 2010; Koedoot et al., 2019). As a comparison, human ER positive breast cancer cell line (with lower metastatic potential) MCF7 was included in this study (Saceda et al., 1988; Sun, Xu, Luo, & Li, 2016). Using these three cell lines, we examined the transcript abundance of *YAP*, the expression of total YAP protein, and phosphorylated (p) YAP protein. YAP transcripts were more abundant in MDA-MB-231 (over 2 folds) and Hs 578T (1.5 folds) compared with MCF7, as indicated by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) results (Fig. 1A). The expression level of total YAP was notably higher in MDA-MB-231 cells whereas there was little difference in pYAPSer127 expression among the three cell lines (Fig. 1B). Other than YAP expression, we further assessed YAP activity by analyzing the transcripts of the YAP target gene *ANKRD1* using qRT-PCR. Fig. 1C showed that *ANKRD1*

transcripts were upregulated by over 35 folds in MDA-MB-231 cells compared with MCF7 cells.

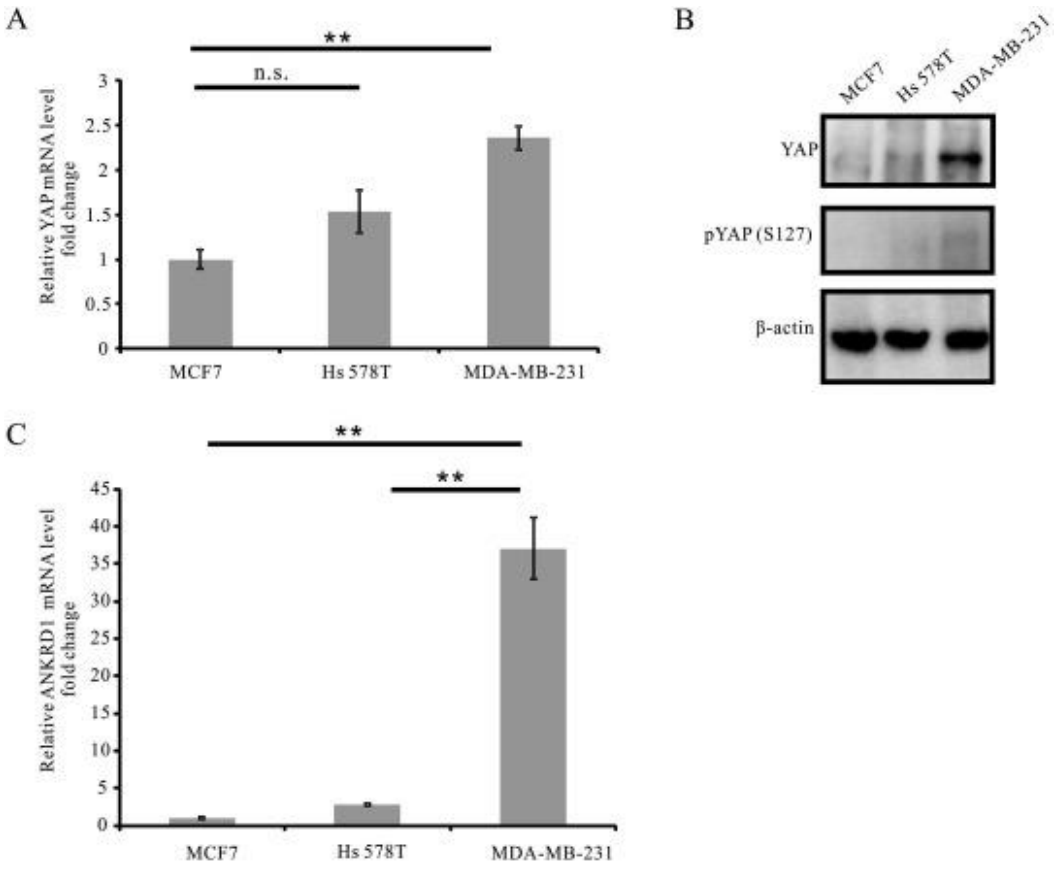

Figure 2.1 The profiling analysis of YAP transcript abundance and protein expression in TNBC cells. (A) qRT-PCR analysis of YAP gene expression in MCF7, Hs 578T and MDA-MB-231 cells; (B) The analysis of YAP and pYAPSer127 protein expression in MCF7, Hs 578T and MDA-MB-231 cells using western blotting; (c) qRT-PCR analysis of ANKRD1 gene expression in MCF7, Hs 578T and MDA-MB-231 cells. Data were represented as means ± SEM of 3 independent experiments with n = 3 each. **P < 0.01, n.s. not significant.

2.3.2 Autophagy promoted YAP nuclear localization and ANKRD1 expression in TNBC cells

YAP promotes autophagy through transcriptional regulation of myosin-II and thus autophagy is crucial in maintaining both the cell survival and proliferative status downstream of the Hippo signaling hubs, YAP–TEAD [11]. In order to

dissect the interplay between autophagy and YAP pathway in the process of cancer metastasis, we triggered the autophagy response by treating the three BC cell lines (MCF7, Hs 578T and MDA-MB-231) with rapamycin (Bhat et al., 2018; Rubinsztein, Gestwicki, Murphy, & Klionsky, 2007) and investigated the rapamycin regulation in YAP signaling. The qRT-PCR data showed no difference in the expression of *YAP* gene between the rapamycin treatment group and control group (Fig. 2A). However, rapamycin treatment increased the expression of YAP target gene *ANKRD1* in MDA-MB-231 (over 2 folds) and Hs 578T (over 1.5 folds) versus MCF7 cells (Fig. 2B). Consistently, compared with DMSO control, autophagy caused YAP to translocate into the nucleus (colocalization of YAP staining with DAPI staining, shown by arrowheads) in TNBC (MDA-MB-231 and Hs 578T) cells (Fig. 2C). In contrast, rapamycin treatment did not alter the subcellular localization of YAP in most of MCF7 cells (Fig. 2C).

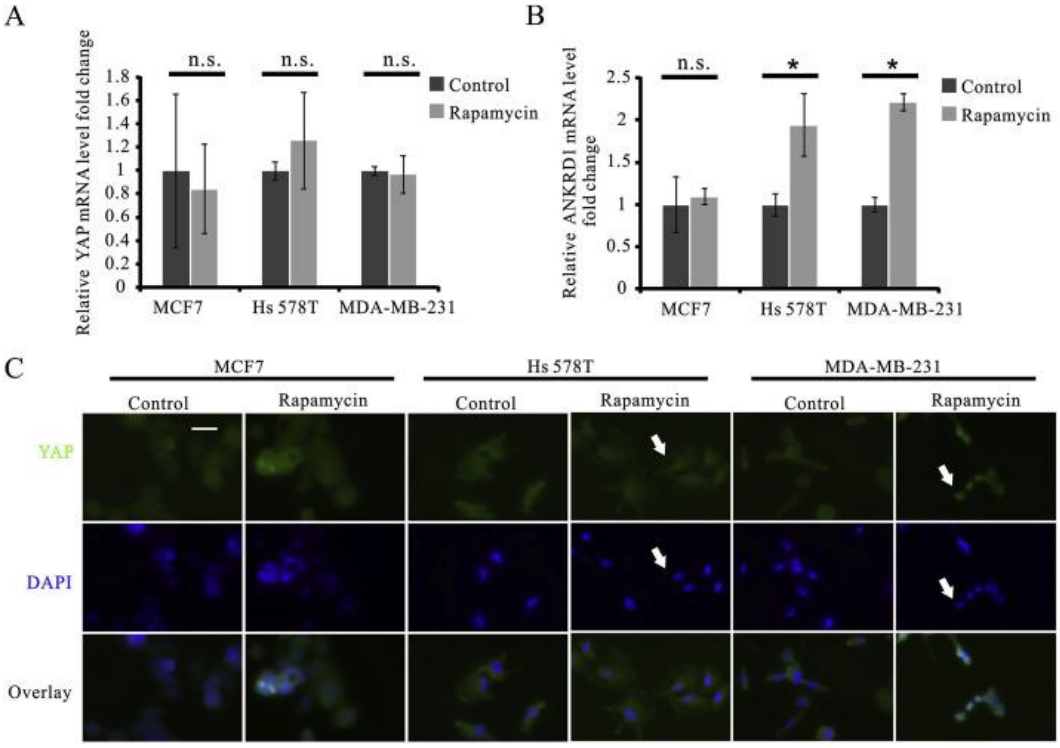

Figure 2.2 The treatment of TNBC cells with rapamycin upregulated ANKRD1 expression and promoted YAP nuclear localization. (A) qRT-PCR analysis of YAP gene expression in MCF7, Hs 578T and MDA-MB-231 cells treated with DMSO control (control) or rapamycin; (B) qRT-PCR analysis of ANKRD1 gene expression in MCF7, Hs 578T and MDA-MB-231 that were treated with DMSO control (control) or rapamycin; (C) The nuclear location of YAP in MCF7, Hs 578T and MDA-MB-231 cells that were treated with DMSO control (control) or rapamycin. YAP and the nuclei were stained in green and blue respectively. Scale bar, 10 μm. The representative cells with YAP localized in the nucleus were highlighted using arrowheads. Data were represented as means ± SEM of 3 independent experiments with n = 3 each. *P < 0.05, n.s. not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3.3 YAP nuclear translocation in TNBC was associated with autophagy response

Although Fig. 2 showed that rapamycin treatment promoted YAP nuclear localization in TNBC cells, it is still unclear whether this is the downstream effect of autophagy or due to the off-target effect of rapamycin. For this reason, we examined the correlation of YAP nuclear localization to LC3-II (autophagy

biomarker) level by double immunofluorescence staining (Fig. 3A) and the quantification of autophagy in YAP positive cells (Fig. 3B). Interestingly, in MDA-MB-231 and Hs 578T cells, a positive correlation between LC3-II signal and YAP expression (LC3-II positive puncta and high YAP expression co-existed in the same cells) was observed (arrowheads in Fig. 3A and CTCF measurement in Fig. 3B). Opposite to TNBC, MCF7 cells exhibited a negative correlation between the YAP and LC3-II channels in that low LC3-II signal (CTCF) was correlated with high YAP expression in the same cells (Fig. 3A-B).

