New mechanisms in cancer metastasis : tumor-endothelial contact-induced angiogenesis and autophagy in the progression of distant metastases

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**NEW MECHANISMS IN CANCER METASTASIS:
TUMOR-ENDOTHELIAL CONTACT-INDUCED ANGIOGENESIS AND
AUTOPHAGY IN THE PROGRESSION OF DISTANT METASTASES**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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AUTOPHAGY IN THE PROGRESSION OF DISTANT METASTASES**

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CONFERENCE PAPERS AND PRESENTATIONS

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ABSTRACT

Cancer is one of the leading causes of death worldwide and is a complicated disease with many manifestations. Angiogenesis and autophagy contribute to the survivability and metastatic ability of cancer cells, thus leading to the progression of the disease. Insights on the mechanisms that drive cancer progression facilitate treatment of the disease. Therefore, this thesis focuses on tumor-endothelial contact-induced angiogenesis and the role of autophagy in the progression of distant metastases, in order to develop therapeutic strategies to halt cancer progression.

I developed a two-dimensional (2D) liver cancer-endothelium co-culture model wherein endothelial cells in direct contact with liver cancer cells were able to differentiate and form tubular structures similar to those plated on matrigel. Growth factors were not the key inducers of angiogenesis, and contact dependent differentiation of endothelial cells were indispensable in the co-culture model. Further investigation of the angiogenic programming mechanism in the co-culture revealed that inhibitors of FAK and Ras/Raf/MEK/ERK pathways effectively inhibited the differentiation of endothelial cells, while the VEGFR inhibitor displayed little effect. Taken together, this co-culture model was able to provide insights on the tumor-endothelium interactions in the differentiation and remodeling of endothelial cells.

In order to increase the relevance of the previous study and to develop a potential platform for the discovery of new anti-angiogenic/anti-tumor agents for liver cancer therapy, I further developed the 2D co-culture model into a three-dimensional (3D) co-culture tumor spheroidal model. Addition of factors and inhibitors, which targets the cellular signaling pathway, to the model system allowed better understanding of mechanisms regulating hepatocellular carcinoma (HCC) development. The 3D model

demonstrated similar protein expressions to a HCC xenograft model, and exhibited upregulation of signaling proteins in the 3D model compared to the 2D model. The effects of several anti-angiogenic agents like sorafenib, sunitinib, and axitinib were analyzed in the 3D co-culture model by utilizing fluorescent proteins, and a FRET-based caspase-3 sensor in the endothelial cell, which can detect apoptosis in real time, allowing for dual multi-screening of anti-angiogenesis and anti-tumor drugs in one-step.

Finally, the autophagic capabilities of lung metastases-derived cell lines that were isolated from mice models were also studied in this thesis. The metastases-derived breast cancer cells experienced increased basal levels of autophagy and underwent stronger autophagy during serum deprivation compared to the parental breast cancer cell line. The metastases-derived cells formed large autophagic vacuoles during serum deprivation and these autophagic vacuoles were discovered to contain autophagic cargo. Inhibition of autophagic flux enhanced the vacuoles, while inhibition to formation of autophagosomes reduced the vacuoles. Furthermore, the formation of the autophagic vacuoles was found to be inhibited by antioxidant and analysis of the parental and metastases-derived cells revealed differences in their antioxidant capabilities. This contributes to increasing evidences of enhanced autophagy capabilities in cancer progression and metastasis, and provides an insight to the role of antioxidant capabilities in regulating autophagy in cancer cells.

Overall, I studied two important aspects in cancer metastasis: angiogenesis and autophagy. I have revealed new insights into contact-dependent cancer angiogenesis and provided evidences of differential autophagic capabilities linked to antioxidant defenses mechanism in cancer metastasis. Discovering unexplored ideologies in these hallmarks may unfold new therapeutic targets in the treatment of cancer.

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ABBREVIATIONS

·OH	Hydroxyl radical
2D	Two-dimensional
2-ME	2-methoxyestradiol
3D	Three-dimensional
AIA	Angiogenesis inhibiting agent
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
Ang	Angiopoietin
ATG	Autophagy related genes
Baf-A1	Bafilomycin-A1
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
C3	Caspase-3
CAF	Cancer-associated fibroblast
CFP	Cyan fluorescence protein
CMA	Chaperone-mediated autophagy
CQ	chloroquine
Dll	Delta-like
DMEM	Dulbecco's Modified Eagle Medium
Drp1	Dynamin-related GTPase
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
EHS	Engelbreth-Holm-Swarm
EMT	epithelial–mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase mitogen
ESCRT	Endosomal sorting complex
ETC	Electron transport chain
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FF	Form factor
FGF	Fibroblast growth factor
FRET	Fluorescence resonance energy transfer
g	gram
GIST	Gastrointestinal stroma tumor
GSH-Px	Glutathione peroxidases
H ₂ O ₂	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factors
HIF	Hypoxia-inducible transcription factors
HSC	Hepatic stellate cells
HSC70	Heat shock cognate 70
l	Litre
IHC	Immunohistochemistry
LC3	The mammalian homolog of ATG8
M	Molar

MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinases
MnSOD	Superoxide dismutase
MnTBAP	Manganese (III) tetrakis (4-benzoic acid)
porphyrin	chloride
mTOR	Mechanistic target of rapamycin
mTorc1	Mammalian target of rapamycin complex 1
MVB	Multivesicular body
NAC	N-acetylcysteine
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NBF	Neutral buffered formalin
NOX	NADPH oxidase
O ₂ ⁻	Mechanistic target of rapamycin
OS	Overall survival
p62/SQSTM1	p62/sequestosome-1
PDGF	Platelet-derived growth factor
PDGFR	Platelet derived growth factor receptor
PE	Phosphatidylethanolamine
PEI	Percutaneous ethanol injection
PFA	Paraformaldehyde
PFS	Progression-free survival
PG	Propyl gallate
PI3K	Phosphoinositide 3-Kinase

PKC	Protein kinase C
PMA	Phorbol myristate acetate
RCC	Renal cell carcinoma
RFA	Radiofrequency ablation
RNS	Reactive nitrogen species
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
TACE	Transarterial chemoembolization
TAM	Tumor associated macrophages
TTP	Time to tumor progression
VDA	Vascular disrupting agent
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSMC	Vascular smooth muscle cells
YFP	Yellow fluorescence protein
$\Delta\Psi M$	Mitochondria membrane potential

CHAPTER 1 Introduction

1.1 Cancer Metastasis

Cancer is the leading cause of death worldwide. Overall, there were 14.1 million new cases and 8.2 million deaths in 2012 [1]. Current focus on cancer research mainly targets the pathogenesis and the treatment of primary tumors, however, ninety percent of deaths from solid tumors are due to the spread of cancer to other parts of the body or metastasis [2]. Metastases arise from coordinated cellular processes where cancer cells escape from their primary location, travel through the bloodstream and colonize at distant organs to form new tumors. Once tumor cells spread to important visceral organs, cancer becomes life threatening and become challenging to control and treat. Delving into cellular and molecular details of the mechanisms underlying the metastatic process may result in development of new therapeutic strategies.

1.1.1 Important Steps in Cancer Metastasis

Metastasis is one of the biggest challenges in curing cancer, and the multi-step process of metastasis has been highly investigated. The process of metastasis consists of linked sequential steps which includes the detachment of tumor cells from primary tumor, migration and invasion into the local host tissue, entry into the blood circulation (intravasation), transition in the blood or lymph, followed by the exit of tumor cells (extravasation) and colonization at distal sites [3]. Only a small subset of tumour cells can overcome these diverse challenges, therefore metastasis is generally an inefficient process [4]. In the initial steps of metastasis, the epithelial–mesenchymal transition (EMT) plays a major role in cancer metastasis, where tumor cells alter their genomic signaling and gain enhanced migratory and invasive potential [5] to invade and

intravasate into the bloodstream. Tumor cells are then disseminated to distant organs via the blood circulatory and the lymphatic system. In the distant organs, tumor cells adhere to capillary walls and extravasate into the organ parenchyma, where favorable microenvironments initiate the proliferation and colonization of new secondary tumors.

1.1.2 Metastasis in Hepatocellular Carcinoma (HCC)

HCC accounts for 90% of all liver cancers [6]. It is the third leading cause of cancer death worldwide [7, 8] with 745,000 deaths in 2012 [1]. In Asia, the incidence rate is the highest, with endemic prevalence of Hepatitis B and C [9]. Among 782,000 new cases diagnosed in 2012, 64% of them were found in Asian countries [10]. Virus infection such as Hepatitis B and C [11], aflatoxin contamination of food (found in peanut butter) and alcohol intake are the main factors for developing HCC [6, 12]. Long-term damage to the liver and scarring of the liver tissue, cirrhosis, also increase the risk of liver cancer [12, 13]. There is an increasing incidence of liver cancer in developed countries such as Japan, Europe and the United States [7]. In the United States, the incidence of HCC has increased by 80% [12], where obesity and diabetes set the induction of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) which eventually translate to liver cancer [7, 14]. The incidence of HCC is expected to rise by 2020 [15], where HCC incidence has tripled in the United States during the past two decades [12]. Mortality rate associated with liver cancer is often due to the poor prognosis associated with HCC, where many patients face tumor unresectability and dissemination of HCC cells to other parts of the body.

1.1.3 Treatment of HCC

For early stage HCC, surgical resection, liver transplantation and radiofrequency ablation (RFA) or percutaneous ethanol injection (PEI) offer potential cure [16]. Surgical resection is currently considered the most curative strategy, however, clinical data suggest that HCC is hard to be removed by surgery due to the presence of liver malignancy in unresectable regions, the presence of multi-tumor lesions, or poor liver functions [17]. The next best treatment option, liver transplantation, is also potentially curative [18], yet globally, organ shortage remains a limiting factor, with a low availability of matching liver. For non-resectable patients, both local ablation methods (RFA and PEI) are feasible as treatments of HCC. PEI uses injection of ethanol to dehydrate and cause necrosis to the tumor, while RFA causes thermal injury to the target tumor by using high electromagnetic energy [19]. Both ablation methods aim at destroying the entire tumor without removing them, therefore are only suited for HCC patients with few small tumor lesions of ≤ 3 cm or a large tumor of ≤ 5 cm [20]. For early stage HCC, the curative treatments mentioned above offers high rates of complete responses and potential cure [18, 20].

Alternative treatments to intermediate stage HCC involve transarterial chemoembolization (TACE) or radioembolization [17, 18]. TACE involves the intra-arterial injection of chemotherapeutic drugs with embolizing agents to induce tumor necrosis at the contact area, while radioembolization refers to the usage of radioactive particles instead of chemotherapeutic drugs [21]. At more developed stages, targeted systematic chemotherapy with sorafenib is the only approved therapy. Although significant, sorafenib have shown small survival benefits with a median survival of 10 months [22]. Therefore, the identification of new anti-cancer drugs is vital in the treatment of HCC.

1.1.4 Metastatic Breast Cancer

Breast cancer is the most common cancer diagnosed in women, with 1.7 million cases, or 25.2% of new women cancer cases diagnosed in the breast in 2012 [1]. Breast cancer is the second most common cause of cancer death in women [23], with 522,000 death in 2012. Although breast cancer has high incidence rate, the mortality rate is low with 14.7% in all female cancer death. However, 90% deaths in breast cancer patients are due to the invasion of metastatic breast cancer through the lymphatic route to the lungs and bone, the most frequent metastasis sites of breast cancer.

1.2 Angiogenesis and Metastasis

Angiogenesis or the development of new blood vessels is crucial in many physiological processes such as embryonic development, reproduction and wound healing, as well as a variety of diseases such as cancer, diabetic retinopathy and rheumatoid arthritis. Angiogenesis is a highly regulated complex process, with brief periods of activation followed by complete inhibition. In tumor angiogenesis, continuous activation of angiogenic pathway is triggered by the over-expression of pro-angiogenic factors by tumor cells. Tumor-host cell interactions in the tumor microenvironment also encourage the formation of tumor vasculature, and enhances the metastatic capabilities of tumor cells to invade into the blood vessels and spread to other parts of the body. Angiogenesis supports tumor growth by providing nutrients and oxygen, and aids in the dissemination of cancer cells to other parts of the body. As most cancer deaths are due to the metastasis, this thesis will focus on the importance of angiogenesis in metastasis.

1.2.1 Angiogenesis as a Critical Step in Metastasis

Although small tumors can receive nutrients by diffusion, tumors require an adequate blood supply for to grow bigger than 1-2 mm [24]. Angiogenesis is required for expansion of the tumor colony, allowing for the somatic evolution of a genetically diversified cancer cell population capable of completing the metastatic process. Furthermore, the blood circulatory system is the primary means for dissemination of cancer cells to distant organs, and angiogenesis provides a means for tumor cells to enter the blood stream. Angiogenesis-deficient mice have displayed an absence of metastasis [25] and the inhibition of angiogenesis has shown a reduction in metastasis under mice models [26, 27]. The vascular stage is associated with tumors capable of metastasizing, and studies have reported the prognostic significance of vasculatures in primary tumor such as lung [28], breast [29] and liver [30, 31]. The number and density of microvessels in different human cancers directly correlate with their invasive potential and metastatic capabilities [24], and a prospective study of over 400 melanoma patients identified vascularity as the most important determinant in overall survival [32]. Tumor vasculatures are pathologically different compared to normal vasculatures, and tumor cells are able to enter the circulatory system much easily due to these morphological differences, which will be discussed in the next chapters.

1.2.2 The Angiogenesis Cascade

Angiogenesis is regulated by both pro-angiogenic and anti-angiogenic factors. The induction of an ‘angiogenic switch’ is when the balance of pro and anti-angiogenic factors tips in favor of pro-angiogenesis (Figure 1-1). The angiogenic switch triggers quiescence vasculature into perivascular detachment and vessel dilation, followed by angiogenic sprouting, formation and maturation of new vessels, and finally the

recruitment of perivascular cells [24]. Pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF) and platelet-derived growth factor (PDGF) are able to stimulate angiogenesis, while natural inhibitors such as the statins are able to keep ECs in a quiescence state (Figure 1-1).

Figure 1-1. The angiogenic balance. The angiogenic switch is affected by pro- and anti-angiogenic factors. When the balance tips in favor of pro-angiogenic factors, the angiogenic switch is triggered and ECs undergo angiogenesis. Figure reprinted from reference [24] with permission from Nature Publishing Group.

When a quiescent vessel senses the release of an angiogenic signal such as VEGF or PDGF, pericytes first detach from the vessel wall which is mediated by angiopoietin-2, and remove themselves from the basement membrane by proteolytic degradation mediated by matrix metalloproteinases (MMPs) [25]. Existing vessels dilates as ECs loosen their junctions. Blood vessels become highly permeable in response to VEGF,

and plasma proteins escape the leaky vessels to lay down a provisional basement membrane. Integrin signaling triggered by interactions with the matrix then assist ECs to migrate into the scaffold. Furthermore, ECs proliferate when more angiogenic factors are released by protease degradation of the extracellular matrix (ECM). A tip cell then facilitates the sprouting of the pre-existing vessel by Notch ligands Dll4 and Jagged-1 to form vascular networks and are equipped with filopodia to sense environmental cues. Neighboring cells assume supporting roles as stalk cells by differentiating and elongating while multiplying and trailing after the tip cell. Upon establishing a blood vessel, the blood vessel has to mature and stabilize to form functional blood vessels. Pericytes are recruited by PDGF to cover nascent blood vessels and ECs resume its quiescence state with the removal angiogenic cues. However, pathological angiogenesis occurs in tumor and ECs are always activated by over expression of VEGF and are seldom in quiescence state. The differences between physiological and pathological angiogenesis will be highlighted in chapter 1.2.3.

1.2.3 Tumor Angiogenesis

Physiological angiogenesis and pathological angiogenesis (or tumor angiogenesis) differs in certain aspects. The tightly regulated process controlled by pro- and anti-angiogenic factors is dysregulated in tumor angiogenesis. In normal angiogenesis, new blood vessels form rapidly from quiescence vasculatures, mature and become stable. In tumor angiogenesis, however, the blood vessels do not return to quiescence state, have delayed maturation and continue to grow new blood vessels. They are irregular compared to normal blood vessels, and have disordered and chaotic architectures. The vascular structures are aberrant and dilated, with abnormal branching characteristics and often leads to dead ends [26]. The vascular networks are also often leaky due to loose

association with pericytes, which is frequently absent [24]. These features are mainly due to the disproportionate expression of pro- and anti-angiogenic factors within the tumor microenvironment. Overexpression of VEGF, contributes to vascular permeability and continuous growth of vasculatures, and tumor hypoxia further complicates the angiogenic response [24].

Tumor angiogenesis is especially important for the sustained growth of tumors and dissemination of cancer cells. Tumors that lack microvessels are limited in size and become necrotic or apoptotic. Angiogenesis is one of the hallmarks of cancer which has been well studied [27]. By gaining access to the host vasculature system, tumors are able to fulfil their needs for oxygen and nutrients, and also remove metabolic wastes, allowing them to grow into sizes of larger than 1 mm [26]. These tumor vasculatures are leaky in nature, so tumor cells are able to fall into the vessels or intravasate into the vessels to be disseminated to other parts of the body. Tumor angiogenesis therefore promotes metastasis, and studies have also found that the vasculatures in primary tumor has prognostic significance in some tumors such as lung [28], breast [29] and liver [30, 31]. Hence, anti-angiogenesis has been identified as a potential therapeutic mechanism in the approach for the treatment and prevention of progression of many cancer types such as HCC [30, 32], advanced cervical cancer [33] and advanced non-small-cell lung cancer [28].

1.2.4 Angiogenesis in HCC

The progression of HCC requires the formation of new blood vessels. HCC is an excellent model to study the interplay between tumor cells and ECs as HCC is identified as one of the most vascular solid tumors, with hyper vascular invasion [34] and a significantly elevated level of VEGF in serum. The underlying blood vessels provide a

means for increased access of tumor cells into the bloodstream in HCC. Furthermore, Sugino *et al.* have reported interactions of HCC cells with blood vessels are key elements in the metastasis of HCC cells [35]. Understanding interactions between tumor cells and the host stroma would bring about new perspectives regarding the role of tumor microenvironment in cancer metastasis. I focus on HCC cell lines to study tumor-angiogenesis due to its hypervascularity and poor prognosis relating to metastasis spread.

