The role of angiopoietin-like 4 (ANGPTL4) in chemoresistance and autophagy during epithelial-mesenchymal transition (EMT)

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**NANYANG
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SINGAPORE

**THE ROLE OF ANGIOPOIETIN-LIKE 4 (ANGPTL4) IN
CHEMORESISTANCE AND AUTOPHAGY DURING
EPITHELIAL-MESENCHYMAL TRANSITION (EMT)**

LIM MIANG KEE, MAEGAN

SCHOOL OF BIOLOGICAL SCIENCES

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Chemoresistance and Autophagy During Epithelial-
Mesenchymal Transition (EMT)**

LIM MIANG KEE, MAEGAN

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from 1 paper(s) published in the following peer-reviewed journal(s) where I was the first and/or corresponding author.

Chapter 3 is published as M.M. K. Lim, J. W. Wee, J. C. Soong, D. Chua, W. R. Tan, M. Lizwan, Y. Li, Z. Teo, W. W. B. Goh, P. Zhu, N. S. Tan. Targeting metabolic flexibility via angiopoietin-like 4 protein sensitizes metastatic cancer cells to chemotherapy drugs. *Molecular Cancer* 17(1):152 (2018). DOI: 10.1186/s12943-018-0904-z.

The contributions of the co-authors are as follows:

- Assoc. Prof. Tan Nguan Soon, Andrew designed, interpreted the data, wrote the manuscript and supervised the entire study
- I designed, performed the experiments and interpreted the data including FACS analysis for *in vivo* animal experiments and energy charge experiments, kinase inhibitor screen, IPA analysis, FACS analysis and wrote the manuscript
- Dr. Pengcheng Zhu designed and performed the experiments and interpreted the data including kinase inhibitor screen, IPA analysis and energy charge experiments, and wrote the manuscript
- Dr. Ziqiang Teo performed the kinase inhibitor screen and IPA analysis, FACS analysis and chemoresistance analysis
- Mr. Damien Chua performed the FACS analysis and chemoresistance analysis
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- Mr. Yinliang Li performed the FACS analysis and chemoresistance analysis
- Dr. Wilson Wen Bin Goh performed analysis using the TCGA database

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“Science may set limits to knowledge but should not set limits to imagination.” -

Bertrand Russell

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LIST OF ABBREVIATIONS

18S	18S ribosomal RNA
2-NBDG	2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose
4-OHT	4-hydrotamoxifen
α -cANGPTL4	antibody against human cANGPTL4
Δ ANGPTL4	siRNA knockdown of ANGPTL4
ABC	ATP-binding cassette
ABCB1/MDR1/P-gp	ATP-binding cassette transporter B1
ABCC1/MRP1	ATP-binding cassette transporter C1
ABCG2/BCRP	ATP-binding cassette transporter G2
ACL	ATP-citrate lyase
AMBRA1	BECN1-regulated autophagy protein 1
AMPK	AMP-activated protein kinase
ANGPTL4	angiopoietin-like 4
Atg	autophagy-related genes (yeast)
ATG	autophagy-related genes (mammals)
ATP	adenosine triphosphate
BAD	BCL2 associated agonist of cell death
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
Bcl-w	Bcl-2-like protein 2
Bcl-xL	B-cell lymphoma-extra large
BCRP	breast cancer resistance protein
BECN1	beclin-1 gene
BIM	BCL2 like 11
CAFs	cancer-associated fibroblasts
CAMs	cell adhesion molecules
cANGPTL4	C-terminal fragment of angiopoietin-like 4
CCL5	C-C motif chemokine ligand 5
CCL21	C-C motif chemokine ligand 21
CDH1	E-cadherin gene
cFLIP	cellular FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory protein
CMA	chaperone-mediated autophagy
CTLs	cytotoxic T lymphocytes
DDR1	discoidin domain receptor 1
Dox	doxycycline
EBV	Epstein-Barr virus
ECAR	extracellular acidification rate
ECM	extracellular matrix
EGFR/ErbB1	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum

ERBB3	erb-b2 receptor-tyrosine kinase 3
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
FAK	focal adhesion kinase
fANGPTL4	full length native form of angiopoietin-like 4
FAO	fatty acid oxidation
FAS	fatty acid synthase
Fas	fas cell surface death receptor
FGF	fibroblast growth factor
FOXO1	forkhead box O1
FOXO3	forkhead box O3
G6Pase	glucose-6-phosphatase
GLS	glutaminase
GLS2	glutaminase 2
GLUT1	glucose transporter type 1
GLUT4	glucose transporter type 4
GSH	glutathione
GST	glutathione S-transferase
GTP	guanosine triphosphate
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HER2/ErbB2	human epidermal growth factor receptor 2
HIF-1 α	hypoxia-inducible factor 1 α
HPV	human papillomavirus
HSF1	heat-shock transcription factor 1
ICs	inflammatory cells
IL-4	interleukin 4
IL-17	interleukin 17
i.p.	intraperitoneally
IPA	ingenuity pathway analysis
LC3	microtubule-associated proteins 1A/1B light chain 3
LC3-I	cytosolic form of LC3
LC3-II	lipidated form of LC3 conjugated to PE
LCFAs	long chain fatty acids
LDH	lactate dehydrogenase
LPL	lipoprotein lipase
MAP1LC3B	LC-3 gene
Mcl-1	induced myeloid leukemia cell differentiation protein
MDR	multidrug resistance
MDR1	multidrug resistance protein 1
MKN74 ^{Snai1ER}	snai1 inducible MKN74 cells
MKN74 ^{Snai1ER:shANGPTL4}	dual inducible MKN74 cells
MMPs	matrix metalloproteinases

MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9
MRP1	multidrug resistance-associated protein 1
MSCs	mesenchymal stem cells
MTAs	microtubule target agents
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
NADH	nicotinamide adenine dinucleotide
nANGPTL4	N-terminal fragment of angiopoietin-like 4
NF- κ B	nuclear factor kappa B
NK	natural killer
NSCLC	non-small cell lung carcinomas
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
p21	cyclin dependent kinase inhibitor 1A
p53	tumor protein p53
p62/SQSTM1	ubiquitin-binding protein p62/sequestosome-1
PACE4	paired basic amino acid-cleaving enzyme 4
PAS	phagophore assembly site
PC	proprotein convertase
PCD	programmed cell death
PE	phosphatidylethanolamine
PED	phosphoprotein enriched in diabetes protein
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PFKP	phosphofructokinase
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3 kinase
PI3P	phosphatidyl inositol-3-phosphate
P-gp	p-glycoprotein
PKB/Akt	protein kinase B
PKM1	pyruvate kinase isozyme M1
PKM2	pyruvate kinase isozyme M2
PLC	phospholipase C
PPARs	proliferator-activated receptors
PPP	pentose phosphate pathway
PTEN	phosphatase and tensin homolog
qChIP	quantitative chromatin immunoprecipitation
qPCR	quantitative PCR
RB	retinoblastoma protein
RB1CC1/FIP200	RB1 inducible coiled-coil 1
rh-cANGPTL4	recombinant human cANGPTL4
ROS	reactive oxygen species
S6K	ribosomal protein S6 kinase
SCD1	stearoyl-CoA desaturase
SCO2	SCO2 cytochrome c oxidase assembly protein

Slug	snail family transcriptional repressor 2
Snai1	snail family transcriptional repressor 1
Src	sarcoma viral oncogene homolog
STAT1	signal transducer and activator of transcription 1
TBP	TATA-binding protein
TCA	tricarboxylic acid
TERT	telomerase reverse transcriptase
TFs	transcription factors
TFEB	transcription factor EB
TFPI-2	tissue factor pathway inhibitor-2
TG	triglyceride
TGFβ	transforming growth factor β
TH1	T helper cells Type 1
TH2	T helper cells Type 2
TIGAR	TP53-induced glycolysis and apoptosis regulator
TME	tumour microenvironment
TSC1/2	tuberous sclerosis 1/2
Twist1	twist family basic helix-loop-helix transcription factor 1
Ubl	ubiquitin-like protein
ULK1/2	unc-51 like autophagy activating kinase 1/2
UPR	unfolded protein response
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
WIPI-1	WD repeat domain phosphoinositide-interacting protein 1
XIAP	X-linked inhibitor of apoptosis
Zeb1/2	zinc finger E-box binding homeobox 1/2
ZFYVE1	zinc finger FYVE domain-containing protein 1

LIST OF PUBLICATIONS IN PEER-REVIEWED JOURNALS

1. **Lim MM**, Wee JW, Soong JC, Chua D, Tan WR, Lizwan M, Li Y, Teo Z, Zhu P, Tan NS. Targeting metabolic flexibility sensitizes metastatic cancer cells to chemotherapy drugs. *Molecular Cancer*. 2018 Oct 20;17(1):152.
2. Teo Z, Sng MK, Chan JSK, **Lim MMK**, Li Y, Li L, Phua T, Lee JYH, Tan ZW, Zhu P, Tan NS. Elevation of adenylate energy charge by angiopoietin-like 4 enhances epithelial-mesenchymal transition by inducing 14-3-3 γ expression. *Oncogene*. 2017 Nov 16;36(46):6408-6419.
3. Phua T, Sng MK, Tan EH, Chee DS, Li Y, Wee JW, Teo Z, Chan JS, **Lim MM**, Tan CK, Zhu P, Arulampalam V, Tan NS. Angiopoietin-like 4 Mediates Colonic Inflammation by Regulating Chemokine Transcript Stability via Tristetraprolin. *Scientific Reports*. 2017 Mar 13;7:44351.

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1. **Lim MM**, Teo Z, Choo CC, Zhu P, Tan NS (2015). Understanding the role of autophagy during metastasis. AACR2015, Philadelphia, USA

THESIS SUMMARY

Cancer is among the leading causes of death worldwide, with cancer metastasis accounting for more than 90% of mortality. Cancer metastasis is often associated with malignancy especially in end stage or stage IV human cancers, and mounting evidence from clinical studies have shown that patients with metastatic cancers often display multidrug resistance (MDR). The mechanism behind the association of cancer metastasis and MDR remains unclear, hence impeding treatment efficacies and providing a major challenge for therapeutic approaches. Emerging studies have shown that the Angiopoietin-like 4 (ANGPTL4) protein plays critical roles in metastatic dissemination of cancer cells via disruption of vascular integrity, conferring resistance to anoikis and enhancing metastatic competency of cancer cells by modulating cellular bioenergetics during epithelial-mesenchymal transition (EMT), an early stage event during cancer metastasis. Our present study demonstrated that ANGPTL4 promotes MDR in cancer cells during EMT by transcriptionally upregulating the expression of ATP-binding cassette (ABC) transporters expression via the Myc and nuclear factor kappa B (NF- κ B) signaling pathways. ANGPTL4 also increases ABC transporter activity, which results in an increase of chemotherapeutic drugs efflux out of cell. Furthermore, ANGPTL4 coordinates metabolic re-wiring in cancer cells undergoing EMT by increasing glucose uptake, glycolysis and lipid metabolism, which in turn provides the cellular adenylate energy charge required to fuel the increased activities of ABC transporters. In vivo and in vitro suppression of ANGPTL4 leads to higher retention of chemotherapeutics intracellularly, and an accumulation of cisplatin-DNA adducts was observed in both primary and metastasized tumors in our xenograft model. Furthermore, we demonstrated that ANGPTL4 supports metabolic reprogramming

during EMT by upregulating autophagy, which may potentially be a rapid source of macromolecules for cancer cells during EMT. In summary, our studies have illustrated that ANGPTL4 is a multifaceted protein that has dynamic functions and plays a critical role in modulating cancer metastasis through conferring the MDR phenotype to metastatic cells via ABC transporter expression and activity, as well as coordinating cellular bioenergetics and increasing cellular adenylate energy charge through regulation of glucose and lipid metabolism and autophagy to provide for energy demanding processes during EMT. Our findings suggest that strategies to suppress ANGPTL4 expression and function, may provide a dynamic therapeutic intervention in overcoming MDR in metastatic cancers.

CHAPTER 1:

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Cancer Overview

Cancer is among the leading causes of death worldwide, with an estimated 14.1 million new cases diagnosed, and 8.2 million cancer-related deaths in 2012. According to statistical analysis published in the World Health Organization (WHO) 2014 World Cancer Report, the 5 most common cancers diagnosed in males are lung cancer (16.7%, with an incidence rate of 34.2 per 100,000), prostate cancer (15.0%, with an incidence rate of 31.1 per 100,000), colorectal cancer (10.0%), stomach cancer (8.5%) and liver cancer (7.5%). In females, the 5 most common cancers diagnosed are breast cancer (25.2%, with an incidence rate of 43.3 per 100,000), colorectal cancer (9.2%, with an incidence rate of 14.3 per 100,000), lung cancer (8.7%), cervical cancer (7.9%) and stomach cancer (4.8%). The 5-year prevalence of cancer among the global population is approximately 8.7 million in the first year, 22.0 million within 3 years and 32.6 million within 5 years of diagnosis (Stewart, 2014). This illustrates the global impact of cancer, and the importance of cancer epidemiology, as well as the development of therapeutics and preventive healthcare.

Cancer epidemiology has identified several major risk factors that promote the development of most common cancers. Approximately 5-10% of cancers manifest from inherited genetic mutations, while the remaining cancers are attributed to environmental carcinogens that include behavioral factors such as dietary choices, physical inactivity, obesity and aging, all of which are also common risk factors for chronic disease development. Unabated alcohol consumption and tobacco usage are also major contributing factors, with the latter being the primary risk factor for oral, throat and lung cancers and responsible for approximately 22% of all cancers

worldwide. Prolonged exposure to other environmental carcinogens, such as asbestos, ionizing radiation and ultraviolet radiation, can be avoided to reduce cancer risks. Additionally, infectious agents such as the human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr virus (EBV) and *Helicobacter pylori* or other parasites can also increase the incidence rate of cancer and are responsible for approximately 15% of new cancers (Stewart, 2014).

As a consequence, it is imperative to understand the causative effects and risk factors associated with a specific cancer type to reduce the incidence rate of cancer initiation. This includes performing screening tests and employing early detection protocols, which can contribute to a better prognosis and diagnosis of cancer at the early stages for patients, thus allowing the selection of a suitable treatment to enhance therapeutic efficiency and survival probability, while reducing tumor burden and mortality and increasing curative probability for patients.

The aim to achieve cancer-free and complete remission for patients is highly dependent on the disease progression and their response to therapies. This emphasizes the need for advances in cancer research to provide a better understanding of the molecular events that transpire in transformed cells, as well as in their surrounding microenvironment, which can contribute greatly to the development of novel therapeutic interventions.

1.1.1 Hallmarks of Cancer Overview

Cancer is a collective term for complex multifactorial diseases that are characterized by uncontrolled growth of transformed cells with the ability to self-sustain in growth signals and desensitize from growth inhibitory signals, evade immune surveillance and apoptosis, promote angiogenesis to obtain nutrients from blood stream and altered

metabolism to sustain its proliferative needs, increase in genomic mutations for a selective growth advantage and eventually obtaining invasive and migratory properties for cancer cells to spread. These cells may potentiate into malignancies if they demonstrate the ability to aggressively progress to and invade the surrounding tissues and metastasize to secondary distant sites. Cancer cells are often controlled by genetic and epigenetic modifications that result in accruing mutations and alterations of the transcriptome, which culminate in the activation of molecular signaling cascades that contribute to the evasion of cellular checkpoints, perturbed homeostasis and uncontrolled cellular proliferation (Chatterjee et al., 2018; You and Jones, 2012). Normal human cells are regulated both spatially and temporally by extrinsic factors and intrinsic signaling cascades to execute coordinated functions. Multiple checkpoints within the cells ensure that homeostasis is maintained, and any deregulation in proliferation and growth will often lead to senescence or apoptosis. Thus, to confer a proliferative advantage over the surrounding normal cells, cancer cells are able to deregulate various signaling cascades with a multistep process to bypass these highly regulated intrinsic checkpoints or hurdles in order to overcome these defense mechanisms and confer a proliferative advantage.

Many studies have illustrated the complexity of tumorigenesis with different cancers that present diverse phenotypes and heterogeneous expression patterns. Hence, understanding the mechanisms behind these malignancies can be a major challenge, with most research efforts focusing on more prevalent cancer types. In 2000, Hanahan and Weinberg consolidated the commonalities of most human cancer studies and classified them into six essential attributes required to drive tumorigenesis (Hanahan and Weinberg, 2000). They proposed that cancer cells (i) require a gain in self-

sufficiency in growth signals, (ii) become insensitive to growth inhibitory signals, (iii) evade programmed cell death, (iv) enable replicative potential to gain immortality, (v) induce angiogenesis to provide nutrients for proliferation and (vi) invade and metastasize to local and distant sites. These attributes were defined as the hallmarks of cancer and provided insight that molded the framework for cancer research for the next decade. These factors will be briefly discussed below.

Growth signals are required to modulate cancer cells between quiescent and active proliferative states. These signals are highly coordinated by extracellular ligands in the form of growth factors, extracellular matrix (ECM) components and cell-to-cell adhesion molecules. In normal cells, signal transduction is conventionally propagated by exogenous ligand activation of transmembrane receptors, followed by an intracellular cascade of signaling pathways to elicit enhanced survivability. In contrast, transformed cells confer self-sufficiency by mitigating their reliance on exogenous growth signals through various methods such as (i) generation of their own growth factors and reducing their dependency from exogenous stimuli (Witsch et al., 2010), (ii) overexpression or truncate transmembrane receptors to render them constitutively active (Di Fiore et al., 1987; Sasaki et al., 2013), (iii) truncated mutant receptors can relieve the dependency of extracellular ligand binding, leading to autophosphorylation and activation of these receptors (Huang et al., 1997), (iv) selectively express specific receptor subtypes to preferentially promote signal transduction in the absence of upstream ligand interaction (Chan et al., 2017; Shoji et al., 2004), or (v) posttranslational modification or overexpression of signaling molecules, such as H-Ras or sarcoma viral oncogene homolog (Src) (Formisano et al., 2014; Wu et al., 2016). Normal cells rely on localized signals from the stroma, which comprise of fibroblasts,

ECM and immune cells, among others, to support cellular growth and function during normal homeostasis (Pickup et al., 2014). Therefore, it is logical for cancer cells in a developing tumor to extend their influence towards stromal cells such as cancer-associated fibroblasts (CAFs), immune inflammatory cells (ICs) and tumor-associated endothelial cells within the tumor cell microenvironment (TME) to provide a sustainable growth signal to drive tumor proliferation (Fouad and Aanei, 2017; Kessenbrock et al., 2010; Massague, 2008)

Apart from sustaining signaling transduction to promote proliferation, cancer cells also require the gain of the ability to evade growth inhibition by suppressing tumor suppressor genes that help maintain cellular homeostasis under nontumorigenic circumstances. Classic examples include the tumor suppressor molecules retinoblastoma protein (RB) and tumor protein p53 (p53). RB is a multifunctional protein that serves as a G1 checkpoint protein during cell cycle progression and transcriptional cofactor. Loss of RB is commonly associated with cell cycle re-entry in quiescent stem cells and, importantly, promotes the proliferation of transformed cells (Burkhart and Sage, 2008). Similarly, p53 functions as a transcription factor that controls cell cycle progression via induced expression of cyclin dependent kinase inhibitor 1A (p21). Mutation of p53 in cancer cells can result in suppression of wild-type p53 functions and suppress apoptosis, along with gain in function that promotes a more aggressive and metastatic phenotype (Muller and Vousden, 2014; Ozaki and Nakagawara, 2011).

Another cellular checkpoint that can adversely affect the proliferation and survival of cancer cells is programmed cell death (PCD) in the form of apoptosis. PCD or apoptosis is a highly regulated cellular mechanism that is essential in normal homeostatic

processes such as embryonic development and cellular turnover in tissues. Factors such as cellular stress, uncontrolled growth signals, deprivation of nutrients, DNA damage or virus infection can lead to activation of the apoptotic program, which results in the clearance of these cells in order to maintain barrier integrity, such as in the case of an epithelium (Elmore, 2007). As the highly proliferative capacity of cancer cells can create a substantial amount of cellular stress, apoptosis could be initiated in these cells. Therefore, transformed cells need to evade this protective surveillance mechanism-induced cell death to enhance their survival, which is made possible by aberrantly deregulating the expression of antiapoptotic molecules (Fernald and Kurokawa, 2013; Lopez and Tait, 2015). For instance, cancer cells often displayed a dysregulated p53 expression, a regulator of proapoptotic molecule BCL2-associated X protein (BAX) (Ozaki and Nakagawara, 2011). Alternatively, dominant negative mutations or loss of expression of tumor suppressor phosphatase and tensin homolog (PTEN) has been associated with inhibition of apoptosis via suppression of proapoptotic molecules Fas cell surface death receptor (FAS) & BCL2 like 11 (BIM), and suppress inactivation of antiapoptotic molecules such as BCL2-associated agonist of cell death (BAD) and X-linked inhibitor of apoptosis (XIAP) (Kim and Mak, 2006).

Thus far, the combined effects of dysregulating growth and inhibitory signal, as well as evading apoptosis enhances the survival of cancer cells but lacks the driving force to confer their uncontrolled proliferative characteristics. Normal cells possess limited replicative potential defined by the Hayflick limit and each cellular division results in a reduction in telomere length at the end of chromosomes until a critical length is reached and cells switch to cellular senescence (Shay, 2016; Shay and Wright, 2000). Thus, cellular senescence is conceptually utilized as a defense mechanism against the

proliferative growth of transformed cells during the benign stage. Telomere shortening in normal cells has been shown to activate wild-type p53, which in this circumstance, functions to induce replicative senescence, apoptosis and suppress tumorigenesis. Interestingly, certain cancer cells express constitutively active mutant p53 which can transactivate telomerase reverse transcriptase (TERT), a part of the telomerase complex and lead to telomere lengthening, resulting in circumvention of cellular senescence and the promotion of infinite replication (Roake and Artandi, 2017; Shay and Wright, 2011; Tomas-Loba et al., 2008).

