THE ROLES OF ANGIOPOIETIN-LIKE 4 IN CANCER METASTASIS

TESI LI ANG

SCHOOL OF BIOLOGICAL SCIENCES

2015
The roles of angiopoietin-like 4 in cancer metastasis.

TEO ZI QIANG

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

2015
ACKNOWLEDGEMENTS

I wish to express my heartfelt gratitude and appreciation to my supervisor, Dr. Tan Nguan Soon Andrew, for his patient mentoring and guidance. His vast knowledge and creative concepts has helped me tremendously to complete what I have set out to achieve. Additionally, his drive and enthusiasm is a constant motivation for me to carry on my research endeavor.

I would also like to thank all my laboratory buddies who made this journey possible. Special thanks go out to Mr Tan Ming Jie and Mr. Chong Han Chung Kelvin for coordinating the laboratory and for their wonderful friendship and guidance. Mr Sng Ming Keat, my fellow PhD candidate, for the wonderful support and friendship you provided me throughout the 4 years. Dr Zhu Pengcheng for his advices and ideas on the experiments; Dr Tan Chek Kun William, Dr Ivan Lam, Dr Carol Tan and all my fellow lab mates for their wonderful support, motivation and the laughers we shared in lab to make it such a conducive environment to work in.

I would like to thank the examiners, Dr Ding Jeak Ling, Dr Stephane Mandard and Dr Tan Suet Mien for making time out of their busy schedules to review my thesis.

I also owe my deepest gratitude to my family and my girlfriend. Without their encouragements and patience, I would not have been able to complete this journey.

Last but not least, I would also like to thank Nanyang Technological University for awarding me the Nanyang President’s Graduate Scholarship to carry out my PhD studies.
# TABLE OF CONTENTS

Abbreviations ..........................................................................................................................6
Publication list ..........................................................................................................................7
Summary ....................................................................................................................................8
Introduction ..............................................................................................................................9

1.1 Cancer overview ...............................................................................................................9
1.1.1 Enabling characteristics .................................................................................................10
1.1.1.1 Genomic instability and mutations ................................................................................10
1.1.1.2 Tumor-promoting inflammation ..................................................................................10
1.1.2 Hallmarks of cancer .........................................................................................................11
1.1.2.1 Sustaining proliferative signaling ..............................................................................11
1.1.2.2 Evading growth suppressors .....................................................................................13
1.1.2.3 Resisting cell death ....................................................................................................14
1.1.2.4 Enabling replicative immortality .............................................................................15
1.1.2.5 Inducing angiogenesis ...............................................................................................16
1.1.2.6 Activating metastasis and invasion .........................................................................17
1.1.3 Emerging hallmarks .........................................................................................................18
1.1.3.1 Evading immune destruction .....................................................................................19

1.2 Cancer metastasis ...........................................................................................................21
1.2.1 Tumor-stroma communication .......................................................................................23
1.2.2 Epithelial to mesenchymal Transition (EMT) in cancer progression ............................27
1.2.2.1 Molecular mechanisms involved in EMT ....................................................................29
1.2.2.2 Hypoxia-induced EMT ...............................................................................................31
1.2.2.3 TGF-β-induced EMT .................................................................................................32

1.3 Cancer metabolism .........................................................................................................35
1.3.1 Warburg effect ..............................................................................................................35
1.3.2 Metabolic plasticity ......................................................................................................37
1.3.3 Modulators of reprogrammed cancer metabolism ..........................................................40
1.3.3.1 MYC ..........................................................................................................................40
In vivo EMT model ............................................................................................................... 87
Chromatin-immunoprecipitation (ChIP) and Re-ChIP. .......................................................... 87
Immunofluorescence staining ............................................................................................... 88
Proximity Ligation Assay (PLA) and analysis ....................................................................... 89
Immunoprecipitation and immunoblot ................................................................................... 89
ATP quantification .................................................................................................................. 90
Energy charge determination ................................................................................................. 90
Glucose uptake assay ............................................................................................................ 91
Metabolic analysis .................................................................................................................. 91
Kinase Inhibitor Array: RNA Isolation, RT-PCR and Real time qPCR ............................... 91
Real-time PCR Primers .......................................................................................................... 92
Statistical Analysis ............................................................................................................... 93
Results ...................................................................................................................................... 94
Generation of in vitro EMT models ..................................................................................... 94
EMT induction alters cancer cell bioenergetics ..................................................................... 95
ANGPTL4 augments EMT ...................................................................................................... 97
ANGPTL4 modulates EMT in vivo ....................................................................................... 100
ANGPTL4 regulates cellular bioenergetics during EMT ....................................................... 104
ANGPTL4 is a molecular driver of EMT metabolic changes ................................................. 107
ANGPTL4 modulates the expression of specific 14-3-3 subtypes ......................................... 110
ANGPTL4:14-3-3γ axis coordinates energy demand during EMT ...................................... 113
The ANGPTL4:14-3-3γ:Snail axis coordinates EMT ............................................................. 115
Mechanistic overview of ANGPTL4:14-3-3 axis coordinating cancer cellular bioenergetics and EMT .................................................................................................................................. 117
Discussion ................................................................................................................................ 119
Chapter 2 .................................................................................................................................. 124
Abstract .................................................................................................................................... 125
Introduction .............................................................................................................................. 126
Materials and methods .......................................................................................................... 128
Antibodies ............................................................................................................................ 128
Human Tumor Samples ...................................................................................................... 128
Cell Cultures ....................................................................................................................... 129
Transient transfections assay ........................................................................................ 129
Suppression of ANGPTL4 by RNA Interference (RNAi) .............................................. 129
Expression and purification of recombinant cANGPTL4 proteins ................................ 130
In Vivo Tumorigenicity and Miles Vascular Permeability Assay .................................... 130
In Vivo Metastasis Assay .................................................................................................. 131
Transendothelial Electrical Resistance (TER) measurement .......................................... 131
Internalization assay ......................................................................................................... 132
Surface Plasmon Resonance (SPR) .................................................................................. 132
Immunofluorescence staining ............................................................................................ 132
Proximity Ligation Assay (PLA) and analysis .................................................................. 133
Protein extraction, immunoprecipitation and immunoblot .............................................. 134
FACS analysis .................................................................................................................... 135
Real-time PCR Primers ..................................................................................................... 135
Statistical Analysis .......................................................................................................... 136

Results ................................................................................................................................. 137

cANGPTL4 is elevated in human tumors ........................................................................ 137
Metastatic tumors has enhanced expression of cANGPTL4 .......................................... 139
cANGPTL4 disrupt endothelial junction integrity ............................................................ 140
cANGPTL4 compromise tumor vasculature in vivo ......................................................... 143
cANGPTL4 interacts with integrin α5β1, VE-cadherin and claudin-5 ............................. 145
cANGPTL4 interacts with the respective junction proteins in in vivo tumor ................. 146
cANGPTL4 modulate vascular integrity temporally ....................................................... 149
cANGPTL4 triggers internalization of integrin α5β1, VE-cadherin and claudin-5 ......... 151
Inhibition of cANGPTL4: integrin α5β1 complex formation delays interaction of cANGPTL4 with VE-Cadherin, claudin-5 and vascular disruption ................. 153
cANGPTL4 instigates vascular disruption via activation of integrin-Rac/PAK signaling axis ............................................................................................................................................. 156
Formation of cANGPTL4:proteins complexes trigger nuclear translocation of β-catenin
..............................................................................................................................................158
cANGPTL4 enhanced metastasis to lungs in vivo ..........................................................160
Mechanistic overview of tumor-secreted cANGPTL4-induced EC junction disruption
..............................................................................................................................................163
Discussion...............................................................................................................................165
Conclusion and Future Direction ......................................................................................170
References..............................................................................................................................173
Abbreviations

4-OHT  4-hydroxytamoxifen
AMPK  AMP-activated protein kinase
ANGPTL4  Angiopoietin-like protein 4
ANG  Angiopoietin
ATP  Adenosine triphosphate
AJ  Adheren Junction
BSA  Bovine serum albumin
CAF  Cancer-associated Fibroblast
CDH1  E-cadherin
ChIP  Chromatin immunoprecipitation
COX2  Cyclooxygenase-2
cANGPTL4  Carboxyl-terminal ANGPTL4
DAPI  4', 6-diamidino-2-phenylindole
DDR1  Discoidin domain receptor 1
EC  Endothelial cells
EMT  Epithelial-mesenchymal transition
ErBb3  Epidermal growth factor receptor 3
fANGPTL4  Full-length ANGPTL4
FACS  Fluorescence activated cell sorter
FBS  Fetal bovine serum
HIF-1α  Hypoxia-induced factor 1 alpha
HRP  Horseradish peroxidase
HMVEC  Human microvascular endothelial cell
IP  Immunoprecipitation
JAM  Junction adhesion molecule
PAK  p21-activated Kinase
PBS  Phosphate buffered saline
PGE₂  Prostaglandin E₂
PK3K  Phosphatidylinositol (4, 5) kinase
PLA  Proximity Ligation Assay
PPAR  Peroxisome proliferator-activated receptors
nANGPTL4  Amino-terminal ANGPTL4
NGS  Normal goat serum
SDS  Sodium dodecyl sulfate
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
SPR  Surface Plasmon Resonance
TGF-β  Transforming growth factor-β
TER  Transendothelial electrical resistance
TJ  Tight Junction
VEC  Vascular-endothelial Cadherin
VEGF  Vascular endothelial growth factor
ZO-1  Zona occludens-1

Page 6 of 218  Confidential  Teo Zi Qiang
Publication list


Conference Abstract


Summary

Metastasis, the spread of cancer cells to distant tissues and/or organs, accounts for more than 90% of the mortality rate associated with cancer rate. Cancer metastasis is a complex, multistep event that began with the cancer cells undergoing EMT to gain motility and invasive capacity, leading to its invasion into the systemic circulation and subsequent dissemination to distal organs. Recently, tumor-secreted Angiopoietin-like 4 (ANGPTL4) was identified as a critical pro-metastatic gene in several cancers. However, the role of ANGPTL4 during metastasis is discordant due to the lack of mechanistic understanding. Our present work highlights dynamic roles of ANGPTL4 during malignant progression, particularly by coordinating EMT and the dissemination of metastatic cancer cells. We observed a striking correlation between ANGPTL4 expressions with tumor grades, suggesting that ANGPTL4 may have important roles during malignant progression. Indeed, we demonstrated that cancer cells are able to exploit ANGPTL4 at multiple stages of malignancy. We showed that cancer cells use ANGPTL4 to manipulate cancer cellular metabolic changes during EMT and synchronizes a metabolic shift necessary to drive EMT initiation through an ANGPTL4:14-3-3 signaling axis. Furthermore, we also demonstrated that tumor-derived ANGPTL4 behaves as a pro-vascular permeability factor where it mediates the vascular disruptive effect through a novel integrin α5β1-induced Rac/PAK signaling axis. These result in the declustering and internalization of endothelial cell-cell junctional proteins that disrupts the vascular integrity and enhances metastasis. Taken together, our findings revealed critical roles for ANGPTL4 during metastatic progression and provided new insights for therapeutic intervention against cancer metastasis.
Introduction

1.1 Cancer overview

Cancer is the leading cause of death worldwide, accounting for more than 10% or 7.6 million deaths yearly (World Health Organization). It is now clear that cancer is no longer a singular disease but a collective syndrome characterized by six unique cancer hallmarks: sustained proliferative signaling, evasion of growth suppressors, replicative immortality, resistance to cell death, induction of angiogenesis, and initiation of invasion and metastasis (Hanahan and Weinberg, 2011; Figure 1).

Figure 1. Hallmarks of cancer. The six unique hallmarks of cancer 1) sustaining proliferative signaling; 2) evading growth suppressors; 3) enabling replicative immortality; 4) resisting cell death; 5) inducing angiogenesis; 6) activating invasion and metastasis. These hallmarks allow the cancer cells to proliferate uncontrollably and to survive under adverse conditions at the expense of the host tissues.
1.1.1 Enabling characteristics

1.1.1.1 Genomic instability and mutations
Cancer development is perplexing and multifactorial. The presence of stringent genomic surveillance and repair system ensures that the rates of spontaneous genomic mutations remain low in each cell generation (Barnes and Lindahl, 2004). When the integrity of the genome is compromised, the system activates repair mechanisms to correct the errors. Importantly, surveillance systems detect and intercept mutagens before they damage the cellular genome (Sigal and Rotter, 2000; Negrini et al., 2010). Pre-malignant cells may sensitize themselves to mutagenic agents to increase the probability of mutations that inactivate critical genes governing the genomic integrity. They may even activate epigenetic repression of these genes to potentiate tumorigenesis (Negrini et al., 2010). Because of these indispensable attributes, inactivation of this system during tumorigenesis enhances the underlying genetic instability to fuel genetic diversity and drive the acquisition of cancer hallmarks. These events facilitate the clonal selection of certain mutant phenotypes that confer selective advantages to pre-malignant cells, which allows them to dominate the local microenvironment and eventually leads to tumor formation (Salk et al., 2010).

1.1.1.2 Tumor-promoting inflammation
The immune system is responsible for protecting the body against foreign or innate invaders where it actively seek and destroy rouge cells that compromised normal tissue homeostasis. Tumors consistently demonstrate varying degree of immune cell infiltration, which suggests that the immune system is actively engaged in eliminating cancerous cells.
Coupled with the cancer hallmark of evading immune surveillance, these observations suggest an anti-tumoral function of the immune system.

Unfortunately, the inflammatory response generated by tumor-infiltrating immune cells drives neoplastic transformation (Grivennikov et al., 2010). Emerging evidence has demonstrated that inflammation induces the release of bioactive molecules that promote tumorigenesis into the tumor microenvironment (Qian and Pollard, 2010; DeNardo et al., 2010). These molecules include growth factors that sustain proliferative signaling, survival factors, pro-angiogenic molecules and matrix-modifying enzymes that may enhance metastasis and invasion (Qian and Pollard, 2010; DeNardo et al., 2010). More importantly, immune cells release large amounts of reactive oxygen species (ROS) to kill cancer cells. However, ROS are also highly mutagenic and can be hijacked by the surrounding cancer cells to augment the mutability of the cancer phenotype (Colotta et al., 2009).

1.1.2 Hallmarks of cancer

1.1.2.1 Sustaining proliferative signaling

During tissue homeostasis, normal cells are able to fine-tune the production and release of growth-inducing signals to undergo controlled proliferation and apoptosis for self-renewal and repair. These growth factors appear to be temporally and spatially regulated, eliciting an effect on neighboring cells in a paracrine manner. The growth factors bind to and activate their cognate cell surface tyrosine kinase receptors to trigger downstream intracellular signaling cascades for regulating cell cycle progression, survival and metabolism. Traditionally, tumor-suppressor genes and proto-oncogenes encode the
components of various signal transduction pathways that control the proliferative capabilities of a cell. However, cancer cells deregulate these intrinsic controls to augment tumorigenesis (Deshpande et al., 2005). Cancer cells are mostly self-sufficient and control their own proliferative destiny. They can synthesize abundant growth factors that activate cognate receptors to sustain proliferative signals (Witsch et al., 2010; Perona, 2006). Alternatively, cancer cells can upregulate the expression of growth factor receptors to increase sensitivity to otherwise limiting growth factors in the tumor microenvironment. Importantly, somatic mutations of proto-oncogenes and their downstream signaling molecules result in a constitutively active cascade that sustains the proliferative potential of cancer cells (Witsch et al., 2010; Perona, 2006). Mutations of the catalytic subunit of phosphoinositide 3-kinase (PI3K) are frequently found in a multitude of cancers, resulting in chronic stimulation of AKT/PKB to hyperactivate proliferative signal transduction (Jiang and Liu, 2009; Yuan and Cantley, 2008). Furthermore, complementary mutations that inactivate tumor suppressor genes that function as negative regulators of proliferation enhance the proliferative phenotype. Loss-of-function of PTEN, a negative regulator of the PI3K signaling cascade, is frequently observed in human tumors. PTEN inactivation prevents the degradation of phosphatidylinositol (3,4,5) trisphosphate (PIP₃), which is a downstream signaling product of PI3K. This event further augments the PI3K/AKT signaling cascade, leading to uncontrolled proliferation (Jiang and Liu, 2009; Yuan and Cantley, 2008).
1.1.2.2 Evading growth suppressors

The chronic activations of growth-promoting signals are insufficient to tip the balance and direct cancer cells into full proliferative mode. Instead, these cancer cells must also bypass growth suppressors that are normally tumor suppressors to override the negative proliferative signals. Two archetypal tumor suppressor genes, retinoblastoma RB and TP53, acts as central controllers to direct cellular fates towards proliferation or programmed cell death (Burkhart and Sage, 2008; Sherr and McCormick, 2002). Whereas RB integrates extracellular growth signals to control proliferation, TP53 acts as an intracellular sensor to maintain genomic integrity and growth potential. In response to severe stresses and/or genomic mutations in normal cells, RB and TP53 can halt the cell cycle progression to repair the damages or trigger apoptosis in order to remove these “altered” cells. In cancer cells, mutations to these critical regulatory genes affect normal homeostasis, resulting in uncontrolled cell proliferation and attenuation of apoptosis and ultimately lead to the formation of a tumor (Hoeijmakers, 2001).

Besides RB and TP53, the formations of tight epithelial junctions are also powerful suppressor of uncontrolled cell growth. “Contact inhibition” is an in vitro phenomenon observed in normal cell propagation, where the formation of a confluent monolayer results in the suppression of cellular proliferation. Yet, such growth inhibitory controls are lost on cancer cells cultured in vitro, suggesting that this mode of growth inhibition could be functionally relevant in vivo to maintain normal tissue homeostasis. The neurofibromatosis type 2 (NF2) tumor suppressor gene, merlin, and liver kinase B1 (LKB1) are implicated in the regulation of contact inhibition (Hezel and Bardeesy, 2008;
Okada et al., 2005; Curto et al., 2007). Upon formation of inter-cellular contacts, merlin stabilizes the inter-cellular epithelial-cadherin (E-cadherin) adhesion and drives the coupling of epidermal growth factor receptor (EGFR) to E-cadherin to suppressor mitogenic signaling, thus executing the contact-dependent suppression of proliferation (Curto et al., 2007). LKB1 functions in similar ways, dictating epithelial cell polarity and integrity (Hezel and Bardeesy, 2008). Importantly, LKB1 has been reported to override the powerful proliferative signals of c-myc, which further substantiated the fact that chronic growth-promoting signals are insufficient to drive full neoplastic proliferation (Partanen et al., 2009). Furthermore, the loss of merlin and LKB1 is associated with several malignancies, which further emphasized the importance of such regulation of proliferation.

1.1.2.3 Resisting cell death

As previously mentioned, when a cell sustains irreversible DNA damage or is exposed to severe cellular stress, tumor suppressors can trigger programmed cell death (apoptosis) to remove the abnormal cell. This phenomenon acts as a natural barrier to deter cancer formation and maintain tissue homeostasis (Lowe et al., 2004; Adams and Cory, 2007). Under normal circumstances, apoptosis is triggered through 2 distinct pathways: the extrinsic pathway, which integrates extracellular pro-apoptotic signaling (FAS ligand/FAS receptor), and the intrinsic pathway, which senses intracellular apoptotic signaling. In this respect, the intrinsic apoptotic pathway is more relevant during tumorigenesis. In the case of severe DNA damage or cellular stress, the p53 protein can upregulate the expression of the pro-apoptotic mediators Bax and Bak, which permeabilize the mitochondrial...
membrane, resulting in the release of a potent caspase stimulator, cytochrome c, into the cytosol (Junttila and Evan, 2009). This process triggers a caspase cascade to induce apoptosis. Cancer cells can circumvent apoptosis through a variety of strategies. As highlighted, genomic instability results in the loss of tumor suppressor functions, in this case, p53, which removes the 'gatekeeper' of intrinsic apoptotic signaling. Similarly, cancer cells may upregulate anti-apoptotic molecules or downregulate pro-apoptotic molecules to resist an apoptotic cell fate and ensure cancer propagation.

1.1.2.4 Enabling replicative immortality

For cancer cells to form a macroscopic tumor, they must develop limitless replicative potential. Under normal cellular homeostasis conditions, cells have limited cellular division cycles that are dictated by the presence of chromosomal end-capping telomeres. The telomeres protect the chromosomal ends and shorten progressively as the cell undergoes successive cell divisions (Blasco, 2005; Artandi and DePinho, 2010). The loss of telomeres results in chromosomal end-to-end fusions, which destabilize the karyotype and signal cell destruction directing normal cells into crisis (Hansel et al., 2006; Kawai et al., 2007). The enzyme telomerase, which helps to maintain telomere length, is absent in normal cells, but is surprisingly overexpressed in the majority of human cancers (Artandi and DePinho, 2010). Interestingly, most neoplastic cells undergo telomere shortening due to their highly proliferative nature and are destroyed before they can contribute to the generation of a macroscopic tumor (Hansel et al., 2006; Kawai et al., 2007). Thus, neoplastic cells that acquire telomerase activity have a selective advantage and obtain
resistance against cellular crisis and apoptosis, resulting in the acquisition of an unlimited replicative capability.

However, obtaining telomerase activity may not be the sole contributor to replicative immortality. The temporal gain of telomerase function is also relevant. As mentioned above (see 1.1.1.1), the initial loss of telomeres can fuel the genomic instability necessary to accumulate cancer-promoting mutations, by activating oncogenes and/or inactivating tumor suppressors to further augment the cancer phenotype (Raynaud et al., 2010). The subsequent gain of telomerase activity can stabilize the mutant genome to potentiate cancer development. Furthermore, emerging studies have highlighted a multitude of functions for telomerase. Telomerase reverse transcriptase (TERT), a subunit of telomerase, has been found to augment the Wnt signaling pathway (Park et al., 2009), promote cellular proliferation and resistance to apoptosis (Kang et al., 2004) and participate in DNA damage repair (Masutomi et al., 2005). These findings suggest that the contribution of telomerase to tumorigenesis could be greater than only maintaining telomeric length.

1.1.2.5 Inducing angiogenesis

Normal tissues are well vascularized to ensure a constant supply of nutrients and the exchange of metabolic waste to maintain normal homeostasis. The body vasculature stops developing and remains largely quiescent except during wound healing, when transient pro-angiogenic signals are activated. However, in cancer, the angiogenesis program is constitutively activated, with constant neovascularization of the tumors, presumably to
supply nutrients and remove metabolic waste to cope with the exponential growth of the neoplastic outgrowths (Baeriswyl and Christofori, 2009; Bergers and Benjamin, 2003).

These angiogenic signals are largely dependent on the nature of the tumor and the surrounding microenvironment (Baeriswyl and Christofori, 2009). Although the underlying mechanisms may vary, they converge on common inductive signals, such as vascular endothelial growth factor (VEGF), angiopoietins (ANGs) and, more recently, angiopoietin-like proteins (ANGPTLs) (Chong et al., 2014; Carmeliet, 2005; Ferrara, 2009). VEGF and ANGs bind to the endothelial cell surface receptors VEGFR and TIE, respectively, to induce angiogenic sprouting. The cognate receptors of ANGPTLs are unknown, and emerging studies suggest a controversial context-dependent role of ANGPTLs during angiogenesis (see 1.5.6.2.2). Interestingly, the tumor angiogenesis program is aberrant, unlike during wound repair, where angiogenesis is tightly controlled. This aberrant program results in irregular vascular sproutings, erratic blood flow and leaky vasculatures that are thought to contribute to the metastatic potential of several cancers (Nagy et al., 2010).

1.1.2.6 Activating metastasis and invasion

The majorities of human cancers are derived from epithelial origins and are characterized by major epithelial features, such as stable epithelial cell-cell junctions and robust cell-matrix adhesion. When these epithelial tumors transit into higher malignancy states with local invasion and distal metastasis, they often transit into a more migratory mesenchymal-like phenotype (Steeg and Theodorescu, 2008; Chiang and Massagué, 2008). The developmental epithelial-to-mesenchymal transition (EMT) program has been
strongly implicated as the main cause of the enhanced invasiveness of epithelial cancer cells when initiating metastasis (See 1.2.2). Recent studies have suggested that EMT can be co-opted by a series of transcriptional programs spearheaded by EMT transcription factors (EMT-TFs) Snail, Slug, Twist and ZEB 1/2 (Klymkowsky and Savagner, 2009; Micalizzi et al., 2010). Cancer cells at the invasive edge of tumors are often mesenchymal-like compared to those within the main tumor body, suggesting that the cross-talk between cancer cells and their corresponding microenvironmental signals may serve as a temporal and spatial guide for the induction of EMT (Hlubek et al., 2007).

Consequently, as cancer cells lose cell-cell contacts, they can intravasate across the leaky tumor vasculature, which is induced by the aberrant tumor angiogenesis program (see 1.1.2.5) for distal dissemination. Fortunately, not all metastatic cancer cells survive dissemination to reach a secondary site for colonization. Even then, those that survived and managed to gain entry into a secondary site may not develop into a macroscopic tumor. Interestingly, those cancer cells that are able to establish a secondary niche may display an inherent plasticity to revert back into the epithelial phenotype to ensure successful colonization, completing the metastatic objective (Hugo et al., 2007).

1.1.3 Emerging hallmarks

The enabling characteristics empower the acquisition of the six distinct cancer hallmarks by cancer cells to enhance their survival, proliferative and metastatic capacities. However, emerging evidence has suggested other functionally distinct qualities that are important for neoplastic developments. Evasion of the body’s immune system and the reprogramming of cancer cellular metabolism are two such distinct traits that may have...
significant implications in the development and progression of cancer, which will be discussed below.

1.1.3.1 Evading immune destruction

The immune system provides a relentless surveillance structure to protect the body against foreign infectious agents and even rogue cells, thus eliminating the potential for incipient neoplastic outgrowth. Therefore, the presence of solid tumors in the body suggests that neoplastic cells have adopted evasive mechanisms to prevent immunological destruction. Similarly, immunocompromised individuals appear to have higher incidences of spontaneous cancers, and immunosuppressed organ transplant recipients have been observed to inherit cancer from the donor (Vajdic and van Leeuwen, 2009; Strauss and Thomas, 2010). These observations suggest that the immune system plays critical roles in suppressing and/or eliminating these spontaneous neoplasms. Tumors that have been infiltrated by killer immune cells, such as cytotoxic T cells or natural killer cells, have a better prognosis (Pagès et al., 2010); conversely, tumors secrete immune-suppressive transforming growth factor-β (TGF-β) (Yang et al., 2010) or recruit regulatory T cells to directly suppress the localized immune response (Mougiakakos et al., 2010). However, given recent advancements in tumor immunology, the mechanisms employed by tumors to evade immune destruction remain unclear.

1.1.3.2 Altered cancer metabolism

The highly proliferative nature of cancer cells undoubtedly impacts their cellular metabolism. The accompanying alterations to the metabolism of proliferative cancer cells have been considered a secondary effect to support the growth and proliferative capability
of cancer cells. However, recent evidence suggests otherwise. Key cancer-promoting pathways have been found to converge to reprogram cancer cell metabolism to support the anabolic requirements of proliferating cancer cells (Cairns et al., 2011). Moreover, many of these metabolic alterations are indispensable for malignant transformation. Recent studies have positioned reprogrammed energy metabolism as an emerging cancer hallmark that extends beyond the sustenance of cancer cell growth and proliferation, even conferring a cytoprotective mechanism via redox scavenging and immunity against microenvironmental insults (Schafer et al., 2009; Vaughn and Deshmukh, 2008; Vander Heiden et al., 2009; Figure 2). These novel adaptations that result from metabolic reprogramming are important and pervasive traits in many cancers and are fundamental to the development and progression of the neoplastic state (Cairns et al., 2011; Hanahan and Weinberg, 2011).

![Diagram of Altered Metabolic Phenotype](image-url)
Figure 2. Determinants of cancer cells metabolism. The metabolic phenotypes of the cancer cells are governed by both intrinsic genetic mutations and the extrinsic microenvironmental conditions. The highly proliferative nature of cancer cells meant that the cancer cells metabolism will have to be rewired to cope with the increased demands for energy, macromolecular biosynthesis and maintenance of the redox status within cancer cells. These adaptations are further enhanced through the response to the surrounding microenvironmental conditions to promote tumorigenesis.

1.2 Cancer metastasis

Metastasis, the spread of cancer cells to distant tissues and/or organs, accounts for more than 90% of the mortality rate associated with cancer rate (Jemal et al., 2011). Metastasis is a multi-step process with the following stages: 1) primary tumor cells undergo EMT to gain motility and invasiveness; 2) these cells may breach the tumor basement membrane to trigger local invasion and subsequently disrupt adjacent endothelial cell-cell contacts to initiate distal metastasis; 3) subsequent transmigration of cancer cells across the endothelial cells of proximal blood vessels (intravasation) provides entry into systemic circulation (Figure 3C); 4) once in the circulation, these tumor emboli evade immune recognition and resist anoikis (programmed cell death due to loss of anchorage) (Figure 3D); 5) these emboli adhere or become lodge in the capillary network of target organs before transmigrating across the endothelium (extravasate) into the surrounding stroma (Figure 3E); and 6) after extravasation, metastatic cancer cells adapt to the new environment and proliferate to establish micro-metastases, ultimately leading to the formation of a secondary tumor (Steeg and Theodorescu, 2008; Chiang and Massagué, 2008; Figure 3F). Notably, EMT and the intra- and extravasation steps are considered the rate-limiting steps that determine the final outcome of metastasis (Orr et al., 2000). These processes require intricate interactions between cancer cells and its tumor.
microenvironment, ultimately resulting in disruption of the endothelial barrier (Mendoza and Khanna, 2009).