1.2.5 Vascular Endothelial Growth Factor (VEGF)

High expression levels of VEGF is able to initiate angiogenesis, which is commonly secreted by many tumors. Tumors secrete elevated levels of VEGF at the rim of necrotic and hypoxic tissues. Hypoxia or lack of oxygen is one of the stimuli leading to the stabilization of hypoxia-inducible transcription factors (HIFs), which in turn lead to the upregulation of VEGF and promoting new blood vessels formation.

Vascular endothelial growth factor, or VEGF, is a signaling protein that promotes angiogenesis. Among the members in the VEGF family (VEGF-A, B, C, D and E), VEGF-A (also commonly known as VEGF) is expressed by many cells. It is a natural cytokine produced by many cell types, including platelets, macrophages and tumor cells. All members of the VEGF binds to the tyrosine kinase receptor VEGFR, which forms dimers and become phosphorylated for downstream signaling pathways. VEGF-A binds to the receptor VEGFR-2, leading to the downstream cascade of massive signaling pathways. These signaling pathways includes the mitogen-activated protein kinase (MAPK) cascade comprising p44/p42 MAPK or extracellular signal-regulated kinase mitogen (ERK) and p38 MAPK, PI3K/Akt/mTOR and the focal adhesion kinase (FAK) (Figure 1-2), which trigger angiogenesis processes such as proliferation, survival, differentiation, migration and vascular permeability [36].

Figure 1-2. Signal transduction pathways mediated by tyrosine kinase receptor focused on angiogenesis. Figure reprinted from reference [36] with permission from Springer .

VEGF-C and D binds to VEGFR-3, which mediates lymphangiogenesis, the formation of lymphatic vessels from existing lymphatic vessels. Both blood vessels and the lymphatic vessels are modes of dissemination of metastatic cancer cells, with similar mechanisms in angiogenesis and lymphangiogenesis. Here, I focus on angiogenesis, which is commonly indicated as the key route in the dissemination of tumor cells, and is identified to be present in most solid tumors. The important role in which VEGF contributes to angiogenesis is well documented by many, and is considered the key factor contributing to tumor angiogenesis and metastasis. As such, the plasma level of VEGF is used as a prognostic indicator for many cancers [37-39].

1.2.6 Other Important Factors in the Regulation of Angiogenesis

i. Soluble Secretory Proteins

As VEGF is highly crucial in the formation of blood vessels, other growth factors contributing to angiogenesis are not as highly investigated. Secretory factors such as

PDGF and FGFs stimulates angiogenesis. The receptors of PDGF (PDGFR) shares limited but significant homology as VEGFR, therefore inhibitors targeting VEGFR are often effective against PDGFR. PDGF mainly recruit pericytes to stabilize ECs during the maturation process. Studies using anti-angiogenesis inhibitors against VEGFR and PDGFR have shown that blocking PDGFR renders vessels more sensitive to VEGF blockade by reducing pericyte coverage [40].

The FGF superfamily was among the first to be discovered with angiogenic properties. Aberrant FGF signaling promotes tumor angiogenesis, however FGF deficiency did not produce vascular defects in knockout mice [41], probably due to compensation of other growth factors mediating angiogenesis. Unsuccessful clinical trials on FGF inhibition has also rendered it to be insignificant in the discovery of anti-angiogenesis agents.

Angiopoietins (Ang) are a class of angiogenic stimuli with agonist and antagonist functions. The Ang ligands and their Tie receptors have been identified as the second vascular tissue-specific tyrosine kinase receptor system [42] after VEGF and its receptors. Ang –Tie signaling has multi-functionality in EC quiescence, vascular remodeling and tumor angiogenesis. The molecular mechanisms of agonistic and antagonistic functions in Ang 2 is still unclear, along with the unexplored signaling mechanisms of Tie 1. These conflicting biological activities of the Ang-Tie system warrants caution when considering them to be therapeutic targets in anti-angiogenesis [25].

ii. Cell-cell communication

The tumor microenvironment mediates tumor angiogenesis with tumor-endothelial interactions at play. Direct cell-cell contact is required for Notch and Wnt signaling. The

Notch and Wnt/ β -catenin signaling pathways are guidance signals in tip and stalk cells during vessel branching. Wnt is able to activate Notch by a reciprocal feedback mechanism as Wnt signaling induces a Notch-like phenotype in ECs [43]. Notch signaling triggers tip or tube phenotypes in ECs, and is critical in the proper construction of the vascular system. Notch receptors (Notch 1, 2, 3 and 4) are paired with Jagged-1, -2 or Delta-like (Dll) 1, 3 or 4 ligands, of which the Jagged-1 and Dll4 has been implicated in tumor angiogenesis. Two publications, Zeng *et al.* and Funahashi *et al.* have reported significant upregulation of Jagged-1 ligands in tumor cells and may be implicated in the direct crosstalk between tumor cells and ECs to promote tumor angiogenesis through MAPK signaling [44, 45]. Notch ligand Dll4 is overexpressed in many tumor ECs, and the expression of Dll4 is positively correlated with VEGF expression. Preliminary investigations have shown that inhibition of Dll4 causes the overgrowth of nonfunctional tumor vasculatures [46]. Jagged-1 ligands expressed by tumor cells engaged in Notch receptor in neighboring ECs which may be important in EC differentiation [47]. Clearly, the function of Notch ligands and receptors is an important area for investigation into the tumor microenvironment and has potential therapeutic applications in tumor angiogenesis.

iii. Extracellular Matrix (ECM)

The ECM is an important component in angiogenesis. ECM is present in the surrounding, where vascular ECs adhere to the ECM to form stable vasculatures. The ECM component includes collagen, fibronectin and laminin, which forms the basement membrane, and various angiogenic cytokines are deposited in this matrix [48, 49]. It serves as a physical scaffold for organization cues in the absence of cell-cell contact [48]. The ECM controls the cytoskeleton of ECs to initiate capillary morphogenesis and

remodel the vasculature through integrin-dependent signaling pathways. ECs adhere to the ECM through integrin receptors present on the cell surface, and activate FAK, triggering multiple downstream signaling pathways such as cell proliferation, survival, angiogenesis, etc. [48]. The ECM components are secreted by ECs mainly; yet, pericytes may secrete some components of the ECM too. The properly assembly of the basement membrane matrix may require supporting pericytes such as fibroblast and muscle cells. In particular, fibroblasts contribute to the ECM by producing fibronectin and facilitating the assembly of the fibrin matrix [50], which regulates neovessel formation by controlling actin reorganization and inducing EC tube morphogenesis. Collagen type I and fibrin gels are some ECM components used to stimulate vascular tube morphogenesis in 3-dimensional (3D) matrices [51, 52].

1.3 Vascular Targeting Strategies

Anti-angiogenesis therapies hold promise in the treatment of many cancers as disruption of the tumor vasculatures leads to a decrease in tumor growth. Unlike chemotherapeutic drugs, anti-angiogenesis drugs are unique cancer-fighting agents aimed at targeting the blood vessels instead of tumor cells. There are two main groups of vascular targeting strategies: vascular disrupting agents (VDAs) and angiogenesis-inhibiting agents (AIAs). VDAs rapidly induce cell death in the established tumor vasculatures, while AIAs inhibit the formation of new blood vessels. Currently, AIAs used in anti-cancer treatments include anti-VEGFR inhibitors such as bevacizumab, and sorafenib [53] for HCC. VDAs such as combrestastain A-4 phosphate disrupt the cytoskeleton of ECs [54], resulting in regression of unstable tumor neovessels and central tumor necrosis [53, 55]. Both VDAs and AIAs are used to target different stages of cancer. AIAs are important during tumor development where neo-angiogenesis take place, whereas, VDAs target

blood vessels that are already established in the tumor, damaging vascularized tumor. Here, I focus on AIAs important for the prevention of new vasculature formation to prevent metastasis.

1.3.1 Angiogenesis Inhibiting Agents (AIA)

In 1971, Judah Folkman first proposed angiogenesis inhibition as a therapeutic strategy against cancer, where he identified angiogenesis as a criterion for the growth of solid tumors and dissemination of cancer cells (metastasis). Since then, many angiogenesis inhibitors have been discovered, with benefits offered to cancer, retinopathies and chronic inflammatory processes, as well as therapeutic angiogenesis for the treatment of ischemic diseases and arteriosclerosis [56].

AIAs, also known as anti-angiogenic agents, are cytostatic in nature. They suppress endothelial growth and are not toxic to tumor cells. AIAs are therefore designed to target early stages of tumor growth or developing metastases [53] with new blood vessels arising from pre-existing vasculatures. AIAs most commonly target VEGF, which has found to be the key driver for angiogenesis. VEGF is overexpressed in most solid tumors such as HCC [30, 31] and small non-lung cancer [57] and inhibition of VEGF can suppress tumor growth [58]. Combination of anti-angiogenesis drugs with chemotherapeutic drugs such as paclitaxel have shown improvement to progression-free survival (PFS) and/or overall survival (OS) in many cancers [58].

There are several FDA approved AIAs currently in the market: bevacizumab, sorafenib, sunitinib, axitinib and pazopanib. Bevacizumab, or Avastin[®] was the first FDA approved anti-angiogenesis inhibitor that has shown efficacy in many metastatic cancers [58]. Unlike most AIAs, bevacizumab is a humanized monoclonal antibody that binds to VEGF-A and blocks the downwards signaling of VEGFR-2 which subsequently

triggers the angiogenesis cascade discussed in chapter 1.2.2. Other AIAs function mainly as anti-tyrosine kinases with different binding capabilities on VEGFR and PDGFR, resulting in massive inactivation of signaling pathways in ECs (Figure 1-2). Part of this thesis focuses on three AIAs, sorafenib, sunitinib and axitinib, which function as anti-tyrosine kinases with differential binding activities to specific receptors, in order to understand and inhibit tumor-induced angiogenesis.

1.3.2 Sorafenib, Sunitinib and Axitinib

Sorafenib is approved for the treatment of advanced RCC, advanced thyroid carcinoma, and has been established as the standard first-line therapy in advanced HCC. It is a multi-kinase inhibitor targeting VEGFR-1, 2 and 3, PDGFR- β , c-Kit, RET and blocks serine/tyrosine kinases Raf-1 and B-Raf. Unlike many anti-angiogenesis inhibitors, sorafenib also targets tumor cells. It is the first oral multikinase inhibitor targeting Raf which affects tumor signaling and the tumor vasculature [59]. Mitogen-activated protein kinase kinase (MEK)/ERK signaling pathway is a crucial pathway for tumor proliferation, survival, induction of cancer stem cell phenotype, EMT and metastasis. The broad spectrum of activities sorafenib elicit on tumor cells is unclear. New mechanisms of actions on apoptosis and EMT are still currently uncovered. Nagai *et al.* revealed that inhibition of the MEK/ERK pathway reduced hepatocyte growth factors (HGF) which induces EMT in HCC cell lines. EMT-related proteins such as zinc finger protein SNAI1 and N-cadherin, was downregulated, preventing the transformation of HCC cell lines and decreasing the invasiveness of HCC cell lines [60]. Fernando *et al.* have also reported the induction of apoptosis in HCC cells by selectively targeting tumor cells producing autocrine signals which protects them from adverse conditions [61]. This effectively sensitizes HCC to sorafenib and protect normal hepatocytes from

apoptosis. The success of sorafenib encourages more molecularly targeting molecules to be discovered for chemotherapy.

Sunitinib is an orally available tyrosine kinase inhibitor approved by the FDA for anti-angiogenesis therapies in metastatic cancer. It inhibits several tyrosine kinase receptors such as VEGFR-1, 2, 3, PDGFR α , β , c-KIT and RET. The broad based specificity towards tyrosine kinase crucial for the angiogenic process makes it suitable to function as an anti-angiogenesis agent. It is currently approved for the treatment of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumor (GIST) [62]. Treatment of HCC with sunitinib has unfortunately seen multiple side effects and high toxicities in clinical trials [63]. The cellular mechanism of sunitinib has been reported with direct cytotoxicity on tumor cells, anti-angiogenic effects resulting in delayed tumour growth or tumour regression, and vascular disruption of existing vessels resulting in tumor necrosis [64].

Sunitinib is currently under clinical trials for other cancer types. Nonetheless, there exist the possibility of employing sunitinib as an anti-angiogenesis agent, combined with other chemotherapeutic drugs for the treatment of HCC in future. Majumder *et al.* have studied the cellular mechanism of sunitinib and its implications in angiogenesis and concluded that sunitinib effectively blocks hepatic stellate cells (HSC) and also blocks both *in vitro* and *ex vivo* angiogenesis by reducing EC formation and decreasing vascular sprouting [65]. Anti-tumor effects of sunitinib have been observed by Bagi *et al.*, with inhibition to HCC proliferation, induction of the wild type p53 tumor suppressor protein, and arrest to the cell cycle at the S/G1 phase [66]. Large areas of necrosis and reduced viable tumor cells was visualized in *in vivo* orthotopic studies with HCC cell lines [66]. Thus, sunitinib can be potentially used as a drug candidate for HCC due to its broad

range of targets to the tumor cell and ECs, although potential toxicity issues have to be taken into consideration.

Axitinib is a tyrosine kinase inhibitor specific for VEGFR-1, 2 and 3, PDGFR and c-Kit [67]. It has high binding affinity towards VEGFR-1, 2 and 3, and PDGFR, with efficacy found at nanomolar concentrations [68, 69]. Hu-Lowe *et al.* have reported that axitinib blocks VEGF-mediated EC survival, inhibits endothelial tube formation in a 3D matrix system, and dose-dependently reduced the phosphorylation of downstream signaling pathways such as Akt, endothelial nitric oxide synthase (eNOS) and ERK, which are targets of VEGFR-2 [69]. Anti-tumor effects associated with anti-angiogenesis effects is demonstrated by axitinib in nonclinical models, with minimal and/or well-tolerated side effects [69]. It is currently approved for the treatment of RCC and in clinical trials for HCC [70]. Kang *et al.* have reported that axitinib has significantly improved PFS (3.6 vs 1.9 months with placebo) and time to tumor progression (TTP, 3.7 vs 1.9 months with placebo) as second-line therapy in HCC [71]. The potential use of axitinib in combination with other chemotherapeutic agents can be best used for maximal anti-tumor efficacy, and multiple clinical trials have demonstrated the efficacy of combination studies with axitinib [72].

1.3.3 Resistance to Anti-Angiogenesis Therapy

In contrast to conventional anticancer drugs, anti-angiogenesis agents are a unique class of drugs in fighting cancer. They do not act directly on cancer cells, but function by limiting the growth of blood vessels. Anti-angiogenesis agents prevent tumors from growing, essentially controlling the size of tumors to achieve a balance as a means of cancer therapy. In the previous decades, tumor angiogenesis has been regarded as one of the many therapeutic means to treat cancer. Secretory factors released by tumor cells

are well investigated as key inducers in tumor angiogenesis. Tumor cells secrete substantial amounts of VEGF to pre-existing blood vessels and initiate vascular sprouting towards the tumor. VEGF is highly expressed in many tumors, and is an essential regulator in angiogenesis. Many research and clinical studies focused on the VEGF pathway to target and limit tumor angiogenesis, with multiple inhibitors targeting VEGF and PDGF discovered and currently approved in the market.

Despite the success of these angiogenic inhibitors, complications and resistance relating to the drugs have been discovered in recent years. Many angiogenesis inhibitors are gradually losing their therapeutic efficacy when used in cancer treatments. Resistance to anti-angiogenesis therapy can be divided into two modes: 1) adaptive resistance and 2) intrinsic, non-responsiveness to anti-angiogenesis therapies (Figure 1-3). In adaptive resistance, clinically available anti-angiogenesis drugs only target the core-signaling pathway of angiogenesis: the VEGF pathway. Different tumors exhibit widely varying susceptibility to VEGF blockade. In addition, tumor cells may secrete many different soluble secretory factors important for angiogenesis; therefore, targeting VEGF may prove to be ineffective in many occasions. The interconnected and compensatory pathways discussed in chapter 1.2.6 limit the effectiveness of inhibiting VEGF signaling alone. Furthermore, cell-cell communication such as Notch and Dll ligands are critical in angiogenesis, and currently no commercial drugs targeting these pathways have been identified yet. Research targeting new mechanisms of angiogenesis would revitalize angiogenesis drugs as a class of drugs for targeting the cancer stroma. In addition, recent clinical treatments have focused on the simultaneous inhibition of multiple angiogenic signaling pathways, aiming to reduce drug resistance to VEGF inhibitors.

Figure 1-3. Modes of resistance to anti-angiogenesis therapy. Figure reprinted from reference [73] with permission from Nature Publishing Group.

In the second mode of resistance, clinical efficacy of anti-angiogenesis drugs may be ineffective in certain cancers. Anti-angiogenesis drugs have found to be more effective in highly vascularized tumors such as RCC, and multiple anti-angiogenesis drugs have been approved for the treatment of RCC [74, 75]. In contrast, less vascularized tumors such as pancreatic cancer have displayed low efficacy or appeared to be resistant to anti-angiogenesis drug treatment [73]. Bevacizumab has been revoked as an anti-angiogenesis drug for treatment in metastatic breast cancer [76]. The inherent angiogenic property of the host organ plays a significant role in determining the efficacy of anti-angiogenesis therapy.

1.3.4 Other Drawbacks of Vascular Targeting Strategies

Many angiogenesis inhibitors have been discovered since anti-angiogenesis was proposed as a treatment strategy in cancer. However, in recent years, several reports have shown that short term usage of anti-angiogenesis drugs may cause accelerated metastasis in tumors [77, 78]. Despite transient vessels stabilization and regression of the tumor, survival is often not prolonged in individuals. Patients undergoing anti-angiogenesis therapies may experience survival benefits of several months; however, the spread of tumor cells to other organs is ultimately inevitable. In addition, anti-angiogenic therapy is in many cases short-lived as withdrawal of treatment induces rapid tumor vessels regrowth and relapse of tumor growth and metastasis. Studies have shown that VEGFR inhibitors only target ECs, triggering the regression of blood vessels, but bequeathing a potential scaffold for the regrowth of tumors [79]. Inai *et al.* demonstrated the preservation of ghost vessels with angiogenesis inhibitors, where pericytes did not degenerate and the vascular basement membrane persisted after ECs degenerated, allowing rapidly tumor vessels regrowth by trailing along empty basement membrane sleeves [80].