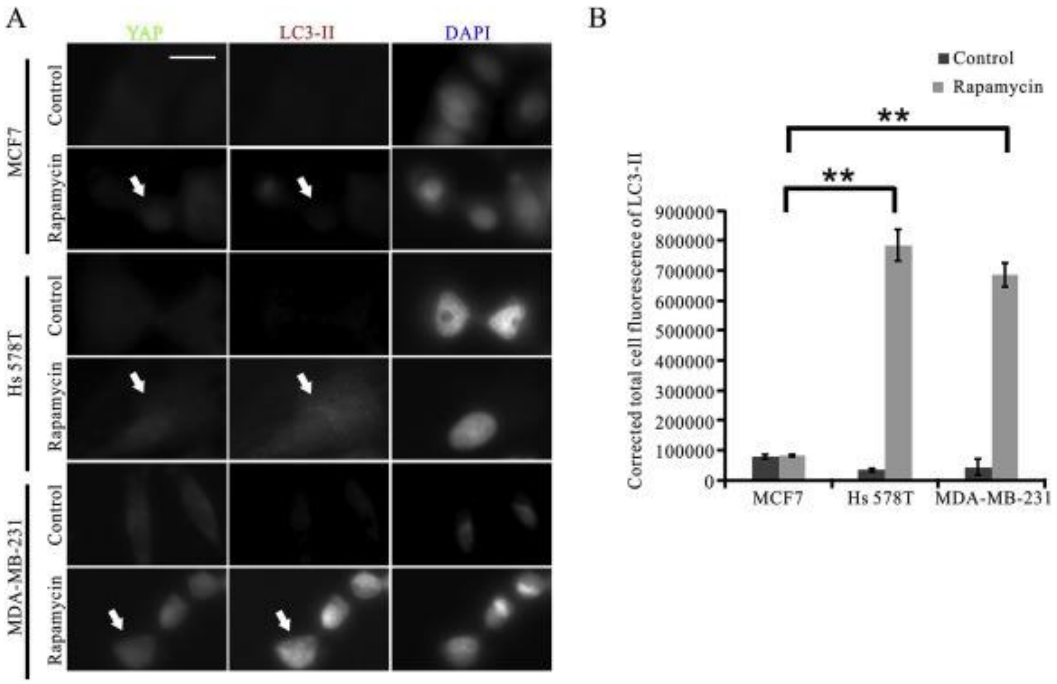

Figure 2.3 TNBC exhibited a positive correlation between autophagy response and YAP expression. (A) The expression of YAP and LC3-II in MCF7, Hs 578T, and MDA-MB-231 cells that were treated with DMSO control (control) or rapamycin. Scale bar, 10 μm; (B) Corrected total cell fluorescence (CTCF) of LC3-II in YAP positive cells from Fig. 3A were analyzed using ImageJ. Data were represented as means ± SD of 3 independent experiments with n = 3 each. **P < 0.01.

2.3.4 Small molecule inhibiting YAP nuclear localization impeded the migration and invasion of TNBC cells

Since autophagy caused YAP to travel into the nucleus in TNBC (Fig. 2 and 3), we sought to understand the biological significance of YAP regulation in cancer metastasis. We applied the small molecule inhibitor dasatinib (Oku et al., 2015) to prevent the YAP nuclear localization (Fig. 4A) and analyzed its effects on the migration and invasion capabilities of TNBC using wound healing assay (Fig. 4B) and the Boyden chamber assay (Fig. 4C). The treatment with dasatinib led to YAP nuclear exclusion and cytoplasmic accumulation in all three BC cell lines (shown by arrowheads, Fig. 4A), which confirmed the inhibition of YAP nuclear localization. As mentioned previously (Chavez et al., 2010), TNBC (MDA-MB-231 and Hs 578T cells) exhibited high motility and invasiveness (Fig. 4B and 4C) compared with ER positive BC line MCF7. In wound healing assay, MDA-MB-231 and Hs 578T quickly migrated to the middle of the scratch gap area after cell seeding, and the scratch gap area subsequently disappeared at 24 hours (Fig. 4B). Following the treatment of dasatinib, the scratch gap area of TNBC (MDA-MB-231 and Hs 578T) cells remained the same at 24 hours (Fig. 4B). In the Boyden chamber assay, the results showed that dasatinib treatment suppressed the invasiveness of TNBC (number of migrated cells dropped from 250 to 30 cells/field in MDA-MB-231 and dropped from 200 to 50 cells/field in Hs 578T approximately) (Fig. 4C). In comparison, MCF7 was not affected significantly by dasatinib treatment in terms of the scratch gap area (Fig. 4B) and number of migrated cells (Fig. 4C).

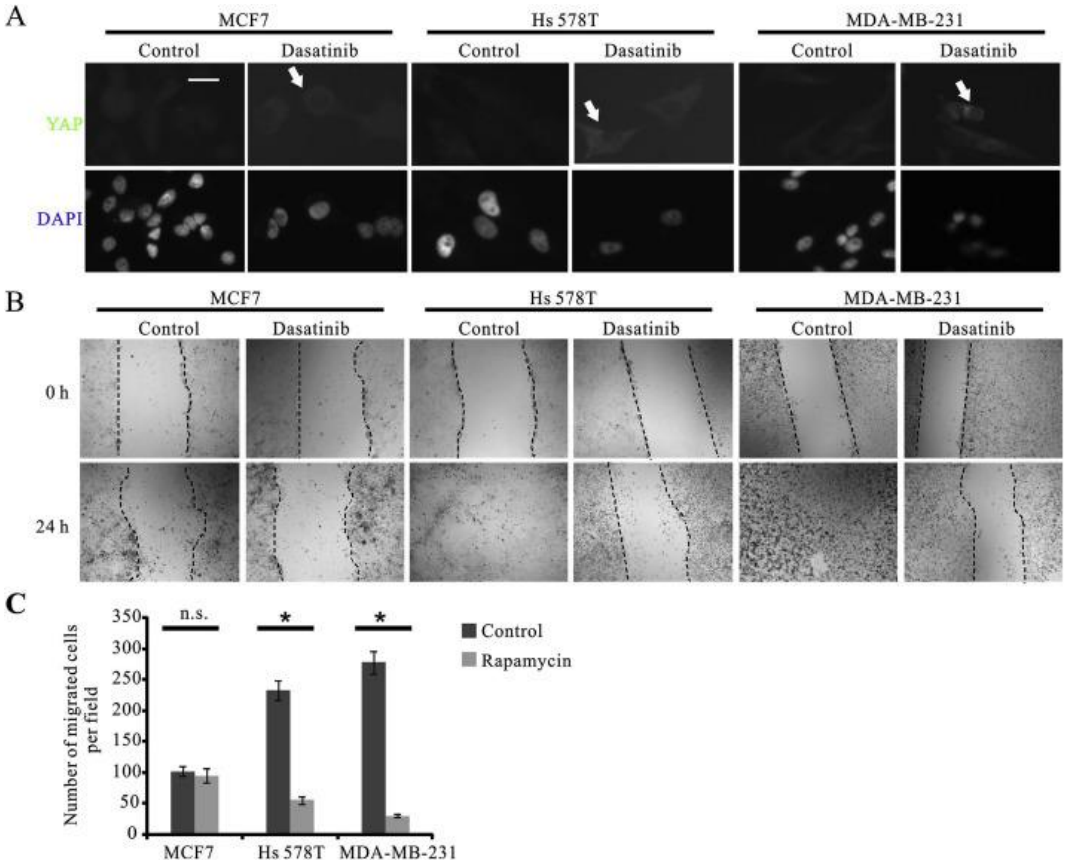

Figure 2.4 Small molecule inhibiting the nuclear localization of YAP impeded cell migration and invasion in TNBC. (A) The inhibition of YAP nuclear localization by small molecule inhibitor dasatinib. MCF7, Hs 578T and MDA-MB-231 cells were treated with DMSO control (control) or 5 μM dasatinib and then YAP subcellular localization in those cells was analyzed by immunofluorescence staining. Scale bars, 10 μm. The cells showing the inhibited nuclear localization were highlighted by arrowheads. (B) The effects of dasatinib treatment on cell migration. MCF7, Hs 578T and MDA-MB-231 cells were treated with DMSO control (control) or 5 μM dasatinib and then cell migration was assessed by wound healing assay. (C) The effects of dasatinib treatment on cell invasion. MCF7, Hs 578T and MDA-MB-231 cells were treated with DMSO control (control) or 5 μM dasatinib and cell invasion was assessed by Boyden chamber assay; Data were represented as means ± SEM of 3 independent experiments with n = 3 each. *P < 0.05, n.s. not significant.

2.4 Discussion

There has been an emerging recognition that autophagy is central to cancer metastasis (Song et al., 2015). Although recent studies demonstrated that YAP activity plays a critical role in cancer progression and metastasis (Lamar et al.,

2012), the upstream signal of YAP activation and its downstream effects on TNBC remained unclear. In this research, we performed the analysis of YAP signaling events in TNBC (YAP expression, YAP nuclear localization, target gene *ANKRD1* expression), dissected the interplay between YAP and cancer metastasis using small molecule inhibitor, and then examined the significance of autophagy-YAP-cancer metastasis axis.

Compared with ER positive BC, *YAP* transcripts and protein levels were notably upregulated in both TNBC cell lines (Fig. 1). However, increased expression of the YAP target gene *ANKRD1* in MDA-MB-231 compared to Hs 578T cells suggested the YAP/TEAD might be a checkpoint that distinguishes between the subtypes of aggressive TNBC.

Although autophagy was shown to be critical in cancer metastasis, the understanding of its role in cancer metastasis was limited to its pro-survival effect (Huang, Wang, & Wang, 2018). In this research, we established the link between autophagy, YAP nuclear translocation and YAP activation (through *ANKRD1* expression) in TNBC (Fig 2). Our data (Fig. 2) suggested autophagy activated YAP signaling in a TNBC-specific manner. The specificity of autophagy-YAP axis to TNBC was also confirmed by the positive correlation between autophagy and YAP activation at the single-cell resolution (Fig. 3). ER positive BC exhibited a distinct pattern from TNBC, a negative correlation between autophagy and YAP activation (Fig. 3). This indicated that autophagy-YAP axis might not exist in ER positive BC.

Given the critical role of autophagy in cancer metastasis (Di Fazio & Matrood, 2018; Galluzzi, Bravo-San Pedro, Levine, Green, & Kroemer, 2017; Mowers et al., 2017) , we investigated the potential links between autophagy-YAP axis and metastatic progression of TNBC. With the prevention of YAP nuclear localization (Fig. 4) (Oku et al., 2015; Sugihara et al., 2018), cell migration and invasiveness in both the TNBC cell lines were significantly suppressed. Again, ER positive BC did not exhibit similar changes (Fig. 4). Thus, autophagy-YAP-metastasis axis might be unique to TNBC in the transduction of autophagy signal into metastatic cancer behaviors.

To summarize, our results suggested that TNBC utilizes autophagy as a mechanism to promote YAP nuclear entry, thus giving the cells invasion and metastatic advantage. Therefore, our study reported on the existence of autophagy-YAP-metastasis axis and its potential role in TNBC. Further investigation into this area will shed light on YAP-targeting therapeutics in treating TNBC patients.

Conflicts of Interest

Authors declare no conflict of interest.

Acknowledgments

This research was supported by Mitacs Accelerate Internship Program. We are grateful to Dr. Juliet Daniel for providing human breast cancer cell lines MDA-MB-231 and Hs 578T.

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Chapter 3

YAP Mechanotransduction and Cancer Metastasis

Previous studies reported that extracellular matrix stiffness regulates cancer progression and malignancy. Metastasis requires numerous mechanisms led by cytoskeleton dynamics and focal adhesion alterations where paxillin, the main component of focal adhesion, is essential for cell migration. The development of circulating breast cancer cells on the metastatic sites has been attributed to increasing matrix stiffness. In addition, YAP is a mechanosensitive transcriptional regulator that, upon activation, it regulates downstream transcription of target genes that enhance the motility of breast cancer cells. However, the role of YAP mechanosensing in the complex interplay between breast cancer and matrix remains unclear. This chapter elaborates from chapter 2 and investigates matrix stiffness as the stimulus to observe its behaviour between YAP signalling and cancer metastasis on the secondary metastatic site. The results from this chapter will shed light on the mechanism of the regulation of cancer cell migration by matrix stiffness in breast cancer metastasis.