Figure 3. Pathogenesis of metastasis. (A) Genetic alteration results in a population of cells gaining the ability to proliferate infinitely, giving rise to the primary tumor; (B) Further proliferation of the tumor mass requires increase nutrients leading to the secretion of pro-angiogenic factors to augment tumor angiogenesis; (C) Tumor progress to invasive stage, group of cancerous cells detach from the primary tumor and invade the surrounding stroma; (D) Eventually the metastatic cells transmigrate across the endothelium of the blood vessels and gain entry into the circulation where they are transported to distant sites before they arrest in the target organs; (E) The metastatic cells that adhered onto the blood vessel then extravasate across the endothelium into the new site; (F) Here, they establish a new metastatic niche for growth of the secondary tumor before events in (B), completing the process of metastasis. Reprinted by permission from Macmillan Publishers Ltd: The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited, copyright 2003
1.2.1 Tumor-stroma communication

Tumors are collections of heterogeneous cell populations that consist of the proliferating cancer cells (parenchyme) and its surrounding stroma that makes up the dynamic tumor microenvironment (Hanahan and Weinberg, 2011). Cancer-associated fibroblasts (CAFs), tumor-infiltrating immune cells, and endothelial cells form the bulk of the reactive tumor microenvironment and emerging evidence suggest that the heterogeneity and interdependency of these cells critically influenced tumor growth and progression (Hanahan and Weinberg, 2011; Figure 4). Intrinsically, the normal stroma components possess an inherent plasticity to elicit rapid adaptive responses to compromised epithelial compartments, for example during wound healing. These adaptive responses are tightly coordinated and self-limiting to preserve tissue integrity and homeostasis (Gabbiani, 2003; Desmoulière et al., 2005). However, these intrinsic controls are lost during neoplastic development and the reactive nature of the stroma is exploited to drive malignant progression (Rowley, 1998). The long-term progression of malignancy suggests that the cancer cells can co-opt and “educate” stromal components to assemble a pro-neoplastic niche, which can in turn enhance the neoplastic potential of the surrounding pre-neoplastic cells (Pollard, 2004; Hanahan and Weinberg, 2011). In order to establish reciprocal cell-cell and cell-matrix communications, the cancer cells and its stromal components must communicate via paracrine signaling crosstalk through growth factors, cytokines and chemokines. These back-and-forth communications can therefore co-evolve the cancer cells and its accompanying microenvironment to dictate multiple cancer hallmarks ultimately resulting in the activation of invasion and metastasis. Conversely, antagonism
of the contributions of the tumor stroma has been shown to hinder cancer progression (Tlsty and Hein, 2001).

The reactive nature of the stromal cells surrounding the pre-neoplastic lesions meant that tumor-infiltrating lymphocytes (TILs) will be recruited to the compromised site to restore normal tissue homeostasis. These TILs produce pro-inflammatory mediators such as tumor necrosis factors (TNF-α), TGF-β and other cytokines to target and destroy tumor cells (Grivennikov et al., 2010). However, cancer cells can exploit these responses to generate a chronic inflammatory environment that promotes tumor growth and progression (Fridlender et al., 2009; de Visser et al., 2006; see section 1.1.1.2). Furthermore, immune and cancer cells also produce pro-angiogenic effectors, such as vascular endothelial growth factor (VEGF) and angiopoietin-like 4 (ANGPTL4), that are hijacked to expand the tumor vasculature in order to support tumor growth and lay the foundation for malignant progression (Bergers and Song, 2005; Hanahan and Weinberg, 2011; see section 1.1.2.5 and 1.1.2.6; Figure 4). Indeed, ANGPTL4 is known to be a pro-metastatic gene that has been shown to augment vascular disruption to facilitate metastatic dissemination of cancer cells, which further underscore the importance of the tumor-stromal interactions (see section 1.5.6; Figure 4).

Other than the TILs, CAFs form the major population in the tumor stroma. They are known to influence cancer cell behavior through a series of growth factors, cytokines and chemokines that enhance the proliferative potential and survival of cancer cells (Hanahan and Weinberg, 2011). CAF-derived epidermal growth factor binds to its cognate receptors on tumor cells to activate downstream Src/ERK and Stat 3 signaling to promote tumor
growth through the transcription of the c-myc gene (Jaganathan et al., 2011; refer to section 1.1.2.1). Moreover, the increasing cross-talk between the cancer cells and its stroma enhance TGF-β activation with detrimental outcomes during malignant progression. The increased TGF-β activation in epithelial carcinoma enhanced their metastatic potential, primarily through the activation of EMT (Mima et al., 2013; Giampieri et al., 2009; Figure 4). Importantly, pro-metastatic matrix-metalloproteinases (MMPs) and TGF-β target genes such as CXCL12 and ANGPTL4 were also found to be elevated in the accompanying CAFs, further highlighting the interdependency of the different cell types in a tumor (Calon et al., 2012; Hawinkels et al., 2014; Navab et al., 2011; Lazennec and Richmond, 2010). Additionally, CAFs have also been shown to provide key metabolic support to cancer cells in order to sustain anabolic growth and potentially facilitate malignant transformation (see section 1.3.2; Figure 4). Clearly, these intricate and complex signaling events between the tumor and other cell types require a better understanding to decipher their biological relationships in tumorigenesis and to develop effective therapies against malignant progression.

In recent years, research investigating cancer cell interactions with the microenvironment has revealed key roles for a new class of proteins known as matricellular proteins, which play multiple roles in tumor progression (Chong et al., 2012; Chiodoni et al., 2010; Bornstein, 2009; Murphy-Ullrich, 2001). Matricellular proteins are a group of extracellular matrix (ECM)-associated glycoproteins that are secreted by tumors and neighboring stromal cells into the extracellular environment (Chiodoni et al., 2010). These proteins modulate cell-matrix interactions and cell functions by interacting with
membrane receptors, proteases, hormones, and other bioeffector molecules, as well as structural matrix proteins such as collagen and vitronectin (Bornstein, 2009). Recent studies have implicated several members of the matricellular protein family in tumor progression, such as osteopontin, secreted proteins that are acidic and rich in cysteine and ANGPTL4 (Goh et al., 2010; Goh et al., 2010; Chong et al., 2012; Chiodoni et al., 2010). In particular, the past decade of research has revealed numerous roles for ANGPTL4 under both physiological and pathological conditions. Originally classified as an adipokine due its predominant role in lipid metabolism, new functions of ANGPTL4 in wound repair, angiogenesis, redox regulation and tumorigenesis have been described (Zhu et al., 2012; Figure 4). Importantly, both hypoxia and TGF-β, which are well-known inducers of EMT, can potently enhance the expression of ANGPTL4 (see section 1.5.2). Therefore, it is tempting to hypothesize that ANGPTL4 may have prominent roles during EMT.
Figure 4. Tumor-stroma communications. The heterogeneous and interdependent cellular populations in the primary tumor form the reactive stroma microenvironment that critically influences tumor growth and progression. These stroma cells are “co-opted” and nurture via paracrine signaling communications with the cancer cells to direct malignant progression. The major population of cancer-associated fibroblasts (CAFs) supplies the cancer cells with high energy substrates like ketone bodies and pyruvates to sustain cancer cells metabolic requirements. Additionally, CAFs have also been shown to promote cancer cells proliferation and EMT progression through EGF and TGF-β respectively. Incidentally, TGF-β and TNF-α produced by the tumor-infiltrating macrophages (TILs) also stimulates cancer cells production of pro-angiogenic VEGF and ANGPTL4 to trigger pathogenic angiogenesis. Furthermore, ANGPTL4 enhances vascular leakiness which potentiate the intravasation of the metastatic cancer cells into the systemic circulation.

1.2.2 Epithelial to mesenchymal Transition (EMT) in cancer progression

Most of the tissues in the human body are epithelial in nature, embedded in a mesenchyme of stromal cells, such as fibroblasts, and endothelial cells that support the...
normal physiological functioning of the system. Epithelial cells are adjoined by
specialized cell-cell junctions that not only maintain the three-dimensional organization of
the tissues but also specify the apical-basolateral polarity of the cells, which is important
for many critical physiological events (Huang et al., 2012). Epithelial cadherin (E-
cadherin, CDH1) is the major determinant of epithelial cell-cell junctions. E-cadherin is
characterized by five extracellular repeats, a transmembrane domain and a cytoplasmic
tail. The extracellular domains are responsible for homophilic interactions with adjacent
E-cadherin on neighboring cells to establish epithelial cell-cell junctions. The
establishment of cellular junctions allows the cytoplasmic tail of E-cadherin to associate
with α- and β-catenin and p120 and tethers the cell junctions to the actin cytoskeleton,
thus stabilizing epithelial integrity (Takeichi, 1990). However, it is noteworthy that E-
cadherin serves not only structural roles but also has important regulatory functions
(Kovacs et al., 2002).

Because most cancers arise from an epithelial origin, the majority tend to manifest an
epithelial phenotype. Thus, it is believed that when epithelial tumor cells undergo
malignant transformation and acquire the ability to metastasize, these epithelial tumor
cells lose cell-cell contacts between adjacent cells and transit into a mesenchymal state,
gaining invasiveness and motility (Klymkowsky and Savagner, 2009; Figure 5). This
observed transformation process highly resembles the EMT, which defines a fundamental
yet paradigmatic model of cellular plasticity that was first identified during
embryogenesis (Savagner, 2001; Goh et al., 2010). The exposure of epithelial cells to
various EMT effector signals (growth factors, cytokines, ROS, and hypoxia, among others)
results in a time-dependent ‘kinetic’ event. The cells must first lose their characteristic cell-cell adhesion structures and transit into a mesenchymal state, accompanied by remodeling of their cytoskeleton from keratin- to vimentin-type intermediate filaments and ultimately gaining motility (Valdés et al., 2002; Stockinger et al., 2001; Figure 5). Accumulating studies of clinical tumors and animal models have provided convincing evidence of the relevance of EMT to metastasis. In particular, many signaling pathways that are primarily involved in embryonic EMT are reactivated during cancer metastasis (Berx et al., 2007; Moustakas and Heldin, 2007). Furthermore, EMT is associated with poor patient prognosis and is involved in promoting tumor invasion, metastasis and therapeutic resistance.

![Figure 5. Schematic overview of EMT.](image)

1.2.2.1 Molecular mechanisms involved in EMT

The initiation of the transition process is an extrinsic event that involves the integration of extracellular EMT effector signals and the modulation of an extensive repertoire of intracellular signaling networks, transcription factors and gene expression levels (Kalluri and Weinberg, 2009; Savagner, 2001). Importantly, the majority of the extracellular

Teo Zi Qiang Confidential Page 29 of 218
signal-induced signaling cascades converge at similar end points: 1) the repression of E-cadherin and 2) the regulation of EMT-related genes such as Snai1, Snai2 and ZEB1 (Cannito et al., 2010; Figure 6). The distinct hallmark of EMT is undoubtedly the down-regulation of E-cadherin, which results in the dissolution of epithelial cell junctions (Thiery and Sleeman, 2006; Kalluri and Weinberg, 2009; Savagner, 2001; Figure 6). Notably, the suppression of E-cadherin is sufficient to elicit a complete EMT event, and many late-stage aggressive cancers display a conditional loss of E-cadherin expression (Perl et al., 1998). Conversely, re-activation of E-cadherin expression can suppress invasion and metastatic dissemination and promote the opposite process, the mesenchymal-to-epithelial transition (MET; Xu et al., 2009).

Most of the EMT-inducing signaling cascade converges at the activation of the zinc finger transcription factor Snai1, which plays a pivotal role in the suppression of the E-cadherin gene during EMT (Cano et al., 2000; Batlle et al., 2000). As a transcription factor, Snai1 repress E-cadherin gene expression through at least 2 mechanisms: 1) recruitment of histone deacetylases and corepressors to modify the chromatin structures (Peinado et al., 2004; Figure 6) and 2) interaction with AJUBA and 14-3-3 to form a stable ternary complex that binds to the E-cadherin promoter, thus silencing gene expression (Hou et al., 2010; Figure 6). Importantly, the overexpression of Snai1 alone pushes epithelial cells into a mesenchymal state with concurrent down-regulation of E-cadherin expression (Cano et al., 2000). Likewise, Snai1 can also stimulate the expression of other mesenchymal genes. In addition to Snai1, other transcription factors, such as Snai2, Twist...
and ZEB-1, are known to repress E-cadherin expression and are relevant to EMT during cancer progression (Bolós et al., 2003; Batlle et al., 2000).

Importantly, the histological heterogeneity of carcinomas suggests that only a selected population of cells undergo EMT (Moustakas and Heldin, 2007). This is supported by studies of colorectal carcinomas in which active canonical Wnt signaling was only observed in tumor cells at the invasive front edge (Brabletz et al., 2005). Indeed, one should not expect to observe collective changes to all mesenchymal and epithelial markers concomitantly. In fact, the cell types involved, effector signals and overall prevailing microenvironmental conditions will influence the spectrum of EMT modifications.

1.2.2.2 Hypoxia-induced EMT

Hypoxia is emerging as one of the most relevant physiological conditions capable of inducing EMT (Gort et al., 2008; Hill et al., 2009). Normal tissues are often perfused in an oxygenated environment with an oxygen partial pressure (pO2) that exceeds 20 mmHg (Semenza, 2003). Hypoxia arises when the tissue oxygenation state falls below 10 mmHg. This phenomenon triggers activation of the oxygen-sensitive family of transcription factors called hypoxia-inducible factors (HIFs) to mediate the hypoxic response (Semenza, 2003). HIFs are a class of bHLH transcription factors that consist of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit that heterodimerizes and binds to hypoxia-response elements (HRE) on DNA to regulate the expression of several target genes (Wang et al., 1995). During embryogenesis, hypoxic niches are often found throughout the embryo to maintain the persistence of stemness, control cellular differentiation and determine cellular fate (Simon and Keith, 2008). In the context of
cancer, it is well established that hypoxia is a recurring characteristic of solid malignant
tumors that are heterogeneously distributed within the neoplastic mass (Dewhirst et al.,
2008). Increasing clinical and experimental evidence suggests that hypoxia exerts positive
selective pressure to enhance the aggressiveness of cancer cells, augmenting their invasive
and metastatic potentials (Vaupel and Mayer, 2007; Rankin and Giaccia, 2008; Semenza,
2003). Remarkably, hypoxia has been shown to directly induce EMT through modulation
of the EMT genes Snail1 and Twist and the subsequent suppression of E-cadherin
expression (Figure 6). This finding elucidates a self-sustaining positive feedback loop that
begins with the induction of Snail1 and represses E-cadherin expression. This process
leads to the dissolution of epithelial cell-cell junctions and the liberation of membrane-
bound β-catenins into the nuclei to trigger the expression of more EMT-related genes,
further stabilizing the EMT phenotype.

1.2.2.3 TGF-β-induced EMT

Transforming growth factor-β (TGF-β) is one of the most effective inducers of EMT
during embryogenesis, wound healing, fibrotic diseases and metastasis (Zavadil and
Böttinger, 2005; Cannito et al., 2010). During oncogenesis, TGF-β signaling is critically
involved in cancer cell proliferation, migration and resistance to apoptosis (Elliott and
Blobe, 2005). Recent studies have also suggested a role for TGF-β in the regulation of the
cancer stem cell phenotype, which is a postulated outcome of EMT that could explain the
recurrence of metastatic disease (Morel et al., 2008; Mani et al., 2008).

TGF-β itself can induce EMT via 2 distinct mechanisms. First, binding of TGF-β to its
cognate receptors TGFβR1 and TGFβR2 triggers the trans-phosphorylation of TGFβR1
by TGFβR2. Activated TGFβR1 subsequently phosphorylates and activates the Smad2/3 complex, which then forms a heterotrimer with Smad4 and translocates into the nucleus to control TGF-β target gene expression (Ikushima and Miyazono, 2010; Zavadil and Böttinger, 2005; Figure 6). Snail1 is a direct target gene of TGF-β, and together with the indirect activation of Snail2 and Twist by the TGF-β signaling cascade, EMT-related genes can repress E-cadherin expression to initiate the EMT event (Thuault et al., 2006; Peinado et al., 2003). Additionally, the expression of a number of mesenchymal genes, such as those of vimentin, fibronectin and α-smooth muscle actin, are directly activated by TGF-β to further enhance the mesenchymal phenotype (Nawshad et al., 2005; Zavadil and Böttinger, 2005; Figure 6). Likewise, the Smad complexes can also interact with ZEB1 or ZEB2 to form a transcriptional repressor on the E-cadherin promoter to further augment EMT (Shirakihara et al., 2007). Second, TGF-β can contribute to the initiation of EMT via a non-Smad dependent pathway. For example, activated TGFβR1 can directly interact with the scaffold protein PAR6, which is an important regulator of epithelial polarity and cell-cell junction integrity. The direct binding of TGFβR1 to PAR6 leads to PAR6 phosphorylation and subsequent ubiquitin-targeted degradation, which contributes to the disruption of cellular junctions (Ozdamar et al., 2005; Figure 6). Interestingly, TGF-β can also activate the PI3K and ERK signaling pathways to upregulate Snail1 expression and further stabilize the EMT process (Bakin et al., 2000; Derynck and Zhang, 2003; Figure 6).
Figure 6. Molecular mechanisms of EMT. The initiation of EMT requires a tight coordination between extracellular signals and intracellular signaling cascades. Hypoxia and TGF-β are 2 major triggers of EMT. Epithelial cancer cells under hypoxic stress (pO₂ < 20mmHg) upregulate hypoxia-inducible factor (HIF) protein expression to potentiate the hypoxic response. Being a transcription factor, HIF enters the nucleus and triggers the gene expression of critical EMT genes like Snail1, Snail2 and Twist1 to initiate the EMT event. Extrinsic TGF-β signals also converged at EMT genes regulations via the SMAD transcription factors complexes and an alternative PI3K signaling cascade. The distinct hallmark of EMT is the suppression of E-cadherin expression by Snail1, Snail2 and Twist1 that activates the transition from an epithelial to mesenchymal state. Snail1 also induces the expression of mesenchymal genes such as vimentin, fibronectin and α-smooth muscle actin to complete the mesenchymal phenotype. Additionally, TGF-β is also able to signal for the degradation of PAR6 to destabilize the epithelial cell-cell junctions to further enhance EMT.
1.3 Cancer metabolism

1.3.1 Warburg effect

The most prevalent altered cancer metabolic phenotype is the Warburg effect. This anomalous cancer cell metabolism was first observed by Otto Warburg. He described cancer cells with a glycolytic phenotype even in the presence of oxygen, which is termed the “Warburg effect” (Weinhouse, 1976). Unlike normal cells, these transformed cells bypass mitochondrial oxidative phosphorylation (OXPHOS) and adopt the less efficient, but rapid, aerobic glycolytic pathway to generate ATP (Figure 7). It appears counterintuitive that cancer cells opt for an inefficient system of ATP generation, as less ATP is generated per glucose molecule during aerobic glycolysis. However, emerging evidence now suggests that this glycolytic phenotype provides energetic and biosynthetic advantages to proliferating cancer cells (DeBerardinis et al., 2008; Jones and Thompson, 2009). Assuming free access to glucose, this switch to aerobic glycolysis generates ATP at an enhanced rate compared with OXPHOS, meeting the elevated energetic requirements of cancer cells. Additionally, the high substrate flux through glycolysis enables the effective shunting of glycolytic intermediates, such as pyruvate and lactate, to other biosynthetic pathways, including those of pyrimidine, glycerol and serine/glycine (Vander Heiden et al., 2009). This in turn facilitates the biosynthesis of macromolecules necessary for the assembly of new cells. Precisely, this abnormal glucose addiction by cancer cells enable the development of a non-invasive clinical positron emission tomography (PET) imaging using radioactive $[^{18}\text{F}]$fluorodeoxyglucose (Czernin and
Phelps, 2002; Gambhir, 2002). This technique allows us to pinpoint regions of enhanced glucose uptake and has been highly effective in identifying various neoplastic outgrowths. The observation of 'aerobic' glycolysis inevitably led to hypotheses regarding mitochondrial defects in cancer that prevent the oxidation of glucose via OXPHOS for ATP generation. Glycolysis is therefore a necessary adaptation employed by cancer cells to maintain their own bioenergetic requirements. However, most tumors retain functional mitochondria for OXPHOS (Weinhouse, 1976; Frezza and Gottlieb, 2009). In the context of proliferating cancer cells, the activities of mitochondria appear to be reprogrammed to maximize anabolic metabolism rather than ATP production (Ward and Thompson, 2012).
Figure 7. Warburg Effect. Under normal condition, quiescent cells metabolize glucose to pyruvate that is then directed into the oxidative phosphorylation (OXPHOS) pathway for ATP generation yielding 36 molecules of ATP per molecule of glucose. Under anaerobic situation, these cells bypass OXPHOS and utilize the inefficient anaerobic glycolysis to directly metabolize glucose into lactate yielding only 4 ATP molecules per glucose. Interestingly, this anaerobic glycolysis method is employed by proliferative cells and cancer cells even under aerobic condition which is known as the ‘Warburg effect’. From Vander Heiden et al., 2009. Reprinted with permission from AAAS

1.3.2 Metabolic plasticity

The dynamic nature of tumorigenesis and the surrounding microenvironment suggests that cancer cells may possess the ability to adapt their metabolism to the prevailing microenvironmental conditions. Glucose utilization (Warburg effect) is a well-established metabolic phenotype in cancer cells. Multiple studies demonstrated that tumor suppressors and oncogenes directly influence glucose metabolism. Precisely, the oncogenic phosphatidylinositol 3-kinase (PI3K)/AKT pathway has been identified as the key promoter of cancer cells’ glucose metabolism (Elstrom et al., 2004; see section 1.3.2.2). Hence, blocking glucose metabolism should be selectively toxic to the cancer cells. However, the inhibition of glucose uptake by the cancer cells does not necessarily correlate to positive patient outcomes, suggesting that these glucose-deprived cancer cells can activate alternative metabolic pathways to compensate for the loss of glucose (Ma et al., 2009; Tan et al., 2007).

Indeed, cancer cells can process fatty acids, amino acids and lactate to fuel their bioenergetic requirements (Cairns et al., 2011). The oncogene c-myc is a key mediator of cancers’ glutamine metabolism. c-myc upregulates the expression of cell surface glutamine transporters and associated enzymes to enhance glutamine metabolism to...
support the bioenergetics and biosynthesis demands of the cancer cells (Gao et al., 2009; Wise et al., 2008). Importantly, glutamine metabolism has been demonstrated to be a vital alternative in glucose-deprived cancer cells. Yang and colleague demonstrated that in the absence of glucose, glioblastoma cells specifically enhance glutamate dehydrogenase (GDH) activities to direct glutamine into the mitochondrial citric-acid (TCA) cycle to sustain viability (Yang et al., 2009). They showed that glioblastoma cells can alternate between glucose- or glutamine-addiction depending on the prevailing glutamine or glucose status, which highlights the metabolic flexibility of cancer cells.

Recent studies have also proposed an alternative oncogenesis hypothesis describing several tumors that harbor 2 distinct subpopulations of cancer cells that utilize different energy metabolic pathways (Kennedy and Dewhirst, 2010; Figure 8). One population consisted of hypoxic cancer cells that metabolized glucose via glycolysis for fuel and secrete lactate, whereas their more oxygenated counterparts preferentially scavenge high energy lactate into the TCA cycle for ATP production and anabolism (Porporato et al., 2011; Feron, 2009; Figure 8). These observations, together with the dynamic nature of the tumor microenvironment, emphasize the intrinsic plasticity of cancer cells to utilize alternative nutrient sources to adapt to fluctuating microenvironments. Similarly, in addition to supporting core cancer characteristics, studies have begun to unravel a metabolic synergy between cancer cells and the associated tumor stroma, in particular CAFs. CAFs appear to have similar metabolic flexibility which allows them to drive cancer progression by providing metabolic fuel. CAFs have been demonstrated to adopt a Warburg-like, highly catabolic metabolism to generate high energy intermediates such as
ketones, lactate and pyruvate, which are exported into the surrounding matrix via MCT4. Correspondingly, cancer cells upregulate MCT1 expression to direct the uptake of these high-energy substrates for anabolism (Martinez-Outschoorn et al., 2014; Sotgia et al., 2011; Figure 8). Given that the tumor microenvironment plays a critical role in selecting aggressive phenotypes, it is conceivable that these metastatic cancer cells may have a distinct metabolic landscape compared with their stationary counterparts.

Figure 8. Metabolic plasticity. Two distinct populations of tumor cells exist as symbiont within a single tumor. The tumor cells further away from blood vessels suffers from hypoxic stress, resulting in the modification of its metabolism to maximize glucose uptake and glycolysis for ATP generation to sustain cellular survival. The resultant lactate is exported out into the tumor microenvironment through monocarboxylate transporter 4 (MCT4). The more oxygenated tumor cells have a metabolic preference for lactate. The excess lactate is taken up by MCT1 and converted by lactate dehydrogenase 1 (LDH1) into pyruvate that is then directed into the TCA cycle to maximize ATP production. Similarly, the CAF also adopts a highly glycolytic and catabolic phenotype to generate high energy intermediates to fuel the anabolic requirements of the cancer cells.
1.3.3 Modulators of reprogrammed cancer metabolism

1.3.3.1 MYC

The v-myelocytomatosis viral oncogene homolog (Myc) is a basic helix-loop-helix transcription factor that heterodimerizes with Max to influence the expression of a plethora of genes (Eilers and Eisenman, 2008). Notably, Myc can differentially regulate a core set of genes that have additive influences on cellular proliferation. Myc can transactivate pro-proliferative transcription factors, such as E2F and cyclins, while simultaneously repressing the expression of cell cycle inhibitors to accelerate cell growth (Eilers and Eisenman, 2008). Furthermore, Myc can augment anabolic protein biosynthesis and mitochondrial biogenesis to complement the requirements of increased cellular proliferation (Patel et al., 2004). Genetic alterations in cancer cells frequently deregulate Myc transcription, resulting in ectopic Myc expression. This phenomenon enables cancer cells to acquire replicative immortality (Hanahan and Weinberg, 2011).

In addition to its pro-proliferative roles, Myc has profound effects on the metabolic phenotype of cancer cells. Myc cooperates with HIF, leading to increased expression and membrane translocation of glucose transporters and several critical glycolytic enzymes, such as hexokinase 2, pyruvate dehydrogenase 1 and lactate dehydrogenase A (Shim et al., 1997; Kim et al., 2007; Osthus et al., 2000; Figure 9). Interestingly, Myc also directly enhances mitochondrial biogenesis and further alters the metabolic landscape of cancer cells (Li et al., 2005). Glutamine is an essential amino acid that is required for robust cell proliferation, and multiple cancers have been shown to be addicted to this amino acid (Lu et al., 2010). Wise et al (Wise et al., 2008; Figure 9) showed that Myc can directly fuel...
this requirement by enhancing glutamine uptake through the induction of the glutamine transporters SLC5A1 and SLC7A1. Myc then promotes glutaminolysis, the catabolic metabolism of glutamine, through the upregulation of glutaminase 1 located in mitochondria (Gao et al., 2009; Figure 9). The catabolism of glutamine into glutamate is used by mitochondria to produce α-ketoglutarate, which feeds into the TCA cycle to further supplement the carbon inputs required for the synthesis of fatty acids and other amino acids (Gao et al., 2009; Figure 9).