Another downside to anti-angiogenesis therapies arise from the perpetual need tumors have of blood vessels. Tumors have unlimited needs for nutrients and oxygen. By employing anti-angiogenesis treatments, blood vessels are severed from the tumor, generating a hypoxic and acidic environment [81]. Excessive waste causes low pH, while lack of oxygen generates a hypoxic condition in the tumor. Both hypoxia and acidosis triggers genomic instability and modifications to protein expressions such as HIF, as tumor cells adapt to the adverse environment. Angiogenesis, Inflammation, EMT, induction of stem cell phenotype, acquired resistance to apoptosis, and autophagy are some survival mechanisms adapted by tumor cells [81]. Anti-angiogenesis drugs

targeting only ECs by regulating the VEGF pathways may elicit tumor progression and enhanced malignancy as tumor cells employ multiple compensatory signaling pathways to acquire new resistance and enhanced metastatic capabilities (Figure 1-4). New counteractive solutions have been proposed to simultaneously employ angiogenesis inhibitors with inhibitors targeting other molecular pathways or chemotherapeutic drugs to effectively target tumor cells.

Figure 1-4. Hypoxia and acidosis resulting in tumor progression. Tumor cells are packed in a dense environment where cells undergo unregulated continual proliferation. Without blood vessels, tumor cells experience hypoxia and acidosis when oxygen is insufficient and excessive metabolic waste are deposited into the surroundings. Hypoxia and acidosis leads to adverse consequences with tumor progression and enhanced metastatic capabilities. Reprinted from reference [81] with permission from Elsevier.

In addition, tumor cells are able to modify the surrounding microenvironment. Key stromal cells include ECs, cancer-associated fibroblast (CAF) and tumor associated

macrophages (TAM). The interactions of these cells and the ECM with tumor cells contribute to many aggressive phenotypes of cancer such as angiogenesis, tumor proliferation and metastasis. New directions in cancer research now aim to elucidate the complex interplay of the tumor cells and its tumor microenvironment, which can be translated into therapeutics targeting cancer-supporting stroma, including ECs. Investigating the tumor microenvironment will produce new insights on how tumor cells modify their microenvironment to stimulate blood vessels growth and tumor progression.

1.3.5 The Future of Tumor Angiogenesis

Although targeting the tumor vasculature has not always improve patient OS clinically, progression free benefits have been achieved in many cases [82]. Clinical evaluations of anti-angiogenesis drugs have been difficult and most studies compare anti-angiogenesis drugs with chemotherapy drugs as an evaluation of their efficacy. The comparison of a cytostatic anti-angiogenesis drug against a cytotoxic chemotherapeutic drug with conventional endpoints such as tumor shrinkage and OS as a mean to assess their efficacies is not reasonable. Clearly, a chemotherapeutic drug targeting the tumor would most probably be much effective a killing tumor cells and prolonging OS. A reappraisal of the design of clinical trials have emphasized the evaluation of anti-angiogenesis drugs by assessing the benefits of a particular chemotherapeutic drug alone compared with the same agent in combination with an anti-angiogenesis drug.

Another challenge faced in tumor angiogenesis is drug resistance observed in clinical applications of angiogenesis inhibitors. The modes of resistance has been discussed in chapter 1.3.3. Previous research focusing on VEGF as the central regulatory role of angiogenesis has brought about much advancement in angiogenesis, but new

mechanisms governing angiogenesis has yet to be explored. The other important factors essential for angiogenesis have been discussed in chapter 1.2.6, and recent studies are now leaning towards cell-cell communication in tumor-endothelial induced angiogenesis. Understanding the function of Notch, Dll ligands and other signaling pathways would bring about new progress in targeting tumor angiogenesis.

Furthermore, there exist a need for better angiogenesis assays or animal model to more effectively evaluate angiogenesis inhibitors. Many *in vitro* assays utilize ECs to study angiogenesis, however no tumor cells are incorporated into the assay and are not representative of tumor angiogenesis. The flaws of these assays will be covered in chapter 1.4.1. Furthermore, animal models are mostly performed by implanting a human tumor xenograft in the subcutaneous space of mouse models and the anti-angiogenic responses of metastases growing on different organ sites are not evaluated appropriately. The inherent angiogenic capacity of the host organ affects the efficacy of anti-angiogenesis drugs and this area has not yet been explored. Recognizing the inherent angiogenic capacities of different target organs may contribute to unanswered question related to the efficacy of anti-angiogenesis drugs in different types of cancer.

Finally, the tumor microenvironment (discussed in chapter 1.3.4) contributes to angiogenesis and metastasis. The future of angiogenesis should be broaden to encompass the knowledge that the host stromal surrounding the tumor support tumorigenesis as tumor cells drive the construction of a niche suitable for cancer development. Tumor vascularization requires the cooperation of multiple cell types in the tumor microenvironment, including ECs, pericytes, mesenchymal stem cells (MSCs), CAFs and TAMs. Targeting the stromal component reeducates the tumor microenvironment, reducing the aggressiveness of tumors.

Sorafenib displays dual inhibitory effects on molecular components Raf/MEK/ERK, VEGFR-2 and PDGFR, prevents angiogenesis in ECs and inactivates signaling pathways critical in tumor cells. Patients receiving sorafenib has shown benefits greater than VEGFR-only inhibitors [82]. Targeting signaling pathways in both tumor cells and the surrounding host stroma destroys the tumor microenvironment, effectively disrupting the pre-constructed tumor niche, and preventing the regrowth of tumors and vessels. The development and success of sorafenib in targeting advanced cancers such as HCC and RCC encourages the discoveries of drugs targeting molecular signaling pathways in both tumor cells and the supporting stromal.

1.4 Tools for Anti-Cancer / Anti-Angiogenesis drug screening

1.4.1 Angiogenesis Assays

In vitro and *in vivo* angiogenesis assays are employed in the screening of anti-angiogenesis drugs. The formation of new tubular structures from quiescent ECs is one of the major steps in angiogenesis. As angiogenesis triggers many different behaviors and morphological alterations, proliferation, differentiation and migration assays are commonly used to examine angiogenesis in *in vitro* experiments. Proliferations assays include MTT assay, cell cycle analysis, and BrdU assay to assess EC proliferation from quiescent primary culture of ECs. However, as transformed ECs with proliferating capabilities have been introduced into the market for convenience, proliferation assays have become a complementary method to examine angiogenesis. Migration assays, which include wound healing assay or the use of Boyden chamber, aim to measure the migrating capability of ECs. However, ECs with transformed capabilities of proliferation are also equipped with migrating capabilities.

Only differentiation assays remain as the standard assay to assess angiogenesis. Angiogenesis can be determined by three popular *in vitro* angiogenesis differentiation assays: matrigel, 3D gel and co-culture [83-85]. Table 1-1 summarizes the advantages and disadvantages of *in vitro* differentiation assays available. Matrigel and 3D gel assays provide an extracellular matrix with pro-angiogenic factors and scaffold attachment to induce tubular formation in ECs, but they are not suitable for studying the effects of tumor cell-EC interactions on promoting angiogenesis. Most co-culture studies exploring angiogenesis mechanisms are only conducted with the host stromal cells and ECs or physiological angiogenesis [86-88]. Only few have investigated the signaling mechanisms triggered when co-culturing ECs with tumor cells. Thus, this will be the focus of the thesis.

Table 1-1. Types of *in vitro* EC differentiation assays to study angiogenesis.

Differentiation assays	Advantages	Disadvantages	Refs
Matrigel assay	Rapid, with formation of tube-like structures	Non ECs are able to form tubes, does not involve tumor cells	[87, 89]
3D gel or sprouting assay	Tubules form in all three dimensions, more closely mimics <i>in vivo</i> situation, require the degradation of matrix – involvement of matrix metalloproteinases	Cell aggregate-based angiogenesis, does not involve tumor cells, difficult to analyze	[89]
Co-culture of ECs with other cells	Formation of microvascular networks, tubules form lumen, mimics <i>in vivo</i> situation	Undefined interactions between EC and other cells	[86, 89]

1.4.2 Matrigel Differentiation Assay

Matrigel differentiation assay is the gold standard to observe EC differentiation. When ECs are seeded on top of a layer of matrigel, they undergo differentiation and elongation, resulting in the formation of tubular networks. Matrigel is a source of ECM, which is an extract from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells [89, 90]. It contains multiple growth factors and a matrix scaffold, allowing cell-matrix adhesion and the induction of multiple angiogenic pathways. This triggers the rapid differentiation of ECs when they are cultured on them. However, the matrigel differentiation assay is able to trigger many types of cell lines to form tubular networks when cultured on top of the layer of matrigel, including breast cancer cells MDA-MB-231, glioblastoma cells U87-MG and primary human fibroblast cells [87]. This differentiation is not limited to ECs only; therefore, interpretation of results should be done with caution. Furthermore, without the addition of tumor cells, tumor angiogenesis or the interactions between tumor and ECs cannot be studied effectively.

1.4.3 Co-Culture Models

Recent developments of co-culture systems between ECs and other cell types allow better understanding of cell-cell communications and molecular cross talk between cell types. These co-culture models are able to provide insights as screening platforms and create the microenvironment necessary to study mechanisms of tumor angiogenesis. Fibroblast and ECs are the most commonly used cell lines for co-cultures, with multiple drug screenings performed on the co-culture. Fibroblasts, being one of the most common stroma cells found in tumors, are responsible for secreting extracellular matrix components necessarily for the construction of a matrix scaffold to support angiogenesis [91, 92]. Drug screening with fibroblast-EC culture allows for the substantial

discoveries of anti-angiogenesis agents, however they do not principally target tumor angiogenesis. In order to discover anti-angiogenesis agents that effectively target the tumor vasculatures, tumor cells need to be incorporated into angiogenesis assays. Table 1-2 summarizes the co-culture models available as angiogenesis assays and their functions.

Table 1-2. Co-culture models of ECs with other cell types

Other cell type	Usage	Models	Function of cell	Refs
Fibroblast	Anti-angiogenic drug screening	Angiogenesis	Secretes necessary matrix components which act as scaffolds for EC differentiation	[86]
Vascular smooth muscle cells (VSMC)	Tissue engineering	Normal, atherosclerotic vessel wall	Pericyte support of vessel wall	[88, 93]
Mesenchymal stem cells	Tissue engineering	Bone tissue engineering	Secrete factors which stimulate proliferation and differentiation of ECs	[86]
Tumor cells Eg, Glioma cells	Extravasation process / tumor angiogenesis	Tumor angiogenesis / metastasis	Secretory factors promoting EC differentiation, Cell-cell interaction with EC through adhesion molecules	[86]

1.4.4 Two-Dimensional (2D) vs Three-Dimensional (3D) Cultures

2D cultures, 3D cultures [94] and experimental mouse models [95] have been used as models in the identification of anti-cancer drugs. However, 2D models often fail to

recapitulate the 3D environment *in vivo* and cells in 2D environment often lose their gene functions [96]. On the other hand, animal models are costly and the results obtained may not be easily interpreted [95]. Utilizing 3D cultures help to bridge the gap between animal model and 2D cell assays, improve drug discovery success rate and minimize cost before pre-clinical trials are performed [96, 97]. 3D cultures are able to create a tumor microenvironment with hypoxia and acidosis as tumor cells are packed in a compact environment, with lack of oxygen and excessive metabolite secreted. Furthermore, studies have revealed that cells in 3D culture consists significant alterations in cell behavior.

1.4.5 Fluorescence Resonance Energy Transfer (FRET) Sensor

Activation of caspase-3 is a central event in apoptosis. To detect the activation of caspase-3 in living cells, Luo *et al.* constructed a FRET-based apoptotic sensor [98] . This sensor consists of a cyan fluorescent protein (CFP) fused to a yellow fluorescent protein (YFP) with a specialized linker containing the caspase-3 cleavage sequence DEVD [98]. As shown in Figure 1-5, when the FRET sensor is excited with an excitation wavelength of 433 nm, CFP acts as a donor, transferring energy via FRET to YFP, which acts as an acceptor. As a result, most of the energy is transfer to YFP and YFP emits light at a wavelength of 535 nm. When cells are undergoing apoptosis and caspase-3 is activated, the DEVD sequence linking the CFP and the YFP is cleaved. This results in the separation of the donor and the acceptor, effectively eliminating the FRET effect. Therefore, when apoptotic cells are excited at an excitation wavelength of 433 nm, FRET effect is abolished and CFP emits light at a wavelength of 480 nm. By measuring the fluorescence emission ratio between YFP and CFP, it is possible to detect the activation of caspase-3 in living cells during apoptosis [98]. In this thesis, cell lines

transfected with the FRET sensor for caspase-3 is utilized for real time detection of apoptosis.

Figure 1-5. Schematic representation of FRET changes with caspase-3 activation. Figure reprinted from reference [99] with permission from The Royal Society of Chemistry.

1.5 Autophagy and Metastasis

Autophagy is a physiological self-degradative process undertaken by cells to degrade proteins and organelles. Autophagy has important homeostatic functions, including regulating cell metabolism, and removing misfolded proteins and damaged organelle. The degradation and turnover of long-lived proteins and organelles provide a source of energy for cell survival when cells undergo cellular stress such as nutrient deprivation. Cells may undergo autophagy to remove damaged organelles and proteins produced by stresses such as hypoxia, fluidic shear stresses and drug treatments. However, prolonged autophagy may also lead to autophagic cell death. Defects in autophagy play significant

roles in pathological diseases such as cancer, neurodegenerative diseases, aging, and infectious diseases [100, 101].

1.5.1 Potential Roles of Autophagy in Metastasis

Autophagy acts as a double-edged sword. Autophagy may promote or impede metastasis depending on the contextual demand placed on the tumor cells in the metastatic cascade. Autophagy may restrict inflammation, indirectly limiting invasion and dissemination of cancer cells at an early stage; on the other hand, autophagy may promote metastasis at later stages as they protect tumor cells from anoikis and fluidic shear stress within the blood circulatory system, and from nutrient deprivation when colonizing at distant organs [102]. Many evidences have indicated that autophagy is an important component of tumorigenesis [103], and modulates cell survival under unfavorable conditions during metastasis. However, the impact of autophagy in cancer metastasis are still not fully clarified in many areas. Many functional abilities such as migration, invasion, drug resistance, oxidative and metabolic capacities are changed in metastatic cancer cells via

Figure 1-6. Potential roles of autophagy during metastasis. Autophagy may promote or inhibit metastasis depending on the contextual demand. Figure reprinted from reference [102] with permission from Elsevier.

the induction of autophagy. Understanding the mechanisms by which autophagy influences cancer progression and metastasis will provide more autophagy-targeting strategies in cancer treatment.

1.5.2 The Process of Autophagy

There are three types of autophagy, namely macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy is an evolutionarily conserved process discovered in yeast, and later found in mammals and eukaryotes. The key structure, an autophagosome, engulfs cellular components (proteins, lipids and organelles) before fusing with a lysosome to form an autolysosome [120]. This cargo is released and degraded by the lysosome's hydrolases, and the resulting macromolecules are recycled back into the cytosol to be reused (Figure 1-7). Microautophagy mostly occurs non-selectively, and involves direct engulfment of the cytoplasm by the lysosome [104]. The lysosomal membrane randomly invaginates and differentiate into autophagic tubes to enclose cytoplasmic contents, subsequently dispatching a microautophagic vesicle into the lumen [105]. However, the selective removal organelles such as peroxisomes, mitochondria and the endoplasmic reticulum may also occur through mitophagy. CMA selectively targets single proteins via heat shock cognate 70 (hsc70) and bind to the lysosome-associated membrane protein type 2A (LAMP-2A), which translocate proteins into the lysosomal lumen [106]. Of the three types of autophagy, macroautophagy (hereafter refer to as autophagy) is the most common type of autophagy and is usually studied, whereas the molecular processes of microautophagy and CMA have remained limited as few investigators have studied these two processes in mammalian cells. Our knowledge of microautophagy and CMA mainly comes from the study of yeast, *Saccharomyces cerevisiae*.

A group of genes that was initially identified in budding yeast, *Saccharomyces cerevisiae*, facilitates autophagy. Currently, more than 30 genes, or autophagy-related genes (ATGs) have been identified and its orthologs discovered in mammals [107]. Autophagy is a dynamic process that involves five sequential steps: initiation, elongation, maturation, fusion and degradation.

Figure 1-7. The process of autophagy in mammalian cells. Figure reprinted from reference [108] with permission from Elsevier.

Organelles and cytoplasmic contents are first sequestered by a phagophore, before the complete elongation of a phagophore to form a double-membraned autophagosome. The elongation process requires the ATG5-ATG12 and ATG8-PE conjugation system. The ATG5-ATG12 complex recruits membrane proteins such as LC3 (the mammalian homolog of ATG8). Modification of the LC3 protein by conjugation to the phospholipid phosphatidylethanolamine (PE) allows the lipidated LC3-II isoform to associate with the autophagosomal membrane [109]. This suggests that LC3-II may be a marker for autophagosome. Next, matured autophagosomes fuse with lysosomes to form autophagolysosomes or autolysosomes, which contains lysosomal hydrolases. In the autolysosomes, lysosomal enzymes degrade the inner membrane of the autophagosome

and the cargo contained within. After degradation, the macromolecules are released back into the cytoplasm for reuse.

1.5.3 Regulatory Pathways of Autophagy

The PI3K/Akt/mTorc1 signaling network is one of the key negative regulators of autophagy in mammalian cells. mTorc1 is the central cell-growth regulator that integrates growth factors and nutrient signaling [110]. Under normal conditions, mTorc1 is activated in the presence of glucose and amino acids, directly phosphorylating serine/threonine-protein kinase ULK1 and ATG13 to inhibit the ULK1-ATG13-FIP200 complex, and preventing the initiation of autophagosome [110, 111]. Under nutrient starvations, mTorc1 is inhibited, thus releasing the ULK1-ATG13-FIP200 complex from its inhibitory phosphorylated state and leading to the induction of autophagy [111].

Another regulator of autophagy is the AMP-activated protein kinase (AMPK). AMPK acts as a key energy sensor and regulates cellular metabolism to maintain energy homeostasis [110]. During periods of metabolic stress, AMPK acts upstream of mTorc1 to inhibit its activity, and initiate autophagy [110, 112]. Furthermore, AMPK can act independently of mTorc1 to directly induce autophagy. During nutritional deprivation, mammalian AMPK is activated by low cellular energy levels, presumably via high AMP concentrations and LKB1 [112], which phosphorylates and activates ULK1 on multiple sites, leading to the activation and induction of autophagy [110, 113].