As cancer cells multiply, the rapid expansion of the tumor mass requires nutrients and oxygen to sustain their high proliferative rate and maintain survival. This can be achieved through angiogenesis by increasing vasculature development through the recruitment of endothelial cells. Vascular endothelial growth factor (VEGF) signaling is commonly involved in the recruitment of endothelial progenitor cells and monocyte migration and differentiation via activation of VEGF receptor 2 (VEGFR2) (Ceballos-Suarez et al., 2006; Goel and Mercurio, 2013). Recruited progenitor endothelial cells can undergo differentiation to form new blood vessels. Importantly, the expression of VEGF ligands is often upregulated in transformed cells, thus promoting angiogenesis in the tumor environment (Saharinen et al., 2011). In addition to this signaling, expression of the proangiogenic fibroblast growth factor (FGF) ligand is also hyperactivated in certain cancer types, which can result in a sustained angiogenesis response (Bono et al., 2013).

Highly proliferative cancer cells accrue in mass rapidly and the limitations of the immediate surroundings may prompt cancer cells to leave this suppressive environment in search of a more conducive environment for them to overcome competition and

expand (Friedl and Wolf, 2003). Most cancer cells originate from epithelial cells which are less motile in nature and require a switch to a mesenchymal motile phenotype through the process of epithelial-mesenchymal transition (EMT) to invade the ECM and metastasize to distant organs and tissues (Easwaran et al., 2014; Meacham and Morrison, 2013). EMT reprogramming is best characterized by the loss of epithelial molecule E-cadherin and upregulation of mesenchymal-associated molecules such as N-cadherin and vimentin, which resulting in the cancer cell possessing enhanced migratory capabilities (Jeanes et al., 2008; Lamouille et al., 2014; Petrova et al., 2016). Additionally, the expression of matrix metalloproteinases (MMPs) is upregulated, which leads to increased degradation of the underlying ECM at the basal region of the epithelia (Pang et al., 2016a). Transcription factors (TFs), including snail family transcriptional repressor 1 (Snai1), snail family transcriptional repressor 2 (Slug), zinc finger E-box binding homeobox 1/2 (Zeb1/2) and twist family basic helix-loop-helix transcription factor 1 (Twist1), are often correlated with EMT reprogramming during normal development and are hyperactivated in various aggressive cancer types, indicating that cancer cells promote their phenotypic changes by deregulating these molecules (Lamouille et al., 2014; Medici et al., 2008).

1.1.2 Emerging Hallmarks of Cancer

In 2011, Hanahan revisited the hallmarks of cancer and included 4 additional novel characteristics that have emerged as essential capabilities that cancer cells acquire to further enhance their malignancy. These include (i) chronic inflammation enhanced tumorigenesis, (ii) evasion of immune surveillance, (iii) genomic instability and (iv) aberrant metabolism (Hanahan and Weinberg, 2011). These additional hallmark

reinforce those defined previously and add to the collective knowledge that cancer cells evolve to overcome challenges and sustain their progression towards malignancy.

During normal homeostasis, inflammation occurs as a defense mechanism in response to damaged cells and external factors such as foreign invasion of pathogens and the introduction of irritants into the organ. In response to signaling molecules secreted by damaged cells, innate immune cells can be recruited to the damaged site via chemotaxis to remove affected cells. While inflammation typically removes damaged cells, a persistent inflammatory response (i.e., chronic inflammation) could promote tumorigenesis as ICs can partake in the TME by releasing growth factors, reactive oxygen species (ROS), MMPs, serine proteases, cytokines and chemokines which support cancer cells survival, tissue remodeling, angiogenesis, invasion and metastasis (DeNardo et al., 2010; McAndrews et al., 2015; Shin et al., 2010). However, it is worth noting that the contributions of inflammatory responses to tumorigenesis are most likely dependent on the biological context. As eloquently depicted by Hanahan *et al.*, this contribution, among others that will be discussed below, is a new emerging concept that has elicited conflicting observations by various groups (Fouad and Aanei, 2017; Hanahan and Weinberg, 2011). For instance, human colon and ovarian tumors infiltrated with killer lymphocytes such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are associated with better prognosis than tumor samples that lack these cells (Nelson, 2008; Pages et al., 2010). Thus, the role of inflammation during aggressive tumorigenesis may vary according to cancer types, and characterizing it requires further investigation.

Similar to the contribution of inflammation, the role of immune evasion is also an emerging concept involved in cancer progression. The immune system constantly

monitors detrimental changes in the body, where aberrant damage to cells through injury and infectious particles such as toxins, viruses and bacteria can lead to the secretion of cytokines and the recruitment of immune cells to the affected site. As mentioned briefly above, cancer cells can recruit immune cells by either secreting cytokines themselves or stimulating fibroblasts in the microenvironment to secrete cytokines. As immune cells exert both anti- and protumor activities, cancer cells must avoid detection and eradication if the cells possess antitumor functions. For instance, while recruitment of TH2 cells has been implicated in aggressive angiogenesis to promote tumor survival, loss of T helper cells Type 1 (TH1) cells in mice led to an increase in cancer progression (Knutson and Disis, 2005). Similarly, cancer cells can evade the antitumor activities of the immune system through a ‘survival of the fittest’ strategy within the heterogeneous tumor bulk, in which highly immunogenic clones were eradicated by the immune system while weakly immunogenic cells, unable to provoke a strong immune response, were retained to allow further tumor growth. An alternative strategy is to suppress cellular functions of immune cells such as CTLs and NK cells through secretion of C-C motif chemokine ligand 21 (CCL21) and transforming growth factor β (TGF β) (Shields et al., 2010; Yang et al., 2010).

The third newly emerging hallmark concept that governs the growth and survivability of cancer cells is the acquisition of mutations to the genome in increasing genome instability. Spontaneous mutation and loss of stability of the genome is governed and monitored by cell cycle checkpoints and molecules involved in the detection and repair of damaged DNA during normal homeostatic maintenance of genomic DNA. However, the increased mutagenesis rate in cancer cells caused by oncogenes could lead to dysfunction of the genome maintenance systems and the acquisition of further aberrant

mutations in the genome; some examples of the latter include loss-of-expression or inactivating mutations in genes associated with cell cycle and DNA repair (Deng, 2006). While p53 can function to shut down cellular activities and promote apoptosis due to such failures, additional mutations arising in p53 could effectively counteract these defense mechanisms, allowing increased survivability of cancer cells (Mackay et al., 2018; Yeo et al., 2016). Additionally, telomere shortening, which often occurs in cancer cells due to their uncontrolled replicative capability, can lead to DNA instability and deregulated expression of DNA segments in the genome. As such, telomerase may potentially function as a tumor suppressor by promoting telomere lengthening and subsequent genomic stability, despite its potential tumor promoting functions, as mentioned above.

Sustenance of heavy cellular proliferation means that cancer cells are required to rapidly metabolize large quantities of nutrients to fuel their growth. As a result, cancer cells significantly increase their glucose uptake through upregulation of the expression of glucose transporter type 1 (GLUT1) (Goodwin et al., 2017; Labak et al., 2016). While normal cells generate ATP energy in the presence of oxygen via cellular respiration by metabolizing glucose intake partly through the glycolysis pathway followed by the tricarboxylic acid (TCA) cycle in the mitochondria, cancer cells conversely generate energy through the glycolysis pathway independent of the presence of oxygen, a process termed aerobic glycolysis by Otto Warburg (Vander Heiden et al., 2009; Ward and Thompson, 2012). This pathway supports enhanced proliferation instead of efficient ATP generation by mitochondria, and generates massive amounts of lactic acid and intermediate glycolytic products which can be diverted to other pathways to generate molecular building blocks, such as nucleotides and amino acids.

Additionally, certain tumors consist of multiple subpopulations of transformed cells at which the lactate byproduct generated by one subpopulation that consistently metabolizes glucose via glycolysis can be utilized as a form of fuel by a second subpopulation of cells (Hensley et al., 2016; Lyssiotis and Kimmelman, 2017). Thus, these observations strongly suggest the importance of reprogramming of energy metabolism pathways as a potential hallmark of cancer progression. This in part is associated with the deregulated expression of oncogenes such as Ras and Myc, and tumor suppressors such as p53 (Dang, 2012; Smith et al., 2016).

To summarize, the hallmarks of cancer defines the essential attributes that cancer cells need to acquire to achieve their distinctive characteristics and ascribed to their malignant progression.

1.2 Cancer Metastasis

Cancer metastasis is the spread of cancer cells from their site of origin to a local or distant site within the body. It is often associated with malignancy especially in end stage or stage IV human cancers, and is the primary cause of mortality in most cases. Tumor cells from a primary tumor may infiltrate the blood or lymphatic system and disseminate throughout the circulatory system until eventually settling at a secondary organ (Valastyan and Weinberg, 2011).

Clinically, most metastatic tumors (also known as secondary tumors) are found in the bone, brain, liver, lungs and lymph nodes and less frequently in other organs, such as skin and muscles. Metastasis is usually associated with poor prognosis, with current available treatments having a low curative rate (Fidler and Kripke, 2015). Therapies are therefore intended to alleviate symptoms and impede progression to prolong the

survival of patients. Metastatic tumors are defined based on their originating tissue, e.g., breast cancer tumors metastasized to lungs are called metastatic breast cancers (unless the origin is unknown), as the secondary tumors commonly display similar characteristics and therapeutic responses as the tumors from which they originated. Classification according to their originating type allows clinicians to better assess and customize follow-up therapeutic management based on the efficiency of previous approaches.

Clinical observations suggest that the metastatic sites are not random in occurrence, as observed by breast cancer's tendency to metastasize to the lymph nodes, bone, brain, liver and lung, while stomach, pancreatic and colorectal cancers preferentially colonize liver and lung (Horak and Steeg, 2005; Nguyen et al., 2009). This brings about notions that cancer cell dissemination is tissue specific and that certain metastatic cancer cells thrive better in specific organs. Stephen Paget first proposed the "soil and seed" theory more than a century ago. Upon reviewing 735 breast cancer autopsy reports, he hypothesized that breast cancers metastasize mainly to liver, ovary and bone in a nonrandom manner and that metastatic cancer cells interact with the microenvironment of specific distant organs that are best compatible for the cells' colonization (Langley and Fidler, 2011; Liu et al., 2017).

Isaiah Fidler further supported this theory by tracking radioactive-labeled melanoma cell dissemination in mice. He demonstrated that only a small population successfully migrated to other organs and that only lung-specific disseminated cells were able to populate (Talmadge and Fidler, 2010). Since then, metastasis research has progressed, and multiple groups have illustrated that tumors comprise heterogeneous cell populations that form a complex network which supports tumor development (Fidler

and Poste, 2008; Langley and Fidler, 2011). Heterogeneity diversifies and confers a selective advantage for metastatic cancer cells to survive during circulation and invade target tissues.

1.2.1 Mode of Dissemination

The process of cancer cell migration is a well-orchestrated complex cascade of events characterized by the following steps: local invasion of primary tumor cells through their basal membrane, penetration through the lymphatic or blood vessels (intravasation), transition through the blood circulation, penetration of the vessel walls to a new site (extravasation) and colonization to form secondary tumors (Figure 1).

Figure 1. Multistep process of invasion and metastasis

Tumor cells become invasive and break through the basal membrane to enter the blood stream. Circulating tumor cells eventually penetrate the vessel walls and metastasize to a new site to form

micrometastasis. Micrometastatic cells adapt and survive to colonize and form secondary tumors (Albert et al., 2008; Chaffer and Weinberg, 2011).

Invasion and metastasis are two hallmarks of cancer proposed during the multistep progression of tumorigenesis. They are essential steps for cancer cells to survive when nutrients become scarce or conditions become limiting (Hanahan and Weinberg, 2000). In the past decade, multiple studies have provided insights into various key players that are involved in driving metastasis, but the exact molecular mechanism of how cancer cells acquire their ability to invade and metastasize is not fully elucidated, with emerging new key players such as fibroblast (Ahirwar et al., 2018; Karagiannis et al., 2012), chemokines (Borsig et al., 2014; Muller et al., 2001), fibronectin (Barbazan et al., 2017) and exosomes (Azmi et al., 2013; Plebanek et al., 2017) supporting the transgression. Moreover, the multiple key processes studied thus far do not solely contribute to the phenomena and are often associated with one another to elicit a coordinated effect.

The process of metastatic dissemination of tumor cells is first defined by their acquisition of the ability to transform from an epithelial cell to a mesenchymal-like cell. This is similar to the EMT process during embryonic development. In normal physiology, epithelial cells are held together by cell adhesion molecules (CAMs), such as integrins and cadherins, to maintain cell-to-cell and cell-to-matrix adhesions, which bind cells together and attach them to the ECM (Farahani et al., 2014; Li et al., 2017a). During the initiation of EMT, E-cadherin expression is significantly reduced, while the expression of mesenchymal markers such as N-cadherin, cadherin-11, Snai1, Slug, matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9) and Vimentin is induced in tumor cells. These alterations in expression change the cell

morphology as the cancer cells are no longer tightly bound together to form epithelial sheets or attached to their ECM (Lamouille et al., 2014).

The next step of metastasis is characterized by the intravasation of cancer cells through their basal membrane. Cancer cells interact and modulate their microenvironment to benefit from products secreted by stromal cells and present in the ECM. Normally, the microenvironment exerts inhibitory effects to repress tumor invasion. However, tumor cells adapt by remodeling their surroundings to conditions more suitable for enhancing their growth, proliferation and invasion. One of the common methods by which tumor cells modify their microenvironment is the secretion of signaling proteins to induce stroma cells and ICs to produce proteolytic enzymes such as cysteine cathepsins, serine proteases and MMPs to degrade the ECM (Joyce and Pollard, 2009). These proteolytic enzymes also function to further support cancer cell invasion by disrupting cell-to-cell adhesion via cleavage of CAMs and ECM proteins, as well as aid in growth factor and cytokine signaling to mediate intravasation (Lamouille et al., 2014)

Cancer cells also promote intravasation by switching the types of integrins expressed. The changes in integrin expression during EMT promote invasion and metastasis by switching their binding affinity from normal ECM to degraded ECM constituents and facilitating cellular migration towards the ECM. This in turn potentiates cell survival mechanisms such as anoikis resistance and antiapoptosis through activation of Src, phosphatidylinositol 3 kinase (PI3K), extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK) signaling upon ECM ligand binding (Zhu et al., 2011). Integrins also promote cell migration by mediating the activation of MMP-2 and MMP-9, and the localization of their proteolytic activity to breakdown type IV collagen (Hood

and Cheresch, 2002). Together, changes in integrin expression moderate degradation of the ECM surrounding the tumor and provide intermediate levels of adhesion that are sufficient for cancer cells to dissociate and migrate. Cancer cells eventually succeed in breaking through the basal membrane and slip through the vessel walls to become circulatory cancer cells.

Cancer cells that enter the circulatory system rarely survive due to various factors: the inability to survive in the circulatory environment due to lack in support of an ECM, high shearing force within the circulatory system and interactions with circulatory immune cells. Less than one per million circulatory cancer cells survives this harsh environment and may eventually exit the blood vessels at a local or distant new site (extravasation) and form a micrometastasis (Lambert et al., 2017). Studies have shown that circulating cancer cells may remain dormant for years before successfully colonizing to form secondary tumors (Joyce and Pollard, 2009). The regulation of metastatic cell dormancy and how these cells eventually undergo extravasation remain unclear. Upon extravasation to a secondary site, these cells will need to adapt and survive their new environment to complete colonization. Most cancer cells do not survive outside of their original site due to unfavorable conditions, but the rare number that do survive successfully form a micrometastasis (Hanahan and Weinberg, 2000).

Establishment of metastasized cancer cells at a new distant site is challenging because they need to adapt to the new microenvironment. Survival is dependent on their ability to acquire new mutations by evading suppressive signals from the new microenvironment and gaining the ability to modify their surroundings to support their growth and promote propagation. Secondary sites for cancer cell migration often exhibit similar characteristics as or contain cell lineages similar to those at the primary

site, suggesting that a selective preference contributes to their survival or the probable inability to colonize other regions containing different lineages. This notion was supported by evidence that metastatic tumors are commonly found in the lungs, liver and bones (Disibio and French, 2008; Leong et al., 2006). However, the exact mechanism of organ selection and colonization has yet to be fully understood. The new microenvironment may be unfavorable for metastatic cells, and so for metastatic cancer cells to survive beyond micrometastasis, they are required to adapt and change their new microenvironment and surrounding ICs to one that is preferential for the metastatic cells to survive and initiate persistent growth to colonize the new site (Lambert et al., 2017). These cells then form the secondary or metastatic tumors that retain the characteristics of their tumor of origin (Hanahan and Weinberg, 2000).

Several findings point towards a specific subset of genes that show predominantly enhanced or reduced in expression in more aggressive or metastatic tumors which indicates a pronounced “enhancing gene signature” (Joyce and Pollard, 2009; Ramaswamy et al., 2003). Elucidating these genes that play a pivotal role in promoting cancer cell invasion and migration may provide a better understanding towards a potential therapeutic approach to inhibit metastasis.

1.2.2 Cadherin Expression

Cadherins maintain cellular architecture by interacting with a variety of binding partners. This process is highly regulated for cells to coordinate cell-to-cell cohesion, cell-to-cell recognition, locomotion, cell polarity and intracellular signal transduction. Loss of E-cadherin expression is the most common alteration characterized in epithelial

cancers and confers their invasive and metastatic properties. The loss of E-cadherin expression is correlated with the prognosis of the malignancy. Research has shown that E-cadherin expression is downregulated by TFs such as Snai1, Slug and Twist1 during EMT in epithelial cancers (Batlle et al., 2000; Cano et al., 2000; Chong et al., 2012), and increasing E-cadherin expression reverses this phenotype (Cavallaro and Christofori, 2004). Knockdown of E-cadherin by siRNA also resulted in an increase in vimentin expression and diminished E-cadherin/ β -catenin expression on the cell membrane, which was accompanied by a more invasive phenotype and increased migratory (Cavallaro and Christofori, 2004; Jeanes et al., 2008). N-cadherin is known to promote cell motility in opposition to E-cadherin, and this may potentially be the main rationale behind epithelial-derived cancers conferring a switch in cadherin expression. Additionally, N-cadherin is expressed by stromal cells and the switch from E-cadherin to N-cadherin fosters the interaction of cancer cells with their endothelial and stromal components and enhances the cells' ability to invade this environment (Hazan et al., 2000).

Emerging studies have reported the lack of E-cadherin repression in Snail-expressing tumors, while aberrant expression of Twist1 and Snail in cell culture did not lead to E-cadherin repression, which indicates no EMT reprogramming. Moreover, Twist1-induced dissemination from organoids required E-cadherin expression, further suggesting that EMT reprogramming may not be a contributor to invasion and metastasis, at least during the early stages of invasion (Shamir et al., 2014; Zhou et al., 2004). Alternatively, transformed cells could undergo partial EMT reprogramming and possess both epithelial and mesenchymal phenotypes during the early phases of

invasion, with only full reprogramming occurring during the later stages of aggressive tumorigenesis (Lundgren et al., 2009; Tran et al., 2011).

Studies of breast cancer cells and ovarian cancer cells that have undergone EMT has illustrated that these cells expressed E-cadherin at an elevated level upon colonization at a distant site (Palen et al., 2016; Xu et al., 2014). This suggests that the expression of the cadherin subtype is transient and dependent on the cellular demands during different metastatic events. Interestingly, E-cadherin expression does not solely enhance cancer cell survival. Emerging studies have demonstrated that apoptosis in invading cancer cells is largely due to E-cadherin expression. E-cadherin was also shown to confer apoptotic drug sensitivity to cancer cells (Ferreira et al., 2012; Lu et al., 2014). This suggests that cancer cells may modulate their E-cadherin expression for functions that deviate from the canonical signaling pathways that have been described thus far.

1.3 Cancer Metabolism

Cancer cells are defined by their uncontrolled proliferation and ability to resist cell death, which contribute to their immortality. To attain these capabilities, cancer cells must first undergo mutations, deregulate signaling pathways to achieve sustained proliferation signaling, evade tumor suppression and immune obliteration, overcome senescence and apoptosis, induce angiogenesis and metastasize. The high rate of growth and proliferation denotes the importance of an abundance of nutrients for cancer cells to survive. To overcome this impediment, cancer cells need to modify their metabolism to meet the increased energy expenditure (DeBerardinis et al., 2008; Hanahan and Weinberg, 2011).