**Figure 9. Myc regulates glucose and glutamine metabolism.** Myc directly regulate genes involved in glucose metabolism such as glucose transporter GLUT1, Hexose kinase 2 (HK2), pyruvate kinase M2 (PKM2) and lactate dehydrogenase A (LDHA) to direct glucose into glycolysis. Similarly, Myc also stimulates the expression of glutamine transporter SLC1A5 and glutaminase (GLS) to promote glutamine metabolism. Here, glutamine is converted into α-ketoglutarate in the mitochondrion and diverted into the TCA cycle to generate malate. Malate is then transported out of the mitochondria into the cytosol where it is metabolize to lactate (glutaminolysis).
1.3.3.2 The PI3K pathway

Phosphatidylinositol 3-kinase (PI3K) belongs to a group of conserved intracellular lipid kinases that integrate extracellular growth signals to direct cell proliferation, survival and metabolism (Engelman et al., 2006). PI3K activation results in the conversion of plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP$_2$) into phosphatidylinositol-3,4,5-bisphosphate (PIP$_3$). PIP$_3$ then recruits various signaling molecules to mediate PI3K downstream functions. Of particular interest is the protein serine-threonine kinase AKT. Activated AKT phosphorylates and control the activities of a multitude of proteins involved in cell proliferation, survival and metabolism (Lawlor and Alessi, 2001). For example, activated AKT can directly promote cell growth by inhibiting forkhead-related transcription factor-1, which suppresses the expression of cell cycle inhibitors such as p27Kip1 and RBL-1 (Brunet et al., 2001). Similarly, the inhibitory phosphorylation of glycogen synthase kinase 3 (GSK3) by AKT stabilizes the pro-proliferative activity of c-myc and cyclin D1 to further enhance cellular proliferation (Vivanco and Sawyers, 2002). Furthermore, AKT phosphorylation of pro-apoptotic Bad allows binding of 14-3-3 proteins and prevents binding of Bad to Bcl-2 family members, thus triggering a survival response (Datta et al., 2000; Datta et al., 1997). Given the magnitude of the influence of the PI3K pathway on cell proliferation, survival and metabolism, it is not surprising that this pathway is one of the most commonly activated oncogenic signaling pathways. The activation of this pathway not only confers pro-proliferative and pro-survival attributes to cancer cells but also reprograms cancer cell metabolism to enhance their oncogenic potential (Plas and Thompson, 2005; Figure 10).
AKT activation drives glucose uptake and glycolysis through the increased expression of glucose transporter SCL2A1 (GLUT1) and activation of the key glycolytic enzymes hexokinase and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 to commit glucose to glycolysis (Elstrom et al., 2004). Additionally, AKT activation stimulates the activity of mTOR through the inhibitory phosphorylation of the negative regulator of mTOR tuberous sclerosis 2 (TSC2). mTOR activation directly stimulates endogenous protein synthesis to supplement the growth requirements of cancer cells (Robey and Hay, 2009). Furthermore, mTOR collaborates with AKT to manipulate the metabolic activities of mitochondria for de novo lipogenesis. Mitochondrial citrate is exported into the cytosol and converted into acetyl-CoA by AKT-activated ATP-citrate lyase to be used for the synthesis of fatty acids, cholesterol and isoprenoids, which provide carbon intermediates to fuel the anabolic and bioenergetic requirements of malignant progression (Robey and Hay, 2009).

1.3.3.3 Adenosine Monophosphate (AMP)-activated protein kinase (AMPK)

AMP-activated protein kinase (AMPK) is the primary sensor and regulator of cellular energy and plays a pivotal role in stress survival in mammalian cells (Hardie et al., 2012). AMPK is a heterotrimer that consists of a catalytic α-subunit and β and γ regulatory subunits that respond to fluctuations in the AMP and ATP ratio in cells. Under conditions of starvation, metabolic or oncogenic stress, when cellular energy homeostasis is compromised (high AMP to ATP ratio), AMPK is activated through phosphorylation of the conserved threonine residue (Thr172) (Carling et al., 2012; Hardie et al., 2012). This activation of AMPK antagonizes anabolism and drives cells to a catabolic phenotype that
generates alternative substrates for ATP production and promotes stress survival (Carling et al., 2012). Thus, any factors that are able to alter cellular energy levels influence AMPK activation.

In the context of cancer, dynamic microenvironmental stresses result in an imbalance in the energy demand/supply that causes cancer cells to rely on AMPK functions (Shackelford and Shaw, 2009). Similarly, the loss of AMPK in normal cells results in the deregulation of cellular bioenergetics, leading to programmed cell death and, surprisingly, resistance to oncogenic transformation (Inoki et al., 2003; Laderoute et al., 2006). More importantly, AMPK has been shown to function synergistically with AKT to coordinate cellular bioenergetics and cell survival in response to various physiological cues (Zhong et al., 2008). Similar to Myc and AKT, AMPK potentiates aerobic glycolysis through the activation of pro-glycolytic enzymes and promotes mitochondrial biogenesis (Mendoza et al., 2012; Yalcin et al., 2009). Interestingly, AMPK activation can also direct cancer cells to harness fatty acid catabolism to enhance ATP generation. AMPK is a critical regulator of fatty acid metabolism through the phosphorylation and inhibition of acetyl-CoA carboxylase-1 and -2, thus suppressing the production of malonyl-CoA, which is required for lipogenesis. The decrease in malonyl-CoA reduces inhibition of carnitine palmitoyltransferase-1, leading to an increase in fatty acid uptake by mitochondria as well as \( \beta \)-oxidation (Zaugg et al., 2011; Steinberg and Kemp, 2009).
Figure 10. Overall schematic of proliferative cell metabolism. This figure demonstrates our understanding of how glycolysis, oxidative phosphorylation, pentose phosphate pathway (PPP) and glutamine metabolism are interconnected in proliferative cells. Critical tumor suppressors and oncogenes are known to influence these major metabolic pathways to manipulate cancer cell metabolism. Activation of PI3K/AKT signaling enhanced the glycolytic phenotype of the cells driving glucose uptake and metabolism. Growth factor activation of tyrosine kinase negatively regulates glycolysis to shunt the accumulated high energy glycolytic intermediates into PPP for macromolecular synthesis and NADPH production. Myc, on the other hand, facilitates glutamine metabolism for amino acids and lipid synthesis. AMPK and p53 function as tumor suppressors to halt cell proliferation upon energy or oxidative stress. Tumor suppressors and oncogenes are in red and green boxes respectively. From Vander Heiden et al., 2009. Reprinted with permission from AAAS.

1.4 The 14-3-3 proteins and cancer

The 14-3-3 proteins are a family of seven highly conserved acidic proteins (β, γ, ε, σ, ζ, τ and η) that are ubiquitously expressed in all eukaryotic organisms (Aitken, 2006). These
proteins are specific phosphoserine/threonine (pS/T)-binding proteins with an extensive interactome that has considerable influence on cell proliferation, apoptosis, metabolism and tumorigenesis (Porter et al., 2006). In cells, 14-3-3 proteins exist as either homo- or hetero-dimers with a single ligand-binding site located in each monomer. Thus, 14-3-3 dimers are able to bind 2 pS/T motifs simultaneously, either on one or 2 separate proteins to modulate their activities (Gardino et al., 2006). Upon binding to its partner(s), 14-3-3 proteins can induce conformational changes in target proteins, modify their stability and/or activities (Morrison, 2009). Additionally, binding of 14-3-3 to target proteins can also protect the phosphate group against phosphatases, disrupt the association of binding targets with other proteins and mask localization signals to compartmentalize these target proteins (Morrison, 2009; Aitken, 2006). In this manner, 14-3-3 proteins can act as a ‘control hub’ to fine-tune a wide spectrum of biological functions.

Unsurprisingly, altered expressions of various isoforms of 14-3-3 have been associated with a multitude of human epithelial cancers, thus supporting their critical roles in cancer initiation and progression. Two 14-3-3 isoforms, 14-3-3σ and ζ, are highly associated with oncogenesis. Emerging evidence also suggests the involvement of 14-3-3γ, η and τ (Freeman and Morrison, 2011; Figure 11). 14-3-3σ has been identified as a tumor suppressor due to its ability to bind and stabilize p53 following DNA damage and to initiate cell cycle arrest (Li et al., 2009; Figure 11). More importantly, 14-3-3σ was recently shown to bind to Snail upon phosphorylation of serine 11. This binding result in the sequestration of Snail in the cytosol, thus abolishing the EMT-inducing transcriptional activities of Snail (Du et al., 2010; Figure 11). Moreover, the loss of 14-3-
3σ expression has also been reported in numerous cancers, lending further credence to its tumor-suppressive abilities.

In contrast, elevated 14-3-3ζ appears to play critical roles throughout ontogeny (Neal and Yu, 2010). During cancer initiation, 14-3-3ζ overexpression has been demonstrated to facilitate the dimerization of B-raf, leading to its constitutive activation and thus allowing cancer cells to acquire infinite replicative abilities (Ritt et al., 2010). Furthermore, binding and stabilization of the p85 subunit of PI3K by 14-3-3ζ enhance the PI3K/AKT signaling cascade, conferring additional pro-survival and proliferative benefits to cancer cells (Neal et al., 2012; Figure 11). When cancer cells undergo EMT to metastasize, disruptions of epithelial cell-cell junctions are mediated by 14-3-3ζ through the stabilization of the TGFβ/SMAD signaling axis, which eventually converges to suppress E-cadherin expression (Lu et al., 2009; Figure 11). Importantly, 14-3-3γ, η and τ have recently been implicated in this EMT initiation event. These three 14-3-3 proteins selectively bind to pT177 on Snail1, which stabilizes and potentiates the E-cadherin-repressive function of Snail1 to initiate cell-cell junction disruption and trigger EMT (Hou et al., 2010; Figure 11).

Given the critical regulatory roles of serine/threonine kinases in central metabolic pathways, it is difficult to discern the involvement of 14-3-3 proteins in the regulation of cellular metabolism. Although the impact of these 14-3-3 proteins on cellular metabolism remains poorly understood, several studies of major metabolic signaling pathways have begun to elucidate the importance of the 14-3-3 protein family. Large-scale affinity purification studies using 14-3-3ζ protein as bait have identified a series of key metabolic...
enzymes involved in glycolysis, fatty acid metabolism, ROS metabolism and autophagy (Meek et al., 2004). This result suggests that 14-3-3 can interact with and perhaps modify the activities of these critical metabolic enzymes to influence metabolism.

Indeed, 14-3-3 proteins are able to modulate the downstream activities of AMPK and PI3K/AKT, which are important regulators of cellular energy. AMPK promotes catabolism, whereas AKT favors anabolism (Engelman et al., 2006; Hardie et al., 2012). These two pathways converge at mTORC1, with opposing functions, to control the metabolic status of cells. When cells are proliferating, the PI3K/AKT pathway predominates by activating mTORC1 through inhibitory phosphorylation of TSC1/2, which generates a 14-3-3 binding site to suppress TSC1/2-mediated inhibition of mTORC1 (Li et al., 2002; Figure 11). Second, AKT can directly phosphorylate the proline-rich AKT substrate 40 (PRAS40), which is an inhibitor of mTORC1 (Sancak et al., 2007). The binding of 14-3-3 to PRAS40 is necessary to relieve the inhibitory effects on mTORC1 (Vander Haar et al., 2007). Once active, mTORC1 activates protein synthesis and inhibits self-degrading autophagic processes (Jung et al., 2009; Wullschleger et al., 2006). As expected, in response to energy stress, the high AMP:ATP ratio triggers AMPK activation, which reverses the anabolic effects of AKT. In this scenario, AMPK phosphorylates and activates TSC1/2. This phosphorylation of TSC1/2 prevents 14-3-3 binding; thus, TSC1/2 is free to exert its inhibitory effect on mTORC1. Additionally, AMPK directly phosphorylates the regulatory-associated protein of TOR (RAPTOR) and facilitates 14-3-3 binding to further suppress the functions of mTORC1 and enhance autophagy (Lee et al., 2010; Gwinn et al., 2008; Figure 11). These observations suggest
that 14-3-3 may function as a precise switch to direct the metabolic fate of cells that may be highly dependent on extracellular cues, which is a relevant phenomenon during tumor progression.

Figure 11. 14-3-3 proteins in cancer progression. The 14-3-3 proteins are specific phosphoserine/threonine (pS/T)-binding proteins ubiquitously expressed in all eukaryotic organisms. These proteins have an extensive interactome with considerable influence on cell proliferation, apoptosis, metabolism and tumorigenesis. 14-3-3ζ and σ are two 14-3-3 proteins that are highly associated with tumorigenesis albeit with opposing functions. 14-3-3σ has tumor suppressive functions similar to that of p53. In this aspect, 14-3-3σ bind to
mdm2 to prevent the degradation of p53 which further extend its tumor suppressive influence. Additionally, 14-3-3σ binds and sequesters pro-EMT Snail and pro-proliferation cdc2/cyclinB in the cytosol to suppress malignant progression. On the other hand, 14-3-3ζ enhances tumorigenesis through the upregulation of various pro-tumorigenic signaling cascades. 14-3-3ζ is found to augment TGF-β-SIP1 signaling to suppress E-cadherin gene expression. 14-3-3ζ can also bind and increase PI3K activation to enhance AKT signaling. Emerging evidence also implicates 14-3-3γ during EMT, where it binds and stabilizes the Snail1-Ajuba complex to suppress E-cadherin expression. Lastly, 14-3-3 is also found to control cellular energetics, with inhibitory effects on TSC2 and mTORC1 complexes that is dependent on the phosphorylation sites.

1.5 ANGPTL

A family of 8 proteins, designated ANGPTL 1-8, was identified in systemic circulation and was found to have structural and amino acid sequence similarity to angiopoietin (ANG) (Katoh and Katoh, 2006; Hato et al., 2008; Quagliarini et al., 2012). There are 4 known ANG proteins, ANG 1-4, and only ANG 1 and 2 play critical modulatory roles in angiogenesis (Brindle et al., 2006). These ANGs are characterized by an N-terminal coiled-coil domain and C-terminal fibrinogen-like domain and participate in interactions with their cognate ANG receptors, Tie 1 and 2, to activate downstream pathways that regulate angiogenesis. ANG 1 plays a pro-angiogenic role in promoting vessel integrity and prevents vascular leakiness through Tie 2 signaling, while ANG 2 antagonizes these effects via competitive inhibition with ANG 1 for binding to Tie 2 (Maisonpierre et al., 1997). Although ANGPTLs are structurally similar to ANG, they do not bind to Tie 1 or 2 to mediate their physiological functions (Hato et al., 2008). These ANGPTLs are presently classified as orphan ligands with no cognate receptors. ANGPTL 3, 4, 6 and 8 are known to play critical roles in lipid and glucose metabolism, and ANGPTL 2, 3, 4, 5 and 7 have been implicated in hematopoietic stem cell regulation (Oike et al., 2005;
Emerging evidence now associates ANGPTL4 with cancer progression; however, its role remains controversial and requires further clarification.

1.5.1 ANGPTL4

ANGPTL4 is characterized by a hydrophobic signal peptide, an N-terminal coiled-coil domain, and a fibrinogen-like C-terminal domain (Kersten et al., 2000). Native full-length ANGPTL4 (fANGPTL4) exists as a dimer or tetramer and can undergo proteolytic processing to generate the N-terminal coiled-coiled fragment (nANGPTL4) and the C-terminal fibrinogen-like domain (cANGPTL4) (Ge et al., 2004; Lei et al., 2011; Figure 12). Although ANGPTL4 is known to be processed proteolytically, the mechanism underlying its cleavage and the importance and roles of the various ANGPTL4 fragments are only beginning to be understood.

The proteolytic cleavage of ANGPTL4 is mediated by the proprotein convertases (PCs) furin, PC5/6, paired basic amino acid-cleaving enzyme 4, and PC7 (Lei et al., 2011; Figure 12). Using Huh7 cells transfected with expression vectors encoding various convertases, Lei et al showed that these convertases cleave human fANGPTL4 at an -RRXR- consensus cleavage site in vitro. However, it remains unclear whether these PCs are indeed responsible for the in vivo processing of fANGPTL4 or whether there is any correlation between the expression of PCs and truncated forms of ANGPTL4 in normal tissues or tumors. Furthermore, whether the proteolytic processing of ANGPTL4 occurs intracellularly or extracellularly remains to be elucidated.
The nANGPTL4 is responsible for oligomeric assembly and binds to lipoprotein lipases (LPL) to inhibit their activities (Ge et al., 2004). Disulfide bond formation facilitates the oligomeric formation of nANGPTL4 and fANGPTL4, which in turn enhances its inhibitory effects on LPL activity (Figure 12). Mutations that prevent oligomerization severely compromise the capacity of the protein to inhibit LPL. The fANGPTL4 can also bind to heparin sulfate proteoglycans to participate in the inhibition of endothelial cell migration and tubule formation (Cazes et al., 2006). However, it remains unknown whether fANGPTL4 and nANGPTL4 directly stimulate intracellular signaling because neither a receptor nor a cell-surface interaction partner has been identified. The cANGPTL4 binds to and activates integrins β1 and β5 to regulate cell migration via the FAK/PAK signaling cascade (Goh et al., 2010). Furthermore, cANGPTL4 can also associate with specific matrix proteins and delay their proteolytic degradation by metalloproteinases (Figure 12). This interaction does not interfere with integrin-matrix protein recognition (Goh et al., 2010). Taken together, these studies suggest that different ANGPTL4 fragments exhibit tissue-dependent functions, raising the question of whether the different fragments of ANGPTL4 have distinct roles in human physiology.
Figure 12. Post-translational processing and the functions of individual fragments of ANGPTL4. Full-length ANGPTL4 (fANGPTL4) consist of a hydrophobic signal peptide, an N-terminal coiled-coil domain, and a fibrinogen-like C-terminal domain. It can be cleaved by proprotein convertases (PC), such as furin, to generate the N-terminal ANGPTL4 (nANGPTL4) and C-terminal ANGPTL4 (cANGPTL4). The fANGPTL4 and nANGPTL4 are involved in lipid metabolism via the regulation of lipoprotein lipase (LPL) activity, albeit lower fANGPTL4 efficiency. The fANGPTL4 can also regulate angiogenesis. In contrast, no metabolic role of cANGPTL4 has been reported.
cANGPTL4 is important during wound repair and recent studies implicate cANGPTL4 in tumor progression through the regulation of oxygen free radical levels.

1.5.2 ANGPTL4 expression and its transcriptional regulation

ANGPTL4 expression is regulated by the nuclear hormone receptors peroxisome proliferator-activated receptor (PPAR) and glucocorticoid receptor (GR) as well as hypoxia and fasting (Yoon et al., 2000; Kim et al., 2000; Kersten, 2005; Koliwad et al., 2009). More recently, early expression profiling studies have detected ANGPTL4 in a variety of organs and tissues, including the skin, intestines, kidneys and, predominantly, adipose tissues and liver (Yoon et al., 2000; Kim et al., 2000; Kersten, 2005). However, little is known regarding the relative expression of the various ANGPTL4 fragments in these tissues. It has recently been shown that adipocytes express post-translational modified fANGPTL4 (~65 kDa), whereas the liver produces both cANGPTL4 (~47 kDa) and nANGPTL4 (~26 kDa) (Yang et al., 2008; Lei et al., 2011). The mechanism underlying such tissue-dependent expression of ANGPTL4 remains unknown.

PPARα, β/δ and γ are ligand-inducible transcription factors that belong to the nuclear hormone receptor family and have been implicated in a variety of physiological processes, such as fatty acid metabolism, cellular differentiation and tumorigenesis (Michalik et al., 2004). Studies have shown that ANGPTL4 is one of the PPAR target genes and contains several putative PPAR response element (PPRE) consensus sequences in its promoter (Inoue et al., 2014). PPARs have been shown to regulate ANGPTL4 expression in various normal tissues, especially in adipose tissue and hepatocytes, while recent evidence has revealed the possible PPAR regulation of ANGPTL4 expression in tumors (Kersten, 2005;
Girroir et al., 2008; Zhu et al., 2011). Several new studies reported that hypoxia and hypoxia-inducible factor 1α (HIF-1α) could induce the expression of ANGPTL4 (Zhu et al., 2011; Li et al., 2011; Kim et al., 2011). Indeed, Zhang et al. identified a functional HIF-1α binding site, 5'-ACGTGCCACCA-3', located 1.6 kb upstream of the human ANGPTL4 gene. This finding was confirmed by an independent group using MHCC-97L and SMMC-7721 cells (Li et al., 2011). More recently, a study by Inoue et al. revealed an intriguing synergistic transcriptional regulation of the ANGPTL4 gene by both PPARβ/δ and the HIF signaling axis. Using a chromosome conformation capture assay, the authors went on to show that this synergistic enhancement was possible due to the spatial proximity of the 2 response elements, which allows a conformational adjustment upon binding of the 2 transcription factors (Inoue et al., 2014). TGF-β, a potent regulator of multiple biological processes, including inflammation, cell proliferation and tissue homeostasis, has also been implicated in modulation of ANGPTL4 expression via the Smad3 signaling pathway; however, a functional Smad3 binding element was not shown (Bierie and Moses, 2006; Padua et al., 2008). Given the implications of the PPARβ/δ, HIF and TGF-β signaling axis in cellular metabolism and tumorigenesis, we propose that ANGPTL4 could have a critical role in coordinating the downstream events controlled by these pathways.

1.5.3 ANGPTL4 and energy metabolism

There is a vast repertoire of knowledge on the metabolic roles of ANGPTL4 in both physiological lipid and glucose metabolism. Both fANGPTL4 and nANGPTL4 have a profound effect on plasma triglyceride (TG) and free fatty acid (FFA) levels. ANGPTL4
overexpression in mice results in a marked inhibition of plasma lipoprotein lipase (LPL), thereby impeding the clearance of plasma TG into adipose tissues and leading to hypertriglyceridemia (Yoshida et al., 2002; Mandard et al., 2006). Conversely, ANGPTL4 deletion enhances LPL activity, leading to a decrease in plasma TG levels and an increase in TG-derived FA uptake into adipose tissue for storage. The LPL inhibition mediated by ANGPTL4 is largely attributed to the ability of ANGPTL4 to bind to and dissociate active LPL dimers into inactive monomers, while ANGPTL4 has also been found to target LPL for PC-mediated proteolysis to further inhibit LPL activity (Sukonina et al., 2006; Lichtenstein et al., 2007; Lei et al., 2011).

Recent studies indicated that ANGPTL4 could be a major physiological regulator of LPL activity during fasting and exercising. Under fasting conditions, starvation signals activate cellular TG metabolism to generate FFA for β-oxidation in order to sustain cellular bioenergetics. As anticipated, this fasting-induced increase in the levels of FFAs, which are potent inducers of ANGPTL4 expression, results in significant upregulation of ANGPTL4 expression and a parallel decrease in LPL activity. The inhibition of LPL-induced plasma TG clearance and the subsequent uptake of cholesterol and FAs into tissues trigger a feedback mechanism by increasing the synthesis of cholesterol and FAs (Lichtenstein et al., 2007). The injection of recombinant ANGPTL4 alone acutely raises plasma FA levels (Yoshida et al., 2002). Moreover, ANGPTL4 can also stimulate lipolysis in adipocytes to further enhance the plasma pool of FFAs. Likewise, recent studies have demonstrated that elevated glucocorticoid levels during fasting are necessary to augment ANGPTL4 expression (Kersten et al., 2009; Kuo et al., 2014). Activated glucocorticoid receptors (GRs) can recruit FoxO1 to directly promote ANGPTL4
expression (Yamada et al., 2006; Kuo et al., 2014). These findings suggest a positive regulatory loop between FFA and ANGPTL4, in which the expression of ANGPTL4 increases plasma FFA levels, resulting in a feedback loop to agonize ANGPTL4 expression.

This ANGPTL4 function is not restricted to plasma LPL, as similar observations have been reported for heart- and muscle-derived LPLs. ANGPTL4 has been shown to protect the heart against oxidative stress after an acute or a prolonged high fat load (Georgiadi et al., 2010). Similar to the observations in adipocytes, in vitro studies of myocytes have revealed that FAs and PPARβ/δ agonists can potentiate ANGPTL4 expression and suppress LPL activities (Robciuc et al., 2012; Catoire et al., 2014). This phenomenon was further confirmed in mice and human skeletal muscles, in which ANGPTL4 expression was found to be elevated during starvation and fasting (Lichtenstein et al., 2007; Kersten et al., 2009). Interestingly, the opposite is true in exercising muscles. Enhanced LPL activities were found to be required to facilitate the uptake of FA fuel to cope with energy generation requirements (Grewe et al., 2000). This result suggests an attenuation of ANGPTL4 expression in exercising skeletal muscles. As anticipated, a recent study demonstrated that ANGPTL4 expression was suppressed in exercising muscles but was elevated in non-exercising muscles. This result suggests that the suppression of local FA uptake may be necessary to direct FFAs to exercising muscles for fuel (Catoire et al., 2014). This selectivity in the expression of ANGPTL4 suggests that the body can manipulate physiological factors to control metabolic flexibility.
ANGPTL4 also plays an important role in glucose metabolism. It was recently discovered that upon re-feeding after starvation, the elevation in insulin levels reduces circulating ANGPTL4, presumably through the phosphorylation and retention of FoxO1 in the cytoplasm by the insulin-stimulated PI3K/AKT signaling cascade, leading to the attenuation of ANGPTL4 transcription (Kuo et al., 2014). Similarly, this effect was abolished in an insulin-resistant state (Yamada et al., 2006). In a separate study, ANGPTL4 was found to potently reduce hyperglycemia and increase glucose sensitivity in diabetic mice via inhibition of hepatic glucose synthesis and the potentiation of insulin-mediated inhibition of gluconeogenesis (Xu et al., 2005). These observations in mice were extended to human diabetic patients, in whom serum ANGPTL4 levels correlate inversely to the blood glucose content, suggesting a role for ANGPTL4 in diabetes (Xu et al., 2005).

ANGPTL4 is a critical player in central regulatory pathways of energy metabolism. Hypothalamic ANGPTL4 was found to regulate hypothalamic AMPK levels. High ANGPTL4 levels suppressed food intake and enhanced energy metabolism, encouraging anorexia (Kim et al., 2010). Collectively, these data highlight the importance of ANGPTL4 in lipid and glucose metabolism and implicate it as a therapeutic target in the treatment of metabolic syndromes.

1.5.4 ANGPTL4 and wound repair

Wound repair is an intricate and highly coordinated process that involves a series of events: inflammation, cell proliferation, matrix remodeling, re-epithelialization, neovascularization and, ultimately, wound closure. At the wound bed, a myriad of cytokines and secreted factors are released temporally and participate in the regulation of
the healing events (Singer and Clark, 1999). As a secreted factor, ANGPTL4 was elevated at the wound site after injury (Goh et al., 2010). Using ANGPTL4-knockout mice, the authors demonstrated that ANGPTL4 deficiency significantly impeded wound healing, suggesting a critical role of ANGPTL4 in response to wounded keratinocytes during the repair process (Goh et al., 2010). The authors went on to show that ANGPTL4 interacted with the major extracellular matrix components vitronectin and fibronectin, protecting them against proteolytic degradation. This effect allowed ANGPTL4 to modulate the availability of the wound site extracellular matrix for the cell-matrix communication necessary to facilitate the wound-healing process (Goh et al., 2010). Furthermore, ANGPTL4 can also interact specifically with integrin β1 and β5 on keratinocytes to enhance cell migration, thus facilitating re-epithelialization events. Binding of ANGPTL4 to integrins activates integrin-mediated intracellular signaling pathways. This process resulted in selective integrin recycling, which enhanced the cell migration process and leads to accelerated wound closure (Goh et al., 2010).

These recent findings were extended to diabetic wound healing. The same authors found that wound healing in ANGPTL4-deficient mice highly resembled that of compromised diabetic wounds, with increased inflammation, impaired angiogenesis and matrix deposition (Chong et al., 2014). Additionally, ANGPTL4 was poorly expressed in diabetic wounds after injury, highlighting the potential influence of ANGPTL4 on diabetic wound healing. Indeed, topical application of recombinant ANGPTL4 rescued the impaired diabetic wound angiogenesis and enhanced keratinocyte migration to augment the overall
wound-healing profile (Chong et al., 2014). These findings highlight the considerable potential of ANGPTL4 in wound healing, particularly in diabetic wound closure.

1.5.5 ANGPTL4 and inflammation

Emerging evidence has suggested important roles for ANGPTL4 in modulating the inflammatory response. Previous study demonstrated that an overload of dietary saturated fats elicited significant acute phase responses in ANGPTL4-deficient mice. This phenomenon led to a severe inflammatory phenotype characterized by the formation of macrophage-derived foam cells and the pathological elevation of inflammatory cytokines (Lichtenstein et al., 2010). These observations suggest that ANGPTL4 may have an anti-inflammatory function during the acute phase response. Consistent with this hypothesis, the restoration of ANGPTL4 expression negated the pro-inflammatory effects induced by saturated fats. Here, macrophage ANGPTL4 induced by fatty acids helped reduce lipid uptake into mesenteric lymph node macrophages, suppressing the expression of pro-inflammatory genes and foam cell formation (Lichtenstein et al., 2010). Moreover, ANGPTL4 overexpression protected against the development of atherosclerosis, which is attributed to the attenuation of foam cell formation (Georgiadi et al., 2013).