During autophagosome biogenesis, class III PI3Ks complex containing Beclin-1, Vps34 and p150 for are responsible for sequestration of autophagosome [114]. Studies have shown that PI3K inhibitors such as 3-methyladenine (3-MA), wortmannin and LY294002 interfere with nucleation of the isolation membrane during autophagy, preventing the formation of autophagosomes [115]. Other regulators of autophagosome

maturation and degradation include the endosomal sorting complex (ESCRT), which had been originally identified for recognition and sorting of ubiquitinated proteins into multivesicular bodies (MVBs) [116]. Autophagosomes are able to fuse with MVB to form a hybrid known as amphisome, which may then fuse with lysosomes to degrade the materials incorporated within [117]. New emerging evidences are showing convergence between autophagy and the endocytic pathways through the MVBs, with uncovered roles of ESCRT machinery in phagophore formation, autophagosome-lysosome fusion and degradation of cargos, however the process is still poorly understood.

1.5.4 Methods to Induce Autophagy

The most common methods to induce autophagy is through nutrient deprivation. Starvation can be achieved via 1) withdrawal of serum, 2) withdrawal of amino acids and 3) withdrawal of both serum and amino acids. Although withdrawal of glucose or suppression of the mTORC1 by using inhibitors such as rapamycin may induce autophagy, both treatments are weaker compared to the withdrawal of serum and amino acids [118]. Autophagy can also be induced by oxidative stress. High reactive oxygen species (ROS) levels may induce autophagy in cells through damage to the DNA and cellular organelles, rendering the removal of damaged organelles necessary through autophagy [119]. Various stress treatments are able to trigger autophagy, such as hypoxia, anoikis and drug treatment, however, in this thesis, nutrient deprivation was used as a means to induce autophagy.

1.5.5 Autophagic Flux

Autophagy is a dynamic process. The process of autophagy includes autophagosome formation and maturation, autolysosome formation, and the degradation steps [120], yet throughout the multi-step process, current research methods only allows for end point detection of each step. For example, LC3B levels can be detected by western blot analysis; however, the mere detection of the number of autophagosomes and the presence of LC3B is insufficient to evaluate the entire autophagy system. In any autophagy assay, an increase in LC3B-II levels may represent an increase in autophagosome or an impairment in the downstream degradation process. It is therefore important to measure autophagic flux or the autophagic degradation activity in a cell to understand whether the entire autophagy process is functional. A method to measure autophagic flux is through the measurement of LC3 turnover. [120].

One of the principal methods to measure autophagic flux in a cell is by monitoring the LC3 turnover. LC3-II is degraded in autolysosomes after fusion with lysosomes. If cells are treated with lysosomal inhibitors such as chloroquine (CQ) or bafilomycin-A₁ (Baf-A1) which prevents the formation of autolysosomes, the degradation of LC3-II is blocked, resulting in the accumulation of LC3-II [121]. Lysosomal turnover of LC3-II, and not its transient amount, reflects the autophagic activity.

Degradation of selective substrates in the autolysosome is also another indicator for autophagic flux. A standard autophagy substrate found in the autolysosome is p62 protein, also known as SQSTM1/sequestosome-1. p62 is selectively incorporated into autophagosomes through direct binding to LC3 proteins and is degraded by autophagy. Therefore, total cellular expression of p62 levels is inversely correlated with the autophagic activity. However, potential limitations have been raised with regards to the level of p62 and its correlation with autophagy. First, p62 can be transcriptionally

upregulated during autophagy, which may potentially lead to error in the interpretations of p62 levels as indicators of autophagic flux. Second, it is not clear whether p62 is solely degraded through autophagy or by other means such as the proteasomal pathway, therefore the measurement of autophagic flux with p62 protein levels should be cautiously interpreted.

Other methods to measure autophagic flux includes the measurement of lysosome-dependent long-lived protein degradation, and marking the LC3 protein with fluorescence protein to determine LC3 degradation or the formation of autophagosome/autolysosomes [108]. As all assays have limitations, the combination of different assays to accurately measure autophagic flux and autophagic activity is recommended.

1.5.6 Starvation and Autophagy

During nutrient deprivation by the withdrawal of amino acids, glucose or growth factors, the nutrient sensing regulator, mTorc1 is inhibited, resulting in the dephosphorylation of Ulk1 from AMPK [113] and activation of ATG13 to form autophagosomes. Other than the inhibition of mTorc1, ROS is triggered during starvation. Starvation-induced upregulation of ROS levels also leads to the activation of AMPK pathway [122], leading to downstream activation of autophagy. The upregulation of ROS during starvation has been reported in many studies, with immediate release of mitochondrial ROS [123, 124] or ROS generated by the NADPH oxidase (NOX) complexes present in the membrane [125, 126]. The mitochondria is important in regulating starvation-induced autophagy [122], however, the direct involvement of mitochondria in autophagy is not yet clearly understood.

1.5.7 The Future of Autophagy

Currently, inhibition of autophagy is viewed as an effective strategy in cancer therapy, and nearly 20 clinical trials are currently registered in the National Cancer Institute exploring anti-autophagy treatments in human cancers [103]. While there exists many uncertainties of how anti-autophagy strategies will fare as a form of cancer treatment, many preclinical data have supported this approach [127]. Inhibition to autophagy by chloroquine treatment was able to impair lymphoma development in mouse models [128] and knockout of essential autophagy genes such as ATG7 has shown attenuation of tumor growth [129]. Current clinical trials will hopefully provide insight into whether this will be a viable therapeutic paradigm.

1.6 Reactive Oxygen Species (ROS) and Autophagy

ROS at physiological levels are important cell signaling molecules. However, they are severely harmful to the cell when present in high concentrations. Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) are highly reactive towards lipids, proteins and DNA [130, 131]. Increased production of ROS leads to massive irreversible damage to DNA and oxidative damage to cellular macromolecules (lipids and proteins) and organelles, ultimately requiring autophagy to engulf and degrade oxidized substances and damaged organelles to reduce oxidative damages. The tight interactions between ROS and autophagy is reflected in two aspects: the induction of autophagy by oxidative stress, and the reduction of ROS by autophagy [132].

1.6.1 ROS and Redox Signaling

It is commonly accepted that the mitochondria is the principal source of ROS in the cell. During oxidative phosphorylation, electrons escape the electron transport chain (ETC)

and O_2^- are produced in the presence of oxygen. O_2^- are spontaneously converted into H_2O_2 by the superoxide dismutase (MnSOD) which is a resident protein in the mitochondria matrix. The relatively stable and membrane diffusible H_2O_2 is then removed by catalase and glutathione peroxidases (GSH-Px) found in the cytosol. Another source of ROS is the NADPH oxidase (NOX) family present on the membrane. NOX enzymes share the capacity to transport electrons across the plasma membrane, generating O_2^- and other forms of ROS in the process [133]. Two other forms of SOD present in the cytosol, the copper/zinc superoxide dismutase (Cu/ZnSOD), converts O_2^- into hydrogen peroxide in the cytosol, before the catalysis of hydrogen peroxide into water. The efficiency of the antioxidant defense determines the levels of ROS [134].

In the late 1990s, evidences point to ROS emerging as a key player in cellular signaling. ROS were able to reversibly modify sulfur-containing residues in proteins, mainly that of cysteine and methionine [134]. In particular, H_2O_2 was able to form S-hydroxylated (S-OH) with the reactive cysteine thiol groups (SH) [135, 136]. Oxidative modifications of these reactive cysteine causes modifications to protein structures and their functions, and are deemed to be molecular switches transduced by the redox signal [134]. Other than proteins, excessive ROS in the cell react with lipids in the cellular membrane, nucleotides in the DNA, triggering oxidative damage and mutations to DNA [137]. They may also cause cross-linking or fragmentation of ribonucleoproteins leading to base modification, rearrangement of DNA sequences, effectively exerting carcinogenic effects [137].

1.6.2 Autophagy Regulation via Redox Signaling

Both O_2^- [138] and H_2O_2 [139] play a role in redox signaling and are crucial in controlled mechanisms in cells as signaling molecules. Chen *et al.* have proposed that O_2^- is the

primary ROS produced during serum, glucose or amino acid deprivation that induce autophagy [138]. Many other evidences have however pointed to H_2O_2 as the key inducer in autophagy, with cysteine protease ATG4 as a direct target for oxidation by H_2O_2 during starvation, which is important for the elongation of phagophore by accumulating the pro-autophagic lipidated LC3-II isoform (Figure 1-8) [140, 141]. This process was reversible by the use of antioxidants such as N-acetylcysteine (NAC) [142]. Many have postulated a direct link between ROS and autophagy, as treatment with antioxidants partially or completely reverse the process, however, to date, only few have shown the exact role or mechanism involved with ROS, both O_2^- and H_2O_2 , in tuning autophagy.

AMPK is another candidate of induction of autophagy by ROS production. AMPK has been proposed to be activated upon H_2O_2 exposure by oxidation of the cysteines located on the α and β -subunits [143, 144]. Activation of AMPK would then trigger the downstream pathways to activate Ulk1 and inhibit mTorc1, leading to autophagy as mentioned in chapter 1.5.3.

Figure 1-8. Model depicting the ROS mediated autophagosome biogenesis. Figure reprinted from reference [145] with permission from Elsevier.

1.6.3 ROS and Mitophagy

ROS released from the mitochondria triggers oxidative damage to DNA, lipids and proteins, posing a threat to the cell integrity. Various defense mechanisms have been developed to remove cellular ROS by upregulation of antioxidant enzymes, or to remove damaged organelles through autophagy. The role for non-selective autophagy in response to ROS generation has been discussed in the previous chapters, however, selectively targeting of organelles can occur, such as mitophagy, or the removal of dysfunctional mitochondria by the autophagosome. High ROS levels within the mitochondria may result in mitochondrial DNA damage, lipid peroxidation and the opening of mitochondrial membrane channels, resulting in the loss of the mitochondria membrane potential ($\Delta\Psi_M$), which is central in mediating oxidative energy metabolism. Sakellariou *et al.* have reported that the increase of mitochondrial H_2O_2 production leads to mitochondrial oxidative damage in intact mitochondria [146].

The accumulation of ROS within the mitochondria causes its dysfunction, leading to mitochondrial fragmentation or fission and mitophagy by Parkin and Pink1. Both Pink1 and Parkin interacts with each other and Pink1 lies upstream of Parkin to trigger the translocation of Parkin when there is a loss of $\Delta\Psi_M$ [147]. Parkin translocate from the cytosol to the depolarized mitochondria under oxidative stress, or mitochondrial DNA damage [148, 149]. LC3 and p62 then interact with Parkin to ubiquitinate outer mitochondrial membrane proteins such as VDAC and Mfn1/2, leading to the sequestration of autophagosomes [150, 151].

In yeast, mitochondrial fission and ATG32, Uth1p and Aup1p are required for efficient mitophagy [152], however their equivalents are yet to be found in mammalian cells. Other proteins such as dynamin-related GTPase (Drp1) is required for mitochondrial fission in mammalian cells, and ATG7 and Ulk-1 which are required for

the formation of autophagosome were found to be important in mitophagy, however genetic knockout of these genes only delayed the clearance of mitochondria [153, 154].

A vicious cycle exists where high mitochondrial ROS damages the mitochondria, leading to more ROS production. The intervention of autophagy or mitophagy assist in the removal of damaged mitochondria, effectively reducing ROS and preventing the loss of cell integrity. Understanding how ROS production leads to mitochondrial dysfunction and its induction of autophagy in metastatic cells would allow a better understanding of how autophagy promotes metastasis.

1.7 Rationale of Project

This thesis mainly focuses on two mechanisms in cancer metastasis: tumor-endothelial contact-induced angiogenesis and autophagy in the progression of distant metastases. Cancer metastasis is the main cause of deaths in many tumors, however, most anti-cancer strategies emphasize on cytotoxic therapies to eliminate cancer cells, without designing effective strategies to prevent metastasis. In this thesis, I focus on the primary route of metastasis, where tumor cells enter the blood circulatory system to disseminate to other secondary organs. Angiogenesis has been implicated in tumor growth and expansion. However, angiogenesis as a critical component of tumor metastasis has been neglected. In many tumors, the vascular density acts as a prognostic indicator of metastatic potential, where highly vascularized tumors form distant metastases at a much rapid pace compared to less angiogenic tumors [155]. HCC is classified as a highly vascularized and metastatic tumor with high number of patients' death worldwide. Therefore, HCC-induced tumor angiogenesis will be the one of the focus in this thesis.

Another key element in metastasis is the ability of tumor cells to survive under adverse conditions. Autophagy has been implicated in many tumor cells with enhanced

adaptability to survive unfavorable conditions instead of undergoing programmed cell death. Our group had previously isolated cell lines derived (231-M1, 231-M1A and 231-M1B) from lung metastases in nude mice model, which have enhanced metastatic abilities compared to their parental cell line (231-C3). These breast cancer cell lines (231-C3, 231-M1 and 231-M1A) were used to study the differences in their autophagic capabilities in order to associate their survivability with their metastatic potential. Tumor cells often face nutrient deprivation after circulation in the bloodstream, while attempting to colonize distant sites. Here, the autophagic capabilities relevant to the metastatic cell lines and autophagy with regards to ROS formation induced by nutrient deprivation are studied.

1.7.1 Hypothesis of Project

Angiogenesis is a critical component in tumor metastasis. Despite the success of these angiogenic inhibitors, complications and resistance relating to the drugs have been discovered in recent years. Many angiogenesis inhibitors are gradually losing their therapeutic efficacy when used in cancer treatments. Current angiogenesis inhibitors are screened and discovered by standard angiogenesis assays (discussed in chapter 1.4.1), however, in most assays, tumor cells are absent and they are not suitable for studying the effects of tumor cell-EC interactions on promoting angiogenesis. Most co-culture studies exploring angiogenesis mechanisms are only conducted with the host stromal cells and ECs or physiological angiogenesis [86-88]. Here, I hypothesize that tumor cells play an important role in tumor angiogenesis, and are indispensable in certain cancer that are highly vascularized. These tumor cells may possess an intrinsic capability to induce tumor angiogenesis. By utilizing co-culture systems of tumor cells

and ECs, *in vitro* tumor angiogenesis can be constructed. The signaling mechanisms triggering tumor angiogenesis via tumor cells would also be investigated.

The crosstalk between mitochondrial ROS production, redox signaling and autophagy is not well understood. Metastatic cells employ autophagy as a survival mechanism to remove potential damage from ROS released from mitochondria. In our model, metastatic cells exhibit high levels of MnSOD as a survival advantage. I hypothesized that ROS play a role in autophagy via the rapid conversion of O_2^- into H_2O_2 by MnSOD, thereby allowing high levels of H_2O_2 to be present as signaling molecules. Autophagy in turn functions as a survival mechanism to protect the cell from cell death under oxidative stress and MnSOD is the key regulator here. By utilizing MnSOD to induce autophagy as a survival mechanism, the mitochondria is effectively removed, reducing ROS formation and maintaining the integrity of the cell. The crosstalk between mitochondrial ROS production, redox signaling and autophagy is investigated to recognize the impact of autophagy on ROS generation, mitochondrial function and its relevance to cancer metastasis.

1.7.2 Overall Aims of PhD Project

Cancer metastasis is the main cause of deaths in many tumors, however, most anti-cancer strategies emphasize on cytotoxic therapies to eliminate cancer cells, without designing effective strategies to prevent metastasis. This thesis aim to study two major mechanisms in cancer metastasis: tumor-endothelial contact-induced angiogenesis and autophagy in the progression of distant metastases. Investigating the complex functional interrelation between the cellular and non-cellular compartments of the tumor microenvironment would lead to the identification of new therapeutic targets.

Tumor-endothelial interaction is a key aspect in the tumor microenvironment and play important roles in tumor angiogenesis. Here, I focus on the approaches in which tumor cells directly induce angiogenesis by cell-cell communication. By understanding the mechanisms in which the tumor microenvironment is constructed, potential effective strategies targeting cancer cells through disrupting the tumor microenvironment and the interactions between tumor and stromal cells can then be developed.

Autophagy has been implicated in the survival of tumor cells during adverse conditions such as nutrient starvation and oxidative stress, which tumor cells face during metastasis and from the microenvironment. Autophagy has been implicated in drug resistance, anoikis, immune response and metabolic stress, all which are important in metastasis and tumor development. Cells that do not undergo cell death due to enhanced autophagic capabilities could form metastatic colonies in other parts of the organs. By uncovering the functional protein regulating the enhanced autophagic capabilities in metastatic breast cancer cells, potential therapeutic targets can be developed to inhibit autophagy and prevent metastasis.

Overall, two mechanisms (angiogenesis and autophagy) related to cancer metastasis have been studied. New insights to angiogenesis and autophagy would be valuable as therapeutic strategies in cancer metastasis. Investigating the complex functional interrelation between the cellular and non-cellular compartments of the tumor microenvironment would lead to the identification of new therapeutic targets.

CHAPTER 2 Materials and Methods

2.1 Antibodies and Reagents

The human hepatocellular carcinoma cell line, HepG2 and human umbilical vein endothelial cell, HUVEC, human breast cancer MCF-7, mouse fibroblast NIH-3T3 and vascular smooth muscle cells (VSMC) were purchased from ATCC (Manassas, USA). Human breast cancer cell line MDA-MB-231 was a generous gift from Prof. Xiaofeng Le from University of Texas, M.D. Anderson Cancer Center (Houston, USA). FRET sensor cell lines 231-C3, 231-M1 and 231-M1A were previously generated by our laboratory. Matrigel was purchased from BD Biosciences (San Jose, CA, USA). All drugs and inhibitors were purchased from Calbiochem (San Diego, USA). Culture media and all antibiotics were purchased from Invitrogen (Carlsbad, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, USA). Vimentin, VEGF and LC3B antibodies were purchased from Abcam. Phospho-Akt (Thr308), Akt, phospho-ERK, ERK, phospho-p70s6k, p70s6k and β -tubulin antibodies were purchased from Cell Signaling (Boston, USA). p62/SQSTM1 antibody was purchased from Novus Biological (Littleton, USA).