In normal cells, glucose is broken down in the cytosol into pyruvate by anaerobic glycolysis. Pyruvate then enters the mitochondria to be converted into acetyl-CoA before it enters the TCA cycle to generate energy in the form of nicotinamide adenine dinucleotide (NADH) and guanosine triphosphate (GTP) or adenosine triphosphate (ATP). These molecules are in turn used to yield ATP via oxidative phosphorylation (OXPHOS) within the mitochondria. In 1956, Otto Warburg first described a phenomenon in which cancer cells preferentially metabolize glucose by glycolysis to lactate, even in the abundance of oxygen. This event was termed the “Warburg effect” and helped pioneer in the field of cancer metabolism (Vander Heiden et al., 2009; Warburg, 1956). In the past decade, research has demonstrated an increase in the uptake and utilization of glucose in different tumors and has provided evidence of the Warburg effect (Figure 2) (Fantin et al., 2006; Maya-Mendoza et al., 2015)

Figure 2. *Quiescent versus Proliferating Cells Metabolism*

(Left) Quiescent cells convert glucose to pyruvate through glycolysis, then further break it down by the TCA cycle and OXPHOS to yield ATPs (as depicted by yellow stars). (Right) Proliferating cells yield most of the ATPs through increased glycolysis. Pyruvate is broken down in the mitochondria to yield citrate (for fatty acid synthesis) or converted to lactate in the cytoplasm to regenerate NAD^+ for glycolysis (DeBerardinis et al., 2008).

Cancer cells rely largely on aerobic glycolysis, which has an ~18-fold lower efficiency of generating ATP compared to OXPHOS. Despite this inefficiency, the metabolic reprogramming towards this energy pathway is a selective advantage for cancer cells to meet their higher energy demands. The initial hypothesis was that the reliance of tumor cells on aerobic glycolysis could be largely due to defective mitochondrial metabolism, but studies have proven otherwise, suggesting that a cohesive effect of different factors contribute to this metabolic switch (DeBerardinis et al., 2008; Vander Heiden et al., 2009).

During tumor development, tumor cells are often subjected to harsh oxygen deprivation i.e., hypoxic conditions due to rapid proliferation and a lack of vascularization. Tumor cells must overcome these challenges by modifying their metabolic gene signatures to shift towards glycolysis. This alternative metabolism may provide a faster turnover of glucose than mitochondrial metabolism and confers tumor cells with a more robust adaptation to their constantly changing environment (Pavlova and Thompson, 2016). Another theory was that cancer cells defy the canonical metabolic pathway driven by a low ATP: ADP ratio and shifts towards anabolic-based metabolism in which glucose is constitutively broken down to supply cancer cells with macromolecules; these molecules in turn form the building blocks for proliferating cells (DeBerardinis et al., 2008). This interpretation suggests that the preferential choice of aerobic glycolysis is not to maximize ATP yield but to prioritize the generation of substrates for nucleotides, proteins and lipid synthesis as well as for NADPH generation through the oxidative pentose phosphate pathway (PPP) (Ward and Thompson, 2012). The increase in glycolysis flux compensates for the reduced ATP yield via its rapid production of ATP compared to rate at which OXPHOS produces ATP. Cancer cells also demonstrated a reliance on aerobic glycolysis for survival, as inhibiting lactate dehydrogenase (LDH), which decreases pyruvate conversion to lactate and increases OXPHOS, also resulted in diminished tumorigenic potential (Fantin et al., 2006).

Changes in oncogene and tumor suppressor expression are a prominent feature of altered metabolism, which typically results in increased glucose uptake, lactate production and glycolysis and decreased OXPHOS. These metabolic changes were speculated to be a downstream consequence of changes in the expression of oncogenes or tumor suppressors (Hsu and Sabatini, 2008). However, emerging evidence suggests

that the main role of oncogene and tumor suppressor gene mutations is to alter metabolism (Ramanathan et al., 2005). Activation of p53 negatively regulates metabolism by increasing mitochondrial metabolism through cytochrome c oxidase assembly protein (SCO2) and glutaminase 2 (GLS2), decreasing glycolysis through p53-induced glycolysis and apoptosis regulator (TIGAR), and reducing glucose uptake via repression of GLUT1 and glucose transporter type 4 (GLUT4) (Lu et al., 2010; Shen et al., 2012). Downregulation of these pathways during tumorigenesis abolishes their inhibitory effects on glycolysis (Bensaad et al., 2006; Matoba et al., 2006). Cells expressing the Myc oncogene also showed upregulation of glucose uptake in cancer cells through the PI3K/Akt pathway as well as regulated glutamine uptake and mitochondrial glutaminolysis through a PI3K/Akt independent pathway (Wise et al., 2008). Myc, Ras and other key oncogenes have also been shown to be involved in the upregulation of other metabolic genes to confer the metabolic switch and allow cancer cells to thrive (Boroughs and DeBerardinis, 2015; DeBerardinis et al., 2008).

Cancer cells also engage in their alternative metabolism by expressing metabolic enzymes with different isoforms or splice variants. A classic widely studied example is the expression of pyruvate kinase isozyme M2 (PKM2) in most tumors. In normal adult cells, pyruvate kinase isozyme M1 (PKM1) is expressed and dephosphorylates phosphoenolpyruvate (PEP) to pyruvate in the last step of glycolysis. During tumorigenesis, the PKM2 isoform is highly expressed over PKM1 and forms dimers or tetramers. Tetrameric PKM2 has a high affinity for PEP and promotes glucose conversion to pyruvate and lactate when in close proximity with other glycolytic enzymes, while the dimeric form has a lower affinity for PEP and negates pyruvate conversion, which retains the glycolysis substrates required for biosynthesis. Cancer

cells mediate the PKM2 tetramer to dimer ratio to facilitate various metabolic substrate production based on their metabolic requirements (Mazurek, 2011; Ye et al., 2012).

It is important to note that, although cancer cells rely on aerobic glycolysis to breakdown glucose, mitochondrial metabolism is not completely abolished. The switch from the efficient OXPHOS system to glycolysis as the primary source of generated ATP indicates that mitochondrial metabolism is reprogrammed to play a different role during tumorigenesis. Studies have shown that PI3K/Akt pathway activation results in increased glucose uptake and glycolysis and is one of the most common signatures found in cancer metabolism (Hu et al., 2016). This pathway regulates both glycolytic and mitochondrial metabolism through different mechanisms: commitment of glucose to glycolysis by activation of phosphofructokinase-1, and the promotion of pyruvate conversion to acetyl-CoA to allow entry into the TCA cycle for biosynthesis. Akt signaling is also involved in citrate metabolism via the promotion of acetyl-CoA export from mitochondria to the cytosol by activating ATP-citrate lyase (ACL) and converting citrate (a negative regulator of glycolysis) to acetyl-CoA (Bauer et al., 2005). Downstream of Akt signaling, inhibition of mammalian target of rapamycin complex 1 (mTORC1) also facilitates protein biosynthesis by transamination of TCA substrates to amino acid precursors (Wong et al., 2015; Zhang et al., 2017).

Another focus in the cancer metabolism field is the study of glutamine metabolism. Glutaminase (GLS) plays a pivotal role in the conversion of glutamine to glutamate, a substrate of the TCA cycle. This pathway is driven by Myc, and it appears that apart from glucose, cancer cells rely on glutamine metabolism to support anaplerosis (the replenishment of TCA cycle substrates) and lactate conversion to produce NADPH for lipid biosynthesis (DeBerardinis et al., 2007; Wise et al., 2008). Glutamine metabolism

may also play a compensatory role when glucose uptake or Akt-facilitated glycolysis is inhibited (Yang et al., 2009).

In recent years, studies have shown that many factors are involved in the shift in energy metabolism, and through collaborative efforts of altering various signaling pathways and gene expression levels, cancer cells are able to defy the canonical metabolic pathway and alter their metabolism to fit their needs. These alterations are not strictly absolute as these metabolic pathways are multifaceted; hence, cells are able to adapt to metabolic changes to obtain their nutrients, building blocks and energy from various pathways. Therefore, more in-depth research is needed to clearly understand the metabolic changes in cancer cells and how these changes can be exploited to limit cancer cells from obtaining their energy and, in turn, restrict their growth and proliferation.

1.4 Angiopoietin-like 4 (ANGPTL4)

Angiopoietin-like 4 (ANGPTL4) is a glycosylated secretory protein that was first identified by three different groups (Kersten et al., 2000; Kim et al., 2000; Yoon et al., 2000). It is induced by peroxisome proliferator-activated receptors (PPARs), fasting and hypoxia and is involved in the regulation of insulin sensitivity, glucose and lipid metabolism. It is encoded by the ANGPTL4 gene on chromosome 19p13.3 in humans. It contains 7 exons and can produce multiple splicing variants (transcript variant 1: NM_139314.3; transcript variant 3: NM_001039667.2; transcript variant 4: NR_104213.1; predicted transcript variant 1: XM_005272484.3; predicted transcript variant 2: XM_005272485.3) (Figure 3). The human ANGPTL4 gene encodes a 406 amino acid protein, while the mouse homolog is 410 amino acids long. The human and

mouse ANGPTL4 genes have 75% nucleotide and 77% amino acid similarity (Kim et al., 2000).

Figure 3. Representative diagram of ANGPTL4 splice variants aligned and mapped onto chromosome 19 using GCSC Browser

ANGPTL4 belongs to the angiopoietin-like family (ANGPTL1-7), the members of which show close structural similarities to the angiopoietin family. The structure of ANGPTL4 consists of a hydrophobic signal peptide and a coiled-coil domain at the N-terminus, a linker region containing a proprotein convertase (PC) cleavage site and a fibrinogen-like C-terminal domain. ANGPTL4 can be expressed in its full-length native form (fANGPTL4, ~52 kDa) or can be proteolytically cleaved by PCs such as furin, PC5/6, PC7 and paired basic amino acid-cleaving enzyme 4 (PACE4) to produce the N-terminal fragment (nANGPTL4, ~15 kDa) and the C-terminal fragment (cANGPTL4, ~37 kDa) (Figure 4). fANGPTL4 and nANGPTL4 are secreted as oligomers while cANGPTL4 is secreted as a monomer (Chomel et al., 2009; Goh et al., 2010a; Goh et al., 2010b).

Figure 4. Schematic diagram of ANGPTL4: cleavage site and downstream pathways

ANGPTL4 can be expressed in full-length, or cleaved into nANGPTL4 and cANGPTL4. Different expression of ANGPTL4 evokes different downstream pathways and functions. nANGPTL4 is known to oligomerize and inhibit LPL, while cANGPTL4 was demonstrated to promote wound closure, tumor cell survival and proliferation (Tan et al., 2012).

ANGPTL4 is predominantly expressed in adipose tissues in both humans and mice, but is also found in the liver, lung, kidney, and blood plasma in mice and the liver and placenta in humans (Kersten et al., 2000). The relative tissue-specific expression of fANGPTL4 and the cleaved fragments nANGPTL4 and cANGPTL4 in human and

mouse tissues have yet to be clearly defined, but recent studies have demonstrated that their tissue-specific expression may be due to their distinct functional roles (Zhu et al., 2012).

1.4.1 Role of ANGPTL4 in Lipid Metabolism

fANGPTL4 and nANGPTL4 are known for their role in lipid metabolism as they are secreted by white adipose tissues during fasting. fANGPTL4 and nANGPTL4 form higher order oligomeric structures through the stabilization of disulfide bonds, and this oligomerization is essential for their function to prevent fatty acid uptake in cells by inhibiting circulating triglycerides (TG) and fatty acid hydrolysis by lipoprotein lipase (LPL) (Ge et al., 2004).

When expressed during fasting, ANGPTL4 has a high affinity to LPL and converts LPL dimers to a stable monomeric form. This results in the irreversible inactivation of the catalytic activity of LPL as it loses its ability to form dimers (Lei et al., 2011; Yin et al., 2009). Moreover, it was shown that the ability of fANGPTL4 and nANGPTL4 to inhibit LPL is different, with the latter exerting a higher inhibitory effect. ANGPTL4 further inhibits LPL by enhancing LPL cleavage through (Dijk et al., 2018). Both mechanisms indicate the capability of ANGPTL4 to contribute to the hypertriglyceridemic effect by raising circulating plasma TG levels via LPL inhibition.

Furthermore, ANGPTL4 also mediates lipid metabolism through this mechanism in various ways. ANGPTL4 has been demonstrated to protect the heart against lipid overload and fatty acid-induced oxidative stress (Georgiadi et al., 2010). It was also ascribed to a reduction in lipid storage in the liver, resulting in a decrease in hepatic cholesterol levels and an increase in cholesterol biosynthesis (Lichtenstein et al., 2007).

ANGPTL4 also prevents lipid-induced proinflammatory effects by protecting mesenteric lymph node macrophages from lipid build-up (Lichtenstein et al., 2010).

1.4.2 Role of ANGPTL4 in Glucose Metabolism

Apart from its role in lipid metabolism, ANGPTL4 has also been implicated in the regulation of glucose metabolism. ANGPTL4 was demonstrated to decrease blood glucose levels, increase insulin sensitivity, and downregulate phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) while upregulating fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD1) (Xu et al., 2005). Type 2 diabetes patients have relatively lower ANGPTL4 concentrations in serum, and treatment with rosiglitazone for 8 weeks raises the levels to those comparable in healthy subjects. These findings suggest that ANGPTL4 plays a role in improving glucose tolerance in diabetic patients and mediates this effect through hepatocytes (Xu et al., 2005). Another group showed that cANGPTL4 plays a key role in energy metabolism, as it suppresses food intake and weight gain and enhances energy metabolism by suppressing hypothalamic AMP-activated protein kinase (AMPK) levels (Kim et al., 2010). Overall, ANGPTL4 modulates lipid and glucose metabolism and may play a critical role in sensing and regulation of energy.

1.4.3 Role of ANGPTL4 in Wound Healing

ANGPTL4 also plays a key role in wound healing, a process that involves a series of well-coordinated events to promote wound closure. The process progresses as follows: inflammation stage, re-epithelialization characterized by keratinocyte proliferation and migration, remodeling and eventual closure. Wound repair is mediated by a coordinated effect of growth factors, cytokines and extracellular proteins found in the wound bed. Research has shown that fANGPTL4 and cANGPTL4 produced by keratinocytes at the

wound site interact with vitronectin and fibronectin to delay their proteolytic degradation by MMPs and thereby coordinate cell-to-matrix communication and promote the availability of local ECM to form integrin-matrix protein complexes (Teo et al., 2017a). The expression of ANGPTL4 also facilitates re-epithelialization by binding to integrins $\beta 1$ and $\beta 5$ and activated integrin-mediated signaling, resulting in integrin recycling and enhanced keratinocyte migration (Goh et al., 2010a; Goh et al., 2010b).

1.4.4 Role of ANGPTL4 in Cancer and Metastasis

The potential role of ANGPTL4 in cancer development and metastasis has only recently begun to emerge but has yet to be clearly defined. Various groups have focused on elucidating the role of ANGPTL4 in angiogenesis but have produced contradictory results. Ito *et al.* demonstrated that ANGPTL4 represses proliferation, neovascularization and vascular leakiness and suggested its antiangiogenic functions (Ito et al., 2003). However, ANGPTL4 has been reported to be expressed within several perinecrotic tumors under hypoxic conditions and results in the development of nodules and vascularization (Le Jan et al., 2003). Other studies have shown evidence of ANGPTL4 engaging in other roles during cancer progression. In human colorectal cancer samples, cANGPTL4 is stimulated by prostaglandin E₂ (PGE₂) during hypoxia to promote tumor proliferation via the signal transducer and activator of transcription 1 (STAT1) pathway (Kim et al., 2011b). Tumor-derived cANGPTL4 interacts with integrins $\beta 1$ and $\beta 5$ to confer anoikis resistance via the FAK pathway, and the ANGPTL4-integrin interaction also modulates O₂- levels to maintain an elevated O₂ :H₂O₂ ratio in tumor cells, which is necessary to activate the Src, PI3K/ protein kinase B (PKB/Akt) and ERK1/2 downstream survival pathways (Zhu et al., 2011). Clearly,

ANGPTL4 may play different roles during cancer development, and the expression of different ANGPTL4 fragments may also contribute to the observed discrepancies.

ANGPTL4 expression was identified as a significant lung metastasis signature (in a univariate analysis) that mediates breast cancer metastasis to the lungs. This was further supported by another study demonstrating the involvement of TGF β in conjunction with a lung metastasis signature in priming breast tumors that metastasize to lung. ANGPTL4 is a downstream target of TGF β via the SMAD signaling pathway, and it was shown that ANGPTL4 is involved in priming breast tumors for lung metastasis by promoting lung seeding through disruption of the endothelium, facilitation of tumor cell passage and retention in the lungs (Padua et al., 2008). More evidence of ANGPTL4 involvement in metastasis is shown by an expression profile of ANGPTL4 in human tumors. The array revealed that elevated ANGPTL4 expression is present in all epithelial tumor samples, and its expression is correlated with the progression of the tumor. Further analysis indicated that cANGPTL4 increases vascular leakiness in tumors and interacts with integrin $\alpha 5\beta 1$, VE-cadherin and claudin-5 to disrupt endothelial continuity via integrin-mediated PAK/Rac signaling to promote tumor metastasis to the lungs (Huang et al., 2011). Hypoxia-inducible factor 1 α (HIF-1 α) also induces ANGPTL4 expression in hepatocellular carcinoma (HCC) cells to mitigate transendothelial migration. Elevated serum levels of ANGPTL4 are detectable in patients with chronic hepatitis B and are at a higher level in HCC patients; the level of ANGPTL4 expression correlates with the metastatic phenotype (Li et al., 2011). Although some studies have shown the antimetastatic role of ANGPTL4 via inhibition of tumor cell migration and invasion as well as of vascular permeability, most efforts have supported its metastatic role in tumor cell dissemination (Galaup et al., 2006).

Further in-depth studies are necessary to provide better insight to elucidate the role of ANGPTL4 in tumor development and metastasis.

Metastasis is often compared to wound healing, as numerous studies have highlighted the resemblance between both events; they share similar hypoxic microenvironments, they have prolonged inflammatory responses, they exhibit an ability to modulate their microenvironments to encourage migration, and they both utilize EMT: keratinocytes engage in re-epithelialization, while cancer cells for migration (Barriere et al., 2015). It is interesting to note that ANGPTL4 is involved in the facilitation of both EMT processes during wound healing and cancer cell migration. Nevertheless, there are still distinct differences between both events: epithelial cells in the chronic wound edge exhibit senescence-like characteristics, while cancer cells undergoing EMT during migration display a more invasive behavior required for dissemination and are often resistant to senescence signals. Cancer is frequently described as a wound that never heals; hence, the study of the underlying mechanisms of both events relating to their similarities and differences may provide better insight into both diseases (Suarez-Carmona et al., 2017).

1.5 Rationale of Current Study

Two of the hallmarks of cancer are invasion and metastasis. In 2008, Padua *et al.* demonstrated that ANGPTL4 is necessary for TGF β priming of breast cancer metastasis to the lungs by disrupting vascular integrity (Padua et al., 2008). To fully investigate the role of ANGPTL4 in tumor progression and its potential role in modulating metastasis, an expression profile covering common benign, malignant and metastatic human tumors was used to examine ANGPTL4 expression. cANGPTL4

expression was found to increase along with tumor progression, especially in individuals with an invasive type.

Our laboratory previously demonstrated that ANGPTL4 is involved in facilitating the increase in vascular permeability by disrupting VE-cadherin and claudin-5 clustering and integrin $\alpha 5 \beta 1$ mediated Rac/PAK signaling, thus increasing vascular leakiness and promoting lung metastasis (Huang et al., 2011). Concurrently, we also showed that tumor-derived ANGPTL4 instigates the elevated levels of ROS, which modulate the activation of the Src, PI3K/PKB α and ERK signaling pathways to confer resistance to anoikis in cancer cells (Zhu et al., 2011). Our recent study illustrated that ANGPTL4 plays a crucial role in coordinating cellular energy flux by enhancing cellular adenylate charge during EMT. ANGPTL4 regulates the expression of 14-3-3 γ through PI3K/Akt and MAPK signaling to modulate cellular bioenergetics and energy demands, which conferring cancer cells with augmented EMT and metastatic competency (Teo et al., 2017b).

Emerging studies have shown an association of cancer cells exhibiting a multidrug resistance (MDR) phenotype during metastasis (Sui et al., 2014; Yang et al., 2003). One of the causes of MDR in cancer cells is the increase in ABC transporter expression and activity to efflux a diverse range of chemotherapeutics. Therefore, we hypothesize that ANGPTL4 may play a role in conferring the MDR phenotype to metastatic cells and sustaining the energy-demanding ABC transporter efflux.

Our ongoing studies indicated that ANGPTL4 plays a role in metabolic reprogramming during EMT and suggested that autophagy may also be involved during cancer metastasis. Various studies have indicated that autophagy is active during the EMT

process, but there are contradictory evidence in its involvement (Catalano et al., 2015; Gugnoni et al., 2016; Pang et al., 2016b). Guided by our microarray data, we hypothesize that autophagy plays a role during cancer cell metastasis, and that ANGPTL4 potentially regulates autophagy to provide enough energy for the high-energy processes during EMT.

CHAPTER 2:

MATERIALS & METHODS

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2.1 Antibodies and Reagents

Antibodies and reagents used for the study as follow: Cisplatin modified DNA antibody [CP9/19], Laminin 5 antibody (Abcam, USA); ABCB1 antibody, E-Cadherin (24E10) antibody (Cell Signaling Technologies, USA); Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, USA); Anti-SNAI2, clone 2B6 (Millipore, USA); Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, USA); ATG5 antibody (Novus, USA), β -Tubulin antibody, GAPDH antibody (Santa-Cruz, USA); LC-3 antibody, 4-hydroxytamoxifen (4-OHT), cisplatin and doxycycline (Dox) (Sigma-Aldrich, USA); Beclin-1 antibody (Pierce, USA); IRdye 680-conjugated secondary antibodies (LI-COR Biosciences, USA); ON-TARGETplus siRNA against ANGPTL4 (Δ ANGPTL4) (GE Dharmacon, USA).