In the same context, bacterial lipopolysaccharide (LPS) has also been found to stimulate ANGPTL4 expression. LPS is a potent activator of toll-like receptor (TLR) signaling pathways to trigger inflammatory responses. Upon exposure to LPS, ANGPTL4 level is upregulated in various tissues, such as the heart, muscles and adipose tissues. Likewise, antagonism of TLR4 signaling results in suppressed ANGPTL4 expression following LPS exposure; TLR4 deficient mice also display suppressed ANGPTL4 expression.
Furthermore, several inflammatory cytokines, including interleukins, TNF-α and IFN-γ, also stimulate ANGPTL4 expression (Lu et al., 2010). More recently, a direct link between the acute phase response and ANGPTL4 expression was identified. The acute phase protein α1-antitrypsin (A1AT) was found to directly upregulate ANGPTL4 expression in an ERK1/2- and PPARγ-dependent manner, directing anti-inflammatory functions to suppress acute injury (Frenzel et al., 2014). In a separate study, Tjeerdema and colleague observed elevations of plasma ANGPTL4 in metabolic syndromes patients with systemic low-grade inflammation. They went on to show that TLR3/4 on the macrophages was potently activated by FFA to augment ANGPTL4 production. Importantly, they demonstrated that acute phase C-reactive protein levels strongly correlate with ANGPTL4 levels (Tjeerdema et al., 2014). These evidences appeared to highlight important roles for ANGPTL4 during the inflammatory response; however, the mechanism by which ANGPTL4 elicits such anti-inflammatory functions remains unclear and warrants further investigations.

1.5.6 ANGPTL4 in human cancer

While information regarding the relative expression of the different ANGPTL4 fragments in the normal tissues is emerging, less is known regarding the expression of different ANGPTL4 fragments in various tumors. To date, four studies have reported the elevated expression of cANGPTL4 in various tumors. Through the overexpression of ANGPTL4 cDNA in B16F0 mouse melanoma cells, Galaup et al demonstrated the presence of a smaller ANGPTL4 fragment with a molecular weight that corresponded to cANGPTL4. Using a monoclonal antibody against cANGPTL4 (mAb11F6C4), Zhu et al showed that
cANGPTL4, but not nANGPTL4, was highly expressed in major epithelial tumors, such as squamous cell carcinoma. Consistent with the above findings, Kim et al showed that colorectal cancer cells only secrete cANGPTL4 proteins. High levels of ANGPTL4 expression in oral and esophageal cancer correlated to enhanced tumorigenesis, advanced clinical stage and poor patient prognosis (Wang et al., 2010; Yi et al., 2013; Tanaka et al., 2015). Elevated expression of ANGPTL4 in colorectal cancer also correlated with shorter disease-free survival rates (Kim et al., 2011). Furthermore, ANGPTL4 downregulation impaired tumor growth and metastasis (Feng et al., 2010; Zhu et al., 2011; Adhikary et al., 2013). In contrast, studies have also demonstrated that increased ANGPTL4 expression inhibited the growth, metastasis and angiogenesis of melanoma and lung, gastric and colorectal tumors (Ito et al., 2003; Galaup et al., 2006; Okochi-Takada et al., 2014). It was further shown that high ANGPTL4 levels impaired tumor cell migration and invasion, thus inhibiting metastasis (Galaup et al., 2006). The reason for this discrepancy is unclear. These findings suggest that the expression and roles of ANGPTL4 may be dependent on the context and tumor type. Despite increasing emphasis on the differential roles of fANGPTL4, cANGPTL4 and nANGPTL4, most studies have not vigorously addressed this biological significance in cancer. Emerging studies have also revealed that post-translational modification of ANGPTL4 can affect its biological functions, adding another level of complexity to our understanding of the role of ANGPTL4 in cancer.

Girrol et al suggested that PPARβ/δ modulated ANGPTL4 expression during tumorigenesis in human breast cancer and melanoma cell lines, while Zhu et al reported no apparent correlation between the expression of PPARs and ANGPTL4 in squamous
cell carcinoma. A recent study suggested that prostaglandin E₂ (PGE₂) transactivated PPARβ/δ to regulate the expression of ANGPTL4 under hypoxic conditions (Kim et al., 2011). HIF-1α and TGF-β were also separately found to control ANGPTL4 expression in tumors (Padua et al., 2008; Zhu et al., 2011; Zhang et al., 2012; Li et al., 2011; Kim et al., 2011; Adhikary et al., 2013). These observations suggest that the dynamic tumor microenvironment may significantly influence the transcriptional regulation of ANGPTL4 in cancer and merits further investigation.

1.5.6.1 ANGPTL4 and tumor growth

A large amount of evidence clearly supports the tumor-promoting role of inflammation in cancer development and progression (Coussens and Werb, 2002). Data from various animal models clearly emphasize that the tumor-promoting micro-environment was indispensable for tumor formation and progression. The tumor micro-environment strongly resembled the wound environment, containing numerous growth factors and inflammatory lipid mediators, such as prostanoids, that allowed cancer cells to co-opt various signaling molecules to promote growth, invasion and metastasis (Coussens and Werb, 2002).

Cyclooxygenase-2 (COX-2) is undetectable in most normal tissues. It is an inducible enzyme that becomes abundant in activated macrophages and other cells at inflammation sites. Recently, COX-2 has been shown to be upregulated in various carcinomas and to have a central role in tumorigenesis. COX-2 expression was elevated in ~50% of colorectal adenomas and 85% of adenocarcinomas, and it was associated with poor survival in patients with colorectal cancer (CRC) (Kim et al., 2011). As anticipated,
prostaglandin E2 (PGE2), a product of COX-2, was the most abundant prostaglandin in CRCs, suggesting a potential tumorigenic role for PGE2. A recent study demonstrated a synergistic effect of PGE2 and hypoxia on the enhanced expression of ANGPTL4 (Kim et al., 2011). Specifically, hypoxia induced the expression of EP1, a PGE2 receptor, in CRC. PGE2 stimulates EP1 receptor signaling to enhance ANGPTL4 expression and cANGPTL4 secretion. ANGPTL4 overexpression and treatment with cANGPTL4 recombinant protein increase colorectal carcinoma cell proliferation and tumor growth in vitro and in vivo (Kim et al., 2011). Notably, the study further showed that cANGPTL4 regulates cancer cell proliferation via STAT1 induction, which is dependent on the NADPH-oxidase-mediated production of superoxide (O2•−), Src and MAPK pathways. This result was consistent with the findings of Zhu et al that demonstrate that cANGPTL4 can alter the cellular O2•−:H2O2 ratio to activate Src and ERK, which enhance tumor cell survival (Zhu et al., 2011). In contrast, it was recently demonstrated that ANGPTL4 functions as a tumor suppressor in gastric carcinomas. The authors showed that gastric tumors expressed low levels of ANGPTL4 and that re-expression of ANGPTL4 in these cancer cells resulted in marked suppression of tumor growth in SCID mice (Okochi-Takada et al., 2014). Interestingly, ANGPTL4 did not affect cancer cell proliferation, growth or survival in vitro. Instead, the tumor suppressive effect of ANGPTL4 has been attributed to its ability to inhibit vascular tube formation, leading to impaired angiogenesis, which is detrimental to tumor growth (Okochi-Takada et al., 2014).

More extensive studies will be needed to elucidate the underlying mechanism by which ANGPTL4 modulates cancer proliferation, including studies to identify the receptors that
mediate the downstream effects of ANGPTL4. This information has provided a focal point for future ANGPTL4 research, highlighting the need to demarcate the different biological functions of c- or nANGPTL4 in cancer progression.

1.5.6.2 ANGPTL4 in tumor invasion and metastasis

Metastasis is the spread of malignant cells to distant organs and is frequently the final event in tumor progression, as metastatic tumors are responsible for the majority of cancer deaths. However, metastasis remains the most poorly understood aspect of cancer (Hanahan and Weinberg, 2011). Metastasis is a complex multi-step process whereby tumor cells locally invade the surrounding tissue and migrate across the vascular endothelium of the lymph and blood systems (intravasation). In circulation, tumor cells must evade immunodetection and resist anoikis before arresting at a distal site. These tumor cells exit the circulation (extravasation) to establish a new secondary tumor (colonization) (Chiang and Massagué, 2008; see 1.2). The fundamental mechanism that is required during these events involves the disruption of vascular integrity, which facilitates the transmigration of tumor cells across the endothelium. Incidentally, ANGPTL4 has been demonstrated to execute critical roles across the different stages of metastasis.

Early studies have suggested that fANGPTL4 inhibited VEGF-induced angiogenesis and vascular leakiness (Ito et al., 2003). This decreased vascular leakiness inhibited the intra- and extravasation of tumor cells in vivo (Galaup et al., 2006). Notably, only a single form of ANGPTL4 has been detected in sera in vivo. Because the molecular weight of the ANGPTL4 fragment was not provided, it was not possible to know whether the detected protein band corresponded to fANGPTL4, nANGTL4 or cANGPTL4. By overexpressing
ANGPTL4 in melanoma cells, Galaup et al (2006) demonstrated the presence of fANGPTL4 and of a smaller ANGPTL4 fragment with a molecular weight corresponding to cANGPTL4 (Galaup et al., 2006). Although ANGPTL4 expression impaired tumor cell migration and invasion, the effect of the individual fragment was not validated.

In contrast, previous studies have demonstrated that ANGPTL4 is highly expressed in metastatic cancers, suggesting that it plays a role in metastatic processes (Minn et al., 2007; Zhu et al., 2011). ANGPTL4 enhanced vascular leakiness and promotes the metastasis of breast tumor cells to the lung and melanoma metastasis to the brain (Padua et al., 2008; Zhang et al., 2012; Izraely et al., 2012). TGF-β-induced elevation of ANGPTL4 levels in breast cancer cells resulted in the disruption of endothelial cell-cell junctions, leading to enhanced vascular leakiness and the transendothelial migration of tumor cells (Padua et al., 2008). Consistent with this observation, ANGPTL4 was found to promote the trans-endothelial migration and metastasis of hepatocellular carcinoma through the upregulation of vascular cell adhesion molecules 1 (VCAM-1) on endothelial cells (EC) and the activation of VCAM-1/integrin β1 signaling (Li et al., 2011). This pro-metastatic function of ANGPTL4 was attributed to an increase in VCAM-1 expression on endothelial cells, which served to facilitate the attachment of metastatic cancer cells in the circulation and their subsequent trans-endothelial migration. Importantly, ANGPTL4 has been linked directly to cancer cell invasion. Breast cancer cell invasiveness was severely compromised when ANGPTL4 expression was silenced. Remarkably, recombinant human cANGPTL4, but not fANGPTL4 or nANGPTL4, restores breast cancer cell invasiveness, although the underlying mechanism(s) is unknown (Adhikary et al., 2013).
Furthermore, clinical studies have correlated ANGPTL4 expression to venous and lymphatic invasion in human gastric, colorectal and esophageal squamous carcinoma. This elevated ANGPTL4 expression has also been strongly linked to advanced clinical stages and poor patient outcomes, further emphasizing the role of ANGPTL4 in tumor metastasis (Shibata et al., 2010; Nakayama et al., 2010; Nakayama et al., 2011; Yi et al., 2013). Additional studies are needed to clarify the pro-metastatic role of ANGPTL4 and its underlying mechanism of action. Previous studies suggest that ANGPTL4 may have both homotypic and heterotypic influences in the tumor microenvironment. In fact, these observations reinforce the importance of delineating the physiological functions of the different ANGPTL4 fragments.

The roles of ANGPTL4 in vascular permeability and metastasis remains controversial. The apparent discrepancies observed in previous studies can be attributed to the different experimental approaches and models utilized in each study. Moreover, the functions of ANGPTL4 may be affected by the microenvironment, suggesting context and tissue-specific activities. Presently, the lack of structural information for ANGPTL4 limits our ability to explain these differences. Unfortunately, numerous studies on the roles of ANGPTL4 in cancer have not addressed the post-translational modification and proteolytic processing of ANGPTL4. For example, the expression of different ANGPTL4 fragments in transgenic mice remains unknown. As discussed above, the differential processing of ANGPTL4 can significantly influence its biological functions in various contexts and may have contributed to the observed discrepancies. This possibility is supported by evidence showing that ANGPTL4 processing in humans appears to be tissue
dependent, as different organs, such as the liver and kidney, utilize different forms of ANGPTL4 (Clement et al., 2011). A recent study has shown that the glycosylated form of ANGPTL4 augments the leakiness of the kidney glomerular epithelium, suggesting that post-translational modifications of ANGPTL4 may direct its functions (Clement et al., 2011). Further in vitro and in vivo experiments are necessary to clarify the role of ANGPTL4 in metastasis.

1.5.6.2.1 ANGPTL4 and anoikis resistance

The abnormal growth pattern of tumor cells indicates myriad physiological stresses that may trigger programmed cell death or apoptosis to halt cancer progression. However, tumor cells evade apoptosis via multiple deregulated mechanisms, and resistance to anoikis is a hallmark of cancer (Hanahan and Weinberg, 2011).

An early study showed that ANGPTL4 acted as a negative regulator of apoptosis, which suggests that cancer cells may employ mechanisms to resist apoptosis (Kim et al., 2000). Indeed, ANGPTL4 has been shown to contribute to anoikis resistance in hepatoma cells grown in a detached state. These hepatoma cells formed a synokis-like multicellular aggregate that was resistant to inducers of apoptosis. The suppression of ANGPTL4 by RNA interference (RNAi) enhanced cancer cell apoptosis and sensitized cells to drug treatment; however, the exact underlying mechanism is unknown (Zhang et al., 2008). Tumor-derived cANGPTL4 was identified as a novel player in a redox-mediated survival mechanism that was acquired by tumor cells to facilitate cancer progression through the modulation of ROS (Zhu et al., 2011). Tumor-derived cANGPTL4 interacts with integrins β1 and β5 to modulate O₂⁻ levels through NADPH oxidase to maintain an
elevated, oncogenic O$_2$·H$_2$O$_2$ ratio in tumors. This cANGPTL4-integrin interaction activates Src kinase by oxidation and consequently stimulates the pro-survival PI3K/PKB$\alpha$ and ERK pathways to sustain attachment-independent survival. Importantly, the suppression of ANGPTL4 via RNAi or immunosuppression by a monoclonal antibody (mAb11F6C4) modulates intracellular ROS generation to attenuate the tumor growth that is associated with enhanced apoptosis in vitro and in vivo (Zhu et al., 2011). Further supporting this finding, it was recently shown that cANGPTL4 mediates the phosphorylation of ERK and Src kinase to upregulate STAT1 expression, leading to enhanced colorectal cancer cell proliferation under hypoxic conditions (Kim et al., 2011). Despite the loss of cell-matrix attachment, the hijacking of integrin-mediated signaling by tumor-derived cANGPTL4 provides an attractive alternative survival mechanism that is important for cancer cells during metastasis. This finding suggests that anticancer strategies focusing on anoikis or redox-based apoptosis in tumors may be viable. However, additional studies are needed to validate the role of cANGPTL4 in anoikis. The effect of cANGPTL4 on other pro-survival pathways during anoikis, such as the JAK/STATs pathway, should also be investigated. Furthermore, EMT, the initiating event of metastasis, appears to be essential for resistance to anoikis. Given the pro-metastatic roles of ANGPTL4 and its involvement in anoikis resistance, it will be interesting to explore the possible roles of ANGPTL4 during EMT.

1.5.6.2.2 ANGPTL4 and angiogenesis

The oxygen consumption of rapidly growing tumor cells may outpace an insufficient oxygen supply, leading to a pathological condition known as hypoxia (Bertout et al.,
The hypoxic environment triggers the onset of pathological angiogenesis, during which new blood vessels are formed to supply oxygen and nutrients and to remove metabolic waste products to support tumor survival. Angiogenesis is a multistep process that is regulated by angiogenic factors such as VEGF, ANG and more recently, ANGPTL4. ANGPTL4 is a prominent gene with a hypoxic signature \textit{in vivo} and has been found to be highly expressed in distant metastases compared with primary tumors or regional metastases (Hu et al., 2009). The increased expression of cANGPTL4 in cancer also correlates highly with the expression of hypoxia-inducible factor 1α (HIF-1α) (See 1.5.6). These observations suggest that ANGPTL4 could be a key modulator in tumor angiogenesis in the hypoxic tumor microenvironment.

A previous study has indicated that ANGPTL4 mRNA and protein levels were elevated in endothelial cells in response to hypoxia and exerted a VEGF-independent pro-angiogenic effect (Le Jan et al., 2003). This finding was further corroborated by a study demonstrating the upregulation of ANGPTL4 by a viral G protein-coupled receptor (vGPCR) in Kaposi’s sarcoma using a transgenic mouse model (Ma et al., 2010). The inhibition of ANGPTL4 reduced neovascularization and vascular leakage \textit{in vitro} as well as vGPCR-induced tumorigenesis \textit{in vivo}. Similar pro-angiogenic properties of ANGPTL4 have been reported in human gliomas and in head and neck carcinomas (Katanasaka et al., 2013; Zhou et al., 2014). Notably, the pathological angiogenesis observed in Kaposi’s sarcoma and gliomas were attributed to tumor-derived ANGPTL4, suggesting a paracrine influence on neovascularization. In contrast, autocrine signaling of endothelial-derived ANGPTL4 following simulation with tumor-derived Semaphorin 4A
has also been described in head and neck cancers (Zhou et al., 2014). Interestingly, all 3 studies highlighted the increase in tumor vasculature leakiness accompanying the pro-angiogenic functions of ANGPTL4 and suggested that this phenomenon could enhance metastatic dissemination. Importantly, all of these findings suggest pro-angiogenic roles for ANGPTL4 during tumorigenesis.

Conversely, anti-angiogenic roles of ANGPTL4 have also been reported. ANGPTL4 acts as an anti-angiogenic factor by inhibiting endothelial cell proliferation, chemotactic activity and tubule formation, which are important processes in vascularization (Okochi-Takada et al., 2014; Ito et al., 2003). These ANGPTL4-mediated effects result in impaired neovascularization, which decreases the number of capillary vessels invading into tumor masses and thus limits tumor growth. Similarly, ANGPTL4 has also been reported to be important in acute myocardial infarction and retinopathy, in which the absence of ANGPTL4 increases vascular angiogenesis and leakiness. Interestingly, the same group also reported a pro-angiogenic effect of ANGPTL4, further complicating understanding of the roles of this protein during angiogenesis (Le Jan et al., 2003; Galaup et al., 2006; Cazes et al., 2006). In a separate study, N-glycosylated cANGPTL4 was found to suppress the Raf/MEK/ERK signaling cascade in endothelial cells, impairing bFGF- and VEGF-induced angiogenesis (Yang et al., 2008). These findings suggest tissue- and context-dependent roles of ANGPTL4 during angiogenesis. Furthermore, the mode of ANGPTL4 induction could also affect its downstream effects in tumor vascularization. The mechanism(s) by which ANGPTL4 mediates angiogenesis remain unresolved, and it is critical that the observed discrepancies are resolved.
1.6 Endothelial cell-cell junctions

The endothelium serves as a selective and semi-permeable barrier between the blood and the underlying tissue. Paracellular and transcellular pathways govern the transport of solutes, blood proteins and cells across the endothelium, controlling endothelial permeability (Komarova and Malik, 2010). Paracellular permeability is maintained by endothelial junctions that connect adjacent endothelial cells (ECs) to a tight monolayer that prevents the movement of large proteins greater than 3 nm across the vessel wall and into the surrounding interstitial space (Bazzoni and Dejana, 2004). Adherens junctions (AJs) and tight junctions (TJs) are the two types of interendothelial junctions present and are critical for the maintenance of endothelial barrier integrity (Bazzoni and Dejana, 2004; Dejana, 2004; Figure 13). AJs are thought to be the most abundant junctions between endothelial cells and play critical roles in regulating paracellular permeability (Corada et al., 1999). Other proteins, such as platelet endothelial cell adhesion molecule (PECAM) and junction adhesion molecule (JAM), are also found in the endothelial junction, but their roles in paracellular permeability remain unclear (Weber et al., 2007; Shasby, 2007). However, recent studies have implicated JAM-C in the destabilization of endothelial junctions by affecting AJ organization (Orlova et al., 2006; Li et al., 2009).

The architecture of the inter-endothelial junction varies in different vascular systems. In the brain, the blood brain barrier (BBB) prevents the movement of molecules across the barrier, and ECs are found to be tightly associated with well-defined TJs and AJs. In normal vessels, AJs and TJs are more loosely arranged, with AJs intermingling with TJs (Bazzoni and Dejana, 2004). During development, AJs are formed in the vasculature...
before TJs and are thought to be more important in regulating EC-EC adhesion (Komarova and Malik, 2010). Homophilic interactions between vascular endothelial cadherin (VEC), the major component of AJs, have been shown to regulate the expression of the TJ-specific adhesion protein claudin-5 at the transcriptional level (Taddei et al., 2008).

1.6.1 Adherens junctions

AJs are formed by the cadherin family of transmembrane proteins, which mediate homophilic adhesion between adjacent ECs. In ECs, cell-to-cell adhesion is mediated by VECs, the expression of which is exclusive to endothelial cells (Lampugnani et al., 1992; Figure 13). Crystal structure studies have revealed that VEC clustering is critical to initiate homophilic head-to-head contacts with VECs expressed on adjacent EC to form AJs (Angst et al., 2001). Deletion of the VEC gene resulted in an embryonic lethal phenotype in mice, and this effect was attributed to vascular collapse. The inhibition of VEC homophilic interactions using an anti-VEC antibody resulted in vascular disruption and impaired angiogenesis (Gory-Fauré et al., 1999; Corada et al., 1999; Corada et al., 2002).

VECs are characterized by five extracellular repeats, a transmembrane domain and a cytoplasmic tail (Takeichi, 1990). The extracellular domains are responsible for homophilic interactions, with extracellular repeats 1 playing an important role in mediating the head-to-head interaction with adjacent VECs (Taveau et al., 2008). Following the establishment of AJs, the cytoplasmic tail of the VEC binds to β-catenin and p120, which in turn recruits α-catenin and zona occludin-1 (ZO-1) to anchor the AJs.
to the actin cytoskeleton, thus stabilizing AJ integrity (Taveau et al., 2008; Figure 13). The binding of β-catenin masks the PEST domain of VEC and prevents ubiquitin ligase from targeting VECs for proteosomal degradation, while p120 stabilizes VEC at the cell surface by preventing internalization (Huber et al., 2001; Davis et al., 2003). In addition to the establishment of AJs, such anchorage also allows the dynamic opening and closing of EC junctions by the actin cytoskeleton to regulate paracellular trafficking (Komarova and Malik, 2010).

1.6.2 Tight junctions

TJs are found at most apical regions of the ECs and appear to mediate the fusion of adjacent EC membranes (Farquhar and Palade, 1963). In the BBB, where paracellular permeability is restricted, the TJs are well developed compared with those found in the peripheral vascular system, which maintains tissue homeostasis through the constant flux of solutes, plasma proteins and leukocytes (Simionescu et al., 1975). TJs cooperate with AJs to control cell-cell adhesion, communication and paracellular permeability (Bazzoni and Dejana, 2004). TJs consist of numerous integral membrane proteins and associated intracellular components. Occludin was the first TJ protein to be identified; it consists of 2 extracellular loops, 4 transmembrane domains and 2 cytoplasmic regions (Furuse et al., 1993). The first extracellular loop is important for mediating intercellular adhesion and maintaining endothelial barrier functions, and the cytoplasmic domain of occludin associates with ZO-1 to anchor TJs to the actin cytoskeleton (Furuse et al., 1994; Van Itallie and Anderson, 1997; Figure 13). In addition to controlling intercellular adhesion, occludin is also involved in leukocyte diapedesis (Huber et al., 2000).
In addition to occludin, claudins have also been identified as major components of TJs. There are over 20 different claudins that participate in epithelial and endothelial TJs; however, only claudin-5 is exclusively expressed in endothelial cells (Morita et al., 1999; Morita et al., 1999). Like occludin, claudins consist of 2 extracellular loops, 4 transmembrane domains and 2 cytoplasmic tails (Morita et al., 1999; Figure 13). Mutation and deletion studies of claudins have underscored their role in regulating inter-endothelial adhesion (Morita et al., 1999). Ectopic expression of claudin-5 in MDCK cells results in the formation of TJs and the establishment of transepithelial electrical resistance (TER), which is an indication of enhanced intercellular adhesion (Furuse et al., 2001). Additionally, mutation of the first extracellular domain (ECD-I) abolishes this effect, highlighting the importance of ECD-I in mediating homophilic interactions between claudin-5 on adjacent cells (Morita et al., 1999). Claudins also interact with ZO-1 and anchor TJs to the cytoskeleton (Itoh et al., 1999).

Junction adhesion molecules (JAMs) are also components of TJs (Figure 13). JAMs include JAM-A, JAM-B and JAM-C, which have potentially contradictory functions (Aurrand-Lions et al., 2001; Arrate et al., 2001). The ectopic expression of JAM-A in Chinese hamster ovary (CHO) cells results in the formation of TJs, and blocking antibodies against JAM-A result in the disruption of TJs and a consequent decrease in TER (Martin-Padura et al., 1998; Liang et al., 2000). In contrast, JAM-C appears to enhance paracellular permeability. Ectopic expression of JAM-C results in increased paracellular permeability in epithelial cells and facilitates the diapedesis of leukocytes,
possibly by disrupting AJs (Aurrand-Lions et al., 2001; Johnson-Léger et al., 2002). The function of JAM-B remains unclear.

Figure 13: Schematic representation of endothelial barrier integrity. Endothelial cells expressing VE-cadherin at the adherens junctions under quiescent conditions. Claudin-5, occludin and JAMs makes up the tight junctions adhesion molecules. The organization of adherens and tight junctions, which include VE-cadherin and claudin-5, respectively, and their crosstalk through ZO-1 and JAMs, controls the integrity of the endothelial barrier. Disruption of the junctions, trigger ZO-1 translocation from the membrane into the cytosol. Reprinted by permission from Macmillan Publishers Ltd: VE-cadherin and claudin-5 it takes two to tango, copyright 2008.

1.6.3 Integrin and EC-EC junction

In addition to AJs and TJs, integrins have also been reported to be important for maintaining endothelial junction integrity. Integrin α2β1 is predominantly expressed by ECs and has been found to localize to EC junctions (Lampugnani et al., 1991). Incidentally, integrin α5β1 was also identified at EC junctions, whereas all other integrins were absent. This observation suggests that integrins regulate cell adhesion and paracellular permeability. Indeed, the localization of these integrins to EC borders depends on the formation of AJs and TJs. Following recruitment to the EC membrane, integrin α5β1 binds to the extracellular matrix, laminin, vitronectin and fibronectin to
modulate EC junction integrity (Lampugnani et al., 1991). Blocking the functions of integrin α5β1 with a neutralizing antibody compromises EC monolayer integrity, leading to an increase in paracellular permeability. However, endothelial lesions were not observed, which suggests that AJs and TJs are not significantly affected (Lampugnani et al., 1991).

1.6.4 Regulation of paracellular permeability

External stimuli, such as VEGF and inflammatory mediators, including TNF-α and histamine can regulate the paracellular permeability of the endothelium to facilitate the transmigration of leukocytes into inflammatory sites, formation of new blood vessels or maintenance of tissue homeostasis (Komarova and Malik, 2010). Such events involve temporal or spatial modulation of paracellular permeability (Dejana et al., 2009). Under pathological conditions, such as chronic inflammation and tumorigenesis, mediators and cytokines that are involved in vascular leakiness are often upregulated, resulting in a chronic increase in paracellular permeability (Vandenbroucke et al., 2008). External mediators involved in modulating paracellular permeability primarily affect the integrity of inter-endothelial junctions via 2 distinct mechanisms. First, these mediators can alter the functions of the AJ component, e.g., VEC, and trigger the dissociation and internalization of the VEC clusters, leading to disruption of AJs. Second, they can alter the actin cytoskeleton to generate mechanical forces that pull adjacent cells apart. The pro-permeability effects of these mediators can either be transient or sustained (Mehta and Malik, 2006). In the case of proinflammatory mediators such as histamine and thrombin, there is a transient increase in paracellular permeability. Histamine binds to its cognate
histamine receptor H1 to activate the downstream src-mediated phosphorylation of VEC, leading to the dissociation of AJs. Thrombin affects PAR-1 on endothelial cells to transiently enhance vascular leakiness (Vu et al., 1991; Pober and Sessa, 2007). In the presence of a pro-angiogenic cytokine such as VEGF, there is a sustained increase in paracellular permeability. VEGF binds to VEGF receptor 2 and sustains the downstream activation of c-src, triggering the phosphorylation of VECs and leading to their dissociation and internalization, which disrupts EC-EC junctions (Bates and Harper, 2002).