2.2 Cell Culture

HUVEC-C3 was generated by transfecting the plasmid DNA of a FRET-based caspase3 (C3) sensor into HUVEC cells [156]. HepG2-DsRed was generated by transfecting the plasmid DNA of pDsRed1-N1 vector into HepG2 cells and isolated from a single clone stably expressing the DsRed1 protein. Both HUVEC-C3 and HepG2-DsRed mono- and co-cultures, and other cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO, USA) supplemented with 10% FBS and 1% penicillin/streptomycin

(PS) at 37 °C and 5% CO₂. 3D co-cultures were maintained in DMEM supplemented with 20% FBS and 1% PS.

2.2 Functional assays

2.2.1 Imaging Microscopy

Cells containing the FRET sensor were captured with an inverted fluorescent microscope (Carl Zeiss, Thornwood, USA). The fluorescent images of C3 cells were separately captured using an excitation wavelength of 436 ± 10 nm and a 455-nm dichroic mirror. The emissions of YFP ($E_m = 535 \pm 15$ nm) and CFP ($E_m = 480 \pm 20$ nm) were recorded with a computer-controlled camera (AxioCam MRm, Carl Zeiss) with the Zen software (Carl Zeiss). Images were merged using the Image Pro Plus software (Media Cybernetics) to produce FRET images.

2.2.2 Determination of Cell Apoptosis with FRET Apoptotic Sensor

Green FRET images indicated live cells, while blue FRET images indicated apoptotic cells. The FRET ratio was calculated in the event that the colors of C3 cell was in dispute. Composite image of both YFP and CFP was used to measure the relative emission ratio of YFP/CFP in each cell. The outline of an individual cell and neighboring background references were traced in Image Pro Plus for measurement of YFP and CFP intensities. For calculation of the YFP/CFP ratio, background value was subtracted from both YFP and CFP channel prior to the subsequent division. To compare the apoptotic rates between different cells at various times, the relative emission ratio of YFP/CFP in each cell was calculated by normalizing the initial FRET ratio to 1.0 at t₀. Cells were considered

apoptotic if their relative FRET ratio was reduced from 1.0 to less than 0.6, which is equivalent to more than a 50% reduction of the FRET effect.

2.2.3 Immunofluorescence Staining

Cell lines were seeded in a 12-well removable chamber (Ibidi, Germany). After washing with PBS, cells were fixed at 4% paraformaldehyde (PFA) for 15 min at room temperature and permeabilized with 0.2% Triton-X 100 for 15 min followed by blocking with 3% BSA/0.3 M glycine/0.1% Triton-X 100 in PBS for 1 hr. After blocking, the cells were incubated at 4°C overnight with the primary antibody. (1:100 for vimentin, 1:50 for LC3B, 1:50 for p62). TRITC-conjugated secondary antibody (Calbiochem, USA) at a dilution of 1:100 was used to stain the cells for 1 h at room temperature. All antibodies were diluted in PBST containing 3% BSA. The nucleus was stained with Hoechst 33342 before cells were mounted onto a coverslip using Mowiol® 4-88 (Calbiochem). Images were captured on a fluorescence microscope (Axio Observer Z1, Carl Zeiss) equipped with a CCD camera (AxioCam MRm, Carl Zeiss)

2.2.4 Actin, Mitochondria and Lysosome Staining

Cells were stained for actin after fixation using phalloidin-rhodamine (Invitrogen) dissolved in methanol for 30 min. No permeabilization was required. Staining for mitochondria and lysosome were performed using MitoTracker® Red CMXRos (Thermo Fisher Scientific) and LysoTracker Red DND-99, both at 500 nM for 15 min, followed by fixation with PFA. Images were captured with a LSM 710 META laser scanning confocal microscope (Carl Zeiss, Thornwood, NY).

2.2.5 Western Blotting

Monocultures of HUVEC-C3, HepG2-DsRed and co-cultures of 2D and 3D cells were collected after culturing for two days. The cells were lysed in RIPA buffer containing protease and phosphatase inhibitors before sonicated on ice. Total protein concentration was measured by the Bio-Rad protein assay (Bio-Rad, Hercules, USA). Proteins were resolved with SDS-PAGE and transferred to PVDF membranes. Primary antibodies (1:1000) were incubated overnight at 4°C, followed by the incubation of secondary antibodies at room temperature for an hour the next day. Both primary and secondary antibodies were diluted in TBST containing 5% BSA. Horseradish peroxidase activity were visualized with Supersignal West Femto maximum sensitivity substrate kit (Thermo Fisher Scientific).

2.2.6 Histological Analysis of Xenografts and 3D Spheroids

3D spheroids were collected into a cryovial. The spheroids were allowed to settle to the bottom of the cryovial before the removal and addition of new medium. Spheroids were fixed in 10% neutral buffered formalin (NBF, Sigma Aldrich) overnight at 4°C, before washing and dehydration. Xenografts were fixed in 10% NBF overnight, washed and rinsed under tap water for a second night, before placing into cassettes that were placed into a tissue basket. The tissue basket was then loaded into tissue processor machine (Leica). After rinsing the xenograft under tap water overnight, tissues were dehydrated in a slow and stepwise pace to prevent distortion of the tissue. The following steps illustrate the complete process.

Table 2-1. Steps for dehydration and clearing

Station	Duration	
	Xenografts	3D spheroids
10% NBF	24 h	24 h at 4°C
Tap water	24 h	-
Distilled water	1 h	1 h
50% Ethanol	1 h	10 min
70% Ethanol	1 h	10 min
90% Ethanol	1 h	10 min
100% Ethanol	1 h	10 min
100% Ethanol	1 h	10 min
100% Ethanol and HistoClear (v:v) 1:1	4 h	30 min
HistoClear	4 h	30 min
HistoClear	2 h	30 min
Wax	1.5 h	30 min
Wax	1.5 h	30 min

Spheroids or xenograft tissues were then embedded in paraffin wax using a tissue embedding machine and made into formalin fixed paraffin embedded (FFPE) tissue and stored as wax blocks. Spheroids and xenograft were sectioned at 5- μ m paraffin sections and captured on polylysine microscopic slides (Thermo Fisher Scientific), using standard protocols (Leica).

2.2.7 Hematoxylin &Eosin (H&E) Staining

Before staining, tissue sections were first deparaffinized with histoclear and then undergo hydration in coplin jars using the following procedures (Table 2-2).

Table 2-2. Steps for deparaffinization and rehydration of slides

Station	Duration (min)
Histoclear	3
Histoclear	3
100% Ethanol	2
100% Ethanol	2
100% Ethanol	2
90% Ethanol	2
70% Ethanol	2
50% Ethanol	2

After hydration, H&E was performed using hematoxylin (Sigma Aldrich) and Eosin Y (Sigma Aldrich), with the following steps (Table 2-3), followed by mounting with Canada balsam.

Table 2-3. Steps for H&E staining

Station	Duration (min unless otherwise stated)
Distilled water	1
Haematoxylin	1
Water	2
Distilled water	1
Differentiation solution	10 sec
Tap water	5-10
Distilled water	1
95% Ethanol	1
Eosin	30 sec
90% Ethanol	15 sec
100% Ethanol	1
100% Ethanol	2
100% Ethanol	2
Histoclear	2
Histoclear	2

2.2.8 Immunohistochemistry (IHC)

IHC analysis was performed on 5-µm sections of 3D co-culture spheroids and xenografts. For IHC, antigens were retrieved using sodium citrate buffer at 100°C for 5 minutes. Slides were then stained with the HRP/DAB Detection IHC Kit (Abcam) according to the manufacturer's instructions. The slides were permeabilized for 15 min with 0.25% Triton X-100 in PBST solution, blocked with H₂O₂ solution to remove endogenous peroxidase activity, and blocked with 3% BSA in PBST. The primary antibodies VEGF, LC3B and p62 were diluted 1:100 in PBST containing 3% BSA and incubated overnight at 4°C. After visualization of the target protein with DAB, counterstaining with hematoxylin was performed with the steps listed below (Table 2-4). The slides were then mounted with Canada balsam. All images were captured with an observer Z1 microscope (Carl Zeiss) attached to a color camera (AxioCam 506, Carl Zeiss).

Table 2-4. Counterstaining with haematoxylin

Station	Duration (min unless otherwise stated)
Haematoxylin	10 sec
Running water	1
DI water	1
Differentiating solution	2 sec
Running water	1
DI water	1

2.2.9 Sample Preparation for Transmission Electron Microscopy

Cells were seeded on petri dish and after respective treatments, cells were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, EMS, Hatfield, USA) in 0.1 M phosphate buffer overnight, before washing 3 times in 0.1M phosphate buffer and post-fixed in 1% osmium tetroxide (EMS) for 1 h. Cells were washed and scrapped off petri

dishes for collection into centrifuge tubes. The samples were then packed in 6% gelatin before re-fixation with 2.5% glutaraldehyde and dehydration in an ethanol gradient, followed by infiltration. The samples were then embedded in araldite 502 resin (Ted Pella Inc., Redding, USA) at 60°C for 1-2 days. The resin blocks were cut with an ultramicrotome (Leica), and 80 nm sections were collected on copper-formvar coated grids, and stained with uranyl acetate. Electron micrographs were examined at 120kV on a Tecnai G2 TEM (FEI, Hillsboro, USA) and images recorded with a Gatan CCD camera.

Table 2-5. Steps for dehydration and infiltration

Station	Duration (min unless otherwise stated)
25% Ethanol	5
50% Ethanol	10
75% Ethanol	10
95% Ethanol	10
100% Ethanol	10
100% Acetone	10
100% Acetone	10
6: 1 ratio of 100% acetone : resin	30
1: 1 ratio of 100% acetone : resin	30
1:6 ratio of 100% acetone : resin	24 h

2.3 Establishing a Co-Culture Model to Study Tumor Angiogenesis

2.3.1 HepG2-DsRed and HUVEC-C3 Co-Culture Assay

Two culture methods were adopted to establish the co-culture model. Firstly, HepG2-DsRed and HUVEC-C3 cells were harvested by trypsin treatment and mixed in various ratios of HepG2-DsRed:HUVEC-C3 before seeding them in 12-well plates (3×10^5 cells/well). Alternatively, a HepG2-DsRed monolayer was established before seeding 1×10^5 HUVEC-C3 cells in the 12-well plate. The medium was replaced every day in

both methods. Alternatively, for side by side cultivation of both cell types, the cells were seeded into two compartments of an Ibidi culture insert (Ibidi, Munich, Germany) and allowed to grow towards each other for 2 days.

2.3.2 HUVEC-C3 Differentiation with Various Treatments to HepG2-DsRed Monolayer

HepG2-DsRed cells were seeded in a petri dish and allowed to grow to confluence before fixation or dried. 100% cold methanol was used to fix the monolayer for 10 min on ice, or 4% PFA solution was added for 15 min at room temperature. The monolayer was then washed thrice with PBS for 10 min each. Alternatively, the monolayer was rinsed rapidly with autoclaved Millipore water and removed of all solutions before drying at 37°C. HUVEC-C3 cells were then seeded on the monolayer. For HepG2-DsRed cell extract, HepG2-DsRed were trypsinized and washed with PBS, followed by the addition of autoclaved Millipore water and sonication before centrifuging at 13,000 rpm for 10 min. The supernatant was then coated on a new petri dish for 30 min before seeding HUVEC-C3 cells.

2.3.3 Eliminating Secretory Factors as Inducers of HUVEC-C3 Differentiation

For the transwell experiments, cells of HUVEC-C3 (control), HepG2-DsRed or HepG2-DsRed-HUVEC-C3 co-culture were seeded in the top chamber of a transwell insert (Costar, Cambridge, UK), with HUVEC-C3 at the bottom chamber. Alternatively, HUVEC-C3 (control), HepG2-DsRed or HepG2-DsRed-HUVEC-C3 co-culture were

cultured in a 60 mm petri dish and the conditioned medium was transferred to HUVEC-C3 cells each day for five days consecutively.

2.3.4 Inhibition of HUVEC-C3 Differentiation with Target Agents

Vascular endothelial growth factor receptor (VEGFR) inhibitor (SU5416), anti-HCC drug (sorafenib), MEK inhibitor (U0126), JNK inhibitor (SP600125), PI3K inhibitor (LY294002), p38 inhibitor (SB202190), Rho-associated protein kinase (ROCK) inhibitor (Y27632), PKC activator (phorbol myristate acetate, PMA), FAK inhibitor (Y15) were used to target various signaling pathways regulating angiogenesis. All inhibitors were tested for a series of concentrations. The optimal concentrations were used for each inhibitor to compare their effects on HUVEC-C3 cells differentiation, with LY294002 at 20 μ M, PMA at 20 nM, SU5416 at 4 μ M, sorafenib at 8 μ M, while the rest were used at a concentration of 10 μ M. A chemotherapy drug paclitaxel was used at a concentration of 200 nM. Randomly selected fields of view were photographed two days post seeding of the co-cultures and images were quantified by the Angiogenesis Analyzer plugin for ImageJ.

2.3.5 Matrigel Differentiation Assay

The conditions used in the co-culture assay was also applied to the matrigel differentiation assay. Matrigel (BD Biosciences) was mixed 1:1 with pre-cooled fresh medium and coated on 24 well plates. The coated wells were then incubated at 37°C for 30 min. 1×10^5 HUVEC-C3 cells pre-treated with various inhibitors or drugs for 3 hr were seeded into each wells and incubated for 5 hr along with the inhibitors or drugs.

The morphological changes of the cells were observed and photographed during this period. Tubular networks were then quantified.

2.3.6 Image Analysis of Tubule Formation

All images were captured with an AxioCam MRm camera attached to an Axio Observer.Z1 microscope (Carl Zeiss) unless otherwise stated. For 2D co-cultures, images were adjusted with Image Pro Plus 6.0 using an Angiogenesis Macro downloadable from MediaCybernetics website to obtain skeletonize images of the

Figure 2-1. Quantification of co-culture images from raw image. Images were skeletonized using Image Pro Plus Angiogenesis Macro followed by analysis in Image J Angiogenesis Analyzer plugin. Green = branches, orange = master segments, red surrounded by blue = junctions.

tubule formations (Figure 2-1, top right). The skeletonized images were then analyzed by the Angiogenesis Analyzer plugin with ImageJ software (Figure 2-1, bottom left) written by Carpentier G. (2012) [157] downloadable from the National Institute of Health website. From the analysis, number of junctions was taken for calculation, number of branches plus number of segments were equivalent to number of tubules, while total branching lengths were equivalent to tubules length (Figure 2-1).

Differentiation of cells were quantified with a modification to the form factor (FF) published by Mendez *et al.* [158]. Briefly, cell shapes were measured by determining their area and circumferences, and analyzed to obtain the FF value equivalent to $4\pi(\text{area})/(\text{perimeter})^2$. Differentiated HUVEC-C3 cells with elongations displayed larger perimeter and smaller area resulting in smaller FF values compared to control cells. A normal distribution of 120 control cells was plotted to find the margin in which 5% of the cell lies. This margin was taken as the basal value where HUVEC-C3 cells with a FF value smaller than the basal value will be considered as differentiated cells.

2.3.7 Transwell Assay

A transwell inset with pore size of 0.4 μm (Corning, USA) was inverted and HUVEC-C3 was seeded on the porous membrane and allowed to attach at 37°C and 5% CO_2 . After 30 min, the transwell inset was set upright and either HUVEC-C3, fibroblast or HepG2-DsRed were added to the upper side of the transwell inset. DMEM was added to the chamber and the transwell was incubated overnight. The morphologies of HUVEC-C3 cells were captured with fluorescence microscope after 24 h, 48 h and 96 h.

2.4 Establishing a 3D Co-Culture Platform for Drug Screening

2.4.1 3D Multicellular Spheroid Culture

The 3D co-culture was performed as previously described [159]. Briefly, HepG2-DsRed and HUVEC-C3 cells were harvested by trypsin treatment and mixed in a ratio of 10:1 HepG2-DsRed: HUVEC-C3 supplemented with 20% FBS and 1% PS before seeding them in non-adhesive round bottom 96-well plates (Sigma-Aldrich) which was pre-coated with 1% Pluronic-F127 (Sigma-Aldrich). The plates were placed in microplate carriers (Thermo Fisher Scientific) and centrifuged at 1,000 g for 5 min in the Sorvall Legend XTR Centrifuge (Thermo Fisher Scientific), before incubated overnight at 37°C, 5% CO₂. All other cell lines, HepG2, MCF-7, MDA-MB-231, NIH-3T3, and VSMC were cultured in a 10:1 ratio of cells to HUVEC-C3 cells, with 2500 cells of each cell line and 250 HUVEC-C3 cells seeded in each well. Spheroids were examined at two days post seeding. A LSM 710 META laser scanning confocal microscope (Carl Zeiss) was used to capture confocal Z-stack images of 5 µm apart for NIH-3T3 cells co-cultured with HUVEC-C3 cells. Confocal images were analyzed using Image J software (NIH, National Institutes of Health, USA), and projected into images with average intensities to display the network formed.

2.4.2 Assessing HUVEC-C3 Differentiation with Target Agents

Vascular endothelial growth factor (VEGF, 100 ng/ml), basic fibroblast growth factor (bFGF, 100 ng/ml) and extracellular matrix (ECM) protein, fibronectin (100 µg/ml) were used as inducers of angiogenesis. SU5416 and sorafenib were used at 10 µM in the initial experiments for inhibitory effects on the co-culture model. Sorafenib was used at concentrations ranging from 1 µM to 40 µM. Sunitinib and axitinib were used at

concentrations of 10 nM to 40 μ M. The corresponding concentrations of the inhibitors were added into the culture medium when the cells were seeded. Images were captured two days post seeding of the co-cultures and images were quantified for network formation. Cells that were not round and had protrusions were counted as differentiated cells.

2.4.3 Quantification of 3D tubule networks

Images of spheroids were captured two days post seeding of the co-cultures and quantified for network formation. Cells that were not round and had protrusions were considered differentiated cells and their protrusions/projections were counted as a tubule. Total tubules were counted in each spheroid for a total of 15 spheroids in three independent experiments.