The Migration of Metastatic Breast Cancer Cells is Regulated by Matrix Stiffness via YAP Signalling

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Status: Published

Full Citation:

Chen, W., Park, S., Patel, C., Bai, Y., Henary, K., Raha, A., Mohammadi, S., You, L, & Geng, F. (2021). The migration of metastatic breast cancer cells is regulated by matrix stiffness via YAP signalling. *Heliyon*, *7*(2), e06252. https://doi.org/10.1016/J.HELIYON.2021.E06252

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Abstract

Matrix stiffness is a driver of breast cancer progression and mechanosensitive transcriptional activator YAP plays an important role in this process. However, the interplay between breast cancer and matrix stiffness, and the significance of this interplay remained largely unknown. Here, we showed an increase in YAP nuclear localization and a higher proliferation rate in both highly metastatic MDA-MB-231 cells and the non-metastatic counterpart MCF-7 cells when they were exposed to the stiff matrix. However, in response to the stiff matrix highly metastatic MDA-MB-231 cells instead of MCF-7 cells exhibited upregulated mobility, which was shown to be YAP-dependent. Consistently, MDA-MB-231 cells exhibited different focal adhesion dynamics from MCF-7 cells in response to matrix stiffness. These results suggested a YAP-dependent mechanism through which matrix stiffness regulates the migratory potential of metastatic breast cancer cells.

Keywords: cell mobility; matrix stiffness; mechanosensing; metastatic breast cancer; YAP

3.1 Introduction

Extracellular matrix (ECM) stiffness is considered to be one of the most influential risk factors for cancer progression (Lee et al., 2019; Martin & Boyd, 2008; McConnell et al., 2016) and regulates cancer malignancy (Provenzano et al., 2008).

As the hallmark of cancer malignancy, metastasis is a multistep process that includes cell migration (L et al., 2019; Ritch, Brandhagen, Goyeneche, & Telleria, 2019), which required numerous mechanisms led by cytoskeleton dynamics and Focal adhesion (FA) alterations (Ritch et al., 2019). FA dynamics is a continuous process involving coordination between FA and actin cytoskeleton and is shown to be essential for cell migration (Hu et al., 2014). As a main component of FA (Crawford, Henry, Clason, Becker, & Hille, 2003), paxillin is essential for cell migration and acts as a molecular adapter aiding in implementing changes in the organization of actin cytoskeleton (Deramaudt et al., 2014).

Breast cancer metastasis of a variety of vital organs, such as bone, is one of the leading causes of breast cancer mortality (Lu & Kang, 2007). However, breast cancer metastasis is regarded as a highly inefficient process due to the fact that less than 0.01% of circulating tumour cells eventually succeed in forming secondary tumours (Langley & Fidler, 2011). Some breast cancer cells remain dormant while other cells become more metastatic in secondary organs (Barkan, Green, & Chambers, 2010). The fate of the metastatic process is determined by a complex series of interactions between breast cancer cells and metastatic sites (Langley &

Fidler, 2011), and thus, it would be critical to understand the mechanisms involved in this complex dialogue.

The development of circulating breast cancer cells on the metastatic sites has been attributed to mechanical factors such as matrix stiffness (Braun et al., 2005). Among the metastatic sites for breast cancer, the stiffness of cellular matrix varies considerably (Ondeck et al., 2019). As a mechanosensitive transcriptional regulator with a significant role in cancer, Yes-associated protein (YAP) set responsiveness to the substrate stiffness (Aragona et al., 2013). The subcellular localization of YAP is in either the cytoplasm or the nucleus, and the latter allows YAP binding and activation of transcriptional enhanced associate domain (TEAD) (Dupont et al., 2011). As the target gene of YAP, connective tissue growth factor (CTGF) enhances the motility of breast cancer cells (P. S. Chen et al., 2007). Through the modulation of YAP signalling, ECM stiffness induces the phenotypic changes of breast cancer, including cell proliferation and migration (Haga, Irahara, Kobayashi, Nakagaki, & Kawabata, 2005; Ishihara et al., 2013; Paszek et al., 2005; Umesh, Rape, Ulrich, & Kumar, 2014). The mechanical cues are transduced intracellularly through YAP, which then enhances cancer metastasis (Zanconato, Cordenonsi, & Piccolo, 2016). However, the role of YAP mechanosensing in the complex interplay between breast cancer and metastatic sites, and its significance to the whole metastatic process remained unclear (Barkan et al., 2010; Guise, 2010; Langley & Fidler, 2007).

Previously we have demonstrated metastatic breast cancer promotes the accumulation of YAP in the nucleus, thus giving the cells metastatic advantage (W. Chen, Bai, Patel, & Geng, 2019). In this paper, we demonstrated that migration of metastatic breast cancer cells is promoted by stiff matrix in a YAP-dependent manner.

3.2 Materials and Methods

3.2.1 Preparation of PDMS Substrates for Cell Culture

Polydimethylsiloxane (PDMS) (Sylgard® 184, Dow Corning) base and crosslinker were mixed at three different ratios (by weight) of 1:5, 1:10, and 1:20 as described previously (Park et al., 2010). Mixtures were poured onto prepared 24-well plates (Thermo Fisher Scientific), cured at room temperature overnight (Figure 1). Resistance to deformation is defined by the Young's elastic modulus, E, obtained by applying a tensile force (stress) to a sample with a defined cross-sectional area and measuring the relative change in length (strain). PDMS stiffness was measured by microindentation using a Biomomentum Mach-1 system (Biomomentum Inc) as described previously (Ireland et al., 2020). The base reagent is known to contain 0.5% xylene, 0.2% ethylbenzene, 60% dimethylvinyl-terminated dimethyl siloxane, 30–60% dimethylvinylated and trimethylated silica and 1–5% tetra (trimethylsiloxy) silane. Crosslinking agent contains 0.19% xylene, 0.1% ethylbenzene, 55–75% dimethyl, methylhydrogen siloxane, 15–35% dimethylvinyl-terminated dimethyl siloxane, 10–30% dimethylvinylated and trimethylated silica and 1–5% tetramethyl tetravinyl cyclotetrasiloxane. The standard ratio for PDMS preparation is 1:10 as per the instruction of PDMS supplier

Dow Corning. 1:5 and 1:20 ratios were chosen to extend PDMS stiffness range in order to identify the responses of breast cancer to matrix stiffness.

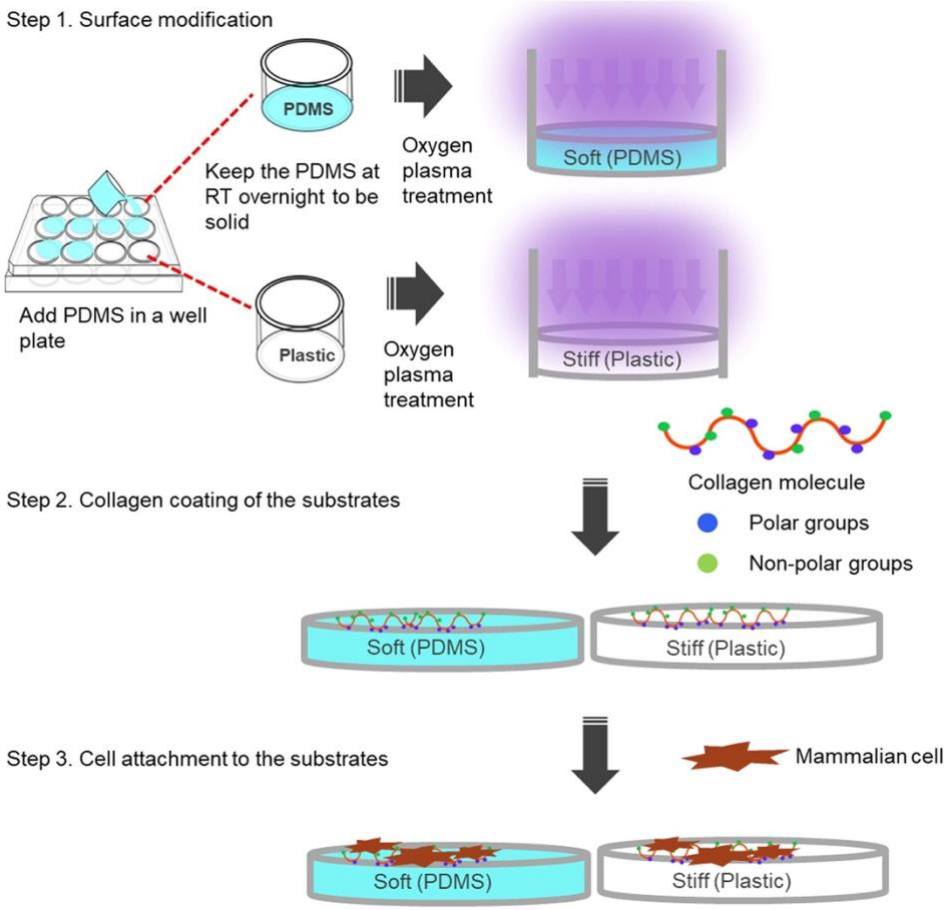

Figure 3.1 The preparation of PDMS and plastic substrates for mammalian cell culture. PDMS and plastic substrates on well plates were subject to plasma treatment and collagen coating prior to the mammalian cell attachment.

3.2.2 Cell Culture and Reagents

Human breast cancer cell line MCF-7 was purchased from American Type Culture Collection (ATCC). MDA-MB-231 cells were kindly provided by Dr. Juliet Daniel at McMaster University. MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific). All

cell cultures were incubated at 37°C with 5% CO2. Both the cell types were grown on PDMS and plastic substrates on 24 well plates (Thermo Fisher Scientific).

3.2.3 RNA Extraction

MCF-7 and MDA-MB-231 cells were seeded and cultured on PDMS and plastic substrates. Then the cells were harvested and total RNAs were extracted using PureLink RNA Mini kit (Thermo Fisher Scientific) according to the manufacturer's directions. RNA concentrations were then measured using Qubit Fluorometer and Qubit RNA Assay Kit (Thermo Fisher Scientific).

3.2.4 qRT-PCR

YAP and CTGF gene expression were analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) with GAPDH as the internal reference gene. SYBR Green Quantitative qRT-PCR kit was obtained from Sigma-Aldrich. YAP gene was amplified using: YAP forward primer: 5'-GCACCTCTGTGTTTTAAGGGTCT-3'; YAP reverse primer: 5'-CAACTTTTGCCCTCCTCCAA-30. CTGF gene was amplified using: CTGF forward primer: 5'-AGGAGTGGGTGTGTGACGA-3'; CTGF reverse primer: 5'-CCAGGCAGTTGGCTCTAATC-3'. GAPDH gene was amplified using: GAPDH forward primer: 5'-CTCCTGCACCACCAACTGCT-3'; GAPDH reverse primer: 5'-GGGCCATCCACAGTCTCCTG-3'. The procedures were described previously (W. Chen et al., 2019).