The intracellular domains of the AJ and TJ integral membrane proteins associate with numerous signal transduction proteins that propagate the effect of external stimuli on paracellular permeability. In particular, the small Rho GTPase Rac and its downstream p21-activated kinase (PAK) have emerged as central regulators of endothelial barrier functions (Stockton et al., 2004; Miyamoto et al., 2004; Hoang et al., 2011). Rac has three isoforms, Rac1, -2 and -3, which switch between active GTP-bound forms or inactive GDP-bound forms. Conversion between forms is controlled by cognate guanine exchange factors (GEFs) and GTPase activating proteins (GAPs) (Olofsson, 1999; Rossman et al., 2005). In ECs, monolayer integrity is controlled predominantly by Rac1 (Tan et al., 2008). The formation of homophilic contacts between VECs results in the recruitment of T-cell lymphoma invasion and metastasis-inducing protein 1 (TIAM-1), a Rac GEF, to EC junctions. Furthermore, Rac-GTP is activated to stabilize EC junctions (Abraham et al., 2009). Similarly, Rac1 activation can prevent the pro-permeability effect of thrombin (Mehta et al., 2001). In contrast, Rac activation by VEGF signaling results in the
destabilization of EC junctions and promotes VEC internalization (Gavard and Gutkind, 2006). However, ANG-I suppresses VEGF-induced paracellular permeability via Rac1 activation, suggesting that the differential roles of Rac in endothelial barrier functions may be dependent on the type of primary stimulus (Hoang et al., 2011). PAK, which functions downstream of Rac, mirror the effects of Rac. In EC, PAK activation augments paracellular permeability, implicating PAK in the regulation of endothelial barrier functions (Stockton et al., 2004). Activated PAK also phosphorylates myosin-light chain kinase, leading to cytoskeletal contractions that affect the integrity of EC-EC junctions (Stockton et al., 2004). These observations suggest that the role of Rac/PAK signaling axis in endothelial barrier functions are unclear and that different subpopulations of Rac may be present to regulate endothelial permeability.
Chapter 1

Metabolic reprogramming by Angiopoietin-like 4 is required for Epithelial-Mesenchymal Transition
Abstract

Epithelial-mesenchymal transition (EMT) induced by microenvironment stimuli can be attributed to the transcriptional regulation of epithelial and mesenchymal phenotypes. Here we show how EMT is coordinated with cancer metabolism, an emerging hallmark of tumorigenesis. Using three in vitro and an in vivo EMT models, we observe that EMT in gastric carcinoma is an energy-demanding process fuelled by ATP derived from glucose metabolism. We further identify ANGPTL4 as an important driver of cancer cell metabolism within a larger gene program for EMT. Unbiased kinase inhibitor screens reveal that ANGPTL4 differentially regulates the expression of the 14-3-3 adaptor protein family. Thus, we present an important mechanism by which ANGPTL4 coordinates cellular energy flux crucial for EMT via an ANGPTL4:14-3-3 signalling axis.
Introduction

Metastasis, the spread of cancer cells to distant tissues and/or organs, is a complex and multi-step process that renders it a challenging therapeutic target (Chiang and Massagué, 2008; Steeg and Theodorescu, 2008). Importantly, metastasis accounts for more than 90% of the cancer mortality rate, highlighting the significance of the metastatic event (Jemal et al., 2011). The initiation of the metastatic process is considered a rate-limiting event in metastasis and highly resembles the epithelial-mesenchymal transition (EMT) that occurs during embryogenesis and wound healing (Chiang and Massagué, 2008; Steeg and Theodorescu, 2008; Orr et al., 2000; Mendoza and Khanna, 2009; Klymkowsky and Savagner, 2009; Savagner, 2001). During cancer EMT, epithelial cells lose their cell polarity and cell-cell adhesions are weakened as they adopt a mesenchymal phenotype that is accompanied by cytoskeletal remodeling, and ultimately gain motility and the ability to invade distant organs (Goh et al., 2010; Greenburg and Hay, 1986). The exponential growth of the primary tumor exposes cancer cells to various microenvironmental stresses, including hypoxia, acidic and inflammatory milieus (Stockinger et al., 2001). Interestingly, while these constraints can kill cancer cells, they also exert selective pressures on cells to exploit adverse microenvironments by modifying their cellular behavior and selecting for a highly invasive phenotype that facilitates metastasis (Valdés et al., 2002). Microenvironmental stimuli can initiate the EMT process by controlling the expression and function of the specific transcription factors like Snail, Slug, Twist and ZEB-1, which are pivotal for EMT (Chiang and Massagué, 2008; Thiery and Sleeman, 2006; Perl et al., 1998). Hence, the attenuation of EMT becomes paramount to target metastasis.
Cancer metabolic reprogramming has been recognized as one of the ten cancer hallmarks (Hanahan and Weinberg, 2011). Our current understanding of cancer metabolism is based primarily on the comparison of the metabolic statuses between primary tumors and normal healthy cells. Cancer cells derive a substantial amount of ATP from aerobic glycolysis, i.e. the Warburg effect, rather than oxidative phosphorylation to support their anabolic growth and proliferation (Vander Heiden et al., 2009). Besides glycolysis, cancer cells are also observed to exploit glutaminolytic flux, amino acid and lipid metabolism, mitochondrial biogenesis, pentose phosphate pathway and macromolecular biosynthesis (Ward and Thompson, 2012; Dang and Semenza, 1999). Recent studies have also suggested that oncogenes and tumor suppressors function as critical modulators of metabolic reprogramming to support tumor progression (Dang et al., 1997; Jones and Thompson, 2009). Hypoxia and TGF-β, which are known initiators of EMT, can also modulate cancer cell metabolism during tumorigenesis (Lunt et al., 2009; Finger and Giaccia, 2010). Thus, it is conceivable that these microenvironmental signals may also alter cancer cell bioenergetics to increase their motility and enhance their invasive capabilities. Despite the significance of EMT in metastasis, little is known about the changes in cellular bioenergetics that occur during EMT and whether such metabolic reprogramming is a functional pre-requisite for EMT. Furthermore, the molecular drivers of EMT metabolic reprogramming and the mechanism that coordinates this change in energy remain unknown.

Our current study aims to identify and define the role of drivers of EMT metabolic reprogramming. Comparative microarray analysis of three *in vitro* EMT models revealed
that genes involved in ROS and metabolic regulations were enriched. Notably, the expression of angiopoietin-like 4 (ANGPTL4), a recently identified pro-metastatic gene was elevated in several gene clusters. We further showed that ANGPTL4 accelerated EMT, while its deficiency delays the EMT process. The underlying mechanism involves the regulation of adaptor protein 14-3-3 to synchronize EMT and changes in cellular bioenergetics.
Materials and Methods

Antibodies

Antibodies for E-cadherin, pan-AKT, p-AKT (S473), p-AKT (T308), AMPK, p-AMPK (T172), TSC2, p-TSC2, mTOR and various 14-3-3 subtypes (Cell Signaling, USA); N-cadherin and Snai2 (Millipore, USA); β-tubulin, Snail1, HIF-1α and 14-3-3σ (Santa Cruz, USA); Vimentin, laminin 332 and mouse monoclonal anti-human cANGPTL4 mAb11F6C4 (Abcam, USA); GLUT-I and IRdye 680-conjugated secondary / antibodies (Thermo Scientific, USA); Alexa Fluor 488-conjugated secondary antibodies and Alexa Fluor 594-conjugated phalloidin (Molecular Probes, USA). Rabbit polyclonal a anti-human cANGPTL4 were produced in-house.

Cell culture

MKN78 human gastric cancer cell line (ATCC, USA) were cultured in RRPMI-1640 supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37 °C. All other cell lines were cultured in DMEM supplemented with 10% FBS. MKN78ΔANGPTL4 were generated as per manufacturer’s protocol (Thermo Scientific, USA) with DhāarmaFECT transfection reagent and Smartpool siRNA against ANGPTL4 previous chαaracterized (Goh et al., 2010). After transfection, MKN78ΔANGPTL4 were rested for 48 h hours before any experiments. For experiments, cells were seeded at a density of 5.25×10³ cell/cm² to allow the formation of individual colonies before indicated treatments were performed.
Retrovirus transduction

MKN78Snai1ER cells were generated using retroviral transduction as previously described (Mani et al., 2008). Briefly, HEK 293T cells were transfected with pCL-10A1 retrovirus packaging vector (Imgenex, USA) and pWZL-Snail-ER (Addgene, USA). The pseudoviruses were collected and used to infect MKN78 cells. Following transduction, cells were selected with 5 μg/mL of blasticidin hydrochloride (Sigma Aldrich, USA).

Generation of dual-inducible MKN78Snai1ER/shANGPTL4 cell line

Doxycycline-inducible pSingle-tTS-shRNA vector carrying either shANGPTL4 or scrambled shRNA was introduced into parent MKN78Snai1ER cells using Fugene HD reagent as per manufacturer protocol (Promega, USA). MKN78Snai1ER/shANGPTL4 and MKN78Snai1ER/shscrambled cells were selected with 1 mg/mL of G418 for G418 resistance (Sigma Aldrich, USA). The efficiency of knockdown was verified by real-time PCR and immunoblotting after treatment with various concentrations of doxycycline (up to 10 μg/mL).

In vitro EMT model

Hypoxia treatments were carried out in hypoxic chamber (Stem cell Technology, USA) purged with 5% CO2 and 95% N2 to obtain O2 concentration at 1% as determined by an O2 sensor. For TGF-β-induced EMT model, cells were treated with 10 ng/mL of TGF-β over a period of 2 days. For direct induction of snail-mediated EMT, MKN78Snai1ER/shANGPTL4 and MKN78Snai1ER cells were exposed to 20 ng/mL of 4-hydroxytamoxifen (4-OHT) over a period of 4 days.
Expression and purification of recombinant cANGPTL4 proteins

The expression and purification of recombinant cANGPTL4 were performed as previously described (Goh et al., 2010).

In vivo EMT model

Six-week-old BALB/c athymic female nude mice (20-22 g) were injected subcutaneously with either $1 \times 10^6$ MKN78Snai1ER/shANGPTL4 or MKN78Snai1ER/shscrambled cells resuspended in matrigel. A week after xenograft was established, each xenograft group was randomly divided into four subgroups. Two subgroups received intraperitoneal injection of 4-OHT (4 mg/kg) and were fed on either normal chow diet or Dox-diet (625 mg/kg, Harlan Laboratories, USA). The other two subgroups received vehicle and similarly fed on the two different diets. Injection was done twice weekly using sunflower seed oil as carrier for 4-OHT. After 8 weeks, the mice were sacrificed and the lungs were harvested for further analysis.

Chromatin-immunoprecipitation (ChIP) and Re-ChIP

ChIP experiments were carried out as previously described with slight modifications (Montagner et al., 2014). Briefly, chromatin was cross-linked using 0.5% formaldehyde for 10 min at 37°C and sonicated in SDS lysis buffer (1% SDS, 10mM EDTA, and 50mM Tris-HCl, pH 8.1) to obtain DNA fragments of 200-500 bp. Ten percent of the supernatant was retained as input, while the remaining amount was immunoprecipitated using anti-Snail antibody (Santa Cruz, USA) and immunocomplexes were affinity precipitated by Protein A/G Sepharose (Santa Cruz, USA). Re-ChIP was performed by subsequent probing with
anti-14-3-3γ antibody (Thermo Scientific, USA). DNA fragments were reverse cross-linked at 65 °C for 6 h. The ChIP primer sequences are listed in the table below.

Table 1. Primer sequences.

<table>
<thead>
<tr>
<th></th>
<th>Forward (5' -&gt; 3')</th>
<th>Reverse (5' -&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail-binding region</td>
<td>TAGAGGGTCACCGCGTCTAT</td>
<td>GGCTGGAGTCTGAACTGACT TC</td>
</tr>
<tr>
<td>Negative Control</td>
<td>TCTAGGAGCAGCAGGAAGG GAG</td>
<td>GTCCCTTTTCATCTCCTGGCA AG</td>
</tr>
</tbody>
</table>

**Immunofluorescence staining**

EMT was identified by immunofluorescence staining for E-cadherin and Snai2. MKN78 cells were fixed with 4% paraformaldehyde for 10 min, permeablized with 0.2% Triton-X 100 for 10 min and blocked with 3% normal goat serum (NGS) containing 0.05% Triton-X 100 in a humidified chamber for 1 h at room temperature. Following three washes in PBS, cells were incubated overnight at 4 °C with either anti-human-E-cadherin (1:200) or Snail1 (1:100) antibodies in 3% NGS. After three washes, cells were incubated for 1 h at room temperature with Alexa488-secondary antibodies (1:250) and counterstained with Alexa594-phalloidin for F-actin and DAPI for nuclei. Immunostainings performed without primary antibodies served as negative controls. Images were acquired using Zeiss LSM710 confocal microscope with a Plan-APOCHROMAT 40x/1.4 oil objective and ZEN2009 software.
Proximity Ligation Assay (PLA) and analysis

MKN78CTRL or MKN78ANGPTL4 (5 x 10³ cells) were subjected to either normoxia or hypoxia for 2 days at 37 °C. At indicated time, MKN78 cells were fixed, permeabilized and blocked similar to immunofluorescence staining. Next, MKN78 cells were incubated with monoclonal mouse-anti human 14-3-3γ antibody (1:200) and monoclonal rabbit anti-human Snail antibody (1:200) in 3% NGS overnight at 4 °C. PLA was carried out as per manufacturer’s protocol (Olink Bioscience, USA). Images were taken using Carl Zeiss confocal microscope LSM710 using a Plan-APOCHROMAT 63x/1.4 oil DIC objective, and ZEN2012 LE software with constant exposure and gain. Number of protein interaction was quantified using the “BlobFinder” (Allalou and Wahlby, 2009).

Immunoprecipitation and immunoblot

For immunoprecipitation, at the indicated time points after treatments, cells were lysed using IP lysis buffer (25mM Tris-HCl pH7.4, 150mM NaCl, 1% NP-40, 1mM EDTA). The lysate was then incubated with indicated antibodies overnight at 4 °C with constant rotation. Following that, the antibodies were affinity precipitated using protein A/G beads (Santa Cruz, USA). Proteins were released by boiling for 10 min in Lamelli’s buffer. Protein extracts were resolved using 12 % SDS-polyacrylamide gel electrophoresis and electrotransferred (25 mM Tris, 192 mM glycine, 20% methanol) onto a low fluorescence PVDF membrane for immunoblot analysis. Membranes were stripped and re-probed for other proteins as described (Yeung and Stanley, 2009). Protein bands were detected using Odyssey CLx Infrared Imaging system (LI-COR, USA).
ATP quantification

ATP level in the cancer cells were quantified using the ATP determination assay kit (Invitrogen, USA) as per manufacturer protocols. Briefly, ATP was released from the cells using somatic cell lysis buffer (Sigma Aldrich, USA) after treatment for indicated time period. ATP levels were assayed and the luminescence readings were taken using a GloMax 20/20 luminometer (Promega, USA). Luminescence readings were plotted against an ATP standard curve to obtain the actual ATP concentration followed by normalization to the protein concentration.

Energy charge determination

The cellular levels of AMP, ADP and ATP were determined by HPLC. Cells were lysed using boiling milli-Q water (3 x 10⁵ cells: 100 μL of water) to precipitate cellular proteins. Supernatant containing the nucleotides were collected for analysis. Nucleotides standards (Sigma Aldrich, USA) were prepared in milli-Q water and resolved on a Shimadzu LC-20AD series Quaternary Gradient HPLC system with PDA detector using a Hypersil ODS 150 mm x 4.6 mm I.D. C18 reverse-phase column (3 μm particle size) and 5 μm Hypersil ODS C18 guard column (Thermo Scientific, USA). Separation of the nucleotides were carried out with a mobile phase containing 60 mmol/L of KH₂PO₄, 0.45 mmol/L of tetrabutylammonium bromide (TBAB) and 1.26 mol/L of acetonitrile. The pH of the mobile phase was adjusted to 3.20 using concentrated phosphoric acid. Retention times of the individual nucleotides are matched to that of the respective nucleotide standards. Areas under the peaks that correspond to the respective nucleotides were tabulated and energy charge was calculated using the formula:

\[
\text{Energy charge} = \frac{\text{[ATP]} + \frac{1}{2}\text{[ADP]}}{\text{[ATP]} + \text{[ADP]} + \text{[AMP]}}
\]
Glucose uptake assay

Briefly, cells were incubated with 10 μM of 2-NBDG for 12 h. The cells were then harvested and subjected to flow cytometry analysis using BD Accuri C6 flow cytometer (BD Biosciences, USA). For in vivo glucose uptake assay, mice were fasted and simultaneously injected with 100 ul of IRDye 800CW 2-DG (1mg/kg) intravenously via the tail vein 24 h before whole animal imaging.

Metabolic analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in real-time using a XF96 flux analyzer (Seahorse Bioscience). Cells were seeded on 96-well microplates. All measurements were normalized against total protein content from cell lysates.

Kinase Inhibitor Array: RNA Isolation, RT-PCR and Real time qPCR

MKN78 cells were treated with 95 different kinase inhibitors (SYN-2103; SSynkinase, Australia) in the absence and presence of rh-cANGPTL4 (10 μg/ml) for 6 hours. RNA was isolated and reverse-transcribed using the iScript Reverse Transcription Supermix (Bio-Rad, USA). qPCR was then carried out in CFX96™ real-time PCR thermocycler (Bio-Rad, USA), using the KAPA™ SYBR qPCR Universal Master Mix (KAPABiosystems).
### Real-time PCR Primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Sequence (5' to 3')</th>
<th>Reverse Sequence (3' to 5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANGPTL4</td>
<td>TCCAACGCCACCCACTTAC</td>
<td>TGAAGTCATCTCACAGTT GACCA</td>
</tr>
<tr>
<td>CDH1</td>
<td>GCGGAGAGCTACACGTTCA</td>
<td>GACCGGTGCAATCTTCAA A</td>
</tr>
<tr>
<td>DDR1</td>
<td>CACTTCGCTCCCTGTGTC</td>
<td>AGAGGTGCGACTGGAACA A</td>
</tr>
<tr>
<td>ERBB3</td>
<td>CTGATCACCGGCCTCAAT</td>
<td>GGAAGACATTGAGCTTCT CTGG</td>
</tr>
<tr>
<td>Snail</td>
<td>GCCCTCAACTGCAATACTGC</td>
<td>CTTCTTGACATCTGAGTGG GTC</td>
</tr>
<tr>
<td>TBP</td>
<td>GCTGGTTATCGGGAGTTGG</td>
<td>ACTGGCCTGCTGTCTTAG AG</td>
</tr>
<tr>
<td>ZEB1</td>
<td>GCCAACAGACCCAGACTGTT</td>
<td>TCTTGCCCTTCCTTTCCT G</td>
</tr>
<tr>
<td>14-3-3α/β</td>
<td>GGCAAAGAGTACCGTGAGAAG CTGGTTGTGT AGCA\TTGG GAATA</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>14-3-3γ</td>
<td>AGCCACTGTGAATGAGGAAC CTGCTCAATGCTACTTGAT GACC</td>
<td></td>
</tr>
<tr>
<td>14-3-3ε</td>
<td>GATTCGGGAATATCGGCAAATGG GCTGGAATGAGGTGTTTG TCC</td>
<td></td>
</tr>
<tr>
<td>14-3-3η</td>
<td>TGGCTGATGGAAACGAAGAAAGAA CCTCTGCTAAGTAGGCGGT AGT</td>
<td></td>
</tr>
<tr>
<td>14-3-3σ</td>
<td>TGACGACAAGAAGCGCATCAT GTAGTGGAAGACGCGAAAA GTTCA</td>
<td></td>
</tr>
<tr>
<td>14-3-3τ</td>
<td>AGGGTCATCTCTAGCATCGAG CCACTTTCTCCCGATTAGTC CTT</td>
<td></td>
</tr>
<tr>
<td>14-3-3ζ</td>
<td>CCTGCATGAAGTCTGTAACTGAG GACCTACGGGCTCCCTACA ACA</td>
<td></td>
</tr>
</tbody>
</table>

Statistical Analysis

Statistical analyses were performed using two-tailed Mann-Whitney tests with SPSS software. All statistical tests were two-sided. \( p \) value of \( \leq 0.05 \) is considered significant.
Results

Generation of in vitro EMT models

To examine whether EMT is accompanied by changes in cancer cell bioenergetics, we established three in vitro EMT models using the polarized gastric cancer cell line MKN78 (Figure 14). Two models were generated using hypoxia (1% O2; Figure 14A) and inflammation (TGF-β; Figure 14B) in order to mimic the microenvironment of stress-induced EMT. The third model utilized the transgenic MKN78 cell line (MKN78snai1ER), which harbors a Snai1ER transgene that allows the direct initiation of EMT by treatment with 4-hydroxytamoxifen (4-OHT; Figure 14C). Upon exposure to either microenvironmental stress or 4-OHT, the epithelial colonies of MKN78 cells progressed into a mesenchymal-like phenotype after 48 h (Figure 14A and B) and 96 h (Figure 14C), respectively. The E-cadherin staining of the epithelial cell borders diminished as early as 24 h and was more pronounced at 48 h when the cancer cell colonies were scattered, adopting a mesenchymal-like phenotype (Figure 14A-C). The reduced expression of E-cadherin corresponded with increased Snai2 expression, which indicated that EMT had occurred (Figure 14A and B). This EMT phenotype was further confirmed using quantitative PCR and immunoblotting for critical EMT genes. Critical epithelial genes (E-cadherin, CDH1; discoidin domain receptor 1, DDR1; receptor-tyrosine kinase, ErBb3) were downregulated, along with concomitant increase in mesenchymal genes expression (Snai1; zinc finger E-box binding homeobox 1, ZEB-1; Figure 14A-C).
Figure 14. **In vitro** EMT models. (A-C, top panel) Immunofluorescence staining of E-cadherin in hypoxia (A), TGF-β treated MKN78 (B) or 4-OHT treated MKN78Snai2ER cells (C) at indicated time intervals. Cells were counterstained with DAPI (blue) for nuclei and phalloidin (red) for actin cytoskeleton. Scale bar: 40 μm. (A-C, middle panel) Relative expression of EMT related genes in MKN78 cells exposed to hypoxia (A), TGF-β (B) or 4-OHT treated MKN78Snai2ER cells (C) at indicated time intervals. Data (means±S.D) from 3 independent experiments. * P<0.05, ** P<0.01. (A-C, bottom panel) Immunodetection of E-cadherin and Snai2 protein in hypoxia (A), TGF-β treated MKN78 (B) or 4-OHT treated MKN78Snai2ER cells (C) at indicated time intervals.

**EMT induction alters cancer cell bioenergetics**

Next, to identify common molecular drivers of EMT, we performed a comparative microarray gene expression analysis of the three EMT models. We observed an enrichment of genes involved in the regulation of metabolic processes across the 3 EMT models over 48 h, in particular, glucose, fatty acids, autophagy, amino acids and ROS.
metabolism (Figure 15A and B). Real-time PCR and western blot analysis further confirmed the changes in the mRNA and protein levels of selected genes involved in individual metabolic processes (Figure 15C, D, F). Additionally, ROS production was enhanced during EMT (Figure 15G). Our observations suggest that EMT is accompanied by changes in genes involved in metabolic reprogramming, and the overall metabolic phenotype acquired by these cancer cells may influence the initiation of metastasis.
Figure 15. EMT induction alters cancer cell bioenergetics. (A, left panel) Top five most represented biological processes in hypoxia (top), TGF-β (middle) and 4-OHT (bottom) induced EMT models. (A, left panel) Top five most enriched metabolic processes. (B) Heatmap representation of commonly regulated genes across the three EMT models over indicated time points. Green: down-regulated; Red: up-regulated. (C-F) Relative expression (C, E) and immunodetection (D-F) of selected genes involved in the four enriched metabolic processes during hypoxia-induced EMT. (G) Fluorescence measurement of the total ROS content of MKN78 or MKN78<sub>Snai1ER</sub> cells after EMT induction.

**ANGPTL4 augments EMT**

To distinguish driver genes of metabolic reprogramming during EMT from differentially expressed genes associated with cellular metabolism, we performed knockdown experiments followed microarray gene analysis. We identify ANGPTL4 as a potential driver of metabolic changes during EMT as supported by several experimental evidence. Detailed gene profile analysis revealed that the expression of ANGPTL4 was elevated in several gene clusters of all EMT models, suggesting that it may play an important role in cellular bioenergetics during EMT. ANGPTL4 has been recently identified as a pro-metastatic gene (Tan et al., 2012). Therefore, we investigated how ANGPTL4 may influence EMT. We showed that the mRNA and protein levels of ANGPTL4, specifically cANGPTL4, were elevated during EMT in three (mRNA) and two (protein) EMT models respectively (Figure 15D). We observed that >50% of identified genes in our comparative microarray analysis of the EMT models were significant altered upon ANGPTL4 suppression (Figure 15B, E). The presence of neutralizing antibodies against cANGPTL4 (α-cANGPTL4) delayed EMT induction in the 3 EMT models (Figure 16A-C). At 48 and 96 h following EMT induction, the cells retained their epithelial-like morphologies characterised by clear E-cadherin staining and reduced nuclear accumulation of Snai2 (Figure 16A-C). Quantitative PCR and immunoblotting revealed a delay in the expression
of critical EMT genes (Figure 16A-C). Furthermore, the depletion of endogenous ANGPTL4 by siRNA similarly delayed the progression of hypoxia-induced EMT, suggesting that ANGPTL4 might be a critical modulator of EMT (Figure 16A). Conversely, MKN78CTRL treated with recombinant human cANGPTL4 (rh-cANGPTL4) resulted in a partial disruption of E-cadherin staining (Figure 16D). Similarly, the analysis of EMT markers revealed marginal repression of epithelial markers and elevation of mesenchymal markers expressions. These observations suggest that ANGPTL4 can augment the mesenchymal phenotype during EMT.
Figure 16. ANGPTL4 augments EMT. (A-D, left panel) Immunofluorescence staining of E-cadherin in hypoxia (A), TGF-β (B), rh-cANGPTL4 (D) treated MKN78 or 4-OHT treated MKN78SnaiER cells (C) in the presence of antibodies against human cANGPTL4 (α-cANGPTL4) at indicated time intervals. Cells were counterstained with DAPI (blue) for nuclei and phalloidin (red) for actin cytoskeleton. Scale bar: 40 μm. (A-D, middle panel) Relative expression of EMT related genes in MKN78 cells exposed to hypoxia (A), TGF-β (B), rh-cANGPTL4 (D) or 4-OHT treated MKN78SnaiER cells (C) at indicated time intervals. Data (means±S.D) from 3 independent experiments. * P<0.05, ** P<0.01. (A-D, right panel) Immunodetection of E-cadherin, N-cadherin and Snai2 protein in hypoxia treated MKN78CTRL or MKN78ANGPTL4 (A), TGF-β (B), rh-cANGPTL4 treated MKN78 (D) or 4-OHT treated MKN78SnaiER cells (C) exposed to α-cANGPTL4 at indicated time intervals.

ANGPTL4 modulates EMT in vivo

To gain further insights into the role of ANGPTL4 and its biological significance in EMT, we created a dual-inducible EMT xenograft mouse model to investigate the influence of ANGPTL4 on EMT. A doxycycline-inducible shANGPTL4 expression plasmid was introduced into the parental MKN78SnaiER cells to generate the dual-inducible MKN78SnaiER:shANGPTL4 cell line prior to xenograft (Figure 17A). In vivo 2-deoxyglucose imaging showed that 4-OHT treated tumors displayed significantly elevated glucose uptake compared to vehicle-treated tumors (Figure 17B). The suppression of ANGPTL4 in 4-OHT-treated tumors lowered 2-DG uptake to comparable level of vehicle-treated tumors (Figure 17B). Consistent with our above findings, 4-OHT-treated tumors’ expressed higher levels of glucose transporter 1 (GLUT1) than vehicle-treated tumors while ANGPTL4 suppression in 4-OHT-treated tumors resulted in decreased GLUT1 expression (Figure 17C). Importantly, ANGPTL4 was found to be considerably elevated in the xenograft tumors that were exposed to 4-OHT, which induced EMT, while mice fed on doxycycline diet (Dox) showed in vivo suppression of ANGPTL4 in
MKN78Snai1ER:shANGPTL4-derived tumors, thus validating the role of ANGPTL4 in metabolic changes during EMT in vivo (Figure 17C).