2.4.4 Quantification of Spheroids

Cells were seeded in 96 well plates at densities ranging from 1,000 – 10,000 cells per spheroids and allowed to form spheroids overnight. The fluorescence emitted by the spheroids was measured using the SpectraMax M5 Microplate Reader (Molecular Device) with excitation wavelength of 554 nm and emission wavelength of 586 nm. The fluorescence intensity emitted by the spheroids were subtracted against background to obtain the fluorescence intensity from each well. The spheroids were then captured under microscope and the diameter of each spheroid was measured. A total of 10 spheroids was measured for each density and averaged in three independent experiments.

2.5 Autophagy in Metastatic Breast Cancer

2.5.1 Starvation of Cells

Cells were seeded in 60 mm petri dishes at 70% confluency and allowed to attach overnight. The next day, the medium was washed and replaced with either EBSS or serum free DMEM and allowed to incubate for 30 h. The

2.5.2 RNA Isolation and Purification

RNA was isolated using the PureLink[®] RNA mini kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, cells were collected and lysed with freshly prepared lysis buffer containing 1% β -ME. The lysate was then centrifuged at 12,000g for 2 min before an equal volume of 70% ethanol was added to the cell homogenate, followed by thorough vortexing. The resulting lysate was transferred to a PureLink RNA mini-spin column before incubated with DNase I at room temperature for 15 min before washing and elution of purified RNA. RNA yield was quantified by Nanodrop at UV absorbance of 260 nm. RNase-free water was used for all procedures.

2.5.3 cDNA Synthesis

cDNA was synthesized from purified RNA using SuperScript[™] Vilo[™] Master Mix (Thermo Fisher Scientific) and according to the manufacturer's instructions. Briefly, 1 μ g of total RNA was added to the enzyme master mix with reaction buffer and topped up with RNase-free water to 20 μ l in a sterile PCR tube. One PCR cycle was performed with an automated Techne TC-412 thermal cycler (Techne, Burlington, USA), with preheated lid at 105°C. The procedure of the cycle is listed in Table 2-6. cDNA was aliquot and stored in -80°C for long term storage.

Table 2-6. PCR cycle from RNA to cDNA

Temperature (°C)	Duration (min)
25	10
42	60
85	5
4	indefinite

2.5.4 RT-PCR

Primers were designed with the software Primer Premier 6.0 (Premier Biosoft International, Palo Alto, USA) for standard PCR assays. The sequences of the primers used are listed in the following table:

Table 2-7. Sequences of Primers

Primer	Primer sequence
p62/SQSTM1 forward primer	5'-GCACACCAAGCTCGCATTC-3'
p62/SQSTM1 reverse primer	5'-GGACTGGAGTTCACCTGTAGAC-3'
β -actin forward primer	5'-ATGATATCGCCGCGCTCG-3'
β -actin reverse primer	5'-CGCTCGGTGAGGATCTTCA-3'

The PCR was performed using GoTaq[®] DNA polymerase (Promega) and according to the manufacturer's instructions. In brief, in a sterile, nuclease-free microcentrifuge tube, GoTaq polymerase was mixed with the reaction buffer and added to 0.25 μ M of forward and reverse primers, before finally adding 1 μ g of template DNA to the mixture. The mixture was topped up with nuclease-free water to 20 μ l, before processed with a Techne TC-412 thermal cycler following the steps below (Table 2-8). The PCR products were collected, separated by 1% agarose gel electrophoresis and visualized with GelRed[™] (Biotium).

Table 2-8. PCR cycle from cDNA to mRNA

Step	Temperature (°C)	Duration (s)	Number of cycles
Initial denaturation	95	120	1
Denaturation	95	30	25
Annealing temperature	p62/SQSTM1 – 56 β -actin – 60	30	
Extension	72	40 (1 min/kb)	
Final Extension	72	300	1
Soak	4	indefinite	1

2.5.5 siRNA Transfection

Silencer Select siRNA against ATG7 (siRNA ID: s20650), silencer select siRNA against MnSOD (SiRNA No.1 ID: s13268, and No.2 ID: s13269) and Silencer Select negative control siRNA were purchased from Ambion (Life Technologies, USA). Before transfection, cells were seeded on 60 mm petri dishes and allowed to attach overnight. Cells were washed and medium replaced with OptiMEM (Life Technologies) before transfected with 100 nM siATG7 or 20 nM of siMnSOD using Lipofectamine RNA/iMAX (Life Technologies), according to the manufacturer's instructions. After incubation for 6 h, the transfection medium was replaced with fresh medium. Cells were starved 48 h after transfection with siATG7. Alternatively, cells were reseeded in new 60 mm petri dishes 24 h after transfection with siMnSOD, and starved 72 h after transfection. Images were taken 30 h after starvation.

2.5.6 Assessing Autophagy with Inhibitors

Cells were seeded in 60 mm petri dishes and allowed to attach overnight in DMEM containing 10% FBS. The next day, cells were washed with serum-free medium before addition of serum-free medium or EBSS to induce autophagy. To study autophagic flux,

50 μ M of CQ was added 3 h before the cells were collected for western blot. To inhibit or induce the formation of vacuoles, various inhibitors were added. 10 μ M of LY294002, 10 μ M of wortmannin, 200 nM of Baf-A1, or 10 μ M of CQ was added to the starvation medium before performing starvation assays with 231-M1A cells.

To study the effects of ROS and MnSOD on vacuole formation, 20 μ M of propyl gallate (PG), 2 mM of NAC, 50 μ M of manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), 20 μ M of 2-methoxyestradiol (2-ME, Sigma-Aldrich) was added to the starvation medium before performing starvation assays with 231-M1A cells. Images were captured at 30 h to check for vacuole formation.

2.6 Statistical Analysis

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

CHAPTER 3 Establishing a Co-Culture Model to Study Tumor Angiogenesis

3.1 Introduction

Angiogenesis is one of the hallmarks in cancer. Many studies have highlighted its significance in the progression of tumor growth and metastasis [160]. Therefore, anti-angiogenesis has been identified as a therapeutic approach for the treatment of many cancers. Tumor cells play important roles in angiogenesis. Many have highlighted the roles of paracrine factors in tumor-induced angiogenesis [161, 162], with VEGF being the key activator in angiogenesis [163]. However, therapeutic drugs targeting VEGF molecules (Avastin) released by cancer cells, or targeting receptors on the surface of ECs (Sunitinib) are not highly effective as single therapeutic agents in liver cancer [73, 164]. In contrast, molecular agents such as sorafenib, which targets multiple signaling pathways, provide inhibition to angiogenesis and tumor growth, and have shown promising therapeutic effects against liver cancer [165, 166]. The underlying mechanism is that common signaling pathways such as PI3K/Akt/mTOR and Ras/Raf/MEK/ERK [167] can be activated by multiple angiogenic factors including growth factors, the extracellular matrix [90, 168], integrins [168, 169] and other guidance molecules [169].

One angiogenic factor that has not been investigated is the physical tumor-endothelium interactions [170, 171]. Although several model systems have been developed that include both tumor cells and ECs, the cell lines were often cultured in spatially separated spaces in the cases of transwell chambers [161], microfluidics [172, 173] and hydrogels in 3D cultures [162, 174]. Even though these systems can be used to evaluate the paracrine factors released by tumor cells on ECs, the cell-cell interactions

will be hard to study in these indirect co-culture models. Here, a novel co-culture model that allows direct interactions between liver cancer cells and ECs is presented, thus facilitating the study of signaling pathways governing blood vessel formation in liver cancer. The EC used is a human umbilical vein endothelial cell line expressing a FRET-based sensor for caspase-3 (HUVEC-C3), which can detect apoptosis in real time [98, 156]. The FRET-based sensor is a recombinant DNA encoding a cyan fluorescent protein (CFP), a yellow fluorescent protein (YFP), and a 16 amino acid-peptide linker containing the cleavage sequence of caspase-3: Asp-Glu-Val-Asp (DEVD) [98]. When HUVEC-C3 cells are alive, excitation of the donor molecule (CFP) leads to the transfer of emission energy to an acceptor molecule (YFP), resulting in green fluorescence emission. When HUVEC-C3 undergo apoptosis, caspase-3 is activated which in turn cleaves the fusion protein of CFP-DEVD-YFP through its linker, abolishing the FRET effect and resulting in a change of emission fluorescence from green to blue. The liver cancer cell line HepG2-DsRed expresses a red fluorescent protein (DsRed). In this study, liver cancer cells and ECs labeled with different fluorescence proteins were cultured together to investigate their interactions. This system modeled hepatocellular carcinoma (HCC) angiogenesis much more accurately, and HUVEC-C3 differentiated only in direct contact with HepG2 cells. The physical interactions between HepG2 and HUVEC-C3 are the key factors in tilting the angiogenic balance and the cellular signaling pathways were investigated to understand the molecular mechanisms of this tumor-endothelial interaction. With the expression of a caspase-3 sensor [156] in HUVEC-C3 cells, the survival of ECs as well as the cytotoxic effects [175] of inhibitors and anticancer drugs were investigated concurrently.

3.2 Objectives and Significance of Research

3.2.1 Objective 1: Establish a Co-Culture Model to Study Tumor Angiogenesis

In many angiogenesis assays, secretory factors or ECM proteins are mainly used as key inducers to trigger EC differentiation and tumor cells are not included. To study tumor-angiogenesis, tumor cells must be present in the *in vitro* assays. HCC cell lines were utilized to induce tumor angiogenesis in a co-culture with ECs. A HCC microenvironment is constructed via the co-culture, which can be used for studying tumor angiogenesis.

3.2.2 Objective 2: Identify the Factors Inducing HUVEC-C3 Differentiation

With an established co-culture representative of tumor angiogenesis, the factors inducing EC differentiation was explored. Secretory factors such as VEGF, ECM support and direct cell-cell communications are some factors governing angiogenesis. Many researchers have identified VEGF as the key factor in angiogenesis; however, the importance of the tumor microenvironment has been overlooked. By identifying factors that directly trigger tumor angiogenesis in a tumor microenvironment, new directions to anti-tumor angiogenesis would be discovered.

3.2.3 Objective 3: Elucidate the Signaling Pathways Regulated By the Factors in HUVEC-C3 Differentiation

Different secretory factors and protein molecules can trigger similar signaling pathways, and cells can develop resistance to drugs binding to single upstream signaling targets.

By identify and blocking downstream signaling pathways important in angiogenesis and EC differentiation, transcription of genes modulating cell behavior can be stopped. Cellular signaling pathways important for tumor angiogenesis were investigated to understand the molecular mechanisms of the tumor-endothelial interaction in the co-culture. Elucidating downstream signaling pathways regulated by activators would present specific biological targets for the identification of small molecules acting as inhibitors.

3.2.4 Objective 4: Identify the Differences between the Fibroblast-EC and Liver Cancer-EC Co-Cultures

The fibroblast-endothelial co-culture has been established since 1999 with screening of anti-angiogenesis agents performed with this assay [86, 87]. Key factors such as ECM protein, fibronectin, induced endothelial morphogenesis in the fibroblast-EC co-culture. The fibroblast-EC co-culture may represent physiological angiogenesis, while the liver cancer-EC co-culture represented tumor angiogenesis. These two co-culture systems were compared to study the differences between normal and tumor angiogenesis.

3.3 Results

3.3.1 Co-Culture of HepG2-DsRed and HUVEC-C3 Result in HUVEC-C3

Cells Differentiation and Formation of Tube-Like Structures

HUVEC-C3 cells that were stably transfected with a FRET sensor for caspase-3 were utilized in most experiments [156, 176, 177]. HUVEC-C3 cells appeared green when alive and blue (Figure 3-1, red arrows) when undergo apoptosis in FRET images. Monocultures of HUVEC-C3 (green when alive, blue when undergoing apoptosis with FRET imaging) and HepG2-DsRed (red) have a cobblestone morphology, associating with each other in small islands (Figure 3-1A). When HUVEC-C3 was co-cultured with HepG2-DsRed, tubular networks were observed with the differentiation of HUVEC-C3 (Figure 3-1B, top right), while HepG2-DsRed remained in their cobblestone morphologies (Figure 3-1B, top left). Elongation and multiple protrusions of HUVEC-C3 was observed in the co-culture (Figure 3-1, bottom right), with few cells undergoing apoptosis (red arrows, Figure 3-1B, top right).

Co-cultures of the cell lines were performed in two ways: 1) establishing a monolayer of HepG2 cells, followed by the addition of HUVEC-C3 cells (Figure 3-1B), and 2) co-culturing both cell lines together in a ratio of 2:1 HepG2-DsRed:HUVEC-C3 cells (Figure 3-1C). No differences were observed between both methods, with equally successful differentiation and tubular networks formed (Figure 3-1B and C). In order to understand the physical interactions, The contact points between HepG2-DsRed and HUVEC-C3 were looked at when the two cell lines were seeded side by side (Figure 3-1D). When both cells migrated towards one another and made physical contact, HUVEC-C3 cells formed protrusions and sprouts into the HepG2-DsRed colony (Figure 3-1D). Both vasculogenesis (Figure 3-1B and C) and sprouting angiogenesis (Figure

3-1D) which are characteristics of tumor angiogenesis [169] were demonstrated when ECs were activated by angiogenic signals from HepG2-DsRed cells.

Figure 3-1. Co-culture of HepG2-DsRed and HUVEC-C3 induces HUVEC-C3 differentiation. (A) Epithelioid morphology of HepG2-DsRed (red) and HUVEC-C3 (green) when cultured alone. Live HUVEC-C3 cells appeared green in the FRET images, while apoptotic cells appeared blue (red arrows). HUVEC-C3 differentiated after two days when (B) a monolayer of HepG2-DsRed was established followed by seeding HUVEC-C3 cells on top and (C) both cells lines were seeded concurrently HUVEC-C3 showed elongation and branching (white arrows in enlarged panel), while HepG2-DsRed remained cobblestone-shaped. Few HUVEC-C3 cells undergo apoptosis (blue, red arrows) in the co-cultures. (D) Sprouting of HUVEC-C3 into HepG2-DsRed could be visualized when both cells were co-cultured side by side.

In addition, co-cultures of other human cell lines with ECs were performed and it was discovered that HUVEC-C3 cells were able to differentiate with the formation of tubule-like structures in co-cultures with liver cells (Table 3-1). HCC cell line HepG2 and hepatic cells L0-2 were able to induce tubule formation, while other human tumor cell types such as breast adenocarcinoma MDA-MB-231 and lung carcinoma A549 were unable to induce tubule formation (Figure 3-2). Although other non-cancerous human

Table 3-1. Evaluation of EC differentiation and tubule formation in co-culture of HUVEC-C3 and other cells

Cell line	Cell type	Tissue	Disease	HUVEC-C3 differentiation	Tubule formation
HepG2	Human epithelial	Liver	Hepatocellular carcinoma	Yes	Yes
HepG2-DsRed	Human epithelial	Liver	Hepatocellular carcinoma	Yes	Yes
SK-N-SH	Human epithelial	Brain	Neuroblastoma	Yes	No
A549	Human epithelial	Lung	Carcinoma	No	No
MDA-MB-231	Human epithelial	Breast	Adenocarcinoma	No	No
BT-474	Human epithelial	Breast	Ductal carcinoma	No	No
L0-2	Human epithelial	Liver	Normal	Yes	Yes
HEK-293	Human epithelial	Kidney	Normal	Yes	Yes
IMR90	Human fibroblast	Lung	Normal	Yes	Yes
VSMC	Human smooth muscle	Vascular smooth muscle	Normal	Yes	No

cell types such as lung fibroblast IMR90 and embryonic kidney cells HEK-293 induced HUVEC-C3 differentiation and formed tubule networks (Table 3-1), HepG2-HUVEC-C3 co-culture were studied as they formed aberrant and chaotic tubular networks representative of tumor angiogenesis.

Figure 3-2. Other human tumor cell types are unable to form vascular networks except for liver cells. Only liver cell types (L0-2 and HepG2) were able to induce tubular networks, while other cell types such as breast cancer cell MDA-MB-231 were unable to form network formations. HUVEC-C3 co-cultured with L0-2 hepatic cells were able to form orderly networks compared to HepG2.

Different ratios of HepG2-DsRed and ECs were tested in the co-culture models and it was found that as long as HepG2-DsRed and HUVEC-C3 cells were cultured together for a period of time (>3 days), the HUVEC-C3 cells were able to differentiate and form tubular networks regardless of the ratio (Figure 3-3A). However to ensure a high degree of HUVEC-C3 differentiation and rapid formation of tubular networks, a fixed ratio of 2:1 for HepG2-DsRed: HUVEC-C3 was used for the subsequent experiments.

HUVEC-C3 elongated and projected outwards to form tubular networks within three days, followed by regression of the networks when cultured for five days (Figure 3-3B). These phenotypic characteristics were similar to the gold standard for EC differentiation, the matrigel differentiation assay, where tubule formation occurred within 5 hr (Figure 3-3C) and regressed in a day.

Figure 3-3. A ratio of 2:1 HepG2-DsRed: HUVEC-C3 induced high degree of differentiation in HUVEC-C3. (A) HepG2-DsRed (red) and HUVEC-C3 (green) were seeded at different ratios and network formations were captured at day two. Apoptotic HUVEC-C3 appeared blue under FRET imaging. 2:1 ratio of HepG2-DsRed:HUVEC-C3 co-culture showed the best tubular formation after two days, with HUVEC-C3 appearing to have the highest degree of differentiation with a high percentage of elongation of HUVEC-C3, while HUVEC-C3 were less differentiated in the other ratios of HepG2-DsRed: HUVEC-C3 co-culture. (B) Tubular

formation showed few network formations after one day, with cells starting to form protrusions and reorganized within the culture. Network formation was achieved after two days of co-culturing at a ratio of 2:1 of HepG2-DsRed:HUVEC-C3 (as observed in panel A). This network formation started to regress by day 5. (C) Comparison of the co-culture model (left) with the matrigel assay (right). Areas of tubules (circles 1, 2, and 3), average tubule length (yellow) and junctions formed (in enlarged panel) are similar in both models by a quick comparison of images.

The tubule networks in the co-culture were comparable to the networks formed when HUVEC-C3 was plated on matrigel. The average length of tubules (yellow), the junctions indicated by the white dot in the enlarged panels, and the areas of tubules formed (circles 1, 2 and 3) were all similar to those in the matrigel (Figure 3-3C). The tubule formations in the co-culture were similar to the matrigel differentiation assay, enabling it to function as a screening platform for anti-angiogenesis agents.