3.2.5 YAP Immunofluorescence Staining

For the analysis of YAP nucleus localization, MCF-7 cells or MDA-MB-231 cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-

100. Then, the cells were washed with PBS and blocked with 5% PBS-BSA at 4°C overnight. Then the cells were incubated with mouse anti human YAP monoclonal antibody (Santa Cruz Biotechnology) followed by fluorescein isothiocynanate (FITC)-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich). Cell nuclei of each sample were stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Thermo Fisher Scientific) and imaged on Olympus inverted phase contrast and fluorescence microscope (IX51S1F-3, Olympus). For paxillin immunofluorescence, mouse anti-human paxillin monoclonal antibody (Thermo Fisher Scientific) was used.

3.2.6 Colocalization Analysis

CellProfiler (www.cellprofiler.org) was used to quantify YAP nuclear/cytoplasmic intensity in immunofluorescence images and YAP/DAPI colocalization was measured via the correlation between the intensities of green and blue channels on a pixel-by-pixel basis across an entire image. The Cell Profiler colocalization pipeline was carried out to calculate the correlation and colocalization (Manders Coefficient) between the pixel intensities.

3.2.7 Gap Closure Assay

1 x10^5 MCF-7 and MDA-MB-231 cells were seeded on PDMS and plastic substrates on 24 well plates. Cells were grown to form a confluent monolayer in the wells before wounding. A sterilized pipette tip was used to generate wounding across the cell monolayer, and the debris was washed with PBS. At varying intervals, the cells migrating into the wounded area were visualized and photographed at 0 and 24 h under an inverted microscope (IX51S1F-3, Olympus).

The distance between cell front was measured at 0 h and 24 h time-points using ImageJ. To measure the average healing speed inside the wound area at each time-point, the following equation was used as described previously (Pijuan et al., 2019).

v (μm/h) = [Distance initial time (μm) – Distance final time (μm)]/Total time (h)

3.2.8. YAP siRNA Transfection

Pre-designed siRNAs (On-Target plus SMART pool targeting Control and YAP) were purchased from Dharmacon. For siRNA transfection experiments, MCF-7, Hs 578T and MDA-MB-231 cells were grown to form a confluent monolayer on 24 well plates, subject to the wounding, and then transfected with 100 nM of the indicated siRNA using DharmaFECT transfection reagent (Dharmacon), following the manufacturer's protocol.

3.2.9 Statistical Analysis

The statistical analysis in this paper was reported as mean ± SD. For the evaluation of differences, unpaired 2-tailed Student’s t-test was performed assuming equal variance. Differences were considered significant at $P < 0.05$.

3.3 Results

3.3.1 The development and stiffness measurement of soft (PDMS) substrates

Silicone elastomers such as PDMS are usually used to form elastic membranes (Mohammed et al., 2019). The intrinsic PDMS properties make it an appropriate choice of polymer for mechanobiology applications (Park et al., 2010). In this research, we examined the effects of matrix stiffness on YAP nucleocytoplasmic shuttling, proliferation and mobility of breast cancer cells that were cultured on

collagen coated soft (PDMS) substrates or stiff (plastic) substrate. The comparative analysis of plastic versus soft polymers has been performed extensively in mechanobiology research (Aragona et al., 2013; Nukuda et al., 2015; Panciera et al., 2020).

PDMS stiffness was adjusted by varying the ratio of base to curing agent used during polymer mixing. Different mixing ratios yield different proportions of crosslinked and uncrosslinked polymers after curing, which vary the stiffness of the cured polymer (Park et al., 2010; Seghir & Arscott, 2015). We fabricated PDMS substrates using three mixing ratios (base to curing agent) of 5:1, 10:1, and 20:1 (Dupont et al., 2011). PDMS and plastic substrates were subject to the surface modification, collagen coating prior to the mammalian cell attachment (Fig. 1). The modulus of elasticity measurements showed that tensile strength of PDMS substrates corelated linearly to the proportion of crosslinker, and the Young's modulus was determined to be 3.28 megapascal (MPa) for the PDMS of 5:1 mixing ratio, 0.92 MPa for the PDMS of 10:1 mixing ratio, and 0.35 MPa for the PDMS of 20:1 mixing ratio (Fig. 2A). As a comparison to soft PDMS substrates, the plastic substrate (tissue culture polystyrene) has a Young's modulus of 1 gigapascal (GPa) (Syed, Karadaghy, & Zustiak, 2015).

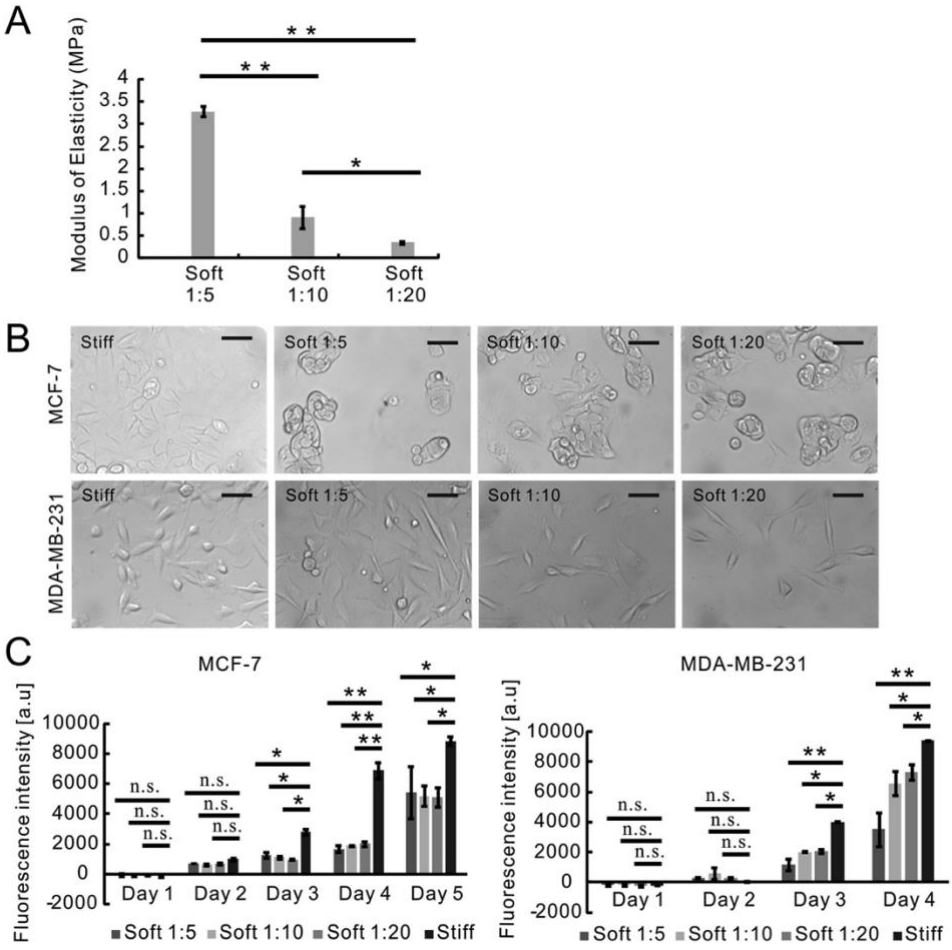

Figure 3.2 Stiff matrix improved proliferation rates of both metastatic and non-metastatic cancer cells. (A) The determination of modulus of elasticity of three types of PDMS substrates. Data were represented as means ± SD (n = 5). ∗∗P < 0.01, ∗P < 0.05. (B) Phase contrast microscopy of MCF-7 and MDA-MB-231 cells that were cultured on plastic (Stiff) or PDMS substrates (Soft 1:5, Soft 1:10, Soft 1:20); Scale bar, 10 μm. (C) The proliferation analysis of MCF-7 and MDA-MB-231 cells that were cultured on plastic (Stiff) or PDMS substrates (Soft 1:5, Soft 1:10, Soft 1:20) using Alamar Blue assay. Data were represented as means ± SD (n = 3). ∗∗P < 0.01, ∗P < 0.05, n.s. not significant.

3.3.2 Matrix stiffness altered the morphology of non-metastatic breast cancer cells instead of metastatic breast cancer cells

As shown in Fig. 1, we performed the surface modification on stiff (plastic) and soft (PDMS) substrates by oxygen plasma treatment and collagen coating (Fig. 1). To investigate the effects of matrix stiffness on breast cancer behaviours, we used highly metastatic breast cancer cell line (MDA-MB-231) and non-metastatic

counterpart cell line (MCF-7) (Liu et al., 2019). Phase contrast microscopy demonstrated that MDA-MB-231 cells exhibited the spindle-shaped morphologies both on stiff (plastic) and soft (PDMS) substrates (Fig. 2B). MCF-7 cells on stiff substrate were flat with cobblestone-like morphology (Fig. 2B). By comparison, MCF-7 cells on soft (PDMS) substrates acquired spherical morphology and formed aggregates (Fig. 2B).

3.3.3 Both metastatic and non-metastatic cancer cells gained higher proliferation rates in response to stiff matrix

The metastatic potentials of MCF-7 and MDA-MB-231 cells were characterized in our previous studies (W. Chen et al., 2019), and their proliferation rates on different substrates were compared using Alamar Blue assay in Fig. 2C. The results showed that MDA-MB-231 cells acquired a significantly higher growth rate on the plastic substrate compared with all PDMS substrates (Fig. 2C). However, both MCF-7 and MDA-MB-231 cells maintained a similar proliferation rate among the three types of PDMS substrates (Fig. 2C).

3.3.4 Metastatic breast cancer cells instead of non-metastatic counterpart acquired migratory advantage from stiff matrix

Then we sought to understand the effects of matrix stiffness on migratory capacity of breast cancer cells with distinct metastatic potential. Thus, we employed the gap closure assay to assess the mobility of highly metastatic MDA-MB-231 and Hs 578T cells in comparison to non-metastatic MCF-7 cells when they were grown on stiff and soft substrates. The images of the gap closure assay were shown in Figure 3A and quantified in Figure 3B. MCF-7 cells that were cultured on soft

(PDMS) and stiff (plastic) substrates exhibited a similar rate of gap closure, which was reflected by the average healing speed (Figure 3B). In comparison, Hs 578T and MDA-MB-231 cells on the stiff (plastic) substrate displayed a higher average healing speed than the cells grown on the soft (PDMS) substrates (Figure 3B). However, there was no significant difference in the average healing speed of cells between stiff and soft substrates when MCF-7, Hs 578T and MDA-MB-231 cells were transfected with siRNA targeting against YAP (Figure 3B).