Histological analysis of the tumor sections revealed that the control tumors were compact with clear encapsulations of the tumor mass compared with tumors that were exposed to 4-OHT, which displayed a dispersed phenotype (Figure 17D). This dispersed phenotype was diminished after the in vivo suppression of ANGPTL4 via Dox-diet (Figure 17D). We further showed that the 4-OHT-treated tumors displayed a loss of E-cadherin staining at the cell-cell borders together with clear disruption of laminin 332 compared with vehicle control treated tumors or 4-OHT-treated ANGPTL4-deficient tumors (Figure 17D). These observations suggest that some of the cancer cells have undergone EMT. Gelatin zymography revealed elevated activities of pro-invasive matrix metalloproteinases (MMPs)-2 and -9 in 4-OHT-treated tumors, which indicated an aggressive tumor phenotype (Figure 17E). Indeed, mice injected with 4-OHT and on a normal diet harboured significantly more lung metastases compared with either the vehicle control mice or mice treated with 4-OHT fed on Dox diet (Figure 17F). This observation was confirmed by the real-time PCR quantification of human TBP mRNA to identify the presence of human cancer cells in mouse lung sections after 4-OHT induction (Figure 17F). Finally, immunofluorescence analysis of benign and metastatic mouse skin tumor sections revealed that metastatic tumors displayed high ANGPTL4 expression that was correlated to a diminished E-cadherin expression, which underscores the importance of ANGPTL4 during EMT (Figure 17G). Taken together, our observations highlights pro-
EMT role(s) of ANGPTL4 and suggested a novel role of ANGPTL4 in EMT-associated cellular bioenergetics.
ANGPTL4 modulates EMT in vivo. (A) Relative ANGPTL4 expression in MKN78snaiiE R :shANGPTL4 cells at indicated doxycycline concentration. (B) In vivo 2-DG fluorescence imaging of mice bearing tumors that received indicated treatments. (C) Representative macroscopic images of tumors and immunodetection of ANGPTL4 and Glut1 from tumor lysates exposed to the indicated treatments. (D) Representative H&E images and immunofluorescence images of E-cadherin and laminin 332 from MKN78snaiiE R :shANGPTL4 tumor sections exposed to indicated treatments. Scale bar: 40 μm (top) and 100 μm (bottom). (E) Gelatin zymography and quantifications of MMPs activities in indicated tumors. (F) Representative macroscopic images of lungs harbouring MKN78snaiiE R :shANGPTL4 micrometastases and relative expression of human TBP gene from indicated lungs. Black arrows in (F) indicate micrometastases (n=5). (G) Immunofluorescence staining of cANGPTL4 and E-cadherin in benign and metastatic skin tumors. Scale bar: 100 μm

ANGPTL4 regulates cellular bioenergetics during EMT

ANGPTL4 has well-established roles in systemic glucose and lipid metabolism (Kersten, 2014). However, its function in cellular energy homeostasis, particularly during EMT, is unknown. Given our observations above, we questioned if ANGPTL4 can control cellular bioenergetics. During EMT, we observed increased glucose uptake in MKN78CTRL and MKN78snaiiE R cells (Figure 18A, B). Treatment with either α-cANGPTL4 or siRNA against ANGPTL4 (siANGPTL4) diminished glucose uptake (Figure 18A, B). Exogenous rh-cANGPTL4 alone resulted in an elevation of glucose uptake during EMT progression and this effect was associated with an increase in the protein expression of glucose transporter 1 protein (Figure 18A, 19C). Cancer cells are known to exploit aerobic glycolysis to metabolize glucose to lactate instead of pyruvate, resulting in the acidification of the extracellular environment. Interestingly, we detected an increase in extracellular acidification rate, suggesting that glycolysis was augmented during EMT and was dependent on ANGPTL4 (Figure 18C).
To gain further insights, we decided to investigate the energy charge status of the cells, which is indicative of the cellular metabolic activities (Live and Kaminskas, 1975; Ridge, 1972; Atkinson and Walton, 1967). We observed increased energy charges in MKN78CTRL and MKN78SnailER cells during EMT progression, which mirrored the increased glycolytic activity (Figure 19D, F). This increase in energy charge can be suppressed using either α-cANGPTL4 or siANGPTL4, while the addition of rh-cANGPTL4 alone augmented energy charge status during EMT (Figure 18D-F). Importantly, in vivo tumors showed similar elevation of energy charge status (~ 1.5 fold) during EMT progression and this elevation can be diminished through the depletion of ANGPTL4 (Figure 19G). Notably, the changes in cellular energy charge corresponded to the intracellular ATP level measurements, lending further credence to the important role of ANGPTL4 in regulating cellular bioenergetics during EMT (Figure 18H-J).
ANGPTL4 is a molecular driver of EMT metabolic changes

The bioenergetics regulatory role of ANGPTL4 prompted us to examine if ANGPTL4 can modulate the two major bioenergetics regulatory nodes, the PI3K/AKT and AMPK pathways. Hypoxia- and 4-OHT- induced EMT resulted in phospho-activation of AKT and AMPK (Figure 19A, B). Exogenous rh-cANGPTL4 alone also triggered AKT and AMPK activation, while the depletion of ANGPTL4 opposed this effect during hypoxia-induced EMT (Figure 19A, C). Analysis of our in vivo EMT tumors revealed similar phospho-activation of AKT and AMPK that is dependent on ANGPTL4 (Figure 19D). These observations suggest that ANGPTL4 may modulate cellular bioenergetics via PI3K/AKT and/or AMPK pathways. Therefore, dominant-negative and constitutive forms of AKT and AMPK were introduced into MKN78CTRL and MKN78Snai1ER cells (Figure 19E). Constitutive AKT (caAKT) increased energy charge (~1.2 fold) in 4-OHT- and hypoxia-induced EMT models (Figure 19F, G). Conversely, dominant-negative AKT (dnAKT) suppressed the energy charge increase during EMT, thus delaying EMT progression (Figure 19F, G). In the absence of EMT-inducing signals, the colony morphology of MKN78 cells harboring caAKT (MKN78caAKT) and dnAKT (MKN78dnAKT) resembles that of MKN78CTRL cells, albeit lower E-cadherin expression in
MKN78_{\text{caAKT}} cells (Figure 19J). However, under hypoxia, these modified MKN78 cells exhibited a more pronounced EMT phenotype, evident from the mesenchymal morphology and absence of E-cadherin, as early as 24 h after hypoxia exposure as compared to 48 h in cognate MKN78_{\text{CTRL}} cells (Figure 19J, K). Similarly, constitutive (caAMPK) increased energy charge while dominant-negative AMPK (dnAMPK) suppressed energy charge elevation in hypoxia- and 4-OHT-induced EMT (Figure 19H-I). Immunofluorescence staining for E-cadherin of MKN78_{caAMPK} also showed diminished signal 24 h after EMT induction (Figure 19J). Altogether, these observations indicate that the consolidation of cellular bioenergetics may be a pre-requisite for EMT and implicates ANGPTL4 as the molecular controller of cellular bioenergetics to drive EMT.
Figure 19. ANGPTL4 is a molecular driver of EMT metabolic changes. (A-D) Immunodetection of indicated proteins in hypoxia treated MKN78_CTRL or MKN78_AANGPTL4 (A), 4-OHT treated MKN78_SnaI cells (B), rh-cANGPTL4 treated MKN78_CTRL (C) and tumors at indicated time points. (E) Immunodetection of AKT and AMPK in MKN78 cells after indicated overexpression. (F-I) Energy charge measurement in MKN78_CTRL (F, H) or MKN78_SnaI cells harboring the respective modification using HPLC after indicated treatments. * P<0.05; ** P<0.01. (J) Immunofluorescence...
staining of E-cadherin in MKN78 cells with the indicated modification under normoxia or hypoxia. Cells were counterstained with DAPI (blue) for nuclei. Scale bar: 50 μm. (K) Immunodetection of E-cadherin and Snail1 in hypoxia treated MKN78 cells harboring the respective modifications.

ANGPTL4 modulates the expression of specific 14-3-3 subtypes

The 14-3-3 proteins interact with phosphorylated Ser and Thr residues on numerous proteins to regulate many mammalian cellular events (Bridges and Moorhead, 2005). The expression of specific 14-3-3 proteins were elevated by ANGPTL4 during wound healing to enable keratinocytes to adopt a more migratory mesenchymal phenotype and to provide resistance to anoikis in cancer cells; the former is highly reminiscent of EMT and the latter is an essential prerequisite for tumor metastasis (Goh et al., 2010; Zhu et al., 2011; Kim et al., 2012). Thus, we examined the expression of all seven isotypes of 14-3-3 proteins during EMT. In response to EMT-inducing signals, MKN78CTRL and MKN78Snai1ER cells displayed an increased expression of 14-3-3γ, η, τ and a decreased expression of 14-3-3σ, while the expression of 14-3-3β, ε and ζ remained relatively unchanged (Figure 20A, B). Interestingly, the suppression of ANGPTL4 resulted in a significant decrease in the expression of 14-3-3γ compared to 14-3-3η and τ, whereas the exposure of MKN78CTRL cells to rh-cANGPTL4 enhanced 14-3-3γ expression (Figure 20A, C). Quantitative real-time PCR also reveals that the expression of 14-3-3γ, η, and τ mRNA was increased as early as 6 hours following exposure to rh-cANGPTL4 alone (Figure 20D). These observations suggest that ANGPTL4 can modulate the expression of specific 14-3-3 isotypes during EMT.
Different members of 14-3-3 protein family play different roles to coordinate numerous cellular functions (Bridges and Moorhead, 2005), however, little is known about the mechanisms that regulate their mRNA expression. To obtain an insight on which signaling pathways may be involved in ANGPTL4-mediated expression of 14-3-3 isotypes, we performed an unbiased kinase inhibitor array to identify key signalling mediators whose inhibition would suppress ANGPTL4-mediated upregulation of 14-3-3 expression (Figure 20E). With our qPCR data followed by Ingenuity Pathway Analysis, we found that majority of the involved kinases converged on the PI3K/AKT and MAPK signalling cascades (Figure 20E). Several of the downstream transcription factors previously implicated in cancer progression were identified (Figure 20E). Immunoblot analysis showed that three of these transcription factors, namely CREB, cFOS and Stat3, were phosphor-activated during EMT (Figure 20F). These findings suggest that ANGPTL4 could coordinate energy demand during EMT via the differential regulation of specific 14-3-3 isotypes.
Figure 20. ANGPTL4 modulates the expression of specific 14-3-3 isotypes. (A-C) Immunodetection of various 14-3-3 isoforms in MKN78CTRL, MKN78ANGPTL4 and MKN78snai1ER cells under hypoxia- (A) and 4-OHT-induced (B) EMT or in the presence of rh-cANGPTL4 (C) at indicated time intervals. (D) Relative expression of various 14-3-3 isoforms in MKN78 cells treated with rh-cANGPTL4 alone after 6 hours. Data (means±S.D) from 3 independent experiments. * P<0.05, ** P<0.01. (E) Analysis map of kinases (blue) whose inhibition attenuates ANGPTL4-induced 14-3-3 expression and possible downstream transcriptional regulators (red). (F) Immunodetection of identified transcriptional regulators in (E) in MKN78CTRL under hypoxia or treated with rh-cANGPTL4. Immunoblots of phospho-proteins are from different membranes.
ANGPTL4:14-3-3γ axis coordinates energy demand during EMT

We then interrogate whether key signaling mediators of cancer cell metabolism and pivotal EMT transcription factors can interact with 14-3-3 using ANIA database (ANnotation and Integrated Analysis of the 14-3-3 interactome (Tinti et al., 2014). Interestingly, our analysis revealed that tuberin sclerosis complex (TSC2) and Snail transcription factor were highly ranked potential interacting partners of 14-3-3. The phosphorylation status of TSC2 regulates the catabolic/anabolic switch in cells, making it a key signaling mediator of cancer cell metabolism (Ward and Thompson, 2012).

Hypoxia- and 4-OHT-induced EMT resulted in an increase in phosphorylation of TSC2 at Ser939 (TSC2s939), indicating TSC2 inhibition (Figure 21A, B). Similar phosphorylation of TSC2 was also observed after application of rh-cANGPTL4 (Figure 21C). Co-immunoprecipitation and proximity ligation assay (PLA) showed an increase 14-3-3γ:TSCs939 complex formation, further confirming that TSC2 was suppressed upon EMT (Figure 21D, E, F). Conversely, ANGPTL4 deficiency did not result in significant alteration of total TSC2 expression but diminished TSC2s939 phosphorylation and subsequent 14-3-3 binding (Figure 21A, D, E, F). No differences were observed in the levels of 14-3-3γ:pTSC2 between wild-type versus si-control MKN78 cells. These observations further underline the importance of ANGPTL4:14-3-3 as a critical controller of the observed bioenergetics changes upon EMT.
Figure 21. The ANGPTL4:14-3-3γ axis coordinates energy demand during EMT. (A-C) Immunodetection of indicated proteins in hypoxia treated MKN78CTRL or MKN78siANGPTL4 (A), 4-OHT treated MKN78siANGPTL4 (B) and rh-cANGPTL4 treated MKN78CTRL (n=3). (D) Immunodetection of 14-3-3γ with TSC2 and TSC2s939 after immunoprecipitation with TSC2 antibody (n=3). (E, F) Proximity ligation assay (PLA) and quantification of 14-3-3γ:TSC2s939 interactions in MKN78CTRL and MKN78siANGPTL4 cells (E) or EMT tumors (F) under indicated treatments. PLA signals are in red. MKN78 cells were counterstained with dapi (blue) for nuclei and alexa-488 phalloidin for actin cytoskeleton. Scale bar: 40 μm. Data (means±S.D) from 3 independent experiments. * P<0.05, ** P<0.01.
The ANGPTL4:14-3-3γ:Snail axis coordinates EMT

The interaction of 14-3-3 with Snail is known to stabilized Snail binding on the E-cadherin promoter to facilitate transcriptional repression during EMT (Hou et al., 2010). In vitro co-immunoprecipitation and proximity ligation assay (PLA) of Snail and 14-3-3 showed a significant increase in 14-3-3γ:Snail complex formation during hypoxia-induced EMT. This interaction was diminished in the absence of ANGPTL4 (Normoxia MKN78CTRL versus MKN78ANGPTL4: 1.63±0.96 vs 1.72±0.58; Hypoxia MKN78CTRL versus MKN78ANGPTL4: 6.73±1.06 vs 2.31±0.3 PLA spots/nucleus; Figure 22A-C). No change in complex formation between Snail with 14-3-3η or τ was observed. 14-3-3β which does not interact with Snail served as a negative control (Figure 22A). PLA analysis revealed significantly more 14-3-3γ:Snail complexes in 4-OHT-treated tumors than in vehicle-treated tumors from mice fed on a normal diet (Normal diet, 4-OHT versus vehicle: 0.79±0.05 vs 0.12±0.07 PLA spots/nucleus; Figure 22D). Tumor sections from 4-OHT-treated mice fed with a Dox diet displayed fewer 14-3-3γ:Snail interactions than mice on normal diet (Dox diet, 4-OHT: 0.24±0.07, 0.23±0.13 PLA spots/nucleus; Figure 22D). We further showed that reduction of the 14-3-3γ:Snail complex in MKN78ANGPTL4 resulted in a decrease in Snail occupancy of the E-cadherin promoter during hypoxia-induced EMT, suggesting that the repression of E-cadherin gene expression was impaired; thus, EMT progression was delayed in the absence of ANGPTL4 (Figure 22E). Altogether, our data revealed a hitherto unknown mechanism by which ANGPTL4 coordinates EMT-associated energy demand via 14-3-3γ expression and also offer additional mechanistic perspective on ANGPTL4:14-3-3:Snail regulation of EMT.
Figure 22. The ANGPTL4:14-3-3γ:Snail axis coordinates EMT. (A, B) Immunodetection of indicated 14-3-3 isoforms with Snail after immunoprecipitation with either Snail or pan 14-3-3 antibodies. (C, D) Proximity ligation assay (PLA) and quantification of 14-3-3γ and Snail1 interactions in MKN78CTRL and MKN78ANGPTL4 cells (C) or xenograft tumor biopsies (D) under indicated treatments. PLA signals are in red. MKN78 cells were counterstained with dapi (blue) for nuclei and alexa-488 phalloidin for...
actin cytoskeleton. Scale bar: 40 μm (C), 50 μm (D). Data (means±S.D) from 3 independent experiments. * P<0.05, ** P<0.01. (E) Representative ChIP results using anti-Snail (E, top panel) followed by re-ChIP with anti-14-3-3γ (E, bottom panel) performed in MKN78CTRL and MKN78ANGPTL4 cells under normoxia or hypoxia. Preimmune serum (IgG) served as control.

Mechanistic overview of ANGPTL4:14-3-3 axis coordinating cancer cellular bioenergetics and EMT

We showed that microenvironment signals induce cancer cells’ EMT. We observed an increase in cancer cells metabolic activities during EMT, together with the characteristic increase in mesenchymal markers and decrease in epithelial markers. Importantly, we identified that ANGPTL4 was a key player during EMT. We showed that ANGPTL4 activated AKT to enhance cancer cells metabolic activities. Similarly, ANGPTL4 was also demonstrated to increase specific 14-3-3 isotype to coordinate cellular bioenergetics crucial for EMT. The 14-3-3 proteins bound to and inactivated TSC2, which resulted in the enhancement of cellular metabolic activities. Concurrently, 14-3-3 proteins bound and stabilized Snail binding to E-cadherin promoter, allowing the repression of the critical epithelial protein to further augment EMT.
Figure 23. Mechanistic overview of ANGPTL4:14-3-3 axis coordinating cancer cellular bioenergetics and EMT. Cancer cells undergoing EMT displayed an increased in metabolic activities, together with an increased in mesenchymal markers and decreased in epithelial markers. During EMT, ANGPTL4 is elevated. The presence of ANGPTL4 activates AKT to upregulates cancer cell metabolism either directly or via suppression of TSC2 activities. These overwrite the inhibitory influence of TSC2 on cancer cell metabolic activities. Notably, ANGPTL4 is able induce the expression of specific 14-3-3 protein isotype to coordinate the change in cellular bioenergetics and EMT. The ANGPTL4:14-3-3 axis synchronizes the suppression of TSC2 and the stabilization of Snail to enhance cancer cell metabolic activities and epithelial genes suppression to augment EMT.
Discussion

Epithelial-mesenchymal transition (EMT) is a rate-limiting step in the initiation of metastasis and confers invasive characteristic to cancer cells (Klymkowsky and Savagner, 2009). The exposure of cancer cells to microenvironmental stresses, such as hypoxia and inflammation, acts as triggers that select for EMT-competent cancer cells (Chaffer and Weinberg, 2011). Incidentally, these stresses also modify cancer cell metabolism to sustain their growth potential (Dang, 2012). However, whether such metabolic reprogramming is a prerequisite for EMT remains unclear. Our microarray analysis of three EMT models revealed an enrichment of genes involved in autophagy, glucose metabolism and ROS production. Thus, it is conceivable that there might be molecular drivers that modulates metabolic reprogramming and primes these cancer cells for EMT competency. Our findings showed that ANGPTL4 is a driver of metabolic changes during EMT and that it synchronizes metabolic shift necessary for EMT through ANGPTL4:14-3-3 signaling axis.

ANGPTL4 is a secreted glycoprotein that has multi-faceted roles during metastasis. Malignant tumors expressed elevated levels of ANGPTL4, indicating a potential role of ANGPTL4 in malignant progression (Tan et al., 2012; Padua et al., 2008; Zhu et al., 2011; Huang et al., 2011). We identify ANGPTL4 as a molecular driver of metabolic changes during EMT as supported by several experimental evidences. First, detailed microarray gene profile analysis revealed that the expression of ANGPTL4 was elevated in several gene clusters related to cellular metabolism, and its deficiency led to >50% changes in gene expression profile. Second, in vitro and in vivo EMT models showed that cancer
cells deficient in ANGPTL4 were unable to elevate cellular energy charge during EMT leading to reduced metastasis. Indeed, molecular and biochemical analysis confirmed the down- and up-regulation of epithelial and mesenchymal markers, respectively. Histological analysis of xenografted EMT tumors also showed increased MMP activities and a disrupted laminin332 phenotype. Third, ANGPTL4 activated the pro-glycolysis AKT and AMPK pathways to enhance the glycolytic phenotype and thus increase cellular energy charge necessary for EMT progression. In EMT, the observed activation of AKT by ANGPTL4 could be used to sustain energy usage by increasing ATP generation through glycolysis. Finally, ANGPTL4 regulated the expression of specific 14-3-3 proteins, which coordinated energy supply for EMT. We showed that the ANGPTL4:14-3-3:TSC2s939 signaling axis accelerated EMT competency. Furthermore, ANGPTL4 modulated Snail complex stabilization through a 14-3-3γ-mediated interaction with the Snail complex to influence the repression of E-cadherin gene expression and thus augmented EMT. Lending further support, previous studies showed that ANGPTL4 triggered the 14-3-3 adaptor protein to sequester the pro-apoptotic Bad protein from mitochondria, conferring resistance to anoikis and favoring tumor survival and growth (Zhu et al., 2011; Terada and Nwariaku, 2011). Recent work also showed that ROS linked glucose metabolism to breast cancer stem cell and EMT phenotype (Schieber and Chandel, 2013; Dong et al., 2013). Coincidentally, ANGPTL4 also engaged integrin-dependent survival signals by activation of the NADPH oxidase Nox1 and bypasses anoikis by controlling ROS (Terada and Nwariaku, 2011; Zhu et al., 2011). These findings indicate an important role of ANGPTL4 as a molecular driver of cellular metabolism during EMT to promote metastasis.
Metastasis of cancer cells requires multiple cooperative hallmark alterations (Chaffer and Weinberg, 2011; Chiang and Massagué, 2008). Interestingly, the functions of many target proteins involved the acquisition of these hallmarks is known to be regulated by the 14-3-3 protein family (Mhawech, 2005; Bridges and Moorhead, 2005). The expression of 14-3-3 protein was also found to be aberrantly upregulated in several tumor types (Hermeking, 2003; Freeman and Morrison, 2011; Tzivion et al., 2006). Previously, we showed that ANGPTL4 binds and activates integrin β1 and/or β5 to exert its downstream effect (Goh et al., 2010; Zhu et al., 2011; Huang et al., 2011). In a separate study on minimal change disease (MCD), the authors showed that circulating pools of Angptl4 reduced proteinuria by interacting with glomerular endothelial αvβ3 integrin. Blocking the Angptl4–β3 integrin interaction via neutralizing integrin β3 antibody or global knockout of Angptl4 or β3 integrin delayed recovery from peak proteinuria in animal models (Clement et al. 2014). Therefore, it is highly probable that during EMT, ANGPTL4 binds and activates integrin and the downstream AKT signaling cascade to augment EMT. In this respect, ANGPTL4 may regulate the expression of specific isoforms of 14-3-3 via integrin activation, and contributed to an important new signaling axis that coordinated multiple biological processes for metastasis. Similarly, neutralizing antibodies against integrin or depletion of endogenous ANGPTL4 can suppress ANGPTL4-mediated activation of integrin and its downstream functions, lending further credence to the ANGPTL4:integrin regulation of specific 14-3-3 isoforms (Goh et al., 2010; Zhu et al., 2011; Huang et al., 2011; Clement et al., 2014). An outcome of this ANGPTL4-mediated regulation of 14-3-3 was the reprogramming of cancer cell bioenergetics through TSC2 inhibition. This process coordinates catabolic processes such as glycolysis and autophagy to provide the necessary
substrates for the PI3K/AKT signaling pathways to signal for anabolic metabolism and primed cancer cells for EMT. Moreover, neutralizing antibody inhibition of ANGPTL4 decreased 14-3-3γ expression, which mitigated the reprogramming event and hindered EMT. Additionally, we found that ANGPTL4 deficiency diminished 14-3-3γ:Snail complex formation, which served to stabilize Snail binding to the E-cadherin gene promoter. Critically, the loss of E-cadherin has been reported to trigger a glycolytic switch that favors EMT (Sousa et al., 2014; Chu et al., 2013). This less efficient but faster mode of ATP production may be preferred to meet the increased energy demands of cancer cells undergoing EMT in order to sustain cytoskeletal restructuring and the migratory and invasive capabilities of the cells. In addition, 14-3-3 targets the mitochondrial core apoptotic machinery though the binding BCL-2 family members (Zhu et al., 2011; Porter et al., 2006). ANGPTL4:14-3-3 is also involved in rapid integrin recycling during cell migration in wound healing, a biological process also important for metastasis (Goh et al., 2010).

ANGPTL4 has prominent roles in systemic lipid and glucose metabolism, and has been implicated in multiple metabolic diseases such as atherosclerosis, obesity and diabetes (Zhu et al., 2012). Our study showed that cancer cell metabolic reprogramming is a pivotal event during EMT progression. Our finding identifies ANGPTL4:14-3-3 signaling axis that coordinates multiple biological processes for metastasis. We revealed a hitherto unknown role for ANGPTL4 as a molecular driver of cellular metabolic reprogramming in EMT to promote malignant transformation. The peripheral and cellular metabolic roles of ANGPTL4 would have far-reaching consequences. Emerging evidence suggested a strong
correlation between metabolic diseases and tumorigenesis and ANGPTL4 could be a major player in these events (Joshi et al., 2015; Gallagher and LeRoith, 2015; De Pergola and Silvestris, 2013). Further investigations on ANGPTL4 will be necessary to clarify the functions of the different ANGPTL4 fragments and their association with cellular metabolism. Taken together, our finding suggest a cellular metabolic role for ANGPTL4 and provide novel insights into the cancer cell metabolic reprogramming which is a pivotal event for EMT progression.
Chapter 2

Angiopoietin-like 4 modulates vascular junction integrity by integrin signaling and disruption of intercellular VE-cadherin and claudin-5 clusters.


All sections are contributed by candidate except for Figure 24A-C; Figure 25D, E; Figure 26D, E; Figure 27; Figure 30; Figure 31B, E; Figure 33C; Figure 34; Table 1
Abstract

Vascular disruption induced by tumor-secreted permeability factors enhances metastasis. Recently, tumor-secreted Angiopoietin-like 4 (ANGPTL4) was identified as a critical pro-metastatic gene in several cancers. However, the role of ANGPTL4 in vascular leakiness and metastasis is discordant due to the lack of mechanistic understanding on how ANGPTL4 control vascular integrity. Herein, we showed that the carboxyl terminal ANGPTL4 (cANGPTL4) interacts with three novel partners, integrin α5β1, VE-cadherin (VEC) and claudin-5 on endothelial cells to trigger vascular disruption. The cANGPTL4 interaction with integrin α5β1 activates the downstream Rac/PAK signaling axis to weaken cellular contacts. This facilitates subsequent cANGPTL4: VEC and claudin-5 complex formation to disrupt the vascular junctions. Inhibition of these complexes formation impedes the vascular disruption. The vascular disruptive and pro-metastatic effects of cANGPTL4 was further validated with in vivo vascular permeability and metastasis assays performed using ANGPTL4-knockout and wild-type mice injected with either ANGPTL4-knockdown or control cells. Altogether, our findings underscore the role of ANGPTL4 in vascular disruption and have direct implications for targeting cANGPTL4 to treat cancer and other vascular pathologies.
Introduction

Our findings in chapter 1 identified an autocrine role for ANGPTL4 during tumor progression to enhance EMT competency. Given that ANGPTL4 is a secreted matricellular protein, we hypothesized that it may have paracrine functions to further potentiate cancer development. The primary tumor microenvironment contains a variety of secreted factors produced by the cancer cells and the associated stromal cells that promotes metastasis through the selection of highly invasive phenotypes (Bernards and Weinberg, 2002). The resultant ability to colonize distant organs depends on both the metastatic potential of the cancer cells as well as its interactions with the endothelium to gain entry into the distant site (Gupta and Massagué, 2006). Traversing of the metastatic cells across the endothelium has been widely recognized as the rate-limiting event for metastatic dissemination. This process entails a series of complex cell-cell and cell-matrix interactions ultimately leading to the disruption of the endothelium (Orr et al., 2000). However, the mechanism that governs the endothelial disruptive process that is pivotal to the spread of the metastatic cells requires further understanding.

Intact endothelial junction integrity, maintained by the AJs and TJs, remains the major hurdle for metastasis. In cancer metastasis, the function of these TJs and AJs are often compromised leading to enhanced paracellular permeability (Dejana et al., 2009). Studies on endothelial paracellular permeability revealed that blocking antibodies against the AJs and TJs components are able to disrupt the endothelial barrier function through hindering the AJ and TJ protein interactions (Lampugnani et al., 1992; Corada et al., 2001). Furthermore, integrin α5β1 was also found to be localized to the EC-EC junctions and are
responsible for the maintenance of the barrier integrity. Through using blocking antibodies against integrin β1 and integrin α5β1, it was demonstrated that the endothelial barrier integrity is perturbed (Lampugnani et al., 1991). It is therefore conceivable that cancer cells may employ analogous mechanisms to disrupt the endothelial junctions and augment endothelial permeability to facilitate metastasis. In this respect, ANGPTL4 has been implicated in cancer metastasis (Padua et al., 2008); but its precise role remains controversial. ANGPTL4 was found to inhibit cancer metastasis by preserving the endothelial barrier integrity (Ito et al., 2003; Galaup et al., 2006). Yet, ANGPTL4 was recently identified as a critical pro-metastatic gene in breast cancer metastasis to the lungs and suggested that ANGPTL4 could be a key player in the metastatic process (Kersten et al., 2000). Clinical studies have also correlate ANGPTL4 expression to venous and lymphatic invasion of the gastric and colorectal cancer cells, further emphasizing ANGPTL4 role in metastasis (Nakayama et al., 2010; Nakayama et al., 2011). Interestingly, ANGPTL4 undergo proteolytic processing to generate the nANGPTL4 and cANGPTL4, whether the truncated ANGPTL4 fragments play specific roles in cancer metastasis remains unclear (Ge et al., 2004). Our recent work revealed that tumors expressed high levels of cANGPTL4. We showed that the tumor cells employed the cANGPTL4-integrin mediated signaling to maintain an oncogenic O₂⁻:H₂O₂ ratio to confer anoikis resistance (Zhu et al., 2011). Yet, despite the evidence implicating ANGPTL4 in cancer metastasis, the specific role of ANGPTL4 in modulating the endothelial barrier integrity remains unclear, which is an aim of this chapter.
Materials and methods

Antibodies

Antibodies for PAK1 and pPAK1 (Ser199/Ser204) (Cell Signaling, USA), integrin β1 [JB1A], α5β1 [JB55], β3[2008] and c-Jun (Millipore, USA); CD29/activated integrin β1 [HUTS-21] (BD, USA); β-tubulin, β-catenin [12F7], EGFR [1005] (Santa Cruz Biotechnologies, USA); VE-Cadherin [BV9], Claudin-5 (Abcam, USA); CD31 (DAKO, Denmark); Occludin (Invitrogen, USA); ZO-1(Zymed Laboratories, USA); Tie 1, Tie 2, JAM-C, β-tubulin, horseradish peroxidase (HRP)-conjugated goat anti-mouse, HRP-conjugated goat anti-rabbit, HRP-conjugated donkey anti-goat (Santa Cruz Biotechnologies, USA); mouse monoclonal anti-human cANGPTL4 mAb4A11H5 and rabbit polyclonal anti-human cANGPTL4 were produced in-house (Goh et al., 2010); Secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG were also used (Molecular Probes, USA).