Recombinant human cANGPTL4 (rh-cANGPTL4) were expressed and purified in-house as previously described (Goh et al., 2010a).

Mouse monoclonal anti-human cANGPTL4 antibodies (α -cANGPTL4) were produced in-house and its specificity were previously described (Goh et al., 2010a).

2.2 Cell Lines and Cell Culture

Human gastric carcinoma cell line MKN74 (ATCC, USA) was maintained in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% fetal bovine serum (FBS). Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

To generate a tamoxifen inducible MKN74 cell line that expresses the Snai1-ER transgene (MKN74_{Snai1ER}), retrovirus transduction protocol was used as previously

described (Teo et al., 2017b). Following transduction, cells were selected with 5 µg/mL of blasticidin hydrochloride (Sigma-Aldrich, USA).

Dual-inducible MKN74_{Snai1ER:shANGPTL4} cells were generated by introducing doxycycline (Dox)-inducible pSingle-tTs-shRNA vector (containing either shANGPTL4 or scrambled shRNA) into MKN74_{Snai1ER} using Fugene HD (Promega, USA) as per manufacturer's protocol. MKN74_{Snai1ER:shANGPTL4} cells were selected with 1 mg/mL of G418 (Sigma Aldrich, USA) and knockdown efficiency was verified by quantitative PCR and immunoblotting analysis upon exposure to 10 µg/mL of doxycycline.

Transient knockdown of ANGPTL4 was generated using ON-TARGETplus siRNA (GE Dharmacon, USA) to generate MKN74 Δ ANGPTL4 and MKN74_{Snai1ER: Δ ANGPTL4}, as per manufacturer's protocol. Knockdown efficiency was verified by quantitative PCR and immunoblotting analysis.

2.3 *In vitro* EMT Models

Three *in vitro* EMT models were used in our study. For the 4-hydrotamoxifen (4-OHT) induced Snai1-mediated EMT model, MKN74_{Snai1ER} and MKN74_{Snai1ER:shANGPTL4} cells were exposure to 20 ng/mL of 4-OHT for 4 days. For hypoxia-induced EMT model, cells were exposed to hypoxic condition carried out in a hypoxic chamber (Stem Cell Technologies, USA) and maintained with a 1% O₂ concentration for 2 days. For TGFβ induced EMT model, cells were exposed to 10 ng/mL of TGF-β1 for 2 days.

For EMT experiments, cells were seeded in 10-cm tissue culture dishes or 6-well plates 2 days prior the start of experiment to allow attachment and formation of individual

colonies. Culture medium was replaced with serum-free RPMI at the start of the experiment. All cells were harvested at their appropriate time point.

2.4 *In vivo* EMT Xenograft Model

All animal experiments were approved by the Nanyang Technological University - Institutional Animal Care and Use Committee (NTU-IACUC), Singapore.

For xenograft experiments, six weeks old NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice were injected subcutaneously in both flanks with 1×10^6 MKN74_{Snai1ER:shANGPTL4} cells reconstituted in serum-free RPMI 1640:matrigel. All mice were injected intraperitoneally (i.p.) with 4-OHT (4mg/kg) after a week. Once xenograft tumors were established, mice were randomly divided into two subgroups and fed on either normal Chow diet or Dox diet (625 mg/kg, Harlan Laboratories, Indianapolis, IN, USA). Cisplatin (4mg/kg) treatment were administered weekly to all mice with a combination of i.p. and intratumoral injections from week 3. Tumor volume were monitored weekly with a vernier caliper and calculated based on the formula $V = 1/2 (L \times W^2)$. Mice were sacrificed from week 5 and tumors, lung and liver were harvested for further analysis.

2.5 Histology Tissue Processing and Embedding

All tissues biopsies harvested from mice were fixed with fresh 4% paraformaldehyde at room temperature for 4 h. Tissues were then dehydrated before embedded in paraffin wax (Leica Microsystems, USA) for paraffin sectioning. Tissues were sectioned at 5 μ m thickness and stored at room temperature till further analysis.

2.6 Animal Tissue Dissociation

Fresh tissue biopsies were harvested from mice, resuspended in DMEM containing collagenase and DNase I, and homogenized into single cell using the gentleMACS

Dissociator (Miltenyi Biotec, Germany) as per manufacturer's protocol. Homogenates were strained using a 70 µm nylon cell strainer and washed with PBS.

2.7 Flow Cytometry

For flow cytometry analysis, single cell suspension obtained from dissociation were resuspended in PBS and blocked with 3% FBS before staining with respective target antibodies or Annexin and Propidium Iodide (BD Biosciences, USA) and incubate on ice. Cells were then washed and resuspended in PBS for flow cytometry analysis. Flow cytometry was performed using the Accuri C6 Flow Cytometer (BD Biosciences, USA) and analysis was done using the FlowJo X software (FlowJo, USA).

2.8 Immunofluorescence Staining

Paraffin embedded sections were antigen-retrieved with Citrate Buffer (pH6.0) for 15 min, permeabilized with 0.2% Triton X-100 for 10 min, blocked with 3 % normal goat serum (NGS) at room temperature for 1 h. Tissue sections were then incubated overnight with primary antibody (1:100) in a humid chamber at 4°C. Tissue sections were treated with three washes of PBS, before incubation with Alexa Fluor 488-conjugated secondary antibodies (1:200) in dark for 1 h and counterstained with Hoechst Dye for nuclei staining. Immunostaining without primary antibody incubation was performed to serve as negative controls. Images were acquired using Zeiss LSM 700 confocal microscope at 20x magnification and analyzed with ZEN 2012 software.

2.9 RNA Extraction and Quantitative PCR

Total RNA were extracted using TRIzol Reagent (Invitrogen, USA) and incubated with chloroform for phase separation to occur. Samples were then centrifugated at 12,000g at 4°C for 15 min. RNA is then precipitated out of the aqueous phase, solubilized in

DEPC water and reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences, USA) according to the manufacturer's protocol. Quantitative PCR (qPCR) was done using the KAPA SYBR FAST qPCR master mix (Kapa Biosystems, USA) and performed using the C1000 Thermal Cycler with CFX96 Real-Time System module (Biorad Laboratories, USA). Primer sequences are summarized in Table 1. All primers were optimized base on melt curve analysis to ensure a single PCR amplicon was produced.

Table 1. Quantitative PCR primer sequences

qPCR Primers:		
Genes	Forward (5' to 3')	Reverse (5' to 3')
18S	GTAACCCGTTGAACCC CATT	CCATCCAATCGGTAGT AGCG
ABCB1	GGTCTTTCCACTAAAG TCGGAGT	TCCCCTTCAAGATCCAT CC
cANGPTL4	TGGTTTGGCACCTGCA GCCATTC	TGCTGCCATGGGCTGG ATCAAC
CDH1	GCCGAGAGCTACACG TTCA	GACCGGTGCAATCTTC AAA
DDR1	CACTCCGCTCCCTGTG TC	AGAGGTGCGACTGGAA CAA
ERBB3	CTGATCACCGGCCTCA AT	GGAAGACATTGAGCTT CTCTGG
Snai1	GCCTTCAACTGCAAAT ACTGC	CTTCTTGACATCTGAGT GGGTC
TBP	GCTGGTTATCGGGAGT TGG	ACTGGCCTGGTGTCTTA GAG
hTBP	GTGGGGAGCTGTGAT GTGAA	GGGAGGCAAGGGTACA TGAG
ZEB1	GCCAACAGACCAGAC AGTGTT	TCTTGCCCTTCCTTTTC CTG
ATG5	TCTGCACTGTTTCTCT AAGGATGCA	TCCGATTGATGGCCCA AAACTGGT
Beclin1	CTGGACACGAGTTTCA AGATCCT	TGTGGTAAGTAATGGA GCTGTGAGTT
LC3	AGCAGCATCCAACCA AAATC	CTGTGTCCGTTACACCA CAG
SQSTM1	AGGCGCACTACCGCG AT	CGTCACTGGAAAAGGC AACC
qChIP Primers:		

Binding Region	Forward (5' to 3')	Reverse (5' to 3')
pNF- κ B/pCREB	CCAATCCCTAAGCCAT GTAAC	AGGCAGGCAGGCTTGA AAG
pSTAT3/pCJUN	AAATAACTCCCTACTT ACTTCC	AAGGAAATGCTTTCTG CCT
Negative Control	TGCAAGTGGAAAGTC GGAG	TGGAAGTCCGGATTGC TTC

2.10 Kinase Inhibitor Array

MKN74 cells were treated with 95 different kinase inhibitors (SYN-2103; Synkinase, Australia) in the presence and absence of rh-cANGPTL4 (10 μ g/mL) for 6 h. RNA was isolated and reverse transcribed to cDNA and qPCR were performed as described previously.

2.11 Immunoblotting Analysis

Total protein was extracted from cells using ice-cold RIPA buffer. Lysates were left on ice for 10 min before centrifuged at 12,000g for 15 min to remove cell debris. Protein extracts were resolved in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN II electrophoresis cell (Biorad Laboratories, USA), before electro-transferred onto nitrocellulose membrane using Mini Trans-blot module (Biorad Laboratories, USA). Membranes were blocked in Rockland's Blocking Buffer (Rockland Immunochemicals, USA) for 1 h and incubated with respective primary antibodies overnight at 4°C. Membranes were further incubated with IRdye 680-conjugated secondary antibodies and detected using ODYSSEY CLx Infrared Imaging system (LI-COR Biosciences, USA). Protein quantification analysis were done using ImageJ software.

2.12 Human Tumor Biopsies

Human frozen tissue biopsies from head and neck, breast, gastric and colon were purchased from Proteogenex, USA (Table 2).

Table 2. Human tumor biopsies details

Sample Source	Sex	Age	Diagnosis	Histological diagnosis	Grade	TNM	Stage
Breast	F	51	cancer	infiltrating ductal carcinoma	G2	T1cN0M0	I
Breast	F	62	cancer	infiltrating lobular carcinoma	G2	T2N3aM0	IIIC
Breast to ovary	F	59	metastases	infiltrating ductal carcinoma	N/A	recurrent	
Colon	M	66	cancer	adenocarcinoma	G1	T2N0M0	I
Colon	M	80	cancer	adenocarcinoma	G1	T3N1M0	IIIB
Colon to lung	F	42	metastases	adenocarcinoma	N/A	recurrent	
Stomach	M	64	cancer	adenocarcinoma	G2	T2N0M0	IB
Stomach	M	58	cancer	adenocarcinoma	G3	T3N2M0	IIIA
Tongue	M	64	cancer	squamous cell carcinoma	G2	T2N1M0	III
Tongue	M	58	cancer	squamous cell carcinoma	G1	T2N0M0	II

2.13 Fluorescence Efflux Assay

Efflux assay to measure MDR1, MRP and BCRP activities were performed using eFluxx-ID Green Multidrug Resistance Assay Kit (Enzo Lifesciences, USA) as per manufacturer's protocol.

Cells were harvested and resuspended into equal number of single cells (2×10^5 / condition) with RPMI-1640 containing DMSO or specific or specific inhibitors (Verapamil: ABCB1 inhibitor; MK-571: ABCC1 inhibitor; Novobiocin: ABCG2 inhibitor; 200 μ m) and incubated for 10 min at 37°C. eFluxx-ID Green Detection

Reagent was added and incubated for additional 40 min before FACS analysis using BD Accuri C6 flow cytometer (BD Biosciences, USA). Data were analyzed using FlowJo and plotted based on mean intensity.

2.14 Quantitative Chromatin-immunoprecipitation (qChIP)

Quantitative ChIP experiments were carried out as previously described. Sonicated chromatin complexes were immunoprecipitated using antibodies against pCREB, pSTAT3, p65(NF- κ B) or p-cJUN. Immunocomplexes were affinity precipitated by Protein A/G Sepharose (Santa Cruz, USA). The ChIP primer sequences are listed in Table 1.

2.15 Fatty Acid Oxidation Assay

MKN74_{Snai1ER:shANGPTL4} cells were exposed to TGF- β 1 for 2 days to induce EMT before performing fatty acid oxidation (FAO) assay using MitoXpress FAO Kit as per manufacturer's protocol (Sapphire North America, USA). Cells were washed and incubated with either FA or FA-Free Measurement Media with MitoXpress Xtra

reagent. Electron transport chain (ETC) activity is measured by the kinetic output using a fluorescence plate reader.

2.16 Partek and IPA

Microarray data were analyzed using Partek Genomic Suite v6.6 (Partek Inc, USA) and samples were compared using multi-way ANOVA analysis to generate heat map. Ingenuity Pathway Analysis (IPA) software was used to conduct pathway analysis and map downstream signaling molecules.

2.17 Statistical Analysis

Statistical analyses were performed with two-tailed non-parametric Mann-Whitney tests using GraphPad Prism software (GraphPad Software Inc, USA). A p-value of < 0.05 was considered significant (*P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant).

CHAPTER 3:

**ROLE OF ANGPTL4 IN MULTIPLE DRUG
RESISTANCE (MDR) DURING METASTASIS**

CHAPTER 3: ROLE OF ANGPTL4 IN MULTIPLE DRUG RESISTANCE (MDR)

3.1 Introduction

Drug resistance describes the ability of cancer cells to negate the effects of cancer therapeutics and survive. Clinically, many patients develop accretion in resistance to chemotherapy over time, which results in an inability to fully eradicate the tumor or result in recurrence. Combination of drugs or therapies are common strategies utilized to overcome drug resistance and enhance drug efficiencies. However, MDR impedes treatment efficacies and render therapeutic efforts futile. MDR is one of the major challenges for therapeutic care and decreases the curative rate for cancers and conform limitations to therapeutic options.

Cancer cells acquire this capability through two methods: (i) genetic inheritance that confers their resistance, or (ii) modification of their intrinsic signaling and genetical makeup upon exposure to chemotherapeutic agents. Research has shown that development of drug resistance in response to chemotherapy occurs through various mechanisms: enhancing DNA repair, defective apoptosis mechanism, altering target molecules, altering metabolism through enzymatic deactivation and altering membrane transport (Holohan et al., 2013; Housman et al., 2014).

Some chemotherapeutics act by targeting cancer cells DNA and inflicting sufficient damage to initiate apoptosis. Cancer cells can circumvent by gene amplification or upregulation of enzymes involved in DNA repair to overcome this challenge by prevent cell death and restore proliferative capacity. Cancer cells can evade apoptosis via expression of mutant topoisomerase I and II, which promotes DNA repair by reduces

binding efficiency of chemotherapeutic drugs to topoisomerase-DNA complex (Lage et al., 2000). Alternatively, epithelial cancer cells have demonstrated an increase in IL-4 expression, which promotes expression of antiapoptotic proteins such as B-cell lymphoma-extra large (Bcl-xL), BCL2, Cellular FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory protein (cFLIP) and phosphoprotein enriched in diabetes protein (PED), while inhibition of IL-4 reverses this effect (Hallett et al., 2012). BCL2, induced myeloid leukemia cell differentiation protein (Mcl-1), A1/Bf1 and Bcl-2-like protein 2 (Bcl-w) were also commonly observed to be upregulated in cancer cells that are conferred with apoptotic resistance (Adams and Cory, 2018). Alternatively, cancer cells may already possess a dysfunctional apoptotic machinery which nullifies the efficacy of drugs administered as they are unable to drive cancer cells towards cell death.

Chemotherapeutic drugs may also function through tubulin/microtubules disruption during mitosis and hinder vascularization during angiogenesis. Microtubule target agents (MTAs) are one of the major class of chemotherapeutic drugs used for cancer treatment and cancer cells may alter the expression of specific tubulin isotypes or mutant form, which affects cellular sensitivity to MTAs or its binding (Day and Siu, 2016; Mukhtar et al., 2014). Increase stability of microtubules by posttranslational modification of tubulin also alters microtubule dynamics during mitosis and reduce effects of MTAs (Song and Brady, 2015).

Cancer cells can also reduce drug efficiency by modifying the pharmacokinetics of the drugs through altered drug metabolism by preventing their conversion to metabolically active substrates. Modulation of enzymatic levels may also limit bioavailability of drug by increasing plasma clearance. Tumors that demonstrate elevated glutathione S-transferase (GST) and glutathione (GSH) activity promotes cisplatin resistance, while

increase in NRF-2 expression upregulate detoxification of drugs (Allocati et al., 2018; Jaramillo and Zhang, 2013). Overexpression of cytochrome P450 in various cancers is also responsible for increasing metabolic breakdown of a diverse range of chemotherapeutic agents and nullifying their efficacy (Li et al., 2017b; McFadyen et al., 2001).

Cell membrane proteins plays an important role in regulating molecules trafficking in and out of cells and plays critical role in drug therapy. Cancer cells may instigate the reduction of intracellular drug concentration by augmenting expression of the transmembrane ATP-binding cassette (ABC) transporters, a broad-spectrum specificity efflux pump that extrude chemotherapeutic drugs out of cell. There are 7 subfamilies within the family, with increase expression of MRP and MDR/TAP members demonstrating responsibility to this phenotype (Rees et al., 2009).

Various studies have demonstrated the association of ABC transporters with development of MDR in tumors. One of the most extensively studied example of ABC transporter is the ATP-binding cassette transporter B1 (ABCB1). ABCB1, also known as multidrug resistance protein 1 (MDR1) or p-glycoprotein (P-gp), is an ATP-dependent membrane transporter that mediates the active removal of xenobiotics in cells. It is encoded by the *ABCB1* gene on chromosome 7q21.12 in humans, and belong to the MDR/TAP subfamily of the ABC transporters superfamily. ABCB1 is a 170 kDa transmembrane glycoprotein that is glycosylated on its N-terminal. ABCB1 expression is regulated by various transcription factors and signaling pathways including NF- κ B, forkhead box O1 (FOXO1), heat-shock transcription factor 1 (HSF1), as well as the MAPK/ERK pathway, PI3K/Akt pathway and Wnt/ β -catenin pathway. Studies has shown an upregulation of ABCB1 expression by Raf-MAPK signaling pathway

through the activation of phospholipase C (PLC), and downregulation by tissue factor pathway inhibitor-2 (TFPI-2) in HCC (Gottesman and Pastan, 2015; Robey et al., 2018).

Other ABC transporters identified to contribute to MDR include ABCC1, also known as multidrug resistance-associated protein 1 (MRP1) and ABCG2, also known as breast cancer resistance protein (BCRP). ABCC1 is shown to be overexpressed in non-small cell lung carcinomas (NSCLC) (Young et al., 2001), early-stage breast cancers and colorectal cancer studies (Chen et al., 2017; Filipits et al., 2005) while increased expression of ABCG2 is demonstrated in breast cancer cell lines *in vitro* (Doyle and Ross, 2003). ABCG2 also confers resistance to topoisomerase I inhibitors in NSCLC and inhibition of it reverses the effects (Ohtsuka et al., 2010).

In recent years, studies have associated EMT with chemoresistance development. Metastatic tumors expressing Snai1, Slug or Zeb1 are less sensitized to drug treatment while deletion of Snai1, Twist1 or inhibition of EMT inducing pathways such as TGF β or Wnt signaling increases drug sensitivity towards cyclophosphamide and gemcitabine and reduces tumor volume (Yang et al., 2004). However, not all cancer cells that exhibit chemoresistance have metastatic potential. The co-expression of EMT or metastatic phenotype and increase in drug resistance during drug treatment may suggest conferring a selective growth advantage for cancer cells in an EMT-dependent manner (Roche, 2018). This suggests a correlation between drug resistance development and EMT as a potential survival mechanism that cancer cells employ.

Previously, multiple studies have illustrated the pro-metastatic role of ANGPTL4. In our recent study, we have identified ANGPTL4 as a key player that elevates adenylate

energy charge and coordinates the energy demands required during EMT. This confers a metabolic flexibility to induce EMT and enhances metastasis in cancer cells (Teo et al., 2017b). ABC transporters are active ATP-dependent efflux pumps that require ATP binding and hydrolysis to function. This suggests a high energy dependency in cancer cells that utilize ABC transporters to sustain the drug resistance phenotype.

In this study, we examined the expression and activity of ABC transporters during EMT in multiple cancer cell lines and evaluate the role of ANGPTL4 in conferring metabolic flexibility and coordinating energy demands during ABC transporters mediated drug resistance.

3.2 Results

3.2.1 Expression and activity of multiple ABC transporters are upregulated during EMT

ABC transporters play a significant role in the efflux of chemotherapeutic drugs in cancer cells to confer its drug resistance. Multiple studies have highlighted similar observations in metastatic cancers. To establish the relation between ABC transporters and metastasis, we first examined the expression of ABC transporters during EMT, which is an early stage event that is essential for invasion and metastasis. We established three *in vitro* EMT models using the polarized gastric carcinoma cell line MKN74. EMT is either induced by exposure to TGF β or hypoxic condition (1% O₂), which resembles the tumor microenvironment stimuli, or a transgenic MKN74 model that carries a Snai1-ER transgene (MKN74_{Snai1ER}). EMT is initiated in MKN74_{Snai1ER} cells upon exposure to 4-OHT.

Upon EMT induction, MKN74 cells and MKN74^{Snai1ER} cell colonies progress from an epithelial-like morphology into a mesenchymal-like phenotype after 48-96 h, across all three models (Figure 5A-C). Quantification of critical EMT genes showed a reduction of epithelial-associated markers such as E-cadherin (*CDH1*), discoidin domain receptor 1 (*DDR1*) and erb-b2 receptor-tyrosine kinase 3 (*ERBB3*), accompanied by upregulation of mesenchymal-associated markers such as Snai1 (*Snai1*) and Zeb1 (*ZEB1*) were observed in all three models. Immunoblotting also showed a reduction of E-cadherin expression, accompanied by an increase in Slug expression upon EMT induction (Figure 5D-F).