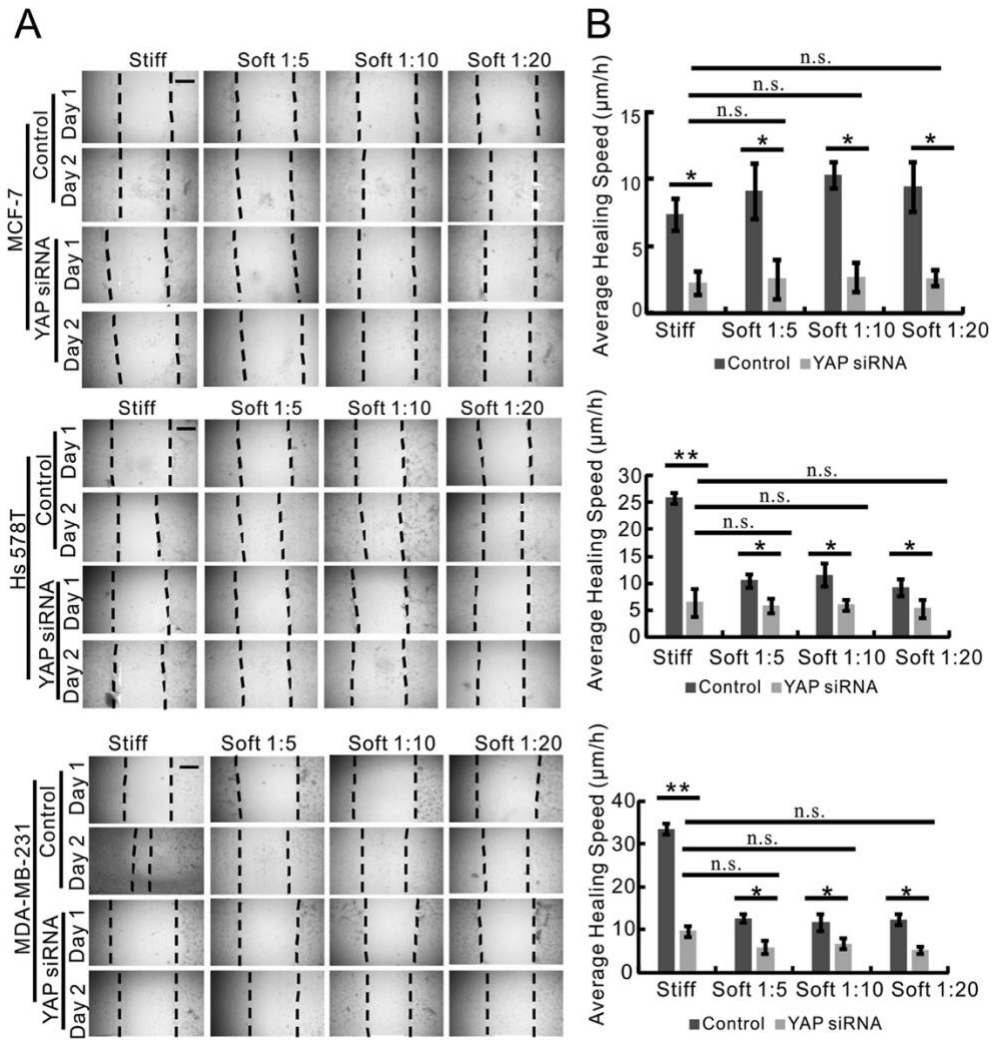

Figure 3.3 Migration of metastatic breast cancer cells was promoted by stiff matrix in a YAP-dependent manner. (A) Time-lapse microscopy images of gap closure of MCF-7, Hs 578T and MDA-MB-231 cells that were cultured on stiff (plastic) or soft (PDMS) substrates (Soft 1:5, Soft 1:10, Soft 1:20) at 0 h (Day 1) and 24 h (Day 2) after scratch was created. The dotted lines define the area lacking cells. Scale bar, 100 μm. (B) The quantification of Gap Closure Assay in Figure 3A using ImageJ. The average healing speed (μm/h) of MCF-7 (top graph), Hs 578T (middle graph) and MDA-MB-231 cells (bottom graph) was shown. Data were represented as means ± SD (n = 3). ∗P < 0.05.

3.3.5 Increased YAP nuclear entry was shown in both metastatic and non-metastatic cancer cells on stiff matrix

To better understand the effects of matrix stiffness on YAP nucleocytoplasmic shuttling, we performed the YAP intracellular localization analysis in MCF-7 and

MDA-MB-231 cells cultivated on soft (PDMS) and stiff (plastic) substrates. Then we conducted fluorescent microscopy of YAP (Fig. 4A, green channel) and DAPI (Fig. 4A, blue channel) and colocalization analysis of the two channels (Fig. 4B) to measure YAP nucleo-cytoplasmic shuttling.

YAP was shown to locate mostly outside the nucleus (Fig. 4A, arrow heads) in both MCF-7 and MDA-MB-231 cells that were grown on soft (PDMS) substrates. When both the cell lines were cultured on stiff (plastic) substrate, YAP was evenly distributed intracellularly (Fig. 4A). Consistently, higher colocalization between YAP and DAPI (YAP nuclear entry) was observed in MCF-7 cells and MDA-MB-231 cells that were cultured on stiff (plastic) substrate (Manders Coefficient 0.9 and 0.7 respectively) (Fig. 4B). Whereas MCF-7 cells and MDA-MB-231 cells grown on soft (PDMS) substrates displayed significantly less YAP and DAPI colocalization (Manders Coefficient close to or less than 0.2).

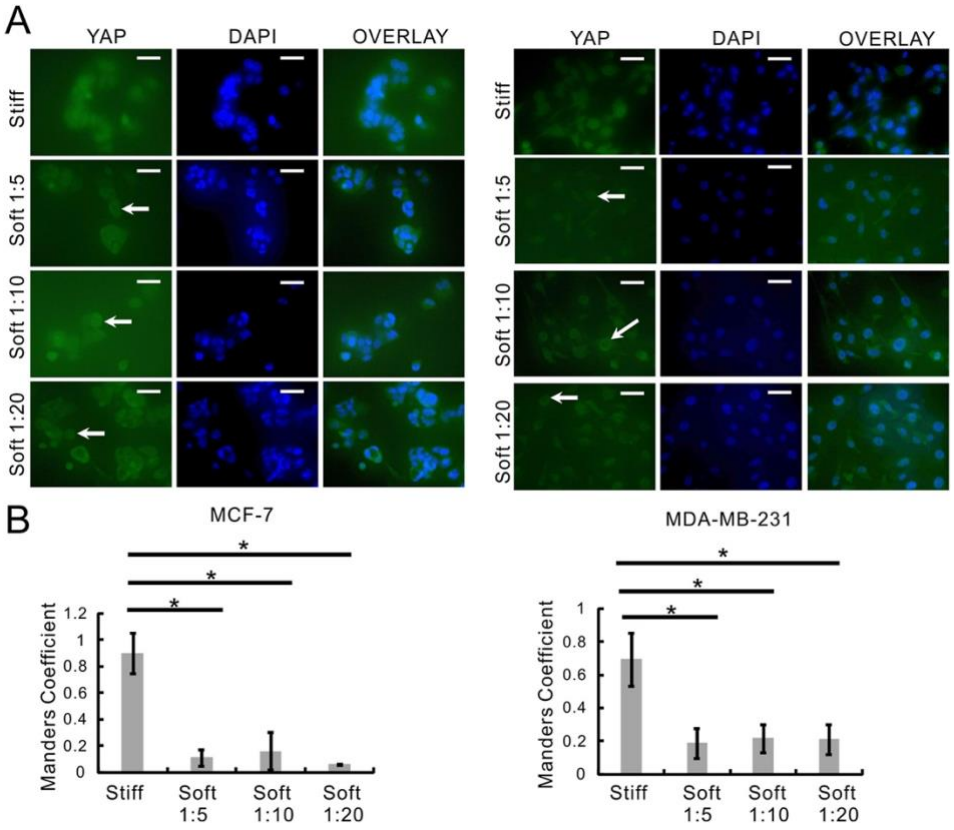

Figure 3.4 Both metastatic and non-metastatic cancer cell lines exhibited increased YAP nuclear entry in response to stiff matrix. (A) MCF-7 and MDA-MB-231 cells were cultured on stiff (plastic) or soft (PDMS) substrates (Soft 1:5, Soft 1:10, Soft 1:20) and then YAP subcellular localization in those cells was analyzed by immunofluorescence staining. The cells showing nuclear exclusion of YAP were highlighted by arrowheads. Field-of-views were selected randomly under each condition and photographed at a magnification of 40 ×. Scale bar, 10 μm; (B) Colocalization analysis of YAP and DAPI in the cells from Figure 4A were analyzed using CellProfiler. Data were represented as means ± SD (n = 3). ∗P < 0.05.

3.3.6 The stiffness difference between PDMS substrates did not affect YAP mRNA expression in metastatic cancer cells

As YAP nucleo-cytoplasmic shuttling was regulated by matrix stiffness (Fig. 4), we sought to identify whether YAP gene expression in MDA-MB-231 cells sensed the stiffness differences between the three PDMS substrates. Due to the low expression of YAP signalling in MCF-7 cells, we conducted the mRNA analysis in MDA-MB-231 cells that were cultivated on PDMS using qRT-PCR analysis. The

results showed no difference in the gene expression of YAP (Fig. 5A, left) and its target gene CTGF (Fig. 5A, right) in MDA-MB-231 cells cultivated on the three types of PDMS substrates. The results indicated that YAP dependent mechanotransduction might not differentiate the range (0.35 MPa to 3.28 MPa) of matrix stiffness.