Human Tumor Samples

Biopsies were subjected to protein and RNA extraction for immunoblotting and qPCR analyses, respectively. Commercial tumor tissue arrays #MTU951 and #MET961 (Pantomics, Inc., USA) were used to study the expression profile of cANGPTL4 in a large human tumor set by immunofluorescence (IF) imaging. The #MTU951 human tumor tissue array contains 40 tumor types, covering most of the common benign, malignant and metastatic tumors originating from 27 anatomic sites, and the #MET961 human cancer metastasis tissue array consists of 48 cases of metastatic cancers from >8 anatomic sites. The tissue arrays were probed with an anti-cANGPTL4 antibody and images acquired as
previously described. Gray scale values (i.e. immunofluorescence signals) were obtained using TissueQuest software (TissueGnostics GmbH).

Cell Cultures

Primary human microvascular endothelial cells (HMVECs; Lonza, Switzerland) were cultured in EndoGRO-MV-VEGF (Millipore, USA), in a humidified atmosphere of 5% CO₂ at 37 °C. Culture surface were pre-coated with 0.1% gelatin in phosphate buffered saline (PBS) as the attachment factor (Cascade Biologies, USA), for 30 minutes prior to the seeding of HMVECs. All other cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere of 5% CO₂ at 37 °C.

Conditioned medium (CM) was obtained from 1 x 10⁵ tumor cells grown in 1 ml of serum-free medium for three days.

Transient transfections assay

Transfections of HMVECs were carried out as per manufacturer’s protocol (Promega, USA) with either constitutive-active Rac1 G12V or dominant-negative Rac1 T17N.

Suppression of ANGPTL4 by RNA Interference (RNAi)

An siRNA against human ANGPTL4 and a scrambled control siRNA were subcloned in the pFIV-H1/U6-puro pFIV/siRNA lentivirus system. The siRNA sequences were previously described (Goh et al., 2010). The correct pFIV siRNA constructs were verified by sequencing using an H1 primer. Pseudovirus purification and transduction were performed. ANGPTL4-knockdown tumor cells were enriched by puromycin selection for

Teo Zi Qiang
Confidential
Page 129 of 218
1 week. The A-5RT3 sub-cell line designated A-5RT3\textsubscript{AANGPTL4}, with the highest knockdown efficiency was chosen in this study, and the non-targeted siRNA transduced line was denoted A-5RT3\textsubscript{CTRL}. Knockdown efficiency of ANGPTL4 and relative expression level of the indicated genes were determined by qPCR and immunoblot as previously described (Goh et al., 2010).

**Expression and purification of recombinant cANGPTL4 proteins**

The expression and purification of full-length ANGPTL4 and recombinant cANGPTL4 were performed as previously described (Goh et al., 2010).

**In Vivo Tumorigenicity and Miles Vascular Permeability Assay**

Six-week-old BALB/c athymic female nude mice (20-22 g) were injected either cANGPTL4 (6 μg/ml) or 0.9% saline buffer was injected intradermally at adjacent locations on the back. To determine vascular permeability, tumor-bearing mice received an intravenous injection of Evans blue dye. Twenty min later, the mice were sacrificed and Evans blue extracted from the tumor and normal muscle as described in Miles vascular permeability assay (Ito et al., 2003). The extravasated dye was extracted using formamide and the amount was quantified by measuring absorbance at 610nm. To determine the tumor vascular volume, 2MDa FITC conjugated dextran (10 mg/kg) was also injected intravenously for 20 min. Fluorescence readings was obtained using Nanodrop 3300 Fluorospectrometer.

For another set of experiments, mice were injected subcutaneously at the interscapular region with either $2 \times 10^6$ or $8 \times 10^6$ cells (A-5RT3\textsubscript{CTRL} or A-5RT3\textsubscript{AANGPTL4}). Same procedures were carried out in C57B/L6J wild-type and ANGPTL4-knockout mice using
B16F10$_{\mathrm{CTRL}}$ or B16F10$_{\Delta\text{ANGPTL4}}$ cells. Injection sites were rotated to avoid site bias. The injected tumor cells were allowed to grow for eight weeks.

**In Vivo Metastasis Assay**

Wild type and ANGPTL4-knockout C57BL/6J mice were injected intravenously with either $5 \times 10^5$ B16F10$_{\mathrm{CTRL}}$ or B16F10$_{\Delta\text{ANGPTL4}}$. For cANGPTL4 treatment, wild type C57BL/6J mice i.v injected with either $2 \times 10^6$ B16F10$_{\mathrm{CTRL}}$ or B16F10$_{\Delta\text{ANGPTL4}}$ cells were treated with PBS or cANGPTL4 (3 mg/kg) thrice weekly. After 3 weeks, the mice were sacrificed and the lungs were harvested for further analyses. Total metastatic burden were quantified by RT-PCR of melanin A. Total mRNA were extracted from lungs and reverse-transcribed as described previously (Goh et al., 2010; Goh et al., 2010).

**Transendothelial Electrical Resistance (TER) measurement**

TER was measured using an electrical cell-substrate impedance sensing system (ECIS, Applied BioPhysics). HMVECs were seeded on sterile 8-well gold-plated electrode arrays precoated with fibronectin at $2 \times 10^5$ cells/well and allowed to adhere and spread for 4 h at 37 °C. Data from the electrical resistance experiments were obtained over the experimental time course at 5 min intervals. Confluent HMVEC monolayers that had stable TERs for 1 h preceding administration of indicated treatments were used. As cells adhered and spread over the microelectrode, TER increased, whereas cell retraction, rounding or loss of adhesion was reflected by a decrease in TER. Resistance values for each microelectrode were normalized as the ratio of measured resistance to baseline resistance and plotted as a function of time.
**Internalization assay**

Confluent HMVECs monolayers were treated with either PBS or cANGPTL4 (6 μg/ml) for 3 h. At indicated time point, internalization assay was carried out as previously described (Goh et al., 2010; Goh et al., 2010).

**Surface Plasmon Resonance (SPR)**

SPR was carried out as previously described using a BIAcore 2000 system (BIAcore, Uppsala, Sweden). Six concentrations (0.16, 0.32, 0.63, 1.25, 2.50 and 5.0 μM) of recombinant integrin β1, first extracellular domain of VE-cadherin (ECD1), extracellular repeat 1 domain of VE-cadherin (EC1), occludin or JAM-A were used. Integrin was expressed in Drosophila S2 cells and various extracellular domains of VE-cadherin and claudin-5 purchased from Abnova. Global fitting of the SPR data was performed as previously described to determine the KD. Pre-injection or preincubation with the indicated antibodies or preimmune IgG was performed to determine specific interactions. Each sensorgram was corrected by subtracting a sensorgram obtained from a reference flow cell with no immobilized protein. The Rmax value was determined to be 283.1 resonance units using anti-cANGTL4 antibodies against the immobilized cANGPTL4. Values are mean±S.D. (n=3)

**Immunofluorescence staining**

Disrupted tight junctions were visualized by immunofluorescence staining for zona occludens-1 (ZO-1). HMVECs were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton-X 100 for 15 min and blocked with 2% bovine serum albumin (BSA) containing 0.1% Triton-X 100 in a humid chamber for 1 h at room
temperature. Cells were incubated overnight at 4 °C with anti-human-ZO-1 antibodies (1:100) in 0.2% BSA. Following two washes in PBS, cells were incubated for 1 h at room temperature with Alexa488-secondary antibodies (1:250). Cells were counterstained with Alexa594-phalloidin for F-actin and DAPI for nuclei. Immunostainings performed without primary antibodies served as negative controls. For β-catenin staining, cells were fixed with 4% paraformaldehyde containing 5% sucrose for 15 min, permeabilized with 0.5% Triton-X 100 in PBS for 4 min and blocked in 5% normal goat serum (NGS) with 0.1% Triton-X 100. Cells were incubated overnight at 4 °C with anti-human β-catenin antibodies (1: 200) in 3% NGS, followed by Alexa594-secondary antibodies. Images were acquired using Zeiss LSM 710 confocal microscope with a 63x objective and ZEN 2009 software.

**Proximity Ligation Assay (PLA) and analysis**

PLA enable the detection, visualization and quantification of protein interactions in both tissue sections and *in vitro* cell lines. A pair of oligonucleotide labeled secondary antibodies (PLA probes) will bind either to the same primary antibodies or 2 primary antibodies that have bound to the sample in close proximity. An individual fluorescent dot will be visualized if the 2 PLA probes are in close proximity.

Confluent HUMVECs (1 x 10⁴ cells) culture was treated with either fresh EndoGRO-MV-VEGF medium or medium supplemented with human recombinant cANGPTL4 (6 μg/mL) and incubated in a humidified atmosphere of 5% CO₂ at 37 °C.

At indicated time, HUMVECs cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, followed by methanol for 5 minutes at -20 °C. Cells were
then blocked in either 3% Normal Goat Serum (NGS) with 0.05% Triton-X or 15% NGS with 0.2% Triton-X for 1 hour at room temperature for VE-cadherin and claudin-5 respectively. Next, HUMVECs were incubated with either polyclonal anti-human cANGPTL4 antibody (1:100) and monoclonal anti-human VE-cadherin antibody (1:25) in 3% NGS overnight at 4 °C or monoclonal anti-human cANGPTL4 antibody (1:10) and polyclonal anti-human claudin-5 antibody (1:200) in 15% NGS overnight at 4 °C. PLA was carried out as per manufacturer protocol (Olink Bioscience, USA). Images were taken using Carl Zeiss confocal microscope LSM 710 using a Plan-APOCHROMAT 63x/1.4 oil DIC objective, and ZEN 2009 LE software with constant exposure and gain. Number of protein interaction was quantified using the “BlobFinder” (Allalou and Wahlby, 2009).

**Protein extraction, immunoprecipitation and immunoblot**

Cell membrane fraction were obtained using ProteoJET™ membrane protein extraction kit (Fermentas, USA) from 5 x 10^6 cells. For immunoprecipitation, HMVECs cells were treated with 6 μg/ml of rh-cANGPTL4 protein. At the indicated time, HMVECs cells were lysed. The lysate was then incubated with an anti-cANGPTL4 antibody overnight at 4 °C. Following that, the antibodies were pulled down using Sepharose protein A. Proteins were released by boiling for 10 min in Lamelli’s buffer. Protein extracts were resolved using 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then electrotransferred (25 mM Tris, 192 mM glycine, 10% methanol, 0.05% SDS) onto a PVDF membrane for immunoblot analysis. Membranes were stripped and re-probed for other proteins as described (Yeung and Stanley, 2009). Protein bands were detected using
Immobilon™ Western Chemiluminescent HRP substrate (Millipore, USA) and exposed onto X-ray film.

For declustering experiments, confluent HMVECs cells were treated with rh-cANGPTL4 protein (3 and 6 μg/ml), 0.25 mM EDTA (as positive control) or control PBS. Membrane fractions were extracted as detailed above and resolved using 8 % native PAGE. Proteins were electrotransferred onto a PVDF membrane under reducing conditions (as above with 10 mM β-mercaptoethanol). Membranes were probed with antibodies against VE-cadherin or claudin-5.

FACS analysis

FACS analysis was carried out according to the manufacturer’s protocol (BD Bioscience, USA). Briefly, cells were washed with cold PBS and then resuspended in 1X binding buffer. Next, 5 μl of FITC-Annexin V and 5 μl propidium iodide was added and the mixture was incubated for 15 min at 25°C in the dark. Four hundred microliters of 1X binding buffer was added before flow cytometry analysis using FACSCalibur and CellQuest software (BD Biosciences, USA).

Real-time PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>CTGGTGGCTGGAATTGACC GCTA</td>
<td>CAAGGGGATATCCACAGAG TACCTTG</td>
</tr>
<tr>
<td>Ribosomal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein L27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>TGGATACAGAACCTTGATG ATGGACA</td>
<td>GGGCTGATGGGATTTCTCTTG</td>
</tr>
<tr>
<td>Melanin A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>ACTCAACCGGCCAGTACTTC</td>
<td>CCATGGGCTGATCAACAT</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Teo Zi Qiang Confidential Page 135 of 218
Statistical Analysis

Statistical analyses were performed using two-tailed Mann-Whitney tests with SPSS software. All statistical tests were two-sided. p value of \( \leq 0.05 \) is considered significant.
Results

cANGPTL4 is elevated in human tumors

To determine the expression profile of cANGPTL4 in human tumors, we performed immunofluorescence imaging using anti-cANGPTL4 antibodies on normal human tissue array and their corresponding tumor tissue array covering benign, malignant and metastatic tumors from various anatomical sites. The cANGPTL4 was found to be elevated in all epithelial tumor samples compared to their corresponding normal tissue samples (Figure 24A-C). Consistent with the observation above, cANGPTL4 protein levels were also found to be elevated in all cancer cell lines that we examined compared to non-tumorigenic cell lines (Figure 24D). These suggest a role for cANGPTL4 in tumorigenesis.
Figure 24. cANGPTL4 is elevated in human tumors. (A, B) Representative H&E (A) and immunofluorescence (B) images of tumor tissues probed with anti-cANGPTL4 antibodies from colon adenocarcinoma. Higher magnification images from 3 random locations are shown. Blue: Dapi, Green: cANGPTL4. (C) Graph of average integrated gray values (relative fluorescence intensity) of cANGPTL4 levels between normal and tumor tissues. Samples from same anatomical sites are grouped. Values (mean±SEM) were calculated from at least 3 randomly chosen microscopic regions. *P < 0.05; **P < 0.01. (D) ELISA measurements of cANGPTL4 protein concentration in conditioned media (CM) of tumorigenic and non-tumorigenic cell lines.
Metastatic tumors has enhanced expression of cANGPTL4

Interestingly, we found that metastatic tumors tend to have higher cANGPTL4 expression, albeit varying levels in different tumor suggesting a role for cANGPTL4 in cancer metastasis (Figure 24C). To further explore these observations clinically, we obtained benign human basal cell carcinoma and metastatic melanoma biopsies. Quantitative real-time PCR (qPCR) and immunoblot analysis revealed that cANGPTL4 mRNA and protein levels are significantly upregulated in these epithelial tumors compared to their perinormal tissues (Figure 25A, B). More importantly, expression of cANGPTL4 was higher in melanoma compared to basal cell carcinoma (Figure 25A, B). This finding was further corroborated when cANGPTL4 protein levels were compared using fine needle aspirates (FNA) from three metastatic breast cancer patients to metastatic and non-tumorigenic cell lines (12.4 μg/ml (FNA), 5.9 μg/ml (metastatic lines) and 0.8 μg/ml (non-tumorigenic lines)) (Figure 25C). These data are consistent to our recent report showing that tumor tissues expressed high levels of cANGPTL4 compared to their surrounding stroma, implicating a role for cANGPTL4 in cancer metastasis (Zhu et al., 2011).
Figure 25. Metastatic tumors have enhanced cANGPTL4 expression. (A, B) Expression levels of cANGPTL4 mRNA (A) and protein (B) in basal cell carcinoma and melanoma paired with their peri-normal tissue samples (n=3). Ribosomal protein 27 (L27) was used as housekeeping reference control for qPCR. β-tubulin served as loading control in immunoblot. (C) Immunodetection of cANGPTL4 in fine needle aspirates (FNA) obtained from metastatic breast cancer patients and CM from 4 tumorigenic (A5RT3, MDA-MB231, MCF7, HT29) and 2 non-tumorigenic cell lines (BJ and HaCaT). Known concentration of rh-cANGPTL4 was used as calibration standards, corresponding to 1-8 μg/ml of cANGPTL4.

cANGPTL4 disrupt endothelial junction integrity

As metastasis is normally associated with vascular leakiness we decided to look into the effect of cANGPTL4 on endothelial junction integrity (Joyce and Pollard, 2009; Desgrosellier and Cheresh, 2010). Metastatic human squamous cell carcinoma A-5RT3 and murine melanoma B16F10 were transduced with either scrambled siRNA (A-5RT3CTRL or B16F10CTRL) or siRNA against ANGPTL4 (A-5RT3AANGPTL4 or B16F10AANGPTL4) to suppress endogenous ANGPTL4 (Figure 26A). Confluent endothelial monolayers were exposed to 3-day old conditioned medium (CM) from A-5RT3CTRL or A-
5RT3_{\text{ANGPTL4}} cell culture. CM from A-5RT3_{\text{CTRL}} but not from A-5RT3_{\text{ANGPTL4}} cell culture was able to cause disruption of the endothelial junctions as evident from the disrupted ZO-1 staining (Figure 26B). More importantly, this disruptive effect can be inhibited by the addition of anti-cANGPTL4 antibody, indicating that cANGPTL4 plays a critical role in vascular disruption (Figure 26B). The EC monolayer junction integrity was also compromised upon treatment with 6 μg/ml of rh-cANGPTL4 regardless of the presence of cyclohexamide or actinomycin, indicating that \textit{de novo} cANGPTL4 protein synthesis was not required (Figure 26C). Same phenotype was also captured using transendothelial electrical resistance (TER) assay where a rapid and dose-dependent increase in paracellular permeability was observed (Figure 26D). The effect was not due to apoptosis of the EC even after treatment with rh-cANGPTL4 for 6 h (Figure 26E). Altogether, these demonstrated that tumor-secreted cANGPTL4 can instigate vascular disruption.
Figure 26. cANGPTL4 disrupt endothelial junction integrity. (A) Relative ANGPTL4 mRNA and protein expression in A5RT3 transfected with either scrambled siRNA (A-5RT3CTRL) or siRNA against ANGPTL4 (A-5RT3AANGPTL4). (B, C) Immunofluorescence staining for ZO-1 in a confluent HMVEC monolayer. HMVEC monolayers were treated with CM from A-5RT3CTRL cells in the presence or absence of anti-cANGPTL4 antibodies and with CM from A-5RT3AANGPTM cells (B); or treated with 6 μg/ml of rh-cANGPTL4 in the presence or absence of cycloheximide and actinomycin for 3 h (C). Cells were counterstained with DAPI (blue) for nuclei and phalloidin (red) for actin cytoskeleton. Scale bar: 40 μm. White arrows indicate endothelial junction lesions. (D) Normalized transendothelial electrical resistance (TER) of confluent HMVEC monolayer treated with varying concentration of rh-cANGPTL4 as indicated. PBS was used as control. (E) FACS analysis of vehicle (PBS)- or rh-cANGPTL4-treated HMVECs stained with annexin V-FITC/propidium iodide (PI). The percentage of apoptotic cells (upper-and lower-right quadrants) is indicated in bold (n=3).

cANGPTL4 compromise tumor vasculature in vivo

We next question the effect of cANGPTL4 on tumorigenesis. A-5RT3CTRL induces large primary tumors by week 8 after injection into immunodeficient mice compared to A-5RT3AANGPTL4 (A-5RT3CTRL 1098±422 mm³ v.s A-5RT3AANGPTL4 551±135mm³, P<0.05, n=6). Miles assays revealed a decreased vascular leakness in A-5RT3AANGPTL4-induced tumors (~5 times lower), quantified by the decreased extravasation of Evans blue dye from the blood vessels (Figure 27A and Table 1). Although A-5RT3CTRL-induced tumors have increased vascular volume, this difference in vascular permeability is independent of the tumor volume and vascular volume (Table 1). Moreover, rh-cANGPTL4 was sufficient to induce vascular leakiness when compared to PBS control (rh-cANGPTL4 0.084±0.014 v.s PBS 0.04 ± 0.01, P<0.05, n=3) (Figure 27B). Consistent with the increased vascular volume of A-5RT3CTRL-induced tumors, EC marker CD31 was also found to be elevated in A-5RT3CTRL- and B16F10CTRL-induced tumors compared to their ANGPTL4-deficient counterpart (Figure 27C, D). Dual immunostaining of cANGPTL4
and CD31 in B16F10-induced tumor confirmed that cANGPTL4 is exclusively expressed by the epithelial tumor cells (Figure 27E). This prompted us to investigate the mechanistic role of cANGPTL4 in encouraging vascular permeability.

**Table 1. ANGPTL4 increases vascular permeability and vascular volume**

<table>
<thead>
<tr>
<th>Tumor (n=15)</th>
<th>OD610 Evans blue/g</th>
<th>Fluorescent unit (FITC)/g</th>
<th>Ratio</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vascular permeability</strong></td>
<td><strong>Vascular volume</strong></td>
<td><strong>VP/VV</strong></td>
<td><strong>Permeability change</strong></td>
<td></td>
</tr>
<tr>
<td>A-5RT3_CTRL</td>
<td>0.76 ± 0.09*</td>
<td>0.82 ± 0.17**</td>
<td>0.927</td>
<td>5.332</td>
</tr>
<tr>
<td>A-5RT3_AANGPTL4</td>
<td>0.073 ± 0.012</td>
<td>0.42 ± 0.09</td>
<td>0.174</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Data represent the mean±SD of 15 animals. *, P < 0.001; **, P < 0.01
Figure 27. cANGPTL4 compromised tumor vasculature in vivo. (A) Evan's blue dye extravasation in A-5RT3CTRL and A-5RT3_{ANGPTL4}-tumors. (B, C) Evan's blue dye extravasation induced by PBS vehicle or rh-cANGPTL4 (B) and quantification of the extravasted dye by measurement of absorbance at 610nm (C). *P < 0.05. (D) Immunodetection of CD31 in A-5RT3CTRL and A-5RT3_{ANGPTL4}-tumors. (E) Immunofluorescence staining of CD31 and cANGPTL4 in B16F10CTRL- and B16F10_{ANGPTL4}-induced tumors. Scale bar: 100 \mu m.

cANGPTL4 interacts with integrin \( \alpha 5\beta 1 \), VE-cadherin and claudin-5

TJ and AJ between the adjacent EC controls the paracellular permeability (Oike et al., 2005). Interestingly, integrin \( \alpha 5\beta 1 \) was also found to be localized to the EC-EC junctions and are known to regulate vascular permeability (Lampugnani et al., 1991). With our knowledge that cANGPTL4 can bind and activate integrin \( \beta 1 \), we hypothesized that cANGPTL4 may interact with these junction proteins to induce vascular leakiness and ultimately vascular disruption. Surface Plasmon Resonance (SPR) analysis reveals that cANGPTL4 interacts with integrin \( \alpha 5\beta 1 \) (\( K_D = 1.26\times10^{-8} \text{ M} \)), VE-cadherin (\( K_D = 1.12\times10^{-7} \text{ M} \)) and claudin-5 (\( K_D = 5.87\times10^{-8} \text{ M} \)) but not with other EC junction proteins like occludin and JAM-A (Figure 28A). Particularly, cANGPTL4 interacts with VE-cadherin EC-1 domain and claudin-5 ECD-1 domain, which are necessary for homophilic interactions between ECs to maintain their paracellular permeability, but not the EC-3 and ECD-2 domains of VE-cadherin and claudin-5 respectively (Figure 28B). Furthermore, these interactions can be blocked using neutralizing antibodies against integrin \( \alpha 5\beta 1 \), VE-Cadherin, claudin-5 and cANGPTL4, suggesting that cANGPTL4 could modulate the vascular permeability via interactions with VE-cadherin and/or claudin-5 (Figure 28C-H).
Figure 28. cANGPTL4 interacts with integrin α5β1, VE-cadherin and claudin-5. (A-H) Sensogram showing binding profiles between immobilized rh-cANGPTL4 and integrin α5β1, VE-cadherin, claudin-5, occludin and JAM-A (A); VE-cadherin extracellular repeat domain 1(EC1), claudin-5 first extracellular domain (ECD1), VE-cadherin EC3 and claudin-5 ECD2 (B); Integrin α5β1 (C), VE-cadherin (D) and claudin-5 (E) pre-blocked with either preimmune IgG or indicated cognate antibodies; Integrin α5β1 (F), VE-cadherin (G) and claudin-5 (H) with the surface pre-blocked with either preimmune IgG or anti-cANGPTL4 antibody.

cANGPTL4 interacts with the respective junction proteins in *in vivo* tumor

Using *in situ* proximity ligation assay (PLA), *in vivo* interaction complexes of cANGPTL4 with integrin α5β1, VE-Cadherin and claudin-5 were also detected in ECs (integrin 22.50±5.01; VE-cadherin 16.43±2.46; claudin-5 12.35±3.44 PLA spots/cell calculated from 250 cells) and A-5RT3_CTRI-induced tumors (Figure 29A, C).
interactions were observed between CD31 and cANGPTL4 or its interacting partners which served as negative control (Figure 29B). The interaction of cANGPTL4 with VE-cadherin and claudin-5 may affect the organization of these junction proteins clusters at the EC junctions to trigger vascular disruption. Therefore, we obtained membrane extracts from confluent ECs treated with rh-cANGPTL4 (3 μg/ml, 6 μg/ml) or EDTA and resolved the extracts under native conditions. The rh-cANGPTL4 treatments resulted in declustering of VE-cadherin and claudin-5 compared to PBS control. EDTA treatment, which disrupts intercellular interactions, served as a positive control (Figure 29D). These findings confirmed that cANGPTL4 interacts with integrin α5β1, VE-cadherin and claudin-5 to modulate vascular integrity.
cANGPTL4 & integrin α5β1

Negative control

CD31 & cANGPTL4

CD31 & Claudin 5

CD31 & VE-cadherin

CD31 & Integrin α5β1

cANGPTL4 & integrin α5β1

cANGPTL4 & VE-cadherin

cANGPTL4 & Claudin 5

cANGPTL4 (negative control)

V 3 μg/ml 6 μg/ml E

claudin5

declustered

clustered

VE-cadherin

claudin5

declustered

VE-cadherin

ATTENTION: The Singapore Copyright Act applies to the use of this document. Nanyang Technological University Library
cANGPTL4 modulate vascular integrity temporally

The observations above prompted us to investigate the mechanism behind cANGPTL4-induced vascular disruption. Kinetic PLA was carried out to clarify the interaction profiles of cANGPTL4 with integrin α5β1, VE-Cadherin and claudin-5 over 180 mins. cANGPTL4:integrin α5β1 interaction complexes was first detected to peak around 30 mins post-rh-cANGPTL4 treatment, followed by cANGPTL4:VE-Cadherin and cANGPTL4:claudin-5 complexes at around 120 mins (Figure 30A). This bimodal interaction profile was further validated with immunoprecipitation assay using antibody against cANGPTL4 (Figure 30B). Treatment with rh-cANGPTL4 did not result in any protein level changes indicating that the differential interaction profile was not due to increase in protein expression (Figure 30C).
Figure 30. cANGPTL4 modulate vascular integrity temporally. (A) *In situ* kinetic PLA detection of complexes between cANGPTL4 and indicated binding partners in HMVECs treated with 6 μg/ml of cANGPTL4. Values (means±S.D.) represent mean fold change in the number of interactions compared to the zero time point, as determined from 3 independent experiments (~600 HMVECs) using BlobFinder. Experiments were terminated when microscopic lesions were observed at 180 min post-cANGPTL4 treatment. (B) Immunodetection of integrin α5β1, VE-cadherin and claudin-5 in anti-cANGPTL4 immunoprecipitates from total protein lysates of HMVECs treated with 6 μg/ml rh-cANGPTL4. (C) Immunoblot of the indicated proteins from total protein lysate of HMVECs treated with rh-cANGPTL4 at the indicated time points. EGFR and β-tubulin were used as loading and transfer controls. Values below respective blots indicated fold-change in the band intensity compared to the time point at 0 h.
cANGPTL4 triggers internalization of integrin α5β1, VE-cadherin and claudin-5

Interestingly, we also observed that cANGPTL4 interaction with the respective junction proteins resulted in the interaction complexes internalization which was not seen in control ECs (Figure 31A, B). Moreover, the internalization of cANGPTL4:integrin α5β1 complexes around 30-60 mins also coincides with a decreased in TER, indicating that this phenomenon marks the initial increase in vascular permeability and may be critical in facilitating the later interactions for vascular disruption (Figure 31A).
A

Time
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Integrin β1 (Internalized) 1.0 1.2 1.3 3.1 7.8 8.2 6.0 4.8 3.4
Integrin β1 (Total) 2.8 2.7 2.8 1.4 1.1 1.1 1.1 1.2 1.1
Integrin α5 (Internalized) 1.0 1.2 1.8 2.3 4.1 5.5 4.2 2.2 2.2
Integrin α5 (Total) 2.1 1.2 0.9 1.0 1.0 1.0 1.0 1.1 0.9 0.9
VE-cadherin (Internalized) 1.0 1.0 1.1 1.1 1.2 1.1 1.2 1.8
VE-cadherin (Total) 2.1 2.4 3.4 5.2 5.3 3.4 3.0 1.0 0.9 1.0
Claudin 5 (Internalized) 1.0 1.0 1.0 0.9 1.1 1.0 1.0 1.0 1.1
Claudin 5 (Total) 0.9 2.1 2.4 2.4 2.3 2.6 4.1 3.8 3.2 1.2
Integrin β3 (Internalized) 1.0 2.3 2.8 3.3 2.5
Integrin β3 (Total) 1.0 2.4 3.8 2.8 2.6
β-tubulin

B

Time
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Integrin β1 (Internalized) 1.0 1.2 1.2 1.3 1.2 1.2 1.2 1.1 1.1
Integrin β1 (Total) 1.3 1.2 1.2 1.2 1.1 1.1 1.3 1.3 1.3 1.2 1.2
Integrin α5 (Internalized) 1.0 1.0 1.0 0.9 1.0 1.1 0.9 1.0 1.0 1.1 0.9
Integrin α5 (Total) 0.9 0.9 0.8 0.9 0.9 0.9 1.0 1.0 1.0 1.1 1.0
VE-cadherin (Internalized) 1.0 1.2 1.1 1.2 1.2 1.1 1.0 0.9 1.3
VE-cadherin (Total) 1.3 1.3 1.2 0.9 0.9 1.1 1.0 1.0 1.0 1.2
Claudin 5 (Internalized) 1.0 1.0 1.0 1.1 1.0 1.0 1.0 1.0 0.8 0.9 1.0
Claudin 5 (Total) 0.8 0.9 1.0 1.0 1.0 1.0 1.1 1.1 1.0 0.9 0.9
Integrin β3 (Internalized) 1.0 2.4 3.8 2.8 2.6
Integrin β3 (Total) 1.0 2.4 3.8 2.8 2.6
β-tubulin
Figure 31. cANGPTL4 triggers internalization of integrin α5β1, VE-cadherin and claudin-5. (A, B) Immunodetection of integrins β1, integrin α5, integrin β3, VE-cadherin and claudin-5 from total protein lysate versus internalized fraction of HMVECs treated with rh-cANGPTL4 (A) or PBS (B). Protein lysates were collected every 10 minutes (0-180 minutes). Values below each band represent the mean fold change in protein expression level compared with the cognate zero time point (n=3). *, P < 0.05; **, P < 0.01.