Next, we examined the expression of ABC transporter genes across the three *in vitro* EMT models by gene expression analysis using Partek Genomics Suite. We observed an enrichment of multiple ABC transporter genes, such as ABCB1, ABCC1, ABCG2 and ABCC6 as early as 24 h (Figure 5G). To verify those data, we performed flow cytometry analysis on MKN74 cells to detect cell surface ABC transporters ABCB1, ABCC1 and ABCG2 and confirmed an increase in expression during TGF- β 1 and hypoxia mediated EMT (Figure 5H). Similar observations were also noted in breast cancer cell line MCF-7 (Figure 5I).

Figure 5. Increased ABC transporters expression during *In vitro* EMT by different stimuli.

(A–C) Immunofluorescence staining of E-cadherin in MKN74 cells exposed to (A) hypoxia, (B) TGF β 1, as well as in (C) MKN74^{Snai1ER} cells treated with 4-OHT at indicated time points. (D–F) Relative fold change in mRNA levels of EMT markers (CDH1, DDR1, ERBB3, Snai1 and ZEB1) (top panel) and immunoblot analysis of E-cadherin and Slug expression (bottom panel) in MKN74 cells exposed to (D)

hypoxia, (E) TGF- β 1, as well as in (F) MKN74^{Snai1ER} cells treated with 4-OHT at indicated time points. TATA-box binding protein (TBP) was used a housekeeping gene for real-time PCR. β -tubulin protein was used a loading and transfer controls for immunoblot analysis. Data are represented as mean $\pm$ s.d. from at least three independent experiments. Loading controls for the immunoblot analyses were from the same sample. (G) Heatmap showing fold change in the mRNA expression of multiple ABC transporters in three in vitro EMT models. n=3 independent experiments (H) FACS analysis of cell surface expression of indicated ABC transporters, ABCB1 (left panel), ABCC1 (middle panel) and ABCG2 (right panel) in hypoxia- and TGF- β 1-induced EMT of MKN74 cells. (I) FACS analysis of cell surface expression of ABCB1 (left panel), ABCC1 (middle panel) and ABCG2 (right panel) in hypoxia- and TGF- β 1-induced EMT of MCF-7 cells. Data are represented as mean $\pm$ s.d. from n=3 independent experiments.

To establish the effects of increased ABC transporters expression during EMT, we examined the drug efflux capacity by an efflux assay, which utilizes a hydrophobic non-fluorescent compound that readily penetrates cell membrane and gets hydrolyzed into a fluorescent dye by intracellular esterases. The fluorescent dye is actively pumped out of the cells in the presence of ABCB1, ABCC1 and ABCG2 but retains in the cytosol in the absence of the three major ABC transporters. The fluorescence signal of the dye is inversely proportional to the ABC transporters activity, and the addition of their respective inhibitors can differentiate individual transporter activity.

Induction of EMT in MKN74 cells and MKN74^{Snai1ER} cells demonstrated a 30-50% decrease in intracellular fluorescence signal, suggesting an increase in ABCB1, ABCC1 and ABCG2 activity during EMT (Figure 6A). Next, we inhibited the activities of each transporter with their respective selective inhibitor: Novobiocin for ABCG2, MK-571 for ABCC1 and Verapamil for ABCB1. Higher fluorescence signals were observed in the presence of Novobiocin or MK-571, indicating that ABCG2 and ABCC1 plays a role in drug resistance in MKN74 during EMT (Figure 6B). This suggests that higher drug efflux is attributed by an increase in multiple ABC transporters expression and activity in cancer cells during EMT, resulting in lower retention of drug concentration within the cells and conferring a survival advantage.

Figure 6. Increased ABC transporters activities during *In vitro* EMT

(A-B) Relative fluorescence signal of efflux assay measuring (A) drug efflux capacity of MKN74 and MKN74Snai1ER cells in all three *in vitro* EMT models, (B) and in the presence of selective ABC transporters inhibitors Novobiocin, MK-571 and Verapamil. Data are represented as mean±s.d. from at least five independent experiments. *P<0.05, **P<0.01.

3.2.2 Elevated expression of ABCB1 and ANGPTL4 correlates with tumor

progression

In our previous study, we identified ANGPTL4 as a key player that elevates adenylate energy charge and coordinate energy demands required during EMT. This confers a metabolic flexibility during EMT initiation and enhance metastasis in cancer cells. Moreover, we showed that immuno-neutralization or knockdown of ANPGTL4 impedes cancer progression into mesenchymal-like phenotype. As ABC transporters require energy in the form of ATP to drive the exportation of drugs, we speculate that ANGPTL4 may play a role in supporting ABC transporters in the process.

We first examined the expression of ANGPTL4 and ABCB1 during EMT in our three *in vitro* EMT models and observed and increase in both cANGPTL4 and ABCB1 expression upon EMT induction (Figure 7A). This increase is also observed in various cancer cell lines such as skin squamous cell carcinoma HSC and II4 cells, hepatocellular carcinoma HepG2 cells and breast cancer MCF-7 cells treated with TGF-β1 (Figure 7B). We further verified this with a human tissue array, with both ABCB1

and cANGPTL4 expression profile consistently elevated with increasing stages of cancer (Figure 7C, Table 2). This reflected a correlation of ABCB1 and cANGPTL4 to cancer progression in a stage-dependent manner.

Figure 7. Elevated expression of ABCB1 and ANGPTL4 correlates with tumor progression

(A-C) Relative protein level of ABCB1 and cANGPTL4 (A) in hypoxia- and TGF-β1-induced EMT of MKN74 and in 4-OHT-induced EMT of MKN74Snail1ER, (B) in TGF-β1-induced EMT of HSC, II4, HepG2 and MCF-7 cells, (C) and in various stages of cancer. Comparison was made against respective stage I samples. Representative immunoblot pictures and densitometric quantification plots are shown. Loading controls (β-tubulin or GAPDH) for the immunoblot analyses were from the same sample. Data are represented as mean±s.d. from n=3 independent experiments. *P<0.05, ** P<0.01.

3.2.3 cANGPTL4 plays a role in Glucose Metabolism during EMT

Next, we evaluated the role of ANGPTL4 in cellular metabolism during EMT. We analyzed the gene expression profile of microarray data (GSE71280) established from three *in vitro* EMT models and collated genes that were enriched across all three models that are involved in metabolic processes (Figure 8A). These genes are associated with ROS metabolism, autophagy, glucose metabolism, amino acid metabolism and fatty acid metabolism. Suppression of ANGPTL4 during all three *in vitro* EMT models resulted in >50% alteration of metabolic gene signatures, suggesting that ANGPTL4 plays a key role in conferring metabolic flexibility during EMT (Figure 8B).

We first validated this finding by performing a glucose uptake assay with 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG), a fluorescent analogue of glucose on various cancer cell lines such as breast cancer MDA-MB-231 cells, adenocarcinomic human alveolar basal epithelial cells A549, skin squamous cell carcinoma A-5RT3 cells and MKN74 (Figure 8C). Reduction of ANGPTL4 expression by immunoneutralization with α -cANGPTL4 antibody or siRNA knockdown (Δ ANGPTL4) resulted in lower uptake of 2-NBDG (Figure 8C).

We then performed a live-cell metabolic assay using the Seahorse XF Analyzer to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Reduction of ANGPTL4 expression by siRNA knockdown (Δ ANGPTL4) demonstrated lower ECAR upon hypoxic-induced EMT, suggesting a reduction in glycolysis in the absence of ANGPTL4 (Figure 8D). A dual inducible MKN74_{Snai1ER:shANGPTL4} cells were generated by introducing doxycycline (Dox)-inducible shANGPTL4 expression plasmid into MKN74_{Snai1ER} cells. MKN74_{Snai1ER:shANGPTL4} cells treated with 4-OHT undergoes EMT, but co-treatment

with Dox suppresses ANGPTL4 expression and results in a reduced ECAR (Figure 8E). Conversely, addition of recombinant cANGPTL4 rescues the effect of ANGPTL4 knockdown and increases ECAR, supporting the theory that ANGPTL4 plays a role in glucose metabolism (Figure 8D).

Figure 8. *cANGPTL4* plays a role in Glucose Metabolism during EMT

(A) Gene enrichment analysis based on gene expression data of three *in vitro* EMT models (GSE71280). (B) Gene expression data of MKN74 cells treated with and without si-RNA against ANGPTL4 in three *in vitro* EMT models. (C) FACS analysis of the fluorescent glucose analog 2-NBDG uptake in MDA-MB-231 (breast) and A549 (lung) cell lines treated with or without neutralizing antibody against ANGPTL4 (α -cANGPTL4) or in control (Scrambled) and ANGPTL4-knockdown (Δ ANGPTL4) A-5RT3 (skin) and MKN74 (stomach) cell lines. Data are represented as mean $\pm$ s.d. from n=5 independent experiments. *P<0.05, ** P<0.01. (D-E) Extracellular acidification rate of (D) control (MKN74) and siRNA ANGPTL4-knockdown (MKN74 Δ ANGPTL4) MKN74 cells and (E) MKN74Snai1ER:shANGPTL4 cells after indicated treatments. Values were normalised to total cellular protein. Values are represented as mean $\pm$ s.d. from at least three independent experiments.

3.2.4 cANGPTL4 plays a role in fatty acid β -oxidation and elevate cellular ATP during EMT

We proceed on to investigate the role of cANGPTL4 in fatty acid metabolism during EMT. Cells degrade long chain fatty acids (LCFAs) in the mitochondria by converting LCFAs to acetyl-CoA through β -oxidation. Acetyl-CoA molecules enters the TCA cycle and ETC to generate ATPs. The fatty acid oxidation (FAO) kit measures β -oxidation of exogeneous oleate, endogenous fatty acid stores, non-long chain fatty substrates and maximum respiratory capacity of cells through OCR. MKN74_{CTRL} cells exposed to TGF- β 1 mediated EMT demonstrated higher respiratory capacity through β -oxidation (Figure 9A). This increase is not observed in MKN74 Δ ANGPTL4 cells but is reinstated in the presence of rh-cANGPTL4.

Following that, we measured the cellular ATP concentration during EMT using an ATP determination assay kit. ATP concentration were determined by luminescence readings plotted against an ATP standard curve and normalized against protein concentration . The intracellular ATP concentration is increased in EMT, but the elevation is abolished in MKN74 and MKN74_{Snai1ER} cells when immunoneutralized with α -cANGPTL4 or in MKN74_{Snai1ER:shANGPTL4} cells (Figure 9B-C). This observation is restored by rh-cANGPTL4 (Figure 9B). Consistent with the microarray data, these finding suggests that ANGPTL4 contributes to the metabolic flexibility within cancer cells that are

undergoing EMT, by influencing glucose and fatty acid metabolism, as well as ATP concentration to support the high energy demanding processes including ABC transporter activities by maintaining the cellular adenylate energy pool.

Figure 9. *cANGPTL4* plays a role in fatty acid β -oxidation and elevation of cellular ATP during EMT

(A) Long chain fatty acid (LCFA) β -oxidation analysis of MKN74 and MKN74 Δ ANGPTL4 cells upon TGF β 1-induced EMT. (B-C) Cellular ATP concentration in (B) MKN74 and MKN74 Δ ANGPTL4 cells and (C) MKN74 Snai1ER and MKN74 $^{Snai1ER:shANGPTL4}$ cells after indicated treatments, ATP concentration was determined from a standard curve using pure ATP. Values were normalized to total cellular protein. Values are represented as mean $\pm$ s.d. from at least three independent experiments.

3.2.5 ANGPTL4-induced elevated cellular ATP fuels ABC transporter efflux during EMT

We have previously shown that ANGPTL4 contributes to cellular metabolism by supporting the cellular adenylate energy pool to provide for the energy demanding

processes during EMT. ABC transporters expression and activities were increased during EMT in cancer cells and they are highly dependent on cellular ATPs. We also showed a concurrent increase of ABCB1 and ANGPTL4 in metastatic cancer cells and their expressions are both correlated to tumor progression.

Therefore, we examined the drug efflux capacity based on reduced cellular ATP levels by the depletion of ANGPTL4. We observed a higher fluorescence dye retention in MKN74 Δ ANGPTL4 cells compared to MKN74 cells (Figure 10A) and this effect is further enhanced in MKN74_{Snai1ER:shANGPTL4} cells when ANGPTL4 was suppressed during EMT induction (Figure 10B). This suggests that ANGPTL4 is required for drug efflux during EMT and the effect is attenuated upon ANGPTL4 depletion.

Next, we investigated the effects of ANGPTL4 on anti-neoplastic drugs and found that the half maximal inhibitory concentration of cisplatin (MKN74_{CTRL} versus MKN74 Δ ANGPTL4: 25.18 ± 3.2 vs 11.07 ± 2.2 μ M) and doxorubicin (MKN74 versus MKN74 Δ ANGPTL4: 0.052 ± 0.009 vs 0.026 ± 0.008 μ M) are drastically reduced in MKN74 Δ ANGPTL4 cells compared to MKN74 cells (Figure 10C). These findings suggest that ANGPTL4 plays a role in supporting increased ABC transporters drug efflux capacity during EMT by providing the energy required in the form of ATP.

Figure 10. ANGPTL4-induced elevated cellular ATP fuels ABC transporter efflux and promote cisplatin and doxorubicin efflux during EMT

(A-B) Relative fluorescent dyes efflux in (A) MKN74 and MKN74 Δ ANGPTL4 cells and (B) MKN74 $_{\text{SnailIER:shANGPTL4}}$ cells after indicated treatments. Values were normalized to total cellular protein. Values are represented as mean $\pm$ s.d. from at least three independent experiments. (C) Graphs showing the IC₅₀ curve of MKN74 (solid line) and MKN74 Δ ANGPTL4 (dotted line) to cisplatin (left panel) and doxorubicin (right panel). Values are represented as mean $\pm$ s.d. from n=5 independent experiments. *P<0.05, ** P<0.01.

3.2.6 Reduced ANGPTL4 in metastatic cancer cells impairs cisplatin efflux in vivo.

So far, we have demonstrated that ANGPTL4 plays a critical role in tumor metastasis during the EMT process. In this study, we have shown the involvement of ANGPTL4 in *in vitro* cancer cells to regulate cellular adenylate energy which supports the increase in drug efflux during EMT by ABC transporters, suggesting that ANGPTL4 plays a

role in conferring the chemoresistance phenotype. To confirm that this observation stays true *in vivo*, we established a xenograft mouse model with MKN74^{Snai1ER:shANGPTL4} cells, and examined the effects of ANGPTL4 during cisplatin treatment of metastasis.

Six weeks old NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice injected subcutaneously in both flanks with 1×10^6 MKN74^{Snai1ER:shANGPTL4} cells to establish the xenograft model. All mice were injected with 4-OHT to induce EMT of the MKN74^{Snai1ER:shANGPTL4} cells. Subsequently, mice were randomly divided into two subgroups and were kept on chow diet (4-OHT:chow) and doxycycline (Dox) containing diet (4-OHT:Dox at 625 mg/kg) respectively (n = 8 per group). All mice were subjected to cisplatin (4mg/kg) treatment with a combination of i.p. and intratumoral injections from week 3. Mice were sacrificed from week 5 and tumors, lung and liver were harvested for analysis (Figure 11A).

We first verified the knockdown effects of ANGPTL4 by Dox-diet within the primary xenograft by qPCR. Dox-diet led to a drastic decrease in ANGPTL4 expression within the primary xenograft, as compared with chow-diet derived xenograft (Figure 11B). Next, we investigated the effects of cisplatin on the xenograft models. Cisplatin is an anti-cancer drug that functions through the interference with DNA replication by forming crosslinks with DNA molecules. This results in the formation of cisplatin-DNA adducts, which damages DNA and results in activation of apoptosis. We quantified the cytotoxic effects of cisplatin by the formation of cisplatin-DNA adducts in human cytokeratin 18 (CK18)-positive cells and percentage of cells undergoing apoptosis. Reduction of ANGPTL4 expression in 4-OHT:Dox xenograft showed that 91% of human CK18-positive cells has detectable levels of cisplatin-DNA adducts, compared with 78% in 4-OHT:chow xenografts (Figure 11C). This reflects a higher accretion of

cisplatin in ANGPTL4 suppressed OHT:Dox xenograft. We also observed a higher percentage of apoptotic cancer cells in 4-OHT:Dox xenografts (67.54%), as compared to 4-OHT:chow xenografts (42.84%) (Figure 11C). We further investigated the tissue morphology of the xenograft sections and found that a clear disruption of basement membrane in 4-OHT:chow xenografts was observed by the absence of laminin 322 staining, while 4-OHT:Dox xenografts had diminished and punctuated laminin (Figure 11D). These findings are consistent with our *in vitro* data thus far.

Moving on, we examined the metastatic potential of MKN74^{Snai1ER:shANGPTL4} cells by analyzing potential metastatic target organs. Using human-specific TATA-binding protein (TBP) primers, we quantified the presence of MKN74^{Snai1ER:shANGPTL4} cells in recipient mouse lung and liver by qPCR. 18S ribosomal RNA (18S) primers that are not species specific are used to normalize both human and mouse cells detected within the organs. Accordant with our xenograft observations, 4-OHT:chow mice demonstrated a higher expression of hTBP levels in their lungs than 4-OHT:Dox mice (Figure 11E). This illustrates that more MKN74^{Snai1ER:shANGPTL4} cells has metastasized to the mice lungs when fed with chow diet, but the metastatic effects are less drastic when ANGPTL4 is suppressed. This finding is less significant in the liver of 4-OHT:chow mice and scarcely detected in 4-OHT:Dox mice, based on the end point of our experiment. We further verified the effects of ANGPTL4 during cisplatin treatment of MKN74^{Snai1ER:shANGPTL4} cells metastasized to the mice lungs by analyzing the expression of cisplatin-DNA adducts. We sorted the cells derived from the lungs of both groups of mice and noted a significantly larger population of human CK18-positive cells in the lungs of 4-OHT:Dox mice (38.6%) than in OHT:chow mice (28.61%). The FACS analysis also revealed that more human CK-18 positive

metastasized cells expressed cisplatin-DNA adducts in the lungs of 4-OHT:Dox mice (83.2%), as compared to OHT:chow mice (55.2%) (Figure 11F). Combining these observations, we demonstrated that *in vivo* suppression of ANGPTL4 expression leads to higher accumulation of cisplatin-DNA adducts in both primary and metastasized tumors, thus reducing metastatic tumor load.

Figure 11. Reduction in ANGPTL4 impairs cisplatin efflux and increases in vivo tumor chemosensitivity.

(A) A schematic illustration of metastatic xenograft model. MKN74^{Snai1ER:shANGPTL4} cells were injected to form primary xenograft tumors (n=6 per experimental subgroup, two tumors per mouse). Metastasis was induced by 4-OHT injections. Mice were fed with either normal chow diet (4-OHT:chow) or Dox-containing diet (4-OHT:dox; 625 mg/kg). Cisplatin treatment (4 mg/kg/week) were administered from week 3 (left panel). Primary xenograft tumors was measured with a pair of vernier calipers with tumor volume calculated by (Length×Width×Width)/2 (right panel). (B-C) (B) Relative mRNA expression of ANGPTL4 and (C) FACS analysis of cisplatin-DNA adduct-positive among CK18+ cells (top panel) and apoptotic (Annexin V-PI positive; bottom panel) cells from 4-OHT:chow and 4-OHT:dox primary xenograft tumors. For qPCR, ribosomal 18S (18S) was used as a housekeeping gene. Values are mean±s.d. from n=3 independent experiments with 5-6 mice per timepoint. (D) Immunofluorescence staining for laminin 332 (green) of 4-OHT:chow and 4-OHT:dox primary xenograft tumors. The nuclei were counterstained with DAPI (blue). Scale bar: 100 μm (top panel). Macroscopic images of lung and liver from mice bearing 4-OHT:chow and 4-OHT:dox primary xenograft tumors. (E) Relative mRNA expression of human TBP in lung (top panel) and liver (bottom panel) tissues of cisplatin-treated xenograft mice. Ribosomal 18S was used as reference gene. Values are mean±s.d. of triplicate runs from 6 mice. (F) FACS analysis cisplatin-adduct+ cells among human CK18+ MKN74 cells that metastasized to the lung tissues of 4-OHT:chow and 4-OHT:dox mice. Data are represented as mean±s.d. from n=3 independent experiments. *P<0.05, ** P<0.01.

3.2.7 Coordinated upregulation of multiple ABC transporters by c-Myc and NF-κB

The concomitant increase of ABC transporters and cANGPTL4 during cancer progression leads us to question if ANGPTL4 can upregulate the expression of these ABC transporters. We first treated MKN74 cells with rh-cANGPTL4 and quantified the mRNA levels of 10 ABC transporters enriched in our EMT models, out of which seven were upregulated in their mRNA expression levels upon 6 h of treatment (Figure 12A).

To elucidate the mechanism by which cANGPTL4 upregulates the seven ABC transporters, MKN74 cells were subjected to a kinase inhibitor array screening, in the presence of rh-cANGPTL4 stimulation. We identified key signaling molecules in the PI3K/AKT, NF-κB and MEK/ERK pathways involved in the upregulation of the ABC transporters by cANGPTL4 (Figure 12B). This is illustrated by the ability of the specific kinase inhibitor to attenuate the upregulation effects of ABC transporters in the presence of rh-cANGPTL4. Conversely, kinase inhibitors that did not attenuate the

upregulation by cANGPTL4 would indicate that those kinases were not involved (Figure 13A).