3.3.2 Phenotypic Characteristics of Activated HUVEC-C3 Cells Induced by HepG2 Cells

To understand the morphological differences in activated HUVEC-C3, the phenotypic characteristics of differentiated HUVEC-C3 induced by HepG2 cells were studied. HUVEC-C3 cells were cultured with HepG2 cells expressing no fluorescence and stained the cells with various markers. When HUVEC-C3 cells were activated by co-culturing with HepG2, HUVEC-C3 cells differentiated and elongated, forming linkages with each other (Figure 3-4A). A prominent feature that was observed in differentiated HUVEC-C3 cells was the change of nuclear morphology from round to oval (Figure 3-4A, enlarged nucleus), whereas in mono-culture of HUVEC-C3, the cells remained cobblestone-shaped with round nuclear morphologies. Other phenotypic changes observed on the differentiated HUVEC-C3 cells included changes to the cytoskeleton

structure and mitochondria redistribution. Staining of phalloidin (Figure 3-4B, left) and MitoTracker (Figure 3-4B, right) showed the actin filaments and mitochondria of HUVEC-C3 spreading outwards along the course of its differentiated path. The phenotypic changes of HUVEC-C3 (Figure 3-4A and B) were due to the interactions with HepG2 cells in the co-culture condition, as these changes were not observed in the HUVEC- C3 mono-culture, nor were they observed in the HepG2 cells in the co-culture.

Figure 3-4. Phenotypic activation of HUVEC-C3 was revealed by immunostaining of mono- and co-cultures. Non-fluorescent HepG2 cells and HUVEC-C3 (green) were co-cultured in a ratio of 2:1 of HepG2:HUVEC-C3 for two days before fixation with paraformaldehyde and stained with the respective markers. (A) HUVEC-C3 (green) elongated and formed linkage with each other when co-cultured with HepG2). The nucleus (blue) was compressed from round to oval within the co-culture (white arrows, enlarged nucleus). (B)

Phalloidin staining (red) showed reorganization of the actin filaments (left) with the actin filaments pulled along the cell (enlarged image). MitoTracker staining (red) showed redistribution of mitochondria (right) throughout the elongated HUVEC-C3 (enlarged image) upon activation by HepG2.

3.3.3 HepG2 as a Physical Support for HUVEC-C3 within the Co-Culture

Next, the interactions between HepG2 and HUVEC-C3 were looked at in detail. Confocal imaging of the co-cultured cells was performed and HepG2-DsRed cell sheets was found to form the bottom layer, with part of HUVEC-C3 cells wedged between. HUVEC-C3 then elongated and formed protrusions above the layer of HepG2-DsRed cells (Figure 3-5).

Figure 3-5. Differentiation of HUVEC-C3 (green) with the physical support from HepG2 cells. Co-culture of HepG2-DsRed and HUVEC-C3 for two days before fixation and visualized by confocal microscopy. Z-stack images showing the relative positions between HepG2-DsRed

and HUVEC-C3. HUVEC-C3 formed protrusions and elongations above HepG2-DsRed cell sheet in co-culture.

Actin staining of the co-culture revealed that HepG2 cells were mainly found attached to the bottom of the plate with HUVEC-C3 cells forming networks above HepG2 cells (Figure 3-6A). This phenomenon was observed regardless of how the two types of cells were seeded: in a mixture or in sequence, where HUVEC-C3 cells were seeded together with HepG2 or after a HepG2 monolayer was formed. Figure 3-6a revealed that HepG2 cells supported HUVEC-C3 from underneath, where the actin filaments of HepG2 moved in perpendicular to HUVEC-C3 elongations (white arrow). Z-stack images showed the actin filaments of HepG2 cells spreading outwards, from its initial attachments on the plate, towards that of HUVEC-C3 cells and supporting it from beneath.

Figure 3-6. Changes to the cytoskeleton in the HUVEC-C3 (green) and HepG2 co-culture. Non-fluorescent HepG2 cells and HUVEC-C3 (green) were co-cultured in a ratio of 2:1 of HepG2:HUVEC-C3 for two days before fixation with paraformaldehyde and stained for (A)

actin or (B) vimentin. Actin staining with phalloidin revealed actin networks (red) of HepG2 running perpendicularly (white arrows) under HUVEC-C3. (C) HepG2 cells stretched out (white arrow) to receive the elongations of HUVEC-C3 (green). Vimentin is expressed in HepG2 but not in HUVEC-C3 cells. Scale bars = 10 μ m.

Staining of the intermediate filament (vimentin) gave a clearer picture of how HepG2 and HUVEC-C3 interacted. Only HepG2 expressed high levels of vimentin while HUVEC-C3 cells did not, allowing us to visualize the structural changes HepG2 cells undertook to accommodate the differentiated HUVEC-C3 cells. As shown in Figure 3-6B, vimentin (red) was initially co-localized near the nucleus (blue) [178] in a dispersed fashion in HepG2 mono-culture. This however changed in the co-culture model, where vimentin within HepG2 cells stretched towards an elongation of the HUVEC-C3 cell. Z-stack images showed the rims of HepG2 incurving to form a trench-like structure, with the arms of HUVEC-C3 resting right in the middle of the curvature formed by HepG2 (Figure 3-6B).

Both actin and vimentin staining revealed the cytoskeleton changes of HepG2 underwent in order to accommodate HUVEC-C3 in the formation of tubular networks. HepG2 acted as physical supports for HUVEC-C3 and there is evident interactions taking place between both cell lines, with morphological adaptations of both cells to accommodate each other.

3.3.4 Physical Contact is Essential for HepG2-induced HUVEC-C3 Differentiation

To demonstrate that HUVEC-C3 differentiation was indeed induced by physical contacts with HepG2, HepG2 cells were inactivated while preserving their physical structures for cell-cell interactions. A monolayer of HepG2-DsRed was first established

Figure 3-7. Physical contact is essential for HUVEC-C3 differentiation. (A) HepG2-DsRed cells were fixed with methanol, PFA or dried rapidly, or the cell extract of HepG2-DsRed was coated on a petri dish before seeding HUVEC-C3. (B) The percentage of differentiated cells was quantified from three independent experiments with at least four observation fields each and compared to the normal co-culture of live HepG2-DsRed and HUVEC-C3 cells. *** $p < 0.005$ vs. control.

before fixing or drying the cells instantaneously, followed by culturing HUVEC-C3 cells on top of the dead HepG2-DsRed cells (Figure 3-7A). The percentages of HUVEC-C3 cells that differentiated under these conditions were quantified by calculating their form factors (FF) (Figure 3-7B). Differentiated cells displayed larger perimeter and smaller area, leading to a lower FF value [158] (equivalent to $4\pi(\text{area})/(\text{perimeter})^2$). Methanol-fixed HepG2-DsRed cells did not induce any differentiation of HUVEC-C3 cells ($4.6 \pm 2.0\%$), which was similar to the control cells ($3.8 \pm 1.20\%$), however $29.7 \pm$

9.3% and $28.9 \pm 6.6\%$ of HUVEC-C3 cells were able to differentiate when HepG2-DsRed cells were fixed with 4% paraformaldehyde or dried respectively (Figure 3-7A and B). The numbers increased to $49.8 \pm 7.7\%$ when the extract of HepG2-DsRed cells was used to coat the surface of the plate before seeding HUVEC-C3 cells. This high level of EC differentiation was also achieved when HUVEC-C3 cells were co-cultured with live HepG2-DsRed cells ($47.2 \pm 3.5\%$, Figure 3-7B).

Fixation of HepG2-DsRed cells with paraformaldehyde or drying the HepG2-DsRed cells could preserve cell surface proteins necessary for inducing HUVEC-C3 differentiation. The cell extract containing the membrane proteins of HepG2-DsRed also resulted in HUVEC-C3 differentiation. However, fixation of HepG2-DsRed cells with methanol altered protein conformations and dissolved lipids which result in the removal of lipoproteins, thus preventing protein-protein interactions between HepG2-DsRed and HUVEC-C3. There is high possibility that the protein that mediated HUVEC-C3 differentiation might be membrane proteins, including lipoproteins on the surface of HepG2 cells.

Figure 3-8 Secretory factors are not the key inducers for HUVEC-C3 differentiation. (A) Conditioned medium of HUVEC-C3, HepG2-DsRed and the co-culture were added to HUVEC-C3, (B) or the cells were cultured in the insert of a transwell with HUVEC-C3 at the bottom of

the well. (C) The percentage of differentiated cells was quantified from three independent experiments with at least four observation fields each.

To determine whether secretory factors were necessary to induce HUVEC-C3 differentiation in the co-culture, HUVEC-C3 cells were cultured with the conditioned media of HepG2-DsRed and that of the co-culture. Furthermore, to ensure that cell densities will not affect HUVEC-C3 differentiation, experiments were conducted with both low (2×10^5 cells / 60 mm dish) and high (6×10^5 cells / 60 mm dish) densities of cells. No significant differentiation was observed (Figure 3-8A and C). This remained true when HUVEC-C3 cells were cultured in the bottom chamber of a transwell at densities of 0.8×10^4 cells / well and 2×10^4 cells/ well with either HepG2-DsRed or the co-culture seeded in the transwell insert (Figure 3-8B and C), allowing paracrine factors to diffuse through to the HUVEC-C3 at the bottom of the well. As no significant differentiation of HUVEC-C3 was observed in both low and high density conditions, only images of HUVEC-C3 cultured at low densities with the conditioned medium and the images of HUVEC-C3 cultured at high densities in transwell experiments are presented.

Physical contact with HepG2 cells was important for HUVEC-C3 differentiation as HUVEC-C3 cells were able to differentiate even when co-cultured with dead HepG2-DsRed cells. Release of growth factors from HepG2-DsRed cells is not the cause of HUVEC-C3 differentiation in the co-culture model.

3.3.5 Investigating the Angiogenic Signaling Pathways in the Co-Culture Model

To investigate the molecular mechanisms of HepG2 induced differentiation of HUVEC-C3, the effects of no serum condition, inhibitors, activators and chemotherapy drugs were tested in the co-culture model. When the cells were cultured without fetal bovine serum (FBS) for two days, HUVEC-C3 maintained their cobblestone morphology, did not differentiate nor undergo apoptosis (Figure 3-9 and Table 3-2). Although essential amino acids and glucose present in the medium can keep the cells alive, growth factors, proteins and a mixture of complex substances within FBS [179] are essential for cell growth and HepG2-induced HUVEC-C3 differentiation.

Figure 3-9. Investigation of signaling molecules important for tubule formation in the co-culture model. 4 μM of VEGFR inhibitor (SU5416), 8 μM of sorafenib, 10 μM MEK inhibitor (U0126), 10 μM of JNK inhibitor (SP600125), 20 μM of PI3K inhibitor (LY294002), 10 μM of p38 inhibitor (SB202190), 10 μM of ROCK inhibitor (Y27632), 20 nM of PKC activator

(PMA), 10 μ M of FAK inhibitor (Y15) and 200 nM of paclitaxel were added to the co-culture model upon cell seeding of HepG2-DsRed (not shown) and HUVEC-C3 (green). Apoptotic HUVEC-C3 appeared blue (red arrows).

Table 3-2. Quantification of tubule network formation under different conditions in the co-culture system

Condition	Number of junctions	Number of tubules	Total tubule length (μ m)	Average network changes	Apoptotic cells (%)	AIA/VDA
Control	620 $\pm$ 56 (100%)	1,111 $\pm$ 85 (100%)	59,426 $\pm$ 1459 (100%)	0.0%	< 5	-
No serum	132 $\pm$ 28 (21.3%)	296 $\pm$ 66 (26.6%)	16,085 $\pm$ 5,759 (27.1%)	-75.0%	< 5	AIA
VEGFR inhibitor SU5416 4 μ M	539 $\pm$ 29 (86.9%)	980 $\pm$ 72 (88.2%)	54,156 $\pm$ 2,519 (86.6%)	-12.8%	< 5	AIA
VEGFR + MEK inhibitor Sorafenib 8 μ M	402 $\pm$ 58 (64.8%)	785 $\pm$ 112 (70.7%)	43,074 $\pm$ 3,588 (72.5%)	-30.7%	< 5	AIA
MEK inhibitor U0126 10 μ M	248 $\pm$ 107 (40.0%)	496 $\pm$ 192 (44.6%)	31,318 $\pm$ 9,503 (52.7%)	-54.2%	< 5	AIA
JNK inhibitor SP600125 10 μ M	449 $\pm$ 19 (72.4%)	846 $\pm$ 41 (76.1%)	45,861 $\pm$ 2,547 (77.2%)	-24.8%	< 5	AIA
PI3K inhibitor LY294002 20 μ M	396 $\pm$ 28 (63.9%)	749 $\pm$ 52 (67.4%)	45,384 $\pm$ 1,998 (76.4%)	-30.8%	< 5	AIA
p38 inhibitor SB202190 10 μ M	697 $\pm$ 75 (112.4%)	1,203 $\pm$ 80 (108.2%)	64,387 $\pm$ 5,801 (108.3%)	+9.6%	< 5	AIA
ROCK inhibitor Y27632 10 μ M	638 $\pm$ 98 (101.2%)	1,081 $\pm$ 125 (97.3%)	60,835 $\pm$ 7,288 (102.4%)	+0.3%	< 5	AIA
PKC activator PMA 20 nM	668 $\pm$ 89 (107.7%)	1,191 $\pm$ 133 (107.2%)	58,677 $\pm$ 5,484 (98.7%)	+4.5%	< 5	AIA
FAK inhibitor Y15 10 μ M	0	0	0	-100.0%	22.2 $\pm$ 10.3	VDA
Chemotherapy drug Paclitaxel 200 nM	0	0	0	-100.0%	24.0 $\pm$ 14.9	VDA

Two anti-angiogenic agents were tested. SU5416 is an inhibitor for vascular endothelial growth factor receptor (VEGFR) which failed clinical trial in phase 3. At 4 μ M, SU5416 did not significantly reduce the tubule formation as the level of network formation averaged from three parameters (number of junctions, number of tubules and total tubule length in μ m) was only reduced by 12.8% (Figure 3-10 and Table 3-2).

At a higher concentration of 8 μ M, SU5416 still did not significantly reduce the tube formation even though the compound formed crystals in the medium. In contrast, sorafenib [166], a clinically used drug for treating HCC and is a multi-kinase inhibitor targeting Raf kinases, VEGFR and platelet derived growth factor receptor (PDGFR), reduced average network formation by 30.7% when 8 μ M of the drug was used for two days (Table 3-2).

MEK inhibitor (U0126) strongly reduced tubule formation by 54.2% (Figure 3-10 and Table 3-2). Most HUVEC-C3 did not differentiate and remained in small clusters. The MEK/ERK pathway is one of the most important signaling pathways in angiogenesis. Inhibiting the Ras/Raf/MEK/ERK pathway as demonstrated by U0126 and sorafenib (Figure 3-9) prevented HUVEC-C3 cell differentiation in the co-culture model.

JNK inhibitor (SP600125) and PI3K inhibitor (LY294002) had moderate inhibitory effect on HUVEC-C3 differentiation with 24.8% and 30.8% reduction on the network formation respectively (Figure 3-10 and Table 3-2). Both inhibitors can regulate survival and migration [180, 181] of ECs which are important for angiogenesis, but are not essential for differentiation.

Three inhibitors used in this study did not affect EC differentiation in the co-culture model. p38 inhibitor (SB202190) did not reduce network formation but rather slightly increased it by 9.6% compared to the control cells at day two (Figure 3-10 and Table

3-2). This result is in agreement with previous findings using p38 MAPK inhibitors including SB202190 that showed that p38 MAPK negatively regulated EC angiogenesis [182, 183].

Figure 3-10. Quantification of tubule formation via signaling molecules important for tubule formation in the co-culture and matrigel model. (A-F) Quantification of tubule formation after addition of various angiogenic mediators and inhibitors in the co-culture model (left) and matrigel differentiation assay (right, refer to Figure 3-9 and Figure 3-12 for images). All images were analyzed with Angiogenesis Analyzer plugin for ImageJ software. Values were normalized to the same areas of observation field. Data shown are means $\pm$ SEM of three independent experiments with 6-9 images selected at random from (A) and Figure 3-12 and analyzed for (A,D) number of junctions, (B,E) number of tubules, and (C,F) total number of tubules (μm). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs. control.

Rho-associated protein kinase (ROCK) inhibitor (Y27632) did not enhance nor suppress tubule formation compared to the control group (Figure 3-9 and Table 3-2).

Some have shown that ROCK signaling is essential for VEGF-mediated angiogenesis [184], while others have shown that ROCK inhibition enhanced sprouting angiogenesis [185, 186]. However, in the co-culture model, ROCK inhibition did not alter tubule formation, as VEGF was not the key inducer in the co-culture model.

Phorbol myristate acetate (PMA), a protein kinase C (PKC) activator and tumor promoter [187], induced morphological changes where cell elongation was observed in both HUVEC-C3 (Figure 3-11) and HepG2-DsRed cells. The tubular networks formed by HUVEC-C3 were incomplete and unconnected (Figure 3-9). Although the computer software, Angiogenesis Analyzer, used in this study characterized the elongated cells as tubular networks with 4.5% increase in tubule formation (Table 3-2), a closer imaging analysis revealed that they are quite different from the differentiated ECs in the control group. This may be due to the ability of PMA to cause HUVEC-C3 differentiation, and not because of their physical interactions with HepG2-DsRed cells.

Most HUVEC-C3 cells did not undergo apoptosis when they were treated with the aforementioned inhibitors. Less than 5% of the treated cells appeared as blue color in the FRET images (Figure 3-9 and Table 3-2) which indicated caspase-3 was not activated. Only FAK inhibitor (Y15) and paclitaxel induced significant apoptosis in HUVEC-C3 cells (blue cells, Figure 3-9) with $22.2 \pm 10.3\%$ and $24.0 \pm 14.9\%$ (Figure 3-10 Table 3-2). The cells appeared round and no tubule formation was observed. HUVEC-C3 emitted blue fluorescence in FRET images, indicating that the FRET effect was completely abolished due to the cleavage of C3 sensor by caspase-3 [98, 156]. FAK inhibitor prevented HUVEC-C3 cells from attaching to the plate, resulting in anoikis [188], where cells undergo apoptosis due to detachment. Paclitaxel on the other hand, triggered cell apoptosis of both HUVEC-C3 (Figure 3-9 and Figure 3-11) and HepG2-

DsRed, demonstrating its ability as a chemotherapeutic drug that targets all proliferating cells.