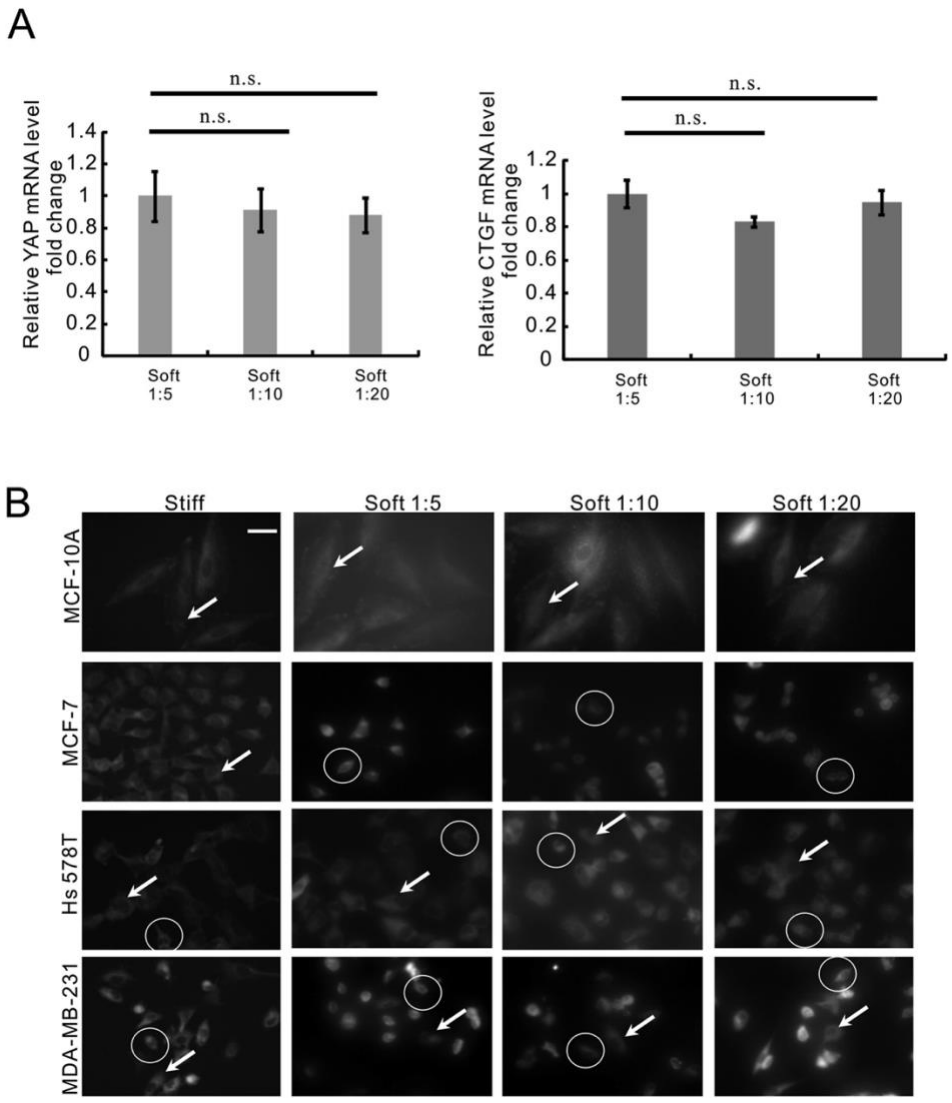

Figure 3.5 Focal adhesion dynamics in metastatic breast cancer cells were regulated by matrix stiffness. (A) qRT-PCR analysis of YAP (left panel) and CTGF (right panel) gene expression in MCF-7 and MDA-MB-231 cells that are cultured on stiff (plastic) or soft (PDMS) substrates (Soft 1:5, Soft 1:10, Soft 1:20). Data were represented as means ± SD

(n = 3). n.s. not significant. (B) The paxillin staining in MCF-10A, MCF-7, Hs 578T and MDA-MB-231 cells that were cultured on stiff (plastic) or soft (PDMS) substrates (Soft 1:5, Soft 1:10, Soft 1:20). Field-of-views were selected randomly under each condition and photographed at a magnification of 40 ×. Scale bar, 10 μm. The representative cells with diffuse staining pattern of paxillin were highlighted using arrowheads and the representative cells with perinuclear staining pattern of paxillin were highlighted using circles.

3.3.7 Focal adhesion dynamics in metastatic breast cancer cells were regulated by matrix stiffness

Figure 3 showed that MDA-MB-231 and Hs 578T cells acquired higher migratory potential on the stiff (plastic) substrate. In order to characterize the FAs dynamics associated with mobility increase of metastatic breast cancer cells, we performed paxillin staining with MCF-10A, MCF-7, Hs 578T, and MDA-MB-231 cells that were cultured on the substrates with different amount of stiffness. A similar diffuse cytoplasmic staining pattern of paxillin was observed in non-cancerous MCF-10A cells cultured on the stiff (plastic) and soft (PDMS) substrates (Figure 5B, highlighted by arrowheads). Metastatic breast cancer cell lines MDA-MB-231 and Hs 578T cells exhibited a mix of both diffuse cytoplasmic staining pattern (Figure 5B, highlighted by arrowheads) and perinuclear staining pattern (Figure 5B, highlighted by circles) when cultured on the stiff (plastic) and soft (PDMS) substrates. Whereas most of MCF-7 cells on the soft substrate (PDMS) displayed perinuclear staining pattern of paxillin (Figure 5B, highlighted by circles) in comparison to the cells on stiff substrate (plastic).

3.4 Discussion

ECM stiffness of metastatic sites is linked to biochemical signalling in breast cancer, which influences the outcome of cancer metastasis (Ulbricht et al., 2013).

Although YAP was shown to be the central hub regulating cancer proliferation and metastasis, the significance of YAP orchestrated mechanotransduction in the outcome of breast cancer metastasis on secondary locations remains unclear (Lamar et al., 2012; Ondeck et al., 2019; Wu & Yang, 2018). In this paper, we identified a YAP-dependent cellular mechanism through which metastatic breast cancer cells enhance their migratory potential on stiff matrix.

YAP is primarily controlled at the level of the nucleocytoplasmic shuttling (Shreberk-Shaked & Oren, 2019). However, the controlling mechanisms over YAP nucleocytoplasmic shuttling orchestrates cancer behaviours are unclear (Dobrokhotov, Samsonov, Sokabe, & Hirata, 2018; Elosegui-Artola et al., 2017; Shreberk-Shaked & Oren, 2019). Most of these mechanical inputs converge on two distinct, yet interdependent signal transduction systems: the Hippo pathway and the state of the actomyosin cytoskeleton (Kofler et al., 2018). Our research characterized the effects of matrix stiffness on breast cancer metastasis via a series of cellular events such as YAP nucleocytoplasmic shutting (Fig.4), alteration of FA dynamics (Fig. 5), cell mobility enhancement (Fig. 3).

This study provided the findings for the understanding of cancer behaviours that are developed in response to matrix stiffness. Firstly, the results showed that migration of metastatic breast cancer cells is regulated by matrix stiffness. And this regulation was not observed in non-metastatic breast cancer cells. Secondly, the results suggested that YAP signalling is required for the regulation of breast cancer cell migration by matrix stiffness.

3.5 Conclusions

To summarize, our study demonstrated that migration of metastatic breast cancer cells is promoted by stiff matrix via YAP signalling. Since initiating cell growth and migration is the most challenging step for disseminating breast cancer cells (Langley & Fidler, 2011), the continued investigation into the regulation of cancer cell migration by matrix stiffness will shed light on the mechanism of breast cancer metastasis.

Conflicts of Interest

Authors declare no conflict of interest.

Acknowledgements

This research was supported by Mitacs Accelerate Program (IT15842). We are grateful to Dr. Juliet Daniel for providing human breast cancer MDA-MB-231 cells.

Author Contributions

W. Chen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper
S. Park: Contributed reagents, materials, analysis tools or data; Performed the experiments
C. Patel: Contributed reagents, materials, analysis tools or data; Performed the experiments
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A. Raha: Analyzed and interpreted the data
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F. Geng: Conceived and designed the experiments; Wrote the paper

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Chapter 4

The Interplay of Autophagy, YAP Mechanotransduction, and Cancer Metastasis

Despite the intense effort over the years, there is limited research investigating how the combined mechanism of autophagy and YAP mechanotransduction is involved in breast cancer metastasis. A study from Totaro et al. recently demonstrated that cellular mechanotransduction regulates autophagy in a YAP/TAZ-dependent manner (Totaro et al., 2019). However, the mechanism in breast cancer metastasis when they metastasize at a secondary site remains unknown. In this chapter, we advance in this direction by introducing two stimuli to breast cancer cells simultaneously, the rapamycin-induced autophagy treatment along with mechanical matrix stiffness. Here, we investigate the convergence of autophagy and cellular mechanotransduction to observe their effect on YAP signalling and cancer metastasis between the primary site and the secondary site.

4.1 Introduction

Breast cancer is the leading cause of cancer-related deaths in females despite many attempts to develop new therapies (Jemal et al., 2009). Among the breast cancer patients, 10-15% have triple-negative breast cancer (TNBC) (Anders & Carey, 2009; Chavez, Garimella, & Lipkowitz, 2010). They relapse more

frequently and have a worse outcome than other subtypes (Anders & Carey, 2009; Chavez et al., 2010).

Extracellular matrix (ECM) stiffness is considered one of the major factors regulating cancer progression and cancer malignancy as they influence cellular behaviour (Dupont, 2016; McConnell et al., 2016; Provenzano et al., 2008). As a hallmark for cancer malignancy, metastasis is a complex process that includes tumour cell invasion, intravasation, survival in the circulation, extravasation, and outgrowth at a secondary site (E. E. Mowers et al., 2017). Breast cancer metastasize to various distant sites, with the brain being one of the most frequent location (Wenjing Chen et al., 2018; Medeiros & Allan, 2019), and is the leading cause for breast cancer mortality. However, breast cancer metastasis is highly inefficient because less than 0.1% of circulating tumour cells (CTCs) will succeed in forming secondary tumours (Langley & Fidler, 2011). In addition, the development of CTCs at a distant metastatic site is driven by the interactions between breast cancer cells and the stiffness of the metastatic site (Braun et al., 2005; Langley & Fidler, 2011). As the matrix stiffness for breast cancer metastatic sites varies, thus, it would be critical to understand the metastatic mechanism on the secondary site.

Autophagy is a highly conserved self-degradative mechanism that has a key role in homeostasis (Galluzzi et al., 2017; Levine, 2007; E. E. Mowers et al., 2017). Various studies using surrogate markers such as the microtubule-associated protein 1A/1B light chain 3-II (LC3-II) reported an association between increased autophagy and metastasis (Lazova et al., 2012; H. Zhao et al., 2013). The

mammalian target of rapamycin (mTOR) signalling is often upregulated in breast cancer, and activation of mTOR1 complex (mTORC1) leads to increased tumour progression and increased autophagy inhibition (Hare & Harvey, 2017). It is known that rapamycin can be used to induce autophagy through inhibition of mTORC1 signalling (Levine & Kroemer, 2008; Rubinsztein et al., 2007).

The Yes-associated protein (YAP) is known as the central regulator of mechanotransduction (Dupont et al., 2011); on the other hand, YAP is a critical downstream effector of the hippo pathway and has a significant role in cancer (Aragona et al., 2013). The subcellular localization of YAP shuttles between the cytoplasm and the nucleus, where the latter allows YAP to bind to the transcriptional enhanced associate domain (TEAD) and mediate downstream target gene expressions that are involved in cell proliferation and migration (Boopathy & Hong, 2019; Meng et al., 2016). Previous studies demonstrate that stiff ECM promotes YAP nuclear localization (Dupont et al., 2011), and increased autophagy is associated with increased YAP activity (Pavel et al., 2018; Totaro et al., 2018). It has also been shown that YAP activity can enhance the metastatic potential of breast cancer (Lamar et al., 2012; T. Wang et al., 2018). However, the combined consequence of autophagy and mechanical factors such as matrix stiffness is not well known.

Our previous studies demonstrated that autophagy promotes TNBC metastasis through YAP nuclear entry (Wei Chen, Bai, Patel, & Geng, 2019), and matrix stiffness regulates TNBC cells migration through YAP signalling (Wei Chen et al.,

2021). Recently, a study using cancer stem cells showed that cellular mechanotransduction regulates autophagy through YAP transcriptional activity (Totaro et al., 2019). However, the interacting effect on TNBC metastasis remains incompletely known. Therefore, we advance from this direction to investigate the role of YAP signalling during the convergence of autophagy and cellular mechanotransduction and their effect on breast cancer metastasis on the primary and the secondary site.