We analyzed the kinases involved using the Ingenuity Pathway Analysis software and noted that these pathways converge and implicate that c-Myc and NF- κ B activation as the imminent downstream event (Figure 13B). We verified the involvement of c-Myc and NF- κ B activation by immunoblotting. Phospho-activation of these key kinases and transcription factors validated their involvement in response to cANGPTL4 stimulation (Figure 12C).

Figure 12. cANGPTL4 regulates transcriptional expression of multiple ABC transporter genes.

(A) Relative fold change in mRNA levels of indicated ABC transporters in MKN74 cells after 6 h of recombinant cANGPTL4 protein (rh-cANGPTL4) treatment. Data are represented as mean $\pm$ s.d. from n=3 independent experiments. *P<0.05, ** P<0.01. n.s. not significant. (B) Relative mRNA level of ABCB1 after treatment with various kinase inhibitors from a Kinase Library (#Synkinase-SYN-2103). Values denotes mean fold change in ABCB1 expression from n=3 independent experiment. (C) Representative immunoblot of total and phosphorylated ERK1/2, PKB/Akt, c-Myc and NF- κ B from MKN74 cells treated with recombinant cANGPTL4. Loading controls for the immunoblot analyses were from the same sample. All qPCR was performed in triplicates. Data are represented as mean $\pm$ s.d. from n=3 independent experiments. *P<0.05, ** P<0.01.

A

B

Figure 13. Coordinated upregulation of multiple ABC transporters by c-Myc and NF- κ B.

(A) A schematic illustration of the rationale behind the kinase inhibitor array, which led up to mapping the pathway in Figure 12B. When MKN74 cells were treated with rh-cANGPTL4, ABC transporter gene expression was increased (left panel). Kinase inhibitors (XXn) that negated this up-regulation of ABC transporters in the presence of rh-cANGPTL4 meant that these kinases were involved in the ANGPTL4-mediated regulation of MDR1 expression (middle panel). Conversely, the addition of kinase inhibitors that did not affect the increase in ABC transporters expression in the presence of rh-cANGPTL4 (right panel) suggested that the target kinase was not involved in ANGPTL4-mediated signaling of ABC transporters. (B) Ingenuity Pathway Analysis (IPA) identified c-Myc and NF- κ B as potential transcriptional factors that regulate the expression of multiple ABC transporters when stimulated with recombinant cANGPTL4 protein. IPA-assisted pathways were mapped following experimental kinase inhibitor screen.

Next, to clarify the potential roles of c-Myc and NF- κ B in the cANGPTL4 mediated upregulation of ABC transporters expression, we performed an *in silico* analysis to identify the putative c-Myc and NF- κ B transcription factor binding sites at the regulatory promoter region of these seven ABC transporters as putative binding sites for NF- κ B and CREB, as well as STAT3 and pCJUN, were too close to be separated for chIP assay (Table 3). Quantitative chromatin immunoprecipitation (qChIP) primers sequences than spun the putative c-Myc and NF- κ B binding sites were either adopted from previously published work by Porro *et al.* or designed from the JASPAR2018 database (Table 1) (Porro et al., 2010).

Table 3. ChIP transcription binding site details

Target Gene	TF	Putative Binding Site Location	Putative Binding Site Sequence	Regulated
<i>ABCB1</i>	NF-κB	-1734	AGGGAGACCCCCT	✓
	c-Myc	-1087	CCATGTGACC	✓
	c-Myc	+1663	CATGTG	✓
<i>ABCC1</i>	NF-κB	-3475	TGGGGTTTCCT	✓
	c-Myc	+245	GCCGCGTGAG	✓
	c-Myc	+933	GCCGCGTGCC	✓
<i>ABCC2</i>	NF-κB	-3676	GGGAAATTCTC	✓
	NF-κB	-2930	GGGTATTTTCA	✗
	c-Myc	-665	CATGTGCC	✗
	c-Myc	+333	GAACAAGTGGT	✗
<i>ABCC4</i>	NF-κB	-2084	TGGGGTTTCACCA	✗
	c-Myc	-746	CATGTG	✓
<i>ABCC5</i>	NF-κB	-2633	TGGTGAAACCCCG	✓
	NF-κB	-1963	GTGAAACCCC	✓
<i>ABCC6</i>	NF-κB	-2427	AGGGGGTACACCC	✗
	NF-κB	-2105	AGGGTGTACCCC	✓
<i>ABCG2</i>	NF-κB	-1774	AGGTGATCCACCT	✓
	c-Myc	-173	GACACGTG	✓

Primer sequences yielding 7 or more on the position-specific scoring matrices were selected for the experiment. Potential c-Myc and NF-κB binding sites at the promotor region of the respective ABC transporter genes selected for the study are shown in Figure 14. Primers sets for these binding sites were evaluated for their amplification efficiency by quantitative PCR using the C1000 Thermal Cycler with CFX96 Real-Time System module. PCR amplification conditions for every primer sets were optimized to ensure a single specific amplicon product, as represented by a single melt peak (Figure 14).

ABCB1

NF- κ B

Fw 5'-CCTGGCTTCACTGGAGTT-3'
Rv 5'-GTCCACCTGGCATTGCTT-3'
Tm 63 °C

c-Myc A

Fw 5'-TCTGCTCAACGAGTTGTATCTTC-3'
Rv 5'-TACCAAGCATTATTATTAAGTTTATGAGG-3'
Tm 65 °C

c-Myc B

Fw 5'-TGGGACATCTGAAATG-3'
Rv 5'-AAGGTACATATTAGGACAAAGTCAATTC-3'
Tm 60 °C

ABCC1

NF- κ B

Fw 5'-CCCAGTAGCTCGATTACA-3'
Rv 5'-GGAGATTGAGACCAAGCTGA-3'
Tm 65 °C

c-Myc A

Fw 5'-ACCGCTCTGGGTACGTG-3'
Rv 5'-GACCCGACCTCCAAAC-3'
Tm 60 °C

c-Myc B

Fw 5'-TGATGTGCCCTACCTGACCTCGG-3'
Rv 5'-AGAGAGAAACAGTCGTGTCCAGATTGCC-3'
Tm 66 °C

ABCC2

NF- κ B A

Fw 5'-CCTGTTAGGTGGGACGTGAT-3'
Rv 5'-GGCAGAGGGATAGGAAAG-3'
Tm 65 °C

NF- κ B B

Fw 5'-TATATGCATGCTGGTTGTAGG-3'
Rv 5'-TCTCCAAGAACTATGGACAAC-3'
Tm 61.6 °C

c-Myc A

Fw 5'-CCAAGGCAGAAGATTGTTGAAGC-3'
Rv 5'-AACAGAGTGGTGGCATCAGTCG-3'
Tm 66 °C

c-Myc B

Fw 5'-GTCTTCGTTCCAGACGAGT-3'
Rv 5'-TCTTGTGTTGGTACCAACCT-3'
Tm 63 °C

ABCC4

NF- κ B

Fw 5'-ACAATATGGTAATATCTCCAGG-3'
Rv 5'-CACCATTGTACATGCTGTGA-3'
Tm 67.8 °C

c-Myc

Fw 5'-CCACTGTTATCTGTAGGCTTTAGGG-3'
Rv 5'-CTTCTCAGGACCAACGACGG-3'
Tm 63 °C

ABCC5

NF- κ B A

Fw 5'-GGTGGATGGATCACAAGGTCT-3'
Rv 5'-CTCTGTCCCTCAGGTTGGAGT-3'
Tm 65 °C

NF- κ B B

Fw 5'-CGTAGCTCACTCCTGTAATCC-3'
Rv 5'-GATCTGGGCTCACTGTAACCTC-3'
Tm 60 °C

ABCC6

NF- κ B A

Fw 5'-ACAATATGGTAATATCTCCAGG-3'
Rv 5'-CACCATTGTATACATGCTGTGA-3'
Tm 65 °C

NF- κ B B

Fw 5'-GGGGCAATATCTCCACAGAA-3'
Rv 5'-GCTGTGACCTCCAGAAAGATA-3'
Tm 60 °C

ABCG2

NF- κ B

Fw 5'-GCCCGGCTAATTTTGTATTTT-3'
Rv 5'-GGCACTACAGGAGAGACTGA-3'
Tm 60 °C

c-Myc

Fw 5'-GTAGTAATCACTCTGGTTCATTCCGTTTCG-3'
Rv 5'-TGCGGCTGGAGGTCACGATGG-3'
Tm 66 °C

Figure 14. Optimisation of qChIP Primers for c-MYC and NF- κ B

The ChIP primer sequences and the annealing temperature (T_m) used for qChIP of the seven ABC transporters were indicated. The melting curve analysis were performed to check the specificities of the qChIPs.

Once the primer sequences were validated, we performed transcription factor binding site enrichment analysis using qChIP to identify the c-Myc and NF- κ B binding sites that potentially regulates ABC transporters expression driven by cANGPTL4. We first treated MKN74 cells with rh-cANGPTL4 in the presence of antibodies against c-Myc and NF- κ Bp65 for 5 h and fixed the cells with formaldehyde to form DNA crosslinks prior to collection. Cells were lysed in SBS lysis buffer, sheared and sonicated to yield DNA fragments ranging from 200-1000 base pairs. The samples were then reverse crosslinked and DNA fragments were recovered. The purified DNA was amplified for qChIP using the optimized primers.

Our data showed a differential transcriptional regulation in both the c-Myc and NF- κ B binding sites for the respective ABC transporters genes (Figure 15). ABCB1, ABCC1 and ABCG2 genes were regulated by both c-Myc and NF- κ B, while ABCC5, ABCC6 and ABCC2 were only regulated by NF- κ B with a 5-fold enrichment. ABCC6 also displayed selective NF- κ B binding affinity as only one of the two putative binding site showed significant enhancement for NF- κ B binding. The ABCC2 gene was predicted *in silico* to be regulated by both c-Myc and NF- κ B but our data suggests that it was only regulated by NF- κ B, and the predicted c-MYC binding site was non-functional. Additionally, no c-MYC binding site was identified in the regulatory region ~3kb up- and downstream of the transcriptional start site of ABCC5 and ABCC6 genes. Similarly, ABCC4 had both c-Myc and NF- κ B putative binding sites but our findings only demonstrated c-Myc regulation.

Figure 15. *ANGPTL4* upregulates ABC transporter expression via *PI3K/AKT*, *MEK/ERK*, *c-Myc* and *NF-κB*

Representative quantitative ChIP assays performed using preimmune IgG (p.i.) or antibodies against phosphorylated NF-κB/p65 and c-Myc in rh-cANGPTL4-treated MKN74 cells. Schematic illustrations show the relative positions of putative NF-κB and Myc binding sites on the regulatory regions of indicated human ABC transporter genes. Putative specific binding sites for respective transcription factors were determined in silico with the Jaspar database. A control region 2 kb upstream of the promoter served as a negative control. Values are represented as mean±s.d. from n=3 independent experiments. *P<0.05, ** P<0.01. n.s., not significant.

To confirm the involvement of c-Myc and NF-κB in cANGPTL4-stimulated signaling, we repeated the experiment by treating MKN74 cells with rh-cANGPTL4 in the presence of siMYC, IKK2 Inhibitor IV, or both siMYC and IKK2 Inhibitor IV for 6 h and quantified the mRNA levels of the 7 ABC transporters. In the presence of siMYC or IKK2 Inhibitor IV, the rh-cANGPTL4 mediated upregulation of the ABC transporters expression were differentially attenuated (Figure 16A). The elevated

expression of ABCB1, ABCC1 and ABCG2 in rh-cANGPTL4-treated MKN74 were reduced when c-Myc and NF- κ B activities were impaired (Figure 16B). In all, our qChIP analysis showed that cANGPTL4 regulates ABC transporters expression through c-Myc and NF- κ B and this enhances the drug resistance capacity during EMT.

Figure 16. rh-cANGPTL4 mediated upregulation of the ABC transporters expression were differentially attenuated by siMYC or IKK2 Inhibitor IV

(A) Relative fold change in mRNA levels of indicated ABC transporters in indicated MKN74 cells after 6 h exposure to recombinant cANGPTL4 protein (rh-cANGPTL4) treatment (left panel). MKN74 cells whose endogenous c-Myc was suppressed by siRNA (siMYC) and/or treated IKK2 Inhibitor IV (IKK2 Inh). We showed that rh-cANGPTL4 upregulated the mRNA level of seven of these ABC transporters. The knockdown efficiency for cMYC was determined by qPCR and western blot (right panel). β -actin as loading control was from the same samples. Data are represented as mean $\pm$ s.d. from n=3 independent experiments. *P<0.05, **P<0.01. n.s. not significant. **(B)** FACS analysis of cell surface expression of ABCB1 (left panel), ABCC1 (middle panel) and ABCG2 (right panel) in recombinant cANGPTL4 (rh-cANGPTL4)-treated MKN74 cells whose c-Myc was suppressed by siRNA (siMYC) and/or NF- κ B activity was inhibited by IKK2 inhibitor (IKK2 Inh; 1 μ M). Data are represented as mean $\pm$ s.d. from 3 independent experiments.

3.2.8 Elevated ANGPTL4 correlates with poorer prognosis in cancer patients

Thus far, our study has demonstrated that ANGPTL4 functions as a key player in modulating cellular bioenergetics and conferring cancer cell resistance to chemotherapy through the upregulation of ABC transporters expression and activity during EMT. To validate our findings, we interrogated the TCGA database to evaluate the clinical relevance of ANGPTL4 and found that 21 out of 68 primary tissue sites displayed ANGPTL4 mutations, with higher representation in the uterus, skin, bladder, esophagus and stomach cancers (Figure 17A). Although ANGPTL4 is not known to be a classic oncogene, analysis from the TCGA database showed that the mutation frequency of ANGPTL4 was approximately 23.56% (82 out of 348 cases), while the mutation frequency of c-Myc and NF- κ B were 40.23% (140 out of 348 cases) and 47.41% (165 out of 348 cases) respectively (Figure 17B). Survival plot of these cases (with the removal of 8 cases that lacked survival data) depicted that patients that carry a mutated ANGPTL4 gene has an increase survival rate (Figure 17C).

Next, we further evaluated the clinical relevance of ANGPTL4 with the survival rate of cancer patients using the Prediction of Clinical Outcomes from Genomic Profiles (PRECOG) database which encompasses 166 cancer expression data sets and overall survival data for ~ 18,000 patients. We found that the unweighted meta-Z score of ANGPTL4 in all human cancers included in the database is 7.6 (P value < 10⁻¹⁰) (data not shown). This supports our findings that high expression of ANGPTL4 is associated with a poor prognosis on the survival rate of cancer patients. Interestingly, most common oncogenes that were amplified in cancers, such as MYC (meta-Z score: 1.76) and SRC (meta-Z score: 1.95) have a lower Z-score. Furthermore, analysis

using the Kaplan-Meier Plotter which assess 18,674 cancer samples and their overall survival data also showed that patients with higher ANGPTL4 expression in cancers such as stomach adenocarcinoma (HR = 1.78), lung adenocarcinoma (HR = 1.77), cervical squamous cell carcinoma (HR = 1.91) and rectum adenocarcinoma (HR = 2.84) had worsen prognosis (Figure 17D).

In all, the bioinformatics analysis of ANGPTL4 in clinical databases complemented our findings and highlighted the significance of ANGPTL4 in cancer development and survival outcome in patients.

Figure 17. Bioinformatics analysis of ANGPTL4 in TCGA database and Kaplan-Meier Plotter.

(A) Frequency distribution for cases with ANGPTL4 mutations across TCGA projects. (B) Frequency distribution of cases in TCGA database containing mutant expression of NF- κ B, c-MYC and ANGPTL4. (C) Survival plot for cases with and without ANGPTL4 in 340 out of 348 cases with NF- κ B and c-MYC alteration. Eight cases were dropped due to lack of survival data. (D) Survival plot of patients expressing ANGPTL4 in various cancers.

3.3 Summary

Cancer is one of the major causes of death globally with metastasis being the leading cause of mortality in cancer diseases. Therapeutic treatment available in the market targeted at various types of cancer has shown drastic improvement over the last decade, overall improving cancer prevalence. However, the curative rate and complete remission is still largely dependent on timely detection, aggressiveness of cancer and patient response to treatment. Cancer patients often develop drug resistance to therapeutics, annihilating their efforts by conferring a selective advantage for the cancer cells to survive and thrive. Therefore, MDR remains one of the major hurdles that needs to be overcome clinically for better advancement in cancer therapy.

Metastasis is a complex multi-step process that is clinically associated with end stage of cancer development and the manifestation is commonly accompanied by MDR, proving its difficulty as a therapeutic target. Studies thus far have shown association of increase in drug resistance via increase of ABC transporters expression in metastatic cancers but the mechanism by which metastatic cancer cells acquire or enhance MDR phenotype remains unclear, as well as a lack of direct evidence to substantiate ABC transporter expression and activities to EMT.

In summary, our study thus far has demonstrated that ABC transporter expression and activity are upregulated during the EMT event of metastasis and this observation is accompanied by an elevated ANGPTL4 expression. The increase in both ABC transporter ABCB1 and cANGPTL4 expression are correlated to tumor progression,

depicting an underlying association of increasing drug resistance phenotype in the presence of ANGPTL4. In our previous studies, we demonstrated that cANGPTL4 is involved in multiple pathways such as attaining anoikis resistance, modulating vascular integrity and coordinating cellular bioenergetics such as increasing cellular adenylate energy to confer cancer cells with a selective advantage for survival and metastasis. Works from other groups also concurred with these theories thus far (Baba et al., 2017; Izraely et al., 2017; Liao et al., 2017).

In this study, we further substantiate our findings and demonstrated that cANGPTL4 plays a vital role in metabolic reprogramming during EMT. cANGPTL4 functions through upregulation of glycolysis and increase in β -oxidation of fatty acids to yield ATP. ANGPTL4 mediated elevation of cellular adenylate energy is necessary to sustain energy demanding processes, including ABC transporter activity during EMT. Reduction of ANGPTL4 in both *in vitro in vivo* metastatic models showed impaired cisplatin efflux, increased apoptosis and reduced metastatic tumor load. ANGPTL4 also potentiates the increase expression of multiple ABC transporters via c-Myc and NF- κ B, coordinating the drug resistance phenotype of cancer cells during EMT.

To sum it up, MDR enhancement through the expression of ABC transporters reduces bioavailability of a diverse range of chemotherapeutic drugs within the cells and limit drug efficacies. Our study illustrates that ANGPTL4 plays a crucial role in metabolic reprogramming and enhanced activation of ABC transporters in cancer cells undergoing EMT. This provides metabolic flexibility and enhance EMT-mediated chemoresistance in metastatic tumor to chemotherapeutic drugs such as cisplatin. Although our study did not elucidate the alteration and activation in all the ABC transporters by ANGPTL4, we demonstrated the involvement of ANGPTL4 in

increasing cellular ATP which is essential for ABC transporter efflux. This suggests that ANGPTL4 may contribute indirectly to the activities of the other ABC transporters through regulation of cellular ATP.

CHAPTER 4:

ROLE OF ANGPTL4 IN AUTOPHAGY DURING

EMT

CHAPTER 4: ROLE OF ANGPTL4 IN AUTOPHAGY DURING EMT

4.1 Introduction

To date, most cancer metabolism studies have been performed on *in vivo* developing tumor models or with *in vitro* cultures that are cancer cells attuned to growth and proliferation. Much less is known about the metabolic changes of cancer cells that are geared towards invasion and migration. During EMT, more anabolic processes are active, which provide cancer cells the impetus to migrate. This results in an increase in their energy demands to sustain anabolism. These processes demand large quantities of ATP and therefore, cancer cells undergoing EMT would require alterations of their metabolic pathways to sustain this energy demand. Moreover, high levels of anabolism require a substantial pool of readily available macromolecules; thus, a potential pathway that cancer cells may rely on to obtain both macromolecules and ATP is autophagy.

Autophagy is a cytosolic degradation pathway that is well conserved throughout evolutionary history across all eukaryotes. It is a catabolic process that occurs at basal levels in all cells and is utilized to breakdown and recycle cellular components such as long-lived or misfolded proteins and damaged organelles via lysosomal degradation. Autophagic levels can be upregulated to play a vital role in cell survival in nutrient-poor conditions by recycling nonessential cellular components during starvation, which enables cells to reuse these components for the synthesis of essential proteins and energy (Mizushima et al., 2010). Research has demonstrated that autophagy also acts as a response to cellular stresses, such as nutrient deprivation, endoplasmic reticulum stress, hypoxia and the buildup of damaged organelles and proteins, and is involved in

many diseases including neurodegenerative disorders, muscular dystrophies, aging, diabetes, infections and cancer (Levine and Klionsky, 2004).

There are three types of autophagy that have been identified to date: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. Each form differs by their specific mechanism for cargo delivery to the lysosome for degradation of the cellular components, as well as by their target specificity and function (Mizushima, 2007). Macroautophagy (herein referred to as autophagy) is the main autophagic pathway utilized by cells to degrade long-lived proteins and organelles and to adapt for survival during cellular stress. Autophagy involves the induction of a pathway via signaling molecules, formation of initial isolation membrane termed phagophore (vesicle nucleation), expansion of the phagophore (vesicle elongation) to form a double-membraned vesicle known as autophagosome that sequesters the cargo, fusion of the autophagosome outer membrane to the lysosome to form autolysosome (vesicle completion), eventual cargo degradation by lysosomal enzymes and macromolecules recycling back into the cytosol (Figure 18) (Mizushima et al., 2010).