Figure 3-11. Assessing the toxicity of signaling molecules important for tubule formation with only the HUVEC-C3 cells. 4 μ M of VEGFR inhibitor (SU5416), 8 μ M of sorafenib, 10 μ M MEK inhibitor (U0126), 10 μ M of JNK inhibitor (SP600125), 20 μ M of PI3K inhibitor (LY294002), 10 μ M of p38 inhibitor (SB202190), 10 μ M of ROCK inhibitor (Y27632), 20 nM of PKC activator (PMA), 10 μ M of FAK inhibitor (Y15) and 200 nM of paclitaxel were added to HUVEC-C3 (green). Apoptotic HUVEC-C3 appeared blue (white arrows).

To ensure that the inhibitors did not affect HUVEC-C3 viability and morphological differentiation, the inhibitors were added to the HUVEC-C3 alone cell culture. Most inhibitors did not induce any morphological changes in HUVEC-C3 alone (Figure 3-11), with only PMA resulting in > 5% elongation of HUVEC-C3. Paclitaxel, on the other hand, resulted in > 5% apoptosis of cells in the HUVEC-C3 culture.

3.3.6 HepG2-HUVEC-C3 Co-Culture as a New Drug Discovery Model in Comparison with the Matrigel-Based Angiogenesis Assay

The co-culture model and the matrigel differentiation assay displayed similar phenotypic characteristics (Figure 3-3C). Matrigel differentiation assay is the gold standard for *in vitro* angiogenesis assay, where support from an ECM is present for EC differentiation. To examine the physical support provided by HepG2-DsRed cells in the co-culture and to compare the differences between matrigel and the co-culture as functional angiogenesis assays, the conditions used in the co-culture were applied to the matrigel differentiation assay, which consist of only HUVEC-C3 cells. The results showed discrepancy from the co-culture model when VEGFR, ROCK inhibitors and no serum conditions were used. HUVEC-C3 cells formed tubules under no serum conditions on the matrigel (6.7% increase in network formation, Figure 3-12 and Table 3-3), however differentiation of HUVEC-C3 was strongly inhibited under no serum conditions in the co-culture model (a reduction of 75.0% tubular networks, Figure 3-9 and Table 3-2). Matrigel, which is a tumor derived matrix, contains growth factors [90] which were necessary for the differentiation of HUVEC-C3, thus allowing HUVEC-C3 to differentiate even without any addition of serum (Figure 3-12).

SU5416 inhibited tubule formation in the matrigel differentiation assay (a reduction of 17% tubule formed, $p < 0.05$ in all three parameters of tubule networks quantified, Figure 3-10D-F), however in the co-culture model, differentiation of HUVEC-C3 was not significantly inhibited (a reduction of 12.8% tubule formed, $p < 0.05$ in only one parameter of tubule network quantified, Figure 3-10A-C). This further confirmed that VEGF is not the key inducer of HUVEC-C3 differentiation in the co-culture model, whereas addition of SU5416 to the matrigel which contain high levels of VEGF could significantly inhibit tubule formation [189].

Figure 3-12. Investigation of signaling molecules important for tubule formation in the matrigel differentiation assay. AIAs and VDAs used in the co-culture model were used in the matrigel differentiation assay. HUVEC-C3 was pretreated with various agents for 3 hr and during the whole assay. 4 μ M of VEGFR inhibitor (SU5416), 8 μ M of sorafenib, 10 μ M of MEK inhibitor (U0126), 10 μ M of JNK inhibitor (SP600125), 20 μ M of PI3K inhibitor (LY294002), 10 μ M of p38 inhibitor (SB202190), 10 μ M of ROCK inhibitor (Y27632), 200 nM of PKC activator (PMA), 10 μ M of FAK inhibitor (Y15) and 200 nM of paclitaxel were added to the HUVEC-C3 seeded on matrigel. Images were taken 5 hr after seeding of HUVEC-C3 on the matrigel.

ROCK inhibitor (Y27632) prevented tubule formations on the matrigel assay (a reduction of 61.8% network formation (Table 3-3). This could be due to the inhibitor preventing VEGF mediated angiogenesis [184] of ECs on the matrigel, however sustained inhibition to the ROCK signaling pathway was unable to inhibit HUVEC-C3 tubular networks formation on the co-culture as the underlying support from HepG2-DsRed cells was the key inducer in HUVEC-C3 differentiation and not that of VEGF.

Table 3-3. Quantification of tubule network formation under different conditions in the matrigel

Condition	Number of junctions	Number of tubules	Total tubule length (μm)	Average network changes	Apoptotic cells (%)	AIA/VDA
Control	211 ± 42 (100%)	365 ± 66 (100%)	21,169 ± 2,864 (100%)	0.0%	< 5	-
No serum	225 ± 48 (106.6%)	381 ± 73 (104.4%)	23,102 ± 3,492 (109.1%)	+6.7%	< 5	AIA
VEGFR inhibitor SU5416 4 μM	169 ± 44 (80.1%)	302 ± 70 (82.8%)	18,247 ± 3,502 (86.2%)	-17.0%	< 5	AIA
VEGFR + MEK inhibitor Sorafenib 8 μM	41 ± 21 (19.4%)	87 ± 40 (23.8%)	6,275 ± 2,889 (29.6%)	-75.7%	< 5	AIA
MEK inhibitor 10 μM U0126	15 ± 8 (7.1%)	34 ± 17 (9.3%)	2,373 ± 1,274 (11.2%)	-90.8%	< 5	AIA
JNK inhibitor 10 μM SP600125	106 ± 42 (50.2%)	191 ± 70 (52.3%)	11,472 ± 3,686 (54.2%)	-47.8%	< 5	AIA
PI3K inhibitor 20 μM LY294002	51 ± 28 (24.2%)	100 ± 45 (27.4%)	6,581 ± 24,97 (31.1%)	-72.4%	< 5	AIA
p38 inhibitor 10 μM SB202190	182 ± 25 (86.3%)	327 ± 38 (89.6%)	19,746 ± 1,883 (93.3%)	-10.3%	< 5	AIA
ROCK inhibitor 10 μM Y27632	66 ± 25 (31.3%)	138 ± 45 (37.8%)	9,630 ± 2,954 (45.5%)	-61.8%	< 5	AIA
PKC activator 20 nM PMA	300 ± 33 (142.2%)	513 ± 133 (140.5%)	27,663 ± 2,282 (130.7%)	+37.8%	< 5	AIA
FAK inhibitor 10 μM Y15	0	0	0	-100.0%	< 5	VDA
Chemotherapy drug Paclitaxel 200 nM	18 ± 16 (8.5%)	43 ± 34 (11.8%)	3,159 ± 2,593 (14.9%)	-88.3%	< 5	VDA

To further demonstrate the importance of physical supports provided by HepG2, the conditioned medium from HepG2 was added to the matrigel consisting of only HUVEC-C3. No significant differences were observed in the network formations when only

HUVEC-C3 was used or when the conditioned medium from HepG2 was supplied to the matrigel as seen in Figure 3-13. This result indicated that the paracrine factors from the HepG2 conditioned medium were not the main angiogenic stimulants in this matrigel system.

Figure 3-13 Comparison of tubule formation in the matrigel differentiation assay without (control) or with the conditioned medium of HepG2-DsRed. (A) DMEM or conditioned medium from HepG2-DsRed was mixed 1:1 in matrigel and coated on petri dish, followed by seeding of HUVEC-C3 with DMEM or the conditioned medium. Images were taken 5 hr after seeding of HUVEC-C3 on the matrigel. Both control and the conditioned medium of HepG2-DsRed showed tubule formation in the matrigel assay. (B-D) Tubule formation was quantified from (A) and data shown are means $\pm$ SEM of three independent experiments with 6-9 images selected at random and analyzed for (B) number of junctions, (C) number of tubules, and (D) total length of tubules (μm).

All other inhibitors had the same suppressing effect on tubule formations both on the matrigel and the co-culture model, with inhibitors (eg, MEK, JNK and PI3K inhibitors) exhibiting their effects much more effectively on the matrigel (Figure 3-12). The physical niche provided by HepG2 cells in the co-culture model protected HUVEC-C3 cells from the inhibitors treatments, which illustrated the true clinical situation in HCC

patients, where anti-VEGF therapies often have transient effects with relapse and tumor progression [73].

These results suggested that the HepG2-HUVEC co-culture model is more representative to liver cancer-induced angiogenesis. Another advantage of this model is having a FRET based-C3 sensor in the ECs, which enabled the identification of vascular disrupting agents (VDAs) [156] and angiogenesis inhibiting agents (AIAs). Furthermore, two days of cell culturing in the co-culture model allowed the identification of VDAs such as paclitaxel, which induced HUVEC-C3 apoptosis on the co-culture (Figure 3-9), and not in the matrigel assay (Figure 3-12). Survival and cytotoxicity to ECs can be monitored with the co-culture model.

3.3.7 Signaling Pathways Undertaken by the HepG2-HUVEC-C3 Co-Culture Model in Mediating Tumor Angiogenesis

A clearer understanding of the molecular signaling pathway (Figure 3-14) within the co-culture is presented with the use of inhibitors and drugs. The inhibitors of the FAK and Ras/Raf/MEK/ERK signaling pathways have prevented HUVEC-C3 differentiation and tubule formation in the co-culture model. The FAK pathway (Figure 3-14) is essential for the survival of HUVEC-C3 cells and regulates many downstream signaling pathways that lead to angiogenic reprogramming within HUVEC-C3 cells. Inhibition of both the PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways have recently displayed promising treatment response to HCC [165] which has also prevent HUVEC-C3 differentiation in the co-culture model, suggesting the potential of the co-culture model as a screening platform for HCC. On the other hand, inhibitors that could only prevent HUVEC-C3 differentiation in the co-culture and not the matrigel system might

not be highly relevant in tumor angiogenesis, where the VEGFR [190] and ROCK [184] inhibitors have failed to elicit an inhibitory response in the co-culture.

Figure 3-14. Signaling pathways and molecular mechanisms mediating HUVEC-C3 differentiation with HepG2-DsRed. Both growth factors and physical interactions are required for proper differentiation of HUVEC-C3 when co-cultured with HepG2, with various signaling pathways triggered by the receptors (left box) and the eventual result of angiogenic programming of ECs (right box). Inhibitors highlighted in red were able to suppress endothelial differentiation and tubule formation in the co-culture system. Of particular importance are the FAK and Raf/Ras/MEK/ERK pathways.

3.3.8 Differences between Fibroblast/Liver Cancer and Endothelial Co-Culture

The importance of physical interactions in the HepG2-HUVEC co-culture was revealed in the previous results. This led to questions raised regarding other types of co-cultures used as angiogenesis assay, namely the fibroblast-EC co-culture. The fibroblast-endothelial co-culture has been discovered to form tubule formations since 1999 [86,

87]. Were physical interactions also the key inducers in the fibroblast-endothelial co-culture? To answer this question, the differences between these two cultures were examined, especially since the key inducers leading to EC differentiation in the fibroblast-endothelial co-culture had been examined in many studies [91, 92, 191]. The fibroblast-ECs co-culture were compared with the liver cancer-EC co-culture in terms of their network formations and the inducer for EC differentiation.

Upon co-cultivation of IMR90 fibroblast with HUVEC-C3, EC differentiation was observed, with HUVEC-C3 differentiating and elongating in one direction (Figure 3-15). The fibroblast-endothelial co-culture formed orderly and circular networks, similar to the matrigel, while the liver cancer-endothelial co-culture form chaotic networks that contained dead ends.

Figure 3-15. Comparison of HUVEC-C3 differentiation in two co-culture models. (A) IMR90 fibroblast – ECs co-culture after 48 h. **(B)** Fibroblast or liver cancer cells co-cultured with HUVEC-C3 after 48 h.

Experiments utilizing the transwell assay were performed with the intention of studying secretory factors in the fibroblast-endothelial co-culture. HUVEC-C3, HepG2-DsRed or IMR90 fibroblast were added into a 0.4 μm transwell insert, with HUVEC-C3 seeded and attached to the underneath of the porous transwell membrane. Secretory factors were able to pass through the porous membrane without direct contact of the cells. By day 1, HUVEC-C3 cells kept in the presence of IMR90 started to differentiate (Figure 3-16, left), while no obvious morphological changes were visualized in the liver-endothelial culture or the endothelial- endothelial culture. This phenomenon remained the same until day 4, when the HUVEC-C3 cells in the liver-endothelial culture displayed altered morphologies when compared to the endothelial-endothelial culture.

Figure 3-16. Differentiation of HUVEC-C3 (green) when co-cultured with fibroblast or HepG2-DsRed cells in different compartments. Transwell assay with HUVEC-C3 co-cultured with itself (left) or fibroblast IMR90 (middle) or liver cancer HepG2-DsRed (right). Only HUVEC-C3 cells attached to the underneath of the transwell membrane is shown.

HUVEC-C3 cells in the HepG2-DsRed culture showed elongations and protrusions, with small contact points seen in HUVEC-C3 cells (Figure 3-16, right)

Interestingly, upon close examination of the HUVEC-C3 kept in the presence of HepG2-DsRed, an unexpected phenomenon was observed. HUVEC-C3 cells were elongating and differentiating at the center of the co-culture, while HUVEC-C3 cells at the rim of the transwell did not show morphological changes. They maintained the same morphology as the HUVEC-C3 cells in the endothelial-endothelial co-culture. Only HUVEC-C3 cells present in the center of the porous membrane and were in contact with HepG2-DsRed displayed small contact points. These cells obtained signal cues directly from HepG2-DsRed cells through the 0.4 μm porous membrane and were differentiating (Figure 3-17). HUVEC-C3 had differentiated by directly interacting with HepG2-DsRed cells through the pores of the membrane, displaying contact points (Figure 3-17 right). These results showed that secretory factors were the main inducers in the

Figure 3-17. HUVEC-C3 displayed morphological differences with and without direct contact with HepG2-DsRed cells. HepG2-DsRed cells were seeded in the transwell insert, and HUVEC-C3 attached to the underneath of the porous membrane. Only cells at the center of the transwell (right) displayed small contact points and differentiation. HUVEC-C3 cells found at the rim of the transwell (left) did not show any morphological changes.

fibroblast-HUVEC-C3 co-culture, while direct contact from HepG2-DsRed cells triggered HUVEC-C3 differentiation in the liver cancer-HUVEC-C3 co-culture.

3.3.9 Fibronectin is the Secretory Inducer in Fibroblast-Endothelial Co-Cultures

The ECM protein, fibronectin, is secreted by fibroblasts in large quantities. Zhou *et al.* have reported the elongation of ECs embedded in 3D gel of cross-linked fibrin where ECs initiate network formations by depositing network fibrils to support the cytoskeletal organization of the cells [192]. Fibronectin polymerization served as a structural scaffold for the network formations of ECs. As hepatocytes have been known to produce fibronectin [193], the expression of fibronectin was studied in the liver cancer-EC co-culture. Immunofluorescence staining of fibronectin was performed on both fibroblast-EC co-culture and liver cancer-EC co-culture. In IMR90 cells, fibril formation were visualized, with structural linkages formed between cells (Figure 3-18A). The fibronectin formed polymerized scaffolds between cells and were not only localized within the cell, but also found in the extracellular spaces. In contrast, no fibril formation was observed in HepG2 cells, and fibronectin was found within the cells in a diffused manner. Western blot analysis of fibronectin showed fibronectin produced in large quantities in IMR90 cells, whereas low amounts of fibronectin were produced in HepG2 and HUVEC cells (Figure 3-18B). Fibronectin may not have been the key inducer in the liver cancer-EC co-culture, while the large amount of fibronectin secreted by fibroblast may have been the key inducer for EC differentiation in the fibroblast-EC co-culture.

Figure 3-18. IMR90 fibroblast cells produce fibronectin, which forms fibril structures whereas HepG2 liver cancer cells does not. (A) Immunostaining of fibronectin (green) in HUVEC, IMR90 and HepG2 cells. (B) Western blot analysis of fibronectin in 50 µg of total cell lysate.

3.4 Discussion

Many have demonstrated EC morphogenesis in liver engineering [170, 194, 195]. However, most focused on liver tissue engineering [194, 195] or introducing new protocols [170] to stimulate tumor angiogenesis. Here, in a co-culture of liver cancer cells and ECs, the ECs were able to undergo morphogenesis, without the use of ECM components or 3D models. This is similar to the layered co-culture of hepatocytes and ECs employed by Harimoto *et al.* and Takayama *et al.* where both studies focused on the up-regulation of liver functions [196] and liver specific genes [197]. However, both did not realized EC morphogenesis or tubular network formations may be induced by the liver cell sheets. Few had studied the heterotypic interactions between liver cells and ECs where liver cells may possess an innate ability to induce EC angiogenesis.

This is the first report of a hepatocyte-EC co-culture model as a 2D platform for studying liver cancer-induced angiogenesis. Interestingly, the aberrant tubular networks formed in this co-culture model is comparable to those *in vivo*, where intravital optical imaging of neovascularization of the liver tumor [198] and other animal models [199] showed similar chaotic and abnormal network formation. I demonstrated the inhibition of the networks with various inhibitors, and as such dissected the molecular signaling pathways in HUVEC-C3 differentiation and presented the co-culture as a drug-screening platform for HCC. VDAs and AIAs can be identified, while the survival and cytotoxicity to ECs can be investigated simultaneously.

The role of liver cancer cells as physical supports in the activation of ECs is suggested here. In contrast to studies performed by Khodarev *et al.*, paracrine factors were the key inducer in HUVEC differentiation where indirect co-culture resulted in phenotypic activation of HUVECs cultured with human glioma cells [161]. Instead of focusing on paracrine factors, the cell-cell interactions was studied in this novel co-culture model.

The physical contacts and not the paracrine factors were the key to EC differentiation. Although the exact mechanism in which HepG2 and HUVEC-C3 cells interact is still unknown, the purpose of this study is to provide evidence that liver cancer cells contributed as physical supports in liver-induced angiogenesis. Characterizing the phenotypic changes in both HUVEC-C3 and HepG2 cells led to valuable insights demonstrating the crosstalk between HepG2 and ECs. The next steps would be to study the upregulated cell surface genes in HUVECs as detected by Takayama *et al.* [197], where integrin may mediate the interactions between hepatocytes and ECs [197]. FAK inhibitor targeting