4.2 Methods

4.2.1 Cell Culture and Reagents

Human breast cancer cells Hs578T cells and MDA-MB-231 cells (both donated by Dr. Juliet Daniel Research Group) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS). All cultures were incubated at 37°C with 5% CO_2. All cell lines were cultured on glass-bottom CytoSoft® PDMS 24 well imaging plates (Advanced BioMatrix) for the preparation of immunofluorescence staining and on CytoSoft® PDMS 6-well plates (Advanced BioMatrix) for the preparation of migration assay. Cells are then subject to rapamycin (Sigma) treatment for induced autophagy.

4.2.2 Preparation of CytoSoft® PDMS Plates for Cell Culture

CytoSoft® PDMS plates with 2 kPa and 32 kPa rigidity were coated for subsequent cell culture. Extracellular matrix coating was prepared by neutralizing Rat Tail Type I Collagen (Gibco) with phosphate-buffered saline (1 X PBS) to a coating concentration of 100 µg/ml. Enough prepared collagen was dispensed into

each well of the plate to coat the surface, then the plates are incubated at room temperature with plate cover on for 45 minutes. Coated surfaces were rinsed two times with culture medium or PBS immediately after incubation, and fresh culture medium was added per well to keep surfaces hydrated before cell culture.

4.2.3 Immunofluorescence Staining and Imaging

Following rapamycin treatment, MDA-MB-231 cells and Hs578T cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 for the analysis of YAP nucleus localization. Then, the cells were washed with PBS and blocked with 5% PBS-BSA at 4°C overnight. Then the cells were incubated with mouse anti-human YAP monoclonal antibody (Santa Cruz Biotechnology) and LC3B antibody in rabbit (Cell Signalling) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich) and Rhodamine (TRITC) – conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch). Cell nuclei of each sample were stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Thermo Fisher Scientific) and imaged on EVOS ™ FL Auto 2 inverted microscope (AMAFD2000, Invitrogen).

4.3 Results

Our previous studies initiated this work when we were investigating the individual scope of autophagy and matrix stiffness on TNBC metastasis. In our first study, we used rapamycin to induce autophagy in TNBC cells pharmaceutically. We demonstrated that TNBC cells use autophagy as a mechanism to promote YAP nuclear localization, contributing to its metastatic cancer behaviours (Wei Chen et al., 2019). Our second study used mechanical factor, matrix stiffness, by seeding

TNBC cells on collagen-coated PDMS substrate of different stiffness. We found that the stiff matrix promotes the migration of metastatic breast cancer cells in a YAP-dependent manner (Wei Chen et al., 2021).

In this chapter, we incorporate both scopes simultaneously as shown in Figure 4.1, which individually, they demonstrated to promote YAP nuclear entry that enhances metastatic behaviour in cells to observe the unknown interacting effect of autophagy and matrix stiffness in YAP biology and future breast cancer behaviour on the secondary site.

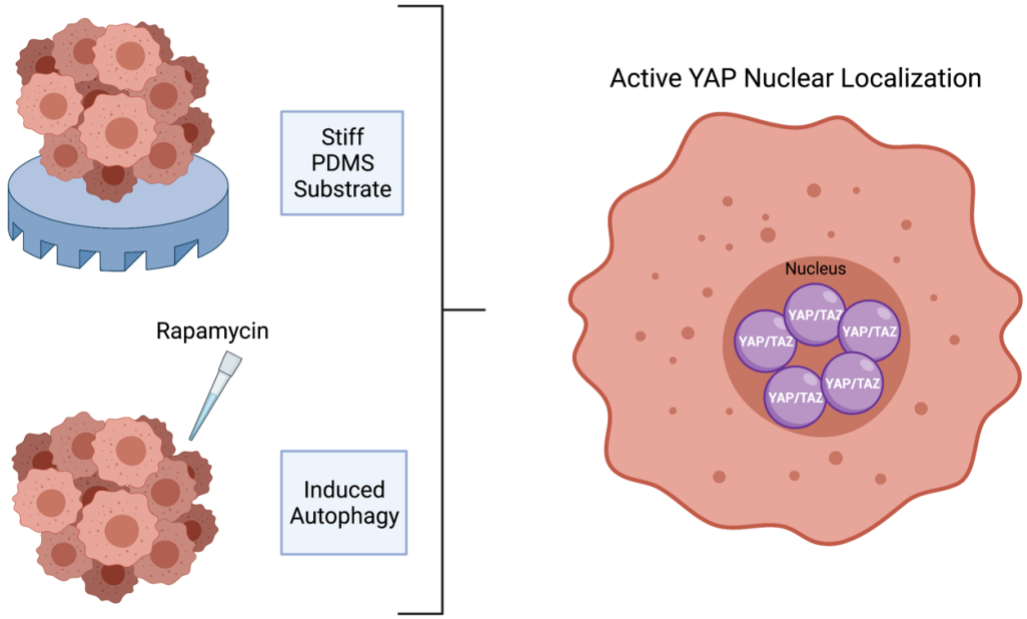

Figure 4.1 Autophagy and matrix stiffness stimulus is shown in previous publications to promote YAP nuclear localization in TNBC. Created with BioRender.com.

First, we cultured TNBC cells, MDA-MB-231 and Hs 578T on 2 kPa and 32 kPa CytoSoft® PDMS plates. The normal glandular breast tissue was shown to have moduli in the range of 0.73 ± 0.18 kPa to 66 ± 17 kPa (Ramião et al., 2016), and

the stiffness of the brain tissue was measured to be in the range of 1-3 kPa (Green et al., 2008; Sack et al., 2008). Thus, in this chapter, we used a 32 kPa CytoSoft® PDMS plate to demonstrate the stiffness of the original site for breast cancer cells and a 2 kPa CytoSoft® PDMS plate to demonstrate the stiffness of the brain tissue, which is one of the significant distant metastatic sites. Then, rapamycin treatment was implemented to induce autophagy within the cells. To verify whether our observation is genuinely due to the effect of autophagy, TNBC cells were also cultured in the same condition and treated with DMSO as control.

As we sought to understand the biological significance of YAP regulation upon both autophagy and matrix stiffness in cancer metastasis, we examined the subcellular localization of YAP to the autophagy verifying marker LC3 by double immunofluorescence staining. We know rapamycin-induced autophagy was activated in the metastatic breast cancer cells as the upregulated autophagic flux was shown by increased LC3 puncta. As seen in Figure 4.2, we observed YAP nuclear entry in MDA-MB-231 cells upon autophagy on both 2 kPa and 32 kPa. However, increased YAP nuclear localization was seen in MDA-MB-231 cells seeded on the stiffer matrix (32 kPa) upon triggering autophagy (Figure 4.2). Similar results were observed in Hs 578T cells where more YAP nuclear translocation was observed on the 32 kPa matrix with induced autophagy (Figure 4.3). Taken together, based on the YAP and LC3 staining, it suggests matrix stiffness and autophagy mediates YAP nuclear translocation collectively.

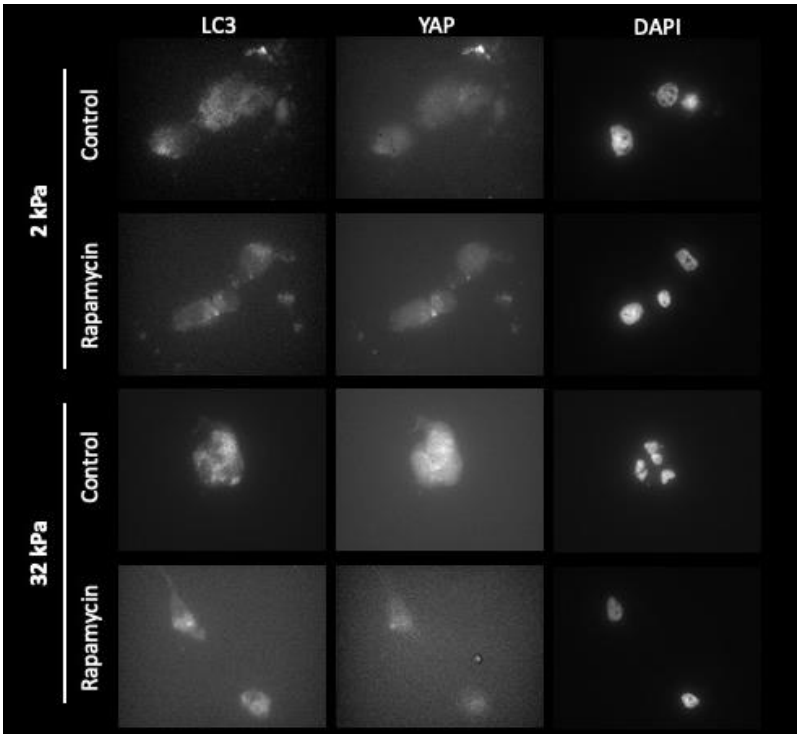

Figure 4.2 MDA-MB-231 cells cultured on brain tissue (2 kPa) and breast tissue (32 kPa) showed stiffer matrix and autophagy together mediated YAP nuclear translocation.

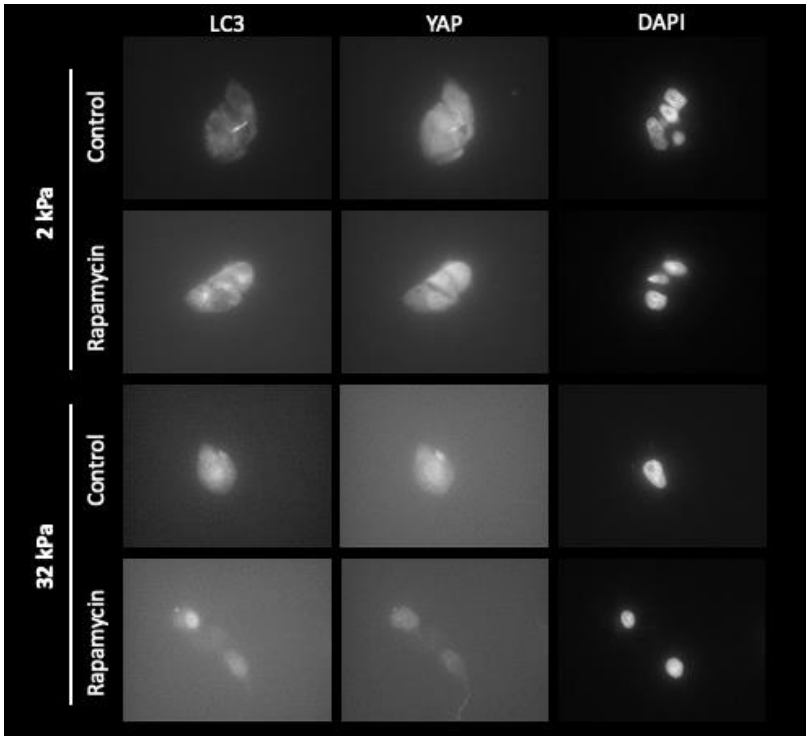

Figure 4.3 Hs 578T cells cultured on brain tissue (2 kPa) and breast tissue (32 kPa) showed matrix stiffness and autophagy mediated YAP translocation together.