Figure 18. Schematic diagram of the autophagy process

Autophagy initiates upon the inhibition of mTOR to form the phagophore. Extension of the phagophore sequesters the cargo and form a complete double membrane autophagosome. The autophagosome then merges with the lysosome to form autophagosome and degrades the cargo. Each step of the process can be inhibited by pharmaceutical drug or knockdown to study the different stages of the autophagic process (Mizushima et al., 2010).

Over the past two decades, studies in the field of autophagy have expanded the discipline rapidly, and the works of many researches have helped to characterize the molecular mechanism by studying autophagy in both yeast and mammalian cells. Although both models have slightly different structures, the integration of knowledge from both models has brought about a better understanding of the process. Autophagy in both yeast and mammals is highly conserved within their morphology, protein framework and core autophagy machinery, with 36 known autophagy-related genes (Atg) identified in both models. The process is complex and requires the participating proteins to be highly coordinated within their interactions for degradation to be complete (Feng et al., 2014).

The core process of autophagy begins with the formation of autophagosomes until fusion of the sequestered cargo with the lysosome to form autolysosome; this process is carried out by the core autophagy machinery, which consists of several components: the Atg1/unc-51 like autophagy activating kinase (ULK) kinase complex, the Atg9 cycling system, the Class III PI3K complex, and two ubiquitin-like conjugation systems Atg8 and Atg12 (Figure 19) (Feng et al., 2014). These proteins are key players in the entire process and are essential for autophagosome formation. In yeast, peri-vacuolar punctate structures known as the phagophore assembly site (PAS) are constitutively present within the cytosol. They act as sites for Atg protein recruitment in a hierarchical manner where nucleation of the isolation membrane begins. In the mammalian model,

the PAS has yet to be defined, but a similar concept exists in the form of colocalized Atg proteins throughout the cytosol (Wong, 2013).

	Yeast	Mammals	Characteristics and functions
Atg1/ULK complex	Atg1	ULK1/2	Ser/Thr protein kinase; phosphorylated by M/TORC1; recruitment of Atg proteins to the PAS
	Atg13	ATG13	Regulatory subunit through phosphorylation by M/TORC1 and/or PKA, linker between Atg1 and Atg17
	Atg17	RB1CC1/FIP200 (functional homolog)	Scaffold protein, ternary complex with Atg29 and Atg31. Phosphorylated by ULK1; scaffold for ULK1/2 and ATG13
	Atg29		Ternary complex with Atg17 and Atg31
	Atg31		Ternary complex with Atg17 and Atg29
	Atg11		Scaffold protein in selective autophagy for PAS organization
		C12orf44/Atg101	Component of the complex with ATG13 and RB1CC1
Atg9 and its cycling system	Atg2	ATG2	Interacts with Atg18
	Atg9	ATG9A/B	Transmembrane protein, directs membrane to the phagophore
	Atg18	WIPI1/2	PtdIns3P-binding protein
PtdIns3K complex	Vps34	PIK3C3/VPS34	PtdIns 3-kinase
	Vps15	PIK3R4/VPS15	Ser/Thr protein kinase
	Vps30/Atg6	BECN1	Component of PtdIns3K complex I and II
	Atg14	ATG14	Component of PtdIns3K complex I
Atg8 Ubl conjugation system	Atg8	LC3A/B/C, GABARAP, GABARAPL1/2	Ubl, conjugated to PE
	Atg7	ATG7	E1-like enzyme
	Atg3	ATG3	E2-like enzyme
	Atg4	ATG4A/B/C/D	Deconjugating enzyme, cysteine proteinase
Atg12 Ubl conjugation system	Atg12	ATG12	Ubl
	Atg7	ATG7	E1-like enzyme
	Atg10	ATG10	E2-like enzyme
	Atg16	ATG16L1	Interacts with Atg5 and Atg12
	Atg5	ATG5	Conjugated by Atg12

Figure 19. The core autophagy machinery proteins

Core autophagic machinery proteins in yeast and mammalian models consisting of the Atg1/ULK complex, Atg9 cycling system, PI3K complex, Atg8 and Atg12 conjugation systems (Feng et al., 2014).

Autophagy is a downstream pathway of the mammalian target of rapamycin (mTOR) inhibition. The molecular mechanism by which autophagosomes are formed has been established, but the involvement of each core machinery protein is still being elucidated. Under nutrient-rich conditions, mTOR negatively regulates Atg1 (a serine/threonine kinase) and Atg13 by phosphorylation. Upon cellular stress, autophagy induction occurs with the inhibition of mTOR, which results in the partial

dephosphorylation of Atg1 and Atg13. Atg1 activates and subsequently binds to Atg13 and Atg17 to form the initial complex for vesicle nucleation. Atg1 also inhibits the phosphorylation and activation of the ribosomal protein S6 kinase (S6K) (He and Klionsky, 2009). In the mammalian model, the formation of the homolog ULK1/2-ATG13- RB1 inducible coiled-coil 1 (RB1CC1/FIP200)-ATG101 complex is nutrient independent. During vegetative conditions, mTORC1 phosphorylates ULK1/2 and ATG13, disrupting interactions between ULK1 and AMPK. ULK1 kinase activity is thus affected by its AMPK-dependent activation during glucose deprivation (Wong, 2013). Starvation results in the release of mTORC1 repression on the complex the activation and subsequent autophosphorylation of ULK1/2. The kinase activity of ULK1/2 is essential for the recruitment of the Class III PI3K complex and phosphorylation of other components within the core machinery, including ATG13, RB1CC1/FIP200, activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) and Beclin1 (Feng et al., 2014).

Activated ULK1 complex dissociates from mTORC1 and binds to the endoplasmic reticulum (ER). The Class III PI3K complex, consisting of Vps34, Vps15, Beclin1 and Atg14L, is activated and recruited to the ER. It converts phospholipids found on the ER membrane surface to phosphatidyl inositol-3-phosphate (PI3P), which is essential for the recruitment of the ATG12-ATG5-ATG16 complex, WD repeat domain phosphoinositide-interacting protein 1 (WIPI-1), zinc finger FYVE domain-containing protein 1 (ZFYVE1) and microtubule-associated proteins 1A/1B light chain 3 (LC3) for the elongation step. ATG12, a ubiquitin-like protein (Ubl), conjugates to ATG5 to form an isopeptide, which in turn binds ATG16 before translocating to the phagophore and complex with the Class III PI3K complex (Wong, 2013). In the presence of the all

. three complexes localized at the ER, LC3 is recruited to interact with phosphatidylethanolamine (PE) and is inserted into the phagophore during the elongation process. This lipidation process converts LC3 from one form (LC3-I) to another (LC3-II) (Mizushima et al., 2010). The phagophore extends in this elongation manner and engulfs the cytosolic region. Once the double membrane structure is merged and sealed, it forms the mature autophagosome which sequesters the cargo. The autophagosomes eventually fuse with lysosomes, the cargo is degraded by lysosomal enzymes, and macromolecules are released through permeases on the lysosomal membrane for recycling (He and Klionsky, 2009).

Studies have shown that Atg proteins may function and interact with other Atg proteins apart from the canonical pathway described, while some Atg proteins are also demonstrated involvement in non-autophagic pathways. The complexity of their relationships deters the progress of comprehending the mechanism of their activation and interactions. For example, ULK1 interaction with LC3 and its related proteins GATE16 and GABARAP was also reported, illustrating the role of ULK1 in mediating autophagy as it relates to autophagy nucleation as well as elongation (Wong, 2013). ULK1 may also potentiate its effects through both kinase-dependent and kinase-independent pathways, as shown when kinase-dead ULK1 could still interact with ATG13 and RB1CC1/FIP200 (Chan et al., 2009). Other studies have demonstrated the involvement of Atg proteins in non-autophagic roles. ULK1/2 was shown to phosphorylate enzymes involved in the glycolysis pathway, which increase glucose uptake and promote metabolism through PPP and reduction of gluconeogenesis (Li et al., 2016). Other Atgs also showed non-autophagic functions through interactions with

GTPases, Rab-GAPs and GEFs, as well as participating in antiviral immune response and exocytosis (Subramani and Malhotra, 2013)

Atg proteins can be regulated by various mechanisms, each differing by their phosphorylation sites to confer a change in activation or inhibition of the protein and its subsequent effects on autophagy. A classic example of an Atg protein regulated by several proteins is ULK1. ULK1 has more than 30 known phosphorylation sites and is hyperphosphorylated under nutrient-rich conditions (Feng et al., 2014). Starvation causes ULK1 dephosphorylation, and mTORC1 is the known master regulator of ULK1, with evidence showing that mTORC1 binds to and phosphorylates ULK1 at S757 to inhibit autophagy induction (Russell et al., 2014). On the other hand, AMPK was reported to be a key driver of autophagy during glucose deprivation. AMPK phosphorylates ULK1 at S555 to promote 14-3-3 and ULK1 binding. AMPK also activates autophagy by removing mTORC1 inhibition via phosphorylation and activation of ULK1 at S317 and S777 (Wong, 2013). The exact mechanistic role of AMPK in regulating ULK1 phosphorylation and whether this regulation promotes or inhibits autophagy is still being elucidated, but the collective results thus far indicate that numerous proteins can regulate ULK1 in different ways by influencing the various phosphorylation sites. In turn, ULK1 also demonstrated a negative feedback regulation of mTOR and AMPK to downregulate autophagy. Other studies have reported an overlap of ULK1 phosphorylation sites by mTORC1 and AMPK, indicating a well-orchestrated regulation of ULK1 activation via the mTORC1 and AMPK pathways (Kim et al., 2011a).

Apart from ULK1, mTORC1 has been demonstrated to inhibit Class III PI3K complex via phosphorylation of ATG14 (Russell et al., 2014). mTORC1 also indirectly regulates

Atg protein expression by phosphorylating the transcription factor EB (TFEB) at S211, which promotes binding to 14-3-3 proteins and is retained in the cytosol. Abolishing this effect during starvation allows TFEB translocation into the nucleus to upregulate the expression of various autophagic and lysosomal genes (Martina et al., 2012). AMPK regulates the Class III PI3K complex by phosphorylating VPS34 and Beclin1, while ATG13 is regulated by mTOR or ULK1/2 via phosphorylation of different residues, which leads to the switch between vegetative and nutrients-poor conditions (Wong, 2013). Beclin1, on the other hand, is regulated by ULK1/2 phosphorylation, as well as by Bcl-2 family proteins. Beclin1 is sequestered by Bcl-2 under nutrient-rich conditions, and phosphorylation of Beclin1 by ULK1/2 activates the Class III PI3K complex (Feng et al., 2014). Another study demonstrated that Akt mediates Beclin1 phosphorylation at S295 in an mTOR-independent manner, which regulates Beclin1/14-3-3/Vimentin complex formation and thus sequesters Beclin1 functional activity and inhibits autophagy during tumorigenesis (Wang et al., 2012). Overall, the regulation of autophagic activation is more complex than the canonical pathway known reported to date, and elucidating these regulatory pathways is critical to fully elucidate the autophagic process.

In recent years, autophagy has been implicated in tumorigenesis. Studies have shown contradictory roles of autophagy, including in both tumor promotion and suppression. Autophagy was first suggested to suppress tumorigenesis when knocking down core autophagic machinery proteins in mouse models resulted in autophagic deficiency and different types of cancer development. Deletion of Beclin1 is common within human breast, ovarian and prostate cancers. Homozygous knockout of Beclin1 in mouse models results in embryonic lethality, while heterozygous knockout mice develop a

spectrum of tumorigenesis phenotypes, including B cell lymphoma, lung adenocarcinoma and HCC (Qu et al., 2003; Yue et al., 2003). Bif-1 knockout mice showed increased incidence of lymphoma and solid tumor development compared to that of wild-type mice (Takahashi et al., 2007). Speculation of tumor development in these two mouse models resulting from the loss of autophagy is debatable because there may be nonautophagic involvement by Beclin1 and Bif-1. However, another study using an Atg5^{-/-} mosaic model provided substantial evidence supporting the potential of autophagy as a tumor suppressor. In these mosaic mice, HCC tumors only developed from Atg5^{-/-} liver cells but not from their neighboring Atg5^{+/+} liver cells (Takamura et al., 2011). Others also provided evidence that autophagy-specific genes such as Atg2B, Atg5 and Atg9B exhibit frameshift mutations in some human gastric and colorectal cancers (Lebovitz et al., 2012). Knockdown of autophagy-associated genes in cell lines also resulted in an increase in chromosomal instability, DNA damage, cellular oxidative stress and cellular transformation (Degenhardt et al., 2006).

Collectively, these findings provide the basis that autophagy functions as a tumor-suppressing mechanism because autophagy is involved in the regulation of cellular integrity in addition to the removal of damaged organelles and the reduction in cellular stress. Compromised autophagy in cells leads to an inability to survive nutrient-poor conditions, the buildup of ER stress and ROS, an increase in chromosomal and cellular damage and the eventual activation of apoptosis. Further evidence supporting the tumor-suppressive role of autophagy is the positive regulation of the process by tumor suppressor genes such as p53, PTEN and tuberous sclerosis 1/2 (TSC1/2), which function to inhibit mTOR, while oncogenic proteins such as mTOR/PI3K/AKT negatively regulate it (Balaburski et al., 2010; Jin, 2005).

Interestingly, autophagy may also be utilized by cancer cells to promote their survival and thus has a tumor-promoting potential. Cancer cells often endure harsh nutrient-deprived conditions, and genomic instability results in a buildup of cellular stresses. Autophagy can be used as a coping mechanism to overcome these obstacles and increase the chances of survival. In Ras-expressing cells, autophagy is required to survive in nutrient-poor environments and facilitate Ras tumor growth, suggesting the role of autophagy in promoting tumorigenesis and the reliance on this pathway to evade metabolic stress and sustain growth (Guo et al., 2011). Another group showed that tumor cells that induced autophagy promotes their survival in p53-induced apoptosis (Amaravadi et al., 2007). Autophagy can also act as a protector for tumor cells in cancer therapies and inhibition of autophagy in combination with chemotherapy induces apoptosis in colon cancer cells (Li et al., 2009).

The reason for the apparent contradictory roles of autophagy is not fully understood, but studies thus far have supported the dual function of autophagy in both suppressing and promoting tumorigenesis. It is not surprising that upon initial tumor development, autophagy forms a checkpoint to detect and suppress cellular transformation. Upon overcoming all the inhibitions, autophagy is then necessary for established tumors to thrive. The dual role of autophagy makes it a complex and difficult therapeutic target because the outcome may be dependent on the cells' current stage of tumorigenesis.

Emerging evidence in recent years has described dysregulation of the autophagic process during EMT. Some studies have shown that autophagy negates the migration and invasion capabilities during EMT by downregulating Snail and Slug expression and concurrently upregulating N-cadherin and R-cadherin in glioblastoma cells. Furthermore, inhibition of autophagy induces EMT and metastasis in gastric cancer

cells by preventing Snail degradation (Catalano et al., 2015). Others have shown that downregulation of autophagy allows ubiquitin-binding protein p62/sequestosome-1 (p62/SQSTM1) to accumulate and stabilize Twist1, which promotes cell proliferation and migration (Gugnoni et al., 2016; Qiang and He, 2014).

In contrast, there are multiple groups that support the theory that autophagy promotes EMT and metastasis (Akalay et al., 2013; Li et al., 2013; Lv et al., 2012; Zhu et al., 2014). The authors showed that inhibiting autophagy prevents nasopharyngeal carcinoma cells from transitioning into a mesenchymal-like phenotype and increases cisplatin efficacy in these cells. Su *et al.* demonstrated that autophagy and EMT are both essential for cancer cells to progress and develop drug resistance (Su et al., 2017). These conflicting data suggest that, similar to autophagy's dual role in cancer progression, autophagy may have dual functions in cancer metastasis. Cancer cells undergoing metastasis may require autophagy as a readily available pool of building blocks to sustain rapid proliferation and the acquisition of mesenchymal-like features, as well as a cell protective signal to suppress metastatic changes.

Another compelling point is that studies on autophagy and metastasis thus far are loosely defined within each cancer model, with a lack of mechanistic clarification of how autophagy is regulated in a spatial and temporal manner. This gap in knowledge might be the key to understanding the roles of autophagy during metastasis and how it may function to either affect tumor malignancy or metastasis, or confer drug resistance. Hence, more work is required to elucidate the roles of the autophagy pathway in cancer progression before it can be a potential target for cancer intervention.

4.2 Results

4.2.1 Autophagic Markers were Enriched During EMT

Multiple studies have demonstrated the involvement of autophagy in cancer migration and metastasis with conflicting results. Metastasis is a complex multi-step process with EMT being an early stage event which cancer cells acquire their migratory capabilities. Thus, we first examine the expression of autophagic genes in our three established *in vitro* EMT models. MKN74 cells were exposed to either hypoxic condition or TGF- β 1, while MKN74^{Snai1ER} cells were treated with 4-OHT to induce EMT. Upon EMT induction, MKN74 cells and MKN74^{Snai1ER} cell colonies progressed into a mesenchymal-like phenotype, across all three models. We validated the EMT induction by quantification of EMT markers expression with quantitative PCR and immunoblotting (Figure 5A-C).

Next, we examined the gene expression profile of genes enriched across the three *in vitro* EMT models using Partek Genomics Suite. We observed an enrichment of multiple gene subsets involved in fatty acid, glucose and ROS metabolisms, and autophagy (Figure 20A, left panel). Further analysis revealed an enrichment of multiple autophagic core machinery genes, such as ULK1, SQSTM1, MAP1LC3B, BECN1, ATG9B, ATG4C, ATG14, ATG13, as well as TSC1 (Figure 20A, right panel). Notably, we observed further increased in the expression of these genes at Day 2 post EMT induction when compared to Day 1. These data suggest that cancer cells undergoing EMT displayed metabolic flexibility, and that autophagy is involved in this process.

To verify this finding, we examined the expression of LC3 by immunoblotting. LC3 is a widely accepted marker of autophagy and is the only known marker to exist on

autophagosomes (Mizushima and Yoshimori, 2007; Mizushima et al., 2010). It is expressed early upon autophagy induction as it is converted from LC3-I to LC3-II during the elongation process of phagophore formation. This conversion allows us to differentiate a functional autophagic process and eliminate a potential build-up of autophagic markers due to a defective autophagic pathway. We observed an increase in the expression of LC3-II in all three models of EMT, accompanied by an increase in Beclin1, Atg 5 and SQSTM1 (Figure 20B-D). Our finding confirmed an increase in expression of multiple autophagic core machinery proteins and an increase in autophagic flux, which is positively regulated when cancer cells undergo EMT induction.

Figure 20. Autophagic markers enriched during EMT

(A) Heatmap showing gene expression data across three *in vitro* EMT models that are involved in glycolysis, autophagy, fatty acid metabolism and ROS production (GSE71280), with fold enrichment for autophagy related genes displayed on right. (B-D) Relative fold change in mRNA levels of autophagy markers (LC3, Beclin1, ATG5, SQSTM1) (bottom panel) and immunoblot analysis of LC3, Beclin1 and ATG5 (top panel) in (B) MKN74_{Snai1ER} treated with 4-OHT, as well as MKN74 cells exposed to (C) hypoxia, (D) TGF- β 1 at indicated time points. (E-F) (E) Immunoblot analysis of LC3 and cANGPTL4 and (F) relative fold change in mRNA levels of autophagy markers (LC3, Beclin1, ATG5, SQSTM1) and cANGPTL4 of MKN74 cells treated with si-ctrl or si-cANGPTL4 and exposed to hypoxia at indicated time points. (G) Relative fold change in mRNA levels of autophagy markers (LC3, Beclin1, ATG5, SQSTM1) of MKN74 cells treated with rh-ANGPTL4 at indicated time point. TBP was used a housekeeping gene for real-time PCR. β -tubulin protein was used a loading and transfer controls for immunoblot analysis. Data are represented as mean $\pm$ s.d. from at least three independent experiments. Loading controls for the immunoblot analyses were from the same sample.

4.2.2 ANGPTL4 Regulates Autophagy During EMT

In our previous chapter, we demonstrated that tumor progression is accompanied by a concomitant increase in cANGPTL4 expression. Similarly, ANGPTL4 expression is also augmented upon EMT induction. We further elucidated that ANGPTL4 plays a critical role in coordinating cellular bioenergetics and regulating metabolism in cancer cells during EMT. As such, we question if ANGPTL4 can regulate the autophagic pathway during EMT.

MKN74 cells were transfected with either scrambled control siRNA (si-ctrl) or siRNA against ANGPTL4 (si-cANGPTL4) prior exposure to hypoxic condition for 48h. An increase in LC3-I and LC3-II expression accompanied by an increase LC3-II conversion was observed in MKN74 cells transfected with si-ctrl, indicating an increase in autophagic flux, while a slight decrease in LC3-II conversion was noted in ANGPTL4-deficient MKN74 (Figure 20E). This finding was further supported by an increase in LC3, Beclin1, ATG5 and SQSTM1 expressions in the MKN74si-ctrl cells after 48 h as evidenced by quantitative PCR. This increase was attenuated in MKN74 cells treated with si-cANGPTL4 (Figure 20F). MKN74 cellsexposed to rh-cANGPTL4 showed an increase of LC3, Beclin1, ATG5 and SQSTM1 expression after 6 h (Figure

20G). Taken together, these observations suggest that ANGPTL4 is involved in the regulation of autophagy during EMT.