Inhibition of cANGPTL4: integrin α5β1 complex formation delays interaction of cANGPTL4 with VE-Cadherin, claudin-5 and vascular disruption

A previous study demonstrated that anti-integrin α5β1 antibody can increase endothelial permeability but insufficient to cause any microscopic lesions (Lampugnani et al., 1991). Based on this, we decided to investigate the importance of the cANGPTL4:integrin α5β1 complex formation. In the presence of monoclonal antibody against integrin α5β1, cANGPTL4 took twice as long to induce vascular disruption compared to cANGPTL4 alone (Figure 32A). Treatment of confluent EC monolayer with integrin α5β1 antibody alone did not induce any visible lesions even after 6 h (Figure 32A). This finding was confirmed with TER, where reduced vascular permeability was observed in cANGPTL4 and integrin α5β1 antibody treated ECs compared to cANGPTL4 treatment alone (Figure 32B). Additionally, we also found that inhibition of cANGPTL4:integrin α5β1 complex formation resulted in delayed interaction of cANGPTL4 with VE-cadherin and claudin-5 (Figure 32C). These data suggest that interactions with these 2 proteins are required for endothelial disruption. Thus, we exposed confluent EC monolayer with rh-cANGPTL4 for 90 mins to allow cANGPTL4:integrin α5β1 complex formation. After 90 mins, rh-cANGPTL4 was then removed. ZO-1 immunostaining showed that cANGPTL4:integrin
α5β1 complex formation alone is unable to trigger EC junctions disruption (Figure 32D). Interestingly, TER analysis revealed that removal of rh-cANGPTL4 reversed the initial endothelial permeability, suggesting the resealing of the endothelial junctions (Figure 32E). Taken together, these information indicate that cANGPTL4 interaction with integrin α5β1 is necessary but not sufficient to induce endothelial disruption. Rather, the initial interactions facilitate the increase in endothelial permeability to allow cANGPTL4 access to VE-cadherin and claudin-5. Binding of cANGPTL4 to VE-cadherin and claudin-5 resulted in their declustering and interfered with their homophilic interactions, ultimately leading to endothelial junction disruption.
Figure 32. Inhibition of cANGPTL4: integrin α5β1 complex formation delays vascular disruption. (A, B and D, E) Immunofluorescence staining for ZO-1 (A, D) and transendothelial electrical resistance (TER) measurement (B, E) in confluent HMVEC monolayer. Cells were treated with either 6 µg/ml of rh-cANGPTL4 in the presence of increasing anti-integrin β1 concentrations (1:100, 1:50) (A, B) or 6 µg/ml of rh-cANGPTL4 followed by removal of exogenous cANGPTL4 at 90 min (D, E). Treatments were for 3 or 6 h. HMVECs were counterstained with DAPI (blue) for nuclei and phalloidin (red) for actin cytoskeleton. Scale bar: 40 µm. White arrows indicate endothelial junction lesions. Data (means±S.D.) from 3 independent experiments. * P < 0.05, ** P < 0.01.
0.05; **, P < 0.01. (C) In situ kinetic PLA detection of cANGPTL4: indicated binding partner complexes in cANGPTL4-treated HMVECs in the presence of anti-integrin β1. Values (means±S.D.) represent mean fold change in the number of interactions compared to the zero time point, as determined from 3 independent experiments (~600 HMVECs) using BlobFinder. Experiments were terminated when microscopic lesions were observed (360 minutes post-cANGPTL4 treatment).

cANGPTL4 instigates vascular disruption via activation of integrin-Rac/PAK signaling axis

Given the pivotal role of the cANGPTL4:integrin α5β1 complexes in initiating the vascular disruptive process, we decided to explore the molecular events that may happen downstream after cANGPTL4 binding. In vitro co-immunoprecipitation of cANGPTL4 with activated integrin β1 (clone Huts-21) revealed that binding of cANGPTL4 activates integrin α5β1, while cANGPTL4 was not found to interact with integrin β3, Tie 1, Tie 2 or JAM-C (Figure 33A, B). Downstream of integrin signaling are Rac1 and PAK, which are well known mediators of endothelial contractility and barrier functions (Stockton et al., 2004; Miyamoto et al., 2004). Our co-immunoprecipitation results showed that Rac1 and PAK are activated upon cANGPTL4 binding to integrin β1 (Figure 33C). With the increased in endothelial permeability coinciding with the increased in cANGPTL4:integrin α5β1 complexes, this suggest that the Rac/PAK signaling axis may be involved in modulating the endothelial permeability and contractility. To test this possibility, ECs are transfected with either constitutive active (G12V) or dominant negative (T17N) Rac1 and allow to grow to confluency before treatment with rh-cANGPTL4. ECs transfected with Rac1(G12V) accelerated the formation of cANGPTL4:VE-cadherin and claudin-5 complexes while Rac1 (T17N) tarried these
complex formations (Figure 33D). These findings further underscore the importance of the Rac/PAK signaling axis downstream of cANGPTL4-activated integrin α5β1, which enhances endothelial permeability that facilitate cANGPTL4 interactions with VE-cadherin and claudin-5 to compromise the EC-EC junction integrity.

A

![Image A]

B

![Image B]

C

![Image C]

D

![Image D]
Figure 33. cANGPTL4 instigate vascular disruption via activation of integrin-Rac/PAK signaling axis. (A, B) Immunodetection of indicated proteins in anti-Huts-21 (A) and anti-cANGPTL4 (B) immunoprecipitates from total protein lysates of rh-cANGPTL4-treated HMVECs (6 μg/ml). IgG immunoprecipitates serves as control. (C) Immunodetection of indicated proteins from anti-Huts-21 immunoprecipitates of HMVECs treated with either vehicle (PBS) or rh-cANGPTL4. Total integrin β1, Rac1 and PAK served as controls. Protein lysates were collected every 20 minutes (0-180 minutes). Values below each band represent the mean fold change in protein expression level from three independent experiments compared with the cognate zero time point. *, P < 0.05; **, P < 0.01.

(D) In situ kinetic PLA detection of cANGPTL4: indicated binding partner complexes in rh-cANGPTL4-treated HMVECs transfected with either constitutive-active Rac1 G12V or dominant-negative Rac1 T17N. Values (means±S.D.) represent mean fold change in the number of interactions compared to the zero time point, as determined from n=3 independent experiments (~600 HMVECs) using BlobFinder. *, P < 0.05; **, P < 0.01.

Formation of cANGPTL4:proteins complexes trigger nuclear translocation of β-catenin

β-catenin are localized at the membrane borders of cells with undisrupted cellular junctions and perturbation or disruption of the cellular junctons results in nuclear translocation of membrane β-catenin (Conacci-Sorrell et al., 2002; Beckers et al., 2008). Therefore we decided to see if β-catenin are translocated into the nucleus upon cANGPTL4-induced endothelial junction disruption. In PBS-treated ECs, β-catenin forms a continuous lining along the membrane as indicated by immunofluorescence and intensity plot, whereas cANGPTL4 treatment resulted in disappearance of the β-catenin lining and nuclear accumulation, indicating cellular junction disruption (Figure 34A). EDTA-treated ECs were used as a positive control (Figure 34A). To better define the translocation of β-catenin from the membrane to the nucleus, we carried out PLA and immunoblot analysis at five critical time points (0 min, 40 min, 90 min, 140 min and 180 min). Immunoblot analysis showed the decreasing membrane localization of β-catenin
over 180 mins, mirrored by the increased in nuclear accumulation of β-catenin (Figure 34B). This observation was also corroborated by our PLA data (Figure 34C). The nuclear accumulation of β-catenin corresponds to the time point where cANGPTL4 induces VE-cadherin and claudin-5 declustering, further confirming the endothelial disruption phenotype. Taken together, these findings indicate that tumor-secreted cANGPTL4 compromise the EC-EC junction via temporal interactions with integrin α5β1, VE-cadherin and claudin-5, leading to nuclear translocation of β-catenin.
Figure 34. Formation of cANGPTL4:proteins complexes trigger nuclear translocation of β-catenin. (A) Immunofluorescence staining for β-catenin (green) in a HMVEC monolayer treated with either 6 μg/ml of rh-cANGPTL4 or 2.5mM EDTA (as positive control) for 3 h. HMVECs were counterstained with DAPI (blue) for nuclei. Scale bar: 40 μm. White arrows indicate endothelial junction lesions associated with reduced β-catenin staining. Representative fluorescence intensity plot of β-catenin and DAPI, indicated by the white dotted line across the nuclei, were quantified using Zen 2009 software (Carl Zeiss). (B) In situ PLA assay for β-catenin and quantification of the number of PLA spots per nuclei of cANGPTL4-treated HMVECs at the indicated time points. (0 min, 1.2±0.8; 40 min, 3±1.23; 90 min, 7.9±1.93; 140 min, 13.3±2.44; 180 min, 17.5±2.68) PLA signals (red) revealed translocation of β-catenin into nuclei at 140 min after treatment (~250 HMVECs). Cells were counterstained with Hoechst dye for nuclei (blue). Scale bar: 40 μm. *, P < 0.05; **, P < 0.01. (C) Immunodetection of β-catenin in membrane, cytosol and nuclei fractions of HMVECs treated with either vehicle (PBS) or cANGPTL4. Protein lysates were collected at the indicated times. Values (means±S.D.) below each band represent the mean fold change in protein expression level compared with the cognate zero time point (n=3). *, P < 0.05; **, P < 0.01.

cANGPTL4 enhanced metastasis to lungs in vivo

To further explore the role of cANGPTL4 and its biological significance in cancer metastasis, we generate two different mice model to test cANGPTL4 impact on lung metastasis. In the first model, we intravenously injected wild-type C57BL/6J (n=12 per group) with B16F10CTRL or B16F10ANGPTL4 (2 x 10^6 cells) and treated them with intravenous doses of rh-cANGPTL4 (6 μg/ml) or PBS. Mice that received B16F10CTRL developed significantly more spontaneous lung metastases compared to mice that received B16F10ANGPTL4 (Figure 35A). Unsurprisingly, intravenous injection of rh-cANGPTL4 induced more lung metastases in both B16F10CTRL- or B16F10ANGPTL4-injected mice (Figure 35A). This was evident from the real-time PCR analysis of melanin A and further corroborated by immunohistostaining of the mice lungs sections (Figure 35B, C). Similar results were also observed using wild-type (ANGPTL4^+/−) and ANGPTL4-knockout (ANGPTL4^-/-) mice intravenously injected with B16F10CTRL or B16F10ANGPTL4 (0.5 x 10^6 cell).
cells). The absence of ANGPTL4 impaired the metastatic potential of B16F10_{CTRL} cells resulting in fewer tumor lung nodules in ANGPTL4^{−/−} mice compared to the ANGPTL4^{+/+} mice (Figure 35D, E). Altogether, our findings consolidated the pro-metastatic role of cANGPTL4 and provided a novel mechanistic insight into cANGPTL4 function in cancer.
Figure 35. cANGPTL4 enhanced metastasis to lungs in vivo. (A-C) Representative macroscopic images of lungs (A); relative expression of melanin A from the indicated lungs (B); representative eosin stained images of lung sections (C) from mice injected intravenously with B16F10CTRL or B16F10cANGPTL4 cells and receiving either PBS or rh-cANGPTL4 injection (3mg/kg) thrice weekly (n=12). Black nodules in (A) and (C) indicate intravasated melanoma (n=5). Scale bar: 100 μm. (D, E) Number of nodules (D) and weights of lungs (E) from wild type and ANGPTL4 knockout C57BL/6J mice intravenously injected with either 5 x 10^5 B16F10CTRL or B16F10cANGPTL4 cells (n=12). **, \( P < 0.01 \); ***, \( P < 0.001 \).

Mechanistic overview of tumor-secreted cANGPTL4-induced EC junction disruption

We showed that tumor-derived cANGPTL4 first interact with integrin α5β1 (30-60 min) leading to the activation of Rac/PAK signaling axis that coincides with the initial increase in vascular permeability(Figure 36-1 and -2).. This event is critical but insufficient to induce disruption of the endothelial cell-cell junctions. Rather, the increase in vascular permeability enables cANGPTL4 to bind to VE-cadherin EC-1 and claudin-5 ECD-1 domains to interfere with the intercellular homophilic interactions (Figure 36-3)..< This results in the declustering of VE-cadherin and claudin-5, leading to the nuclear translocation of β-catenin, indicating complete abolishment of cell-cell contacts (Figure 36-4).
Figure 36. Mechanistic overview of tumor-secreted cANGPTL4-induced EC junction disruption. Schematic diagram of cANGPTL4-mediated disruption of endothelial junctions.

(1) cANGPTL4:integrin α5β1 formation (30-50 min) coincides with (2) the activation of Rac-GTP and pPAK in ECs (40-60 min) and with vascular leakiness; (3) the interaction between cANGPTL4 with VE-cadherin and claudin-5 (120-140 min) results in VE-cadherin and claudin-5 declustering and internalization, leading to disruption of the interendothelial junctions, and stimulates (4) nuclear translocation of β-catenin (180 min).
Discussion

Metastasis requires dynamic cell-cell and cell-matrix communications in order to breach the endothelial barrier and allow the invasion and transmigration of the metastatic cells across the endothelium into the circulation (Joyce and Pollard, 2009). The primary tumor microenvironment contains a myriad of secreted factors that selects for highly invasive phenotypes coupled with pro-permeability factors that compromised the endothelium barrier integrity to facilitate metastasis (Bernards and Weinberg, 2002; Gupta and Massagué, 2006). Hence, detailed understanding of the mechanisms of the pro-permeability factors-induced vascular leakiness could offer new insights for therapeutics strategies. In this aspect, ANGPTL4 was implicated in cancer metastasis through the possible modulation of the endothelial barrier integrity, yet the mechanism remains unknown (Padua et al., 2008). Here, we showed that cANGPTL4 disrupt inter-endothelial junctions via a novel integrin α5β1 mediated Rac/PAK signaling axis followed by cANGPTL4-mediated declustering and internalization of VE-cadherin and claudin-5 resulting in the nuclear accumulation of β-catenin. Furthermore, in vivo vascular permeability and metastasis assay confirmed that cANGPTL4 disrupt the vascular integrity to enhanced vascular permeability and metastasis.

The inter-endothelial junction is maintained by various adhesive proteins located at the AJs and TJs (Bazzoni and Dejana, 2004; Dejana et al., 2009). Additionally, integrin α5β1 was also reported to localize to the inter-endothelial junctions and are important in regulating endothelial barrier functions (Lampugnani et al., 1991). We showed that cANGPTL4 interacted with integrin α5β1, VEC and claudin-5 in a temporal manner to
disrupt the endothelial junctions. Importantly, cANGPTL4 binds to the extracellular domains of VEC and claudin-5 that are necessary for homophilic interactions between corresponding proteins on adjacent endothelial cells, suggesting a physical disruption of the inter-endothelial junctions. The initial interaction between cANGPTL4 and integrin α5β1 activates Rac1 and phosphorylate PAK and this coincides with the increased in vascular permeability as evident from our TER data. This is consistent with Miao et al. (Miao et al., 2002) report that showed an increase Rac1 activation upon binding of integrin α5β1 with its ligands. Furthermore, such increased in Rac1 activity has also previously been linked to endothelium disruption mediated by other pro-permeability factors like VEGF (Gavard and Gutkind, 2006). Thus, the early cANGPTL4:integrin α5β1 interaction may be responsible for initiating the disruption process via modulating actin contractility that weaken cell-cell contacts. Indeed, integrin mediated Rac/PAK signaling axis is reported to regulate EC contractility to control inter-endothelial junction (Stockton et al., 2004). Precisely, we show that constitutive active Rac1 can potentiate, while the dominant negative Rac1 delays the vascular disruptive effects of cANGPTL4. This suggested that tumor cells can exploit this mode of interaction between cANGPTL4 and integrin α5β1 to facilitate cANGPTL4 access to AJs and TJs to induce the declustering of VEC and claudin-5 resulting in the disruption of the endothelial junctions, augmenting metastasis.

Using anti-integrin β1 antibody to prevent cANGPTL4 association with integrin α5β1, we found that the subsequent cANGPTL4:VEC and cANGPTL4:claudin-5 complexes formation were delayed and a concomitant decreased in vascular permeability. Similar
effects were also observed in cANGPTL4-treated ECs expressing dormant-negative Rac1, without affecting the cANGPTL4:integrin α5β1 formation. These indicate that the Rac/PAK signaling downstream of integrin facilitates cANGPTL4 interactions with VEC and claudin-5. Interestingly, by preventing cANGPTL4 interactions with VEC and claudin-5, the initial increased in vascular permeability attributed to the activation of integrin-mediated Rac/PAK signaling did not amount to observable vascular lesions. This further emphasize that the initial Rac/PAK signaling is necessary to potentiate the vascular disruptive process but unable to cause vascular disruption.

The role of Rac1 in endothelial barrier integrity is ambiguous. Studies have indicated that homophilic interactions between VEC recruits active Rac1 resulting in stabilization of the endothelial barrier (Mehta et al., 2001; Abraham et al., 2009). Moreover, angiopoietin-1, a well-known regulator of vascular permeability, binds to its cognate Tie 2 receptor to activate Rac1 and subsequent reduction in VEGF-induced vascular permeability (Hoang et al., 2011). We and others have shown that cANGPTL4 does not bind to angiopoietin receptors Tie1 or Tie2 to mediate its effect downstream. Thus, these indicate that the role of Rac in vascular permeability is complex. The ligand and cellular context that activates Rac1 may be crucial in determining the contextual functions of Rac1 in controlling vascular junction integrity.

The inter-endothelial clustering of VEC and claudin-5, mediated by their extracellular domains, is important in the maintenance of the endothelial barrier functions (Bazzoni and Dejana, 2004; Oike et al., 2005). Therefore, the interference of the homophilic interactions between these adhesive proteins will trigger their declustering and results in
vascular disruption. Accordingly, our data showed that cANGPTL4 can bind to VEC EC1 and claudin-5 ECD1 to trigger the declustering of VEC and claudin-5 to augment the vascular disruptive effect of cANGPTL4. Furthermore, our results also revealed a time-dependent translocation of β-catenin from the membrane into the nuclei of cANGPTL4-treated ECs that coincides with the appearance of vascular lesions, indicative of vascular disruption. The localization of β-catenin at the AJs is essential for the preservation of the stable inter-endothelial barrier functions and this movement of β-catenin from the membrane into the nuclei is reminiscent to the declustering of the AJs and vascular disruption (Conacci-Sorrell et al., 2002; Beckers et al., 2008). Likewise, the nuclear β-catenin can modulate expression of pro-angiogenic genes in ECs that can contribute to cancer metastasis (Skurk et al., 2005; Beckers et al., 2008).

Notably, we also observed an increased in vascular volume in A-5RT3CTRL-induced tumors compared to A-5RT3ANGPTL4-induced tumors, suggesting a pro-angiogenic role for ANGPTL4 in tumor angiogenesis in addition to increasing vascular leakiness to augment metastasis. The roles of ANGPTL4 in angiogenesis are debatable. An early study demonstrated that hypoxic condition elevated ANGPTL4 mRNA and protein levels in ECs and triggered a VEGF-independent angiogenesis program (Le Jan et al., 2003). Similar observations were also made in Kaposi’s sarcoma human gliomas, and head and neck carcinomas where ANGPTL4 induces neovascularization and vascular leakiness (Ma et al., 2010). In contrast, ANGPTL4 was also found to exert anti-angiogenic effects in skin cancer and several vascular-related diseases (Ito et al., 2003; Perdiguerio et al., 2011;
Galaup et al., 2011) These findings suggest that role of ANGPTL4 in tumor angiogenesis could be context dependent.

The controversial role of ANGPTL4 in vascular leakiness and cancer metastasis could be due to the lack of mechanistic understanding of its function. The proteolytic processing of the fANGPTL4 into the nANGPTL4 and cANGPTL4 also highlights the importance to delineate the functions of the individual fragments. We showed that cANGPTL4 behaves as a pro-vascular permeability factor where it mediates the vascular disruptive effect through integrinα5β1-induced Rac/PAK signaling axis and the subsequent declustering and internalization of VEC and claudin-5. Taken together, these observations suggest that the physiological effects of ANGPTL4 could be context dependent and the different fragments may serve different roles. Dysfunctional endothelium is characteristic of various pathological processes such as retinopathy, inflammatory edema and cancer metastasis. Therefore, our findings suggest that targeting cANGPTL4 to impair the metastatic process and treatment of vascular-related disease could be a viable option.
Conclusion and Future Direction

Our present work highlights dynamic roles of ANGPTL4 during malignant progression, particularly by coordinating EMT and the dissemination of metastatic cancer cells. Cancer metastasis is a complex, multistep event that began with the cancer cells undergoing EMT to gain motility and invasive capacity, leading to its invasion into the systemic circulation and subsequent dissemination to distal organs (Savagner, 2001; Mendoza and Khanna, 2009; Chiang and Massagué, 2008). We observed a striking correlation between ANGPTL4 expressions with tumor grades. Benign tumors express high levels of ANGPTL4 compared to normal tissues, while malignant and metastatic tumors express even higher ANGPTL4 levels (Tan et al., 2012; Zhu et al., 2011; Huang et al., 2011). This finding suggests that ANGPTL4 have important roles during malignant progression. Indeed, we demonstrated that cancer cells are able to exploit ANGPTL4 at multiple stages of malignancy. We showed for the first time that cancer cells are able to exploit ANGPTL4 to manipulate cancer cellular metabolic changes during EMT and synchronizes a metabolic shift necessary to drive EMT initiation through an ANGPTL4:14-3-3 signaling axis. Furthermore, we also demonstrated that tumor-derived ANGPTL4 behaves as a pro-vascular permeability factor where it mediates the vascular disruptive effect through a novel integrin α5β1-induced Rac/PAK signaling axis. These result in the declustering and internalization of endothelial cell-cell junctional proteins that disrupts the vascular integrity and enhances metastasis.

Malignant tumors behave like an ecosystem where the heterogeneous cell populations communicate with and react to each other via a complex network of signaling molecules,
extracellular matrix and other mechanical cues (Rowley, 1998; Hanahan and Weinberg, 2011). Importantly, the tumor microenvironment is at the crux of metastasis where the continuous communications between the cancer cells and its microenvironment enable the assembly of a pro-neoplastic niche to enhance the neoplastic potential of the surrounding cells (Pollard, 2004; Hanahan and Weinberg, 2011). The macrophages of the tumor microenvironment play pivotal roles in promoting the growth, invasion, metastasis and therapy resistance of malignant tumor (Noy and Pollard, 2014; Condeelis and Pollard, 2006; Hagemann et al., 2009). The myriad of chemokines, cytokines and growth factors secreted by tumor cells recruits monocytes from the circulation and manipulate monocytes differentiation into tumor-associated macrophages (TAM). Indeed, the tumor microenvironment is mostly immune-protective, providing growth supports and therapeutics resistance to the cancer cells. Thus, understanding the tumor microenvironment and the associated mechanisms that empower malignant development is crucial for future therapeutics interventions.

Under physiological conditions, macrophages can adopt either a pro-inflammatory “M1” phenotype or anti-inflammatory “M2” phenotype in response to microenvironmental stimuli (Sica and Mantovani, 2012). The classically activated M1 TAMs exert a cytotoxic effect on cancer cells, releasing reactive oxygen species, nitrogen intermediates, and inflammatory cytokines that kill cancer cells (Mantovani et al., 2009; Lewis and Pollard, 2006; Kosaka et al., 1991). The alternatively activated M2 TAMs, however, provide a nutritional advantage for cancer cells through the production of a variety of growth factors that promote growth and vascularization of the cancer mass (Mills et al., 2000; Shen et al.,
Thus, a high M1/M2 macrophage ratio is highly correlated with improved prognosis (Riabov et al., 2015; He et al., 2013; Zhang et al., 2014; Edin et al., 2012).

This M1 or M2 switch is thought to be influenced by C-reactive protein (CRP), which is an acute-phase induced serum protein (Volanakis, 2001; Mold et al., 1999; Devaraj and Jialal, 2011). CRP is known to bind to the Fcγ receptors on macrophages to direct them into the pro-inflammatory and cytotoxic M1 phenotype. As such, one would expect the tumor microenvironment to suppress CRP expression and promote M2 TAM to potentiate tumor progression. However, multiple malignant tumors are reported to have high CRP expressions that are associated with poor prognosis (Shin et al., 2015; Imai et al., 2013; Polterauer et al., 2007; Schmid et al., 2007). These tumors have pre-dominant M2 TAM population, suggesting that perhaps, tumor-secreted factors may interfere with the functions of CRP to suppress its pro-inflammatory influences (Tham et al., 2014; Mantovani et al., 2009; Lewis and Pollard, 2006). Interestingly, ANGPTL4 was recently demonstrated to have anti-inflammatory characteristics, where ANGPTL4 was found to protect against atherosclerosis and acute phase response (Tjeerdema et al., 2014; Frenzel et al., 2014; Lu et al., 2010; Georgiadi et al., 2013; Lichtenstein et al., 2010). Importantly, ANGPTL4 level was shown to be closely correlated to CRP levels (Tjeerdema et al., 2014; Frenzel et al., 2014). Furthermore, ANGPTL4 was also found to be significantly elevated in TAMs, although the study did not distinguish M1 or M2 TAMs (Schumann et al., 2015). Since our data indicated high ANGPTL4 expression in malignant tumors, it will be fascinating to investigate if ANGPTL4 can attenuate CRP activities.
References


factor) is a direct glucocorticoid receptor target and participates in glucocorticoid-regulated triglyceride metabolism. J Biol Chem 284, 25593-25601.


positron emission tomography correlates with Akt pathway activity but is not predictive of clinical outcome during mTOR inhibitor therapy. J Clin Oncol 27, 2697-2704.