4.4 Discussion and Conclusion

The stiffness of ECM for different metastatic sites (bone, brain, lung, and lymph node) is linked to biochemical signalling in breast cancer, which influences the outcome of cancer metastasis (Ulbricht et al., 2013). Although YAP plays a critical role in regulating cancer proliferation and metastasis, the significance of YAP coordinated mechanotransduction in response to autophagy in the outcome of breast cancer metastasis remains unclear (Lamar et al., 2012; Ondeck et al., 2019; Wu & Yang, 2018). In this chapter, we identified the synergic effect of autophagy and YAP mechanotransduction in metastatic breast cancer.

Previous studies demonstrated that stiff ECM promotes YAP nuclear translocation (Dupont et al., 2011), and there is a positive association between

autophagy and YAP activity (Pavel et al., 2018; Totaro et al., 2018). In a recent publication by Totaro et al., they described autophagy is mechanically regulated through regulating YAP transcriptional activity (Totaro et al., 2019). However, we generated information from our assumption that 2 kPa demonstrates brain tissue while 32 kPa demonstrates breast tissue, and our results showed autophagy and the response to matrix stiffness synergistically mediated YAP nuclear localization at the primary breast tissue in comparison with the secondary brain tissue as YAP nuclear localization is not as significant on the 2 kPa during autophagy (Figure 4.2 and 4.3). This data suggests that increasing YAP seen on the 32 kPa (breast tissue) promotes cell migration; therefore, metastatic breast cancer cells migrate from the primary breast tissue to a secondary location with softer matrix stiffness, and the metastatic process is halted. As this is a preliminary observation, further investigation is required. YAP knockdown and YAP mutant should be implemented to verify the involvement of YAP activity upon autophagy and mechanotransduction. Moreover, the gap closure assay should be performed to assess the mobility of metastatic breast cancer cells upon the stimuli.

To summarize, our data showed the convergence of autophagy and YAP mechanotransduction in metastatic breast cancer. It suggests mechanotransduction and autophagy together has a synergistic effect to mediated YAP nuclear localization. This data implies that the metastatic breast cancer cells may exhibit different YAP signalling when establishing on a secondary site with different matrix stiffness from the primary site.

Chapter 5

Conclusion

This thesis was inspired by the unclear mechanism in the combined scope of autophagy and cellular mechanotransduction on breast cancer metastasis to provide insight for the development of potential metastatic breast cancer therapeutics. Since breast cancer metastasis to distant locations with different matrix stiffness, it is unclear how and whether the metastatic process will initiate again at the secondary location. Thus, there is no one-size-fits-all conclusion to meet the breast cancer metastasis process on all tissues. Therefore, after extensive investigation, we suggest mechanotransduction and autophagy has a synergistic relationship to mediate YAP nuclear translocation.

In chapter 2, we first started with investigating the effect of autophagy on YAP activity in the context of breast cancer metastasis. Numerous studies have identified an association between increased autophagy and metastasis (Kabeya, 2000; Lefort et al., 2014), but the molecular mechanisms underlying metastatic progression regulated by autophagy remain unclear. A previous study showed YAP is upregulated in cells during induced autophagy, suggesting the role of YAP signalling in autophagy regulation (Liang et al., 2014). However, this correlation is unclear in cancer metastasis. We performed YAP signalling profiling analysis, YAP

subcellular localization, autophagosome formation, and cell invasiveness in TNBC cell lines to investigate the association between autophagy and YAP signalling in TNBC. The results demonstrated that YAP transcriptional and protein expression was significantly upregulated in TNBC. In addition, upon rapamycin-induced autophagy, we observed YAP nuclear localization and increased expression of YAP target gene ANKRD1 in the TNBC. Therefore, in chapter 2, we reported the existence of the autophagy-YAP-metastasis axis in TNBC, and we verified that TNBC uses autophagy to promote YAP nuclear translocation, thus giving the cells invasion and metastatic advantage (Figure 5.1 A).

In chapter 3, we investigated the effect of matrix stiffness on YAP activity in the context of breast cancer metastasis on the primary and secondary metastatic sites. Breast cancer cells can metastasize to various locations with diverse ECM matrix stiffness (Chen, Hoffmann, Liu, & Liu, 2018; Medeiros & Allan, 2019). In addition, the development of CTCs at a metastatic site is guided by the interactions between breast cancer cells and the stiffness of the metastatic site (Braun et al., 2005; Langley & Fidler, 2011). Previous publications reported that increased ECM matrix stiffness enhances YAP nuclear localization (Dupont et al., 2011), in which YAP regulates the migration, invasion, and tumorigenesis of breast cancer cells (Chan et al., 2008). However, the role of YAP mechanosensing remains unclear in the interplay between breast cancer and matrix stiffness (Barkan, Green, & Chambers, 2010; Langley & Fidler, 2007). We prepared PDMS substrates with different stiffness to demonstrate the different matrix stiffness of the metastatic sites,

and performed YAP signalling profiling analysis, YAP immunofluorescence staining and colocalization analysis, YAP siRNA transfection, and gap closure assay to investigate the association between matrix stiffness and YAP signalling in TNBC. Our result showed an increase in nuclear entrance of YAP and a higher proliferation rate in the metastatic MDA-MB-231 cells when they were cultured on the stiff matrix. In addition, the MDA-MD-231 cells displayed YAP-regulated increased mobility in response to the stiff matrix. In summary, this chapter demonstrated that the stiff matrix promotes the migration of metastatic breast cancer cells through YAP signalling (Figure 5.1 B).

Chapter 4 elaborated from the previous chapters to examine the synchronizing involvement of the two stimuli, autophagy and YAP mechanotransduction, in breast cancer metastasis. Breast cancer metastasizes to various distant sites, all of which have distinct matrix stiffness (Wenjing Chen et al., 2018; Medeiros & Allan, 2019). In addition, breast cancer metastasis is highly ineffective because very few CTCs will succeed in forming secondary tumours (Langley & Fidler, 2011). Nonetheless, the mechanism in breast cancer metastasis at a secondary location remains unknown. A recent study demonstrated that cellular mechanotransduction regulates autophagy via YAP signalling (Totaro et al., 2019). However, the convergence of YAP mechanotransduction and autophagy in the context of metastatic breast cancer on the secondary site is unexplored. We performed rapamycin-induced autophagy on TNBC cells cultured on 2 kPa (similar to the stiffness of the brain tissue) and 32 kPa (similar to the stiffness of the breast tissue) CytoSoft® PDMS plates followed

by YAP and LC3 double immunofluorescence staining. The results presented increased YAP nuclear localization in TNBC cells cultured on the stiff 32 kPa matrix upon autophagy; however, there was no significant nuclear localization of YAP in cells seeded on 2 kPa control. This suggests matrix stiffness and autophagy together upregulate YAP nuclear entry (Figure 5.1 C). The data also indicates that the metastatic breast cancer cells may exhibit different YAP signalling upon colonization at a secondary location with a different matrix stiffness from the primary location. Nonetheless, further investigation, such as YAP knockdown and gap closure assay, is required to assess YAP activity in cancer metastasis.

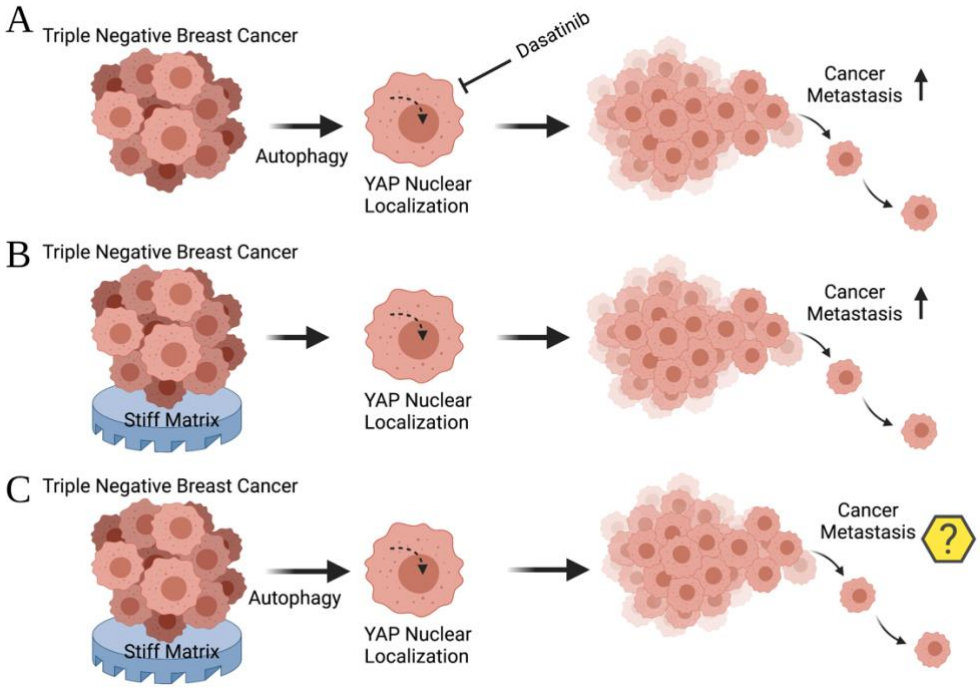

Figure 5.1 Illustration of conclusion from each chapter. (A) Autophagy promotes triple-negative breast cancer metastasis through YAP nuclear localization, (B) matrix stiffness regulates the migration of metastatic breast cancer cells through YAP nuclear localization, and (C) matrix stiffness and autophagy synergistically upregulate YAP nuclear translocation. Created with BioRender.com.

Although we proposed an *in vitro* model to mimic tissue microenvironment in order to study the effect of autophagy and mechanotransduction, there are limitations in our model. To simplify the factors in order to observe the effect of mechanotransduction, we examined breast cancer cells cultured on various matrix stiffnesses to understand how the cells react to the mechanical stimulus. However, tissue microenvironments are not only determined by the matrix stiffnesses; there are many other factors such as the proteins embedded in the tissues and pH of the microenvironment that can be implemented to better simulate the tissue microenvironments. To fully understand the coherent role of autophagy and mechanotransduction and their relationship to breast cancer metastasis, an improved *in vitro* model and future investigation are required. To elucidate the role of YAP in coordinating between the autophagy and mechanotransduction pathway, YAP knockdown or YAP mutants can be implemented in future studies. The gap closure assay and Boyden chamber assay can be performed to assess the mobility and invasion of metastatic breast cancer cells upon the stimuli to determine the invasiveness and migration of the cancer cells. Moreover, photolithography, a process used in microfabrication to pattern on the substrates, can be implemented into our PDMS substrate-making procedure to create a PDMS substrate surface that better simulates the tissue matrix surface for the cell culture model.

In conclusion, our research demonstrated the convergence of autophagy and YAP mechanotransduction in metastatic breast cancer and the potential to initiate metastasis on a secondary location. This will further create the opportunity to

understand YAP biology and the mechanism of breast cancer metastasis that will shed light on YAP-targeting therapeutics in treating metastatic breast cancer patients.

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