4.2.3 ANGPTL4 regulates Autophagy in vivo During Tumor Development

To further strengthen our above findings, we examined the effects of ANGPTL4 in the regulation of autophagy *in vivo* during tumor development. Six weeks old NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice were injected subcutaneously with 1×10^6 MKN74_{Snai1ER:shANGPTL4} cells on both flank of the mouse. Xenografts were allowed to grow for a week until palpable tumors were formed on each flank. Next, the mice were randomly divided into four groups. Two groups were injected intraperitoneally (i.p.) with 4-OHT (4 mg/kg) and fed on chow (4-OHT:Chow) or dox diet (4-OHT:Dox) to induce expression of shRNA against ANGPTL4, while the other two groups were injected with vehicle and fed on chow (Veh:Chow) or dox diet (Veh:Dox). After eight weeks, xenograft tumors were harvested for further analysis (Figure 21A).

Tumor tissues were sectioned and stained for autophagic markers. Regardless of the diet, tumor-bearing mice injected with vehicle, i.e. Veh:Chow and Veh:Dox, showed diffused basal LC3 and Beclin1 staining. Tumors from mice on 4-OHT:Chow regime showed an increase in LC3 staining with punctate formation. Notable, a significant decrease in LC3 staining with lesser punctate formation and diffused basal staining were observed in tumors from mice on 4-OHT:Dox treatment (Figure 21B).

Altogether, these observations indicate that autophagy occurred during EMT *in vitro* and *in vivo*. The deficiency in ANGPTL4 reduces autophagy. Our observation also suggest that, in addition to ANGPTL4, other ANGPTL4-independent pathways may contribute to the increase in autophagic levels during *in vivo* tumor development.

Figure 21. *ANGPTL4* regulates LC3 expression in vivo During Tumor Development

(A) A schematic illustration of metastatic xenograft model. MKN74_{Snai1ER:shANGPTL4} cells were injected to form primary xenograft tumors (n=6 per experimental subgroup, two tumors per mouse). Metastasis

was induced by 4-OHT injections. Mice were fed with either normal chow diet (4-OHT:chow) or Dox-containing diet (4-OHT:dox; 625 mg/kg). Primary xenograft tumors was measured with a pair of vernier calipers with tumor volume calculated by $(\text{Length} \times \text{Width} \times \text{Width})/2$ (right panel). **(B)** Immunofluorescence staining for LC3 (green) of veh:chow, 4-OHT:chow and 4-OHT:dox primary xenograft tumors. The nuclei were counterstained with DAPI (blue). Scale bar: 100 μm (top panel).

4.2.4 ANGPTL4 Induces Autophagy Through MYC, STAT3, STAT1, DDIT3, HIF1 α , FOS, JUN and ID3

ANGPTL4 plays a critical role in cellular metabolism and adenylate charge. We next demonstrated a concomitant increase in expression of autophagic markers and ANGPTL4 during EMT. Guided by these observations, we query how ANGPTL4 alone contributes to the increased autophagy. To this end, MKN74 cells were treated with rh-cANGPTL4 and subjected to an unbiased kinase inhibitor array screening to identify key signaling pathways. MKN74 cells that showed an attenuation in cANGPTL4-mediated upregulated signaling by the kinase inhibitor would indicate the involvement of those specific kinases in mediating cANGPTL4 regulation of autophagy. Cells treated with SNS-314, PIK-90, GCI1746, Lapatinib, BMS-540215, GDC-0941, Rapamycin, XL388, PD173955-Analog1, Quizartinib, GDC0032, SB-216763 and Bosutinib, resulted in a suppression of ANGPTL4-mediated upregulation of autophagic genes expression (Figure 22A). Ingenuity Pathway Analysis suggested the potential involvement of MYC, STAT3, STAT1, DDIT3, HIF1 α , FOS, JUN and ID3 transcription regulators in the signaling cascades by which ANGPTL4 regulates autophagy (Figure 22B).

Figure 22. ANGPTL4 Induces Autophagy Through MYC, STAT3, STAT1, DDIT3, HIF1 α , FOS, JUN and ID3

(A) Relative mRNA level of LC3, Beclin1 and SQSTM1 after treatment with various kinase inhibitors from a Kinase Library (#Synkinase-SYN-2103). Values denotes mean fold change in LC3, Beclin1 and SQSTM1 expression from n=3 independent experiment. **(B)** Ingenuity Pathway Analysis (IPA) identified MYC, STAT3, STAT1, DDIT3, HIF1 α , FOS, JUN and ID3 as potential transcriptional factors that regulate the expression of multiple autophagy genes when stimulated with recombinant cANGPTL4 protein. IPA-assisted pathways were mapped following experimental kinase inhibitor screen.

4.3 Summary

The role of autophagy in tumor development and metastasis remains controversial. Autophagy is a catabolic process that mediates the degradation of non-essential or dysfunctional cellular components. On one hand, the selective degradation of specific EMT proteins seems to be the major molecular mechanism through which autophagy controls EMT (Galluzzi et al., 2015; Qiang and He, 2014). Other studies also showed that autophagy limits the accumulation of autophagy cargo adapter p62/SQSTM1, and indirectly inhibits EMT (Bertrand et al., 2015; Lock et al., 2014). On the other hand, autophagy activation is also used as a cellular strategy to overcome intracellular or microenvironmental stress, including nutrient deprivation, hypoxia and drug effects (Kim et al., 2016). Increased autophagy was also linked to a more mesenchymal stem-like phenotype (Galavotti et al., 2013). Currently, it is not clear to what extent EMT is dependent on autophagy, and thus whether EMT and autophagy act in sequence or in parallel to promote tumor cell invasiveness. We proposed that autophagy may be utilized by cancer cells during EMT as an approach to confer metabolic reprogramming.

In this study, we have demonstrated through our comparative gene analysis that autophagic genes are enriched in all three models of *in vitro* EMT. We also showed an increase in autophagy levels and autophagic flux through the increase of LC3 expression and conversion. These data lend support to a pro-metastatic role for autophagy. Our preliminary data showed that ANGPTL4, a metastasis-related gene, is involved in the upregulation of LC3 expression and conversion, as well as various autophagic core machinery proteins. Silencing of ANGPTL4 decreases this effect similarly in both *in vivo* and *in vitro* although to a lesser extent in the former.

Autophagy induction requires the assembly of basal autophagic proteins to form the autophagosome. Transcriptional expression of autophagic proteins upon induction is a coordinated effort by multiple transcriptional regulators. As shown in our kinase inhibitor screen, multiple signaling pathways are involved in the upregulation of autophagic genes in the presence of cANGPTL4, and these data are consistent with other studies in which upregulation of autophagy is a response to meet cellular needs. This supports the idea that autophagy may be required by cancer cells undergoing EMT and contribute to the high energy-demanding process. As such, our data provides a strong evidence that ANGPTL4 promotes transcriptional regulation of autophagic genes, with MYC as a central coordinated role. Further validation of these preliminary data are required to elucidate the mechanism in which ANGPTL4 utilize to coordinates autophagy. Initial unbiased kinase inhibitor screen revealed the possible involvement of several transcriptional regulators such as MYC, STAT3, STAT1, DDIT3, HIF1 α , FOS, JUN and ID3.

Considering the centralized role of MYC in regulation of proliferative and pro-survival signaling, it was of great interest that IPA of our data showed that most signaling cascades converges on MYC, identifying it as key player. Incidentally, MYC was also showed in our previous chapter to play an important in multidrug resistance via the transcriptional up-regulation of ABC transporter. However, a role for MYC in autophagy is unclear. One group showed that MYC inhibits leukemia cells differentiation through prevention of intracellular ROS build-up and subsequent downregulation of the autophagy pathway (Wu et al., 2018).

Other studies showed that MYC can induce autophagy as a pro-survival mechanism for cancer cells. One group showed that MYC induces autophagy through activation of

unfolded protein response (UPR) to promote their survival from apoptosis (Hart et al., 2012). MYC-dependent JNK1 expression was also demonstrated to induce autophagy through a mTOR-independent pathway by phosphorylation of BCL2 and decreasing BCL2-Beclin1 interaction, resulting in an increase in Beclin1 availability to activate Vps34 and initiate the signaling cascade for autophagosome formation (Toh et al., 2013). Overexpression of MYC or Ras in cancer cells also showed upregulation BNIP3 and LC3 expression through increase activation of AMPK and forkhead box O3 (FOXO3) (Gargini et al., 2016). Interestingly, one study showed that autophagy facilitates cellular proliferation by AMBRA1 mediated dephosphorylation and degradation of MYC (Cianfanelli et al., 2015). Thus far, the potential of ANGPTL4 to increase autophagy seems to indicate the involvement of a MYC-mediated mechanism.

STAT3 is another well-established transcriptional regulator that have dual pro-autophagy and anti-autophagy capacities, regulating autophagy-related genes and proteins differentially. Nuclear STAT3 transcriptionally regulates an array of autophagy-related genes, including BCL2 protein family, LC3, BECN1, PIK3C3, PIK3R1, HIF1A and BNIP3, while cytosolic STAT3 inhibit autophagy by sequestering E1F2AK2, leading to activation of ATG4. STAT3 also acts as a negative regulator of autophagy via its repressive effect on other molecular effectors such as MIR17HG, FOXO1 and FOXO3. (Qin et al., 2015; You et al., 2015). Conversely, STAT3 promotes autophagy during hypoxic condition through upregulation of HIF-1, which leads to activation of BNIP3 (and BNIP3L), that disrupts BCL2-Beclin1 complex through displacement, releasing Beclin1 for autophagy induction (You et al., 2015). IL-6-induced activation of STAT3 promotes expression of EMT markers such as Snai1, Slug and Twist1, but concurrently inhibit the autophagy pathway through sequestering of

Beclin1 in the cytosol and upregulating the negative regulators of autophagy such as BCL2, BCL2L1 and MCL1 (Gugnoni et al., 2016). Interestingly, STAT3-induced EMT was only recently proposed, with upregulation of Twist1 expression as a positive feedback loop to further enhance EMT by promoting IL-6 production and STAT3 activation (Wendt et al., 2014). Thus, the role of STAT3 on autophagy may be context-dependent, and further elucidation of the involvement in STAT3 in autophagy during EMT will provide valuable insights.

The other transcriptional regulators downstream of ANGPTL4 have also been shown in multiple studies to regulate autophagy. STAT1 is a known ULK-1 transcriptional repressor, while Jun inhibits autophagy and potentiates cell death through apoptosis (Goldberg et al., 2017). On the contrary, HIF-1, DDIT3 and FOS are positive regulators of autophagy. FOS binds to the BECN1 promoter region and induce Beclin1 expression, while DDIT3 is known to function through the ATF4-DDIT3-TRIB3-AKT-MTOR axis to induce autophagy (Wang et al., 2015)

Clearly, further experiments will be necessary to winnow the key functional transcriptional regulators, particularly in the relevant microenvironmental stress that stimulates EMT.

CHAPTER 5:

DISCUSSION AND FUTURE DIRECTIONS

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

5.1 Discussion

ANGPTL4 is secreted by tumor cells and its expression level increases with advancing malignancy, especially in invasive disease, suggesting a potential role of ANGPTL4 in tumor progression and invasion. In our previous studies, we identified ANGPTL4 as a key molecular driver that supports cancer development through multiple prosurvival pathways. ANGPTL4 confers anoikis resistance in tumors through an elevated intracellular $O_2(-):H_2O_2$ ratio, which in turn activates the PI3K/PKB α and ERK pathways (Zhu et al., 2011). ANGPTL4 was first demonstrated to promote cancer cell metastasis through the modulation of vascular integrity. ANGPTL4 exerts its prometastatic influence via interaction with integrin $\alpha 5\beta 1$, which triggers Rac1/PAK signaling to disrupt cell-to-cell contacts. ANGPTL4 further promotes endothelial disruption through declustering of VE-cadherin and claudin-5 association, and the combined effects of these functions induced vascular leakiness and facilitated metastasis (Huang et al., 2011).

A mutation harboring a single nucleotide polymorphism (SNP) in cANGPTL4 (T266M cANGPTL4 variant) displayed diminished prometastatic effects owing to reduced binding affinity of ANGPTL4 to integrin $\alpha 5\beta 1$, which manifested as decreased PI3K/PKB α and ERK pathway signaling, increased susceptibility to anoikis, and restored glucose uptake, Glut2 expression and cANGPTL4-mediated increases in adenylate energy charge to normal levels (Tan et al., 2017).

ANGPTL4 also enhances metastasis by coordinating the energy demands required for EMT via PI3K/PKB α and MAPK-regulated increases in 14-3-3 γ expression and

subsequent interactions with TSC2 and Snai1. 14-3-3 γ interacts with Snai1 in the presence of ANGPTL4, which further supports this EMT event by stabilizing Snai1 binding to the E-cadherin promoter and directly represses E-cadherin expression. The ANGPTL4-induced 14-3-3 γ interaction with TSC2 during EMT regulates the energy flux required for the process. This ANGPTL4:14-3-3 γ signaling axis modulates adenylate energy charge and increases the cellular bioenergetics required during EMT (Teo et al., 2017b).

Emerging studies have associated EMT with increased drug resistance, with limited evidence illustrating the underlying mechanism of how both processes are correlated (Housman et al., 2014; Shibue and Weinberg, 2017). Metastatic cancer cells exhibiting MDR heighten the challenge for therapeutic intervention. One of the major methods by which cancer cells acquire MDR traits is through an increase in ABC transporter expression and activity, which enhances a broad efflux of a variety of drugs from cells. In our first study, we wanted to illuminate the definitive relationship between ABC transporter-mediated MDR and EMT. We demonstrated elevated expression and activities of multiple ABC transporters during EMT in our *in vitro* models.

Thus far, little has been shown on the association of ANGPTL4 with the MDR phenotype in cancer. Our previous study identified ANGPTL4 as a key player in coordinating cellular bioenergetics and enhancing adenylate energy charge during EMT. To further support this notion, we demonstrated that cANGPTL4 modulates glucose metabolism and fatty acid β -oxidation to elevate cellular ATP levels during EMT. As ABC transporters are active efflux pumps that require ATP to drive their enzymatic activity, we question whether an ANGPTL4-mediated increase in cellular ATP supports ABC transporter efflux capacity by providing the energy required for the

process. Indeed, we found that suppression of ANGPTL4 *in vitro* and *in vivo* reduced cisplatin efflux by ABC transporters. This suggests that ANGPTL4 plays a crucial role in fostering drug resistance during EMT by providing the ATP necessary to drive ABC transporter efflux.

The enhancement of ABC transporter efflux activities alone is not sufficient to reduce drug concentrations within cancer cells to confer resistance. Increase in ABC transporter expression and activity are required to confer MDR. Interestingly, we observed an increase in ABCB1 expression accompanied by elevated ANGPTL4 expression, which correlated with tumor progression. We investigated whether ANGPTL4 modulates the expression of ABC transporters. Our study showed that the presence of cANGPTL4 can induce an increase in ABC transporter expression. The transcriptional regulation of ABCB1 has been extensively studied, and multiple TFs, such as NK- κ B and p53, directly regulate its expression (Gottesman and Pastan, 2015; Robey et al., 2018). We established c-Myc and NK- κ B as transcriptional regulators of ANGPTL4-induced ABC transporter expression and identified their respective binding sites on 7 of the ABC transporter promoters. Further experiments to illustrate c-Myc- and NK- κ B-induced expression of ABC transporters will better clarify this observation. While we did not analyze all the expression patterns and activities of ABC transporters, we observed that the ANGPTL4-mediated increase in adenylate energy charge potentially supports the efflux capacity of other ABC transporters through ATP. Future studies should include the examination of other ABC transporter expression and activities, and substantiating this claim would provide evidence regarding the extent of the role of ANGPTL4 in drug resistance.

In summary, we demonstrated that ANGPTL4 contributes to the metabolic flexibility of metastatic cancer cells through glucose and lipid metabolism to enhance cellular ATP levels in order to sustain energy-demanding processes such as ABC transporter efflux during EMT. This observation is in line with emerging studies illustrating metabolic reprogramming during EMT by altering expression of metabolic enzymes such as Snail mediated repression of phosphofructokinase (PFKP) which directs glucose metabolism towards the PPP, increases glucose uptake and lactate production, and alters amino acid profiles, mitochondrial activities, TCA cycle flux and OXPHOS (Kim et al., 2017; Vander Heiden and DeBerardinis, 2017). Our gene expression analysis showed changes in ROS metabolism, autophagy, glucose metabolism, amino acid levels and lipid metabolism, suggesting that a global increase in metabolic reprogramming caters to the increased demand of cellular processes during EMT. As we have shown the impact of increased ANGPTL4 on glucose and lipid metabolism in our previous studies, we next question the potential role of ANGPTL4 on the autophagic pathway during EMT.

It is conceivable that cancer cells will require a rapid strategy to generate ATP and macromolecules to sustain elevated anabolic activities during EMT. Anabolic processes demand a large pool of readily available macromolecules. We propose that autophagy is a prime pathway by which cancer cells can depend on the rapid recycling of macromolecules to support anabolism during EMT. In our second study, we examined the expression of key autophagic markers during EMT. We first identified enrichment of autophagic markers in all our EMT models through further analysis of our gene expression array. We further supported this finding by showing an increase in key autophagic markers and autophagic flux as demonstrated by LC3 conversion. This

finding supports the notion that autophagy is upregulated in our EMT models. We examined whether this increase in autophagy is correlated with the previously observed increase in ANGPTL4 expression.

To date, only a few studies have demonstrated a link between ANGPTL4 and autophagy. One group demonstrated an increase in ANGPTL4 expression in the presence of MSL-7, while another group showed ANGPTL4-mediated LPL degradation by the autophagy-lysosomal pathway (Dijk et al., 2016; Lim et al., 2018). These observations are associated with nANGPTL4 and its function as a known lipoprotein lipase inhibitor. Little is known thus far in the association between cANGPTL4 and autophagy. We examined the expression of autophagic markers and autophagic flux and noted a reduction in both *in vitro* and *in vivo* models in the absence of ANGPTL4. We verified these observations with a kinase inhibitor screen under cANGPTL4 stimulation and found that autophagy is induced by cANGPTL4 through various transcriptional regulators, including MYC, STAT3, STAT1, DDIT3, HIF1 α , FOS, JUN and ID3. Autophagy is a complex pathway orchestrated by an array of proteins that form interactions in a highly coordinated manner. To date, studies have shown the regulation of autophagic gene expression by multiple TFs upon autophagy induction. Our study suggests that, indeed, the increase in autophagy during EMT is under the influence of cANGPTL4 through multiple pathways.

Our study thus far only focused on a few key proteins of each component within the autophagic core machinery and does not fully represent the whole pathway. Future studies should include in-depth studies of the autophagy initiation machinery such as the ULK1 complex, incorporate autophagic inhibitors to determine how attenuation of autophagy affects EMT, and elucidate the underlying mechanism of this potential

ANGPTL4-mediated effect and the prometastatic role of autophagy in cancer cells undergoing EMT. Overall, our preliminary study suggests that ANGPTL4 may serve as a major factor in inducing autophagy during EMT.

5.2 Future Directions

Our current study has unveiled new roles for ANGPTL4 during the early events of metastasis. ANGPTL4 promotes multidrug resistance through the transcriptional activation of multiple ABC transporter genes, and enhances cellular energy to fuel their activities. In addition, ANGPTL4 mitigates the oxidative pernicious side effect of an enhanced metabolism by modulating autophagy during EMT.

The role of ANGPTL4 in lipid metabolism is well-characterized via nANGPTL4 binding and inhibition of LPL activities, as well as impeding serum TG clearance, which results in elevated serum TG levels. Emerging studies also implicated a role for ANGPTL4 in glucose metabolism, albeit with conflicting trends. Some groups showed that the suppression of ANGPTL4 in mice improved glucose tolerance, lower blood glucose and increase insulin levels, while others reported the opposite trend (Aryal et al., 2018; Janssen et al., 2018; Wang et al., 2016; Xu et al., 2005). Much less is known about the role of ANGPTL4 in amino acid metabolism. Studies from two groups illustrated a potential role for ANGPTL4 in augmenting enzymatic activities in glucose and amino acid metabolism. Xu *et al.* demonstrated that the overexpression of ANGPTL4 leads to inhibition of PEPCK and G6Pase, both are which essential in gluconeogenesis (Xu et al., 2005). The second group introduced full-length ANGPTL4 into C57BLK6 and db/db mice and performed extensive proteome-profiling on metabolic enzymes in vivo. Multiple metabolic enzymes involved in the amino acid, glycogen metabolism and glucose metabolic pathways were altered. Some of the key

enzymes altered are 3-hydroxyanthranilate 3,4-dioxygenase, phenylalanine hydroxylase, 2-oxoisovalerate dehydrogenase, phosphoglucomutase, fructose-1,6-bisphosphatase, ornithine aminotransferase, ketohexokinase and pyruvate kinase. They also showed that mice treated with ANGPTL4 resulted in enhanced activity of mitochondria respiratory chain complexes II-II and IV (Wang et al., 2007). These findings suggest that ANGPTL4 may have similar capacities in modulating metabolic enzymes and amino acid metabolism during cancer progression.

In summary, ANGPTL4 is a multifaceted protein that has dynamic functions, depending on the progression of the pathologies. Clearly, further mechanistic insights on ANGPTL4-mediated regulation of glucose and amino acid metabolism will be necessary to realize the biological repertoire of ANGPTL4 in cancer progression.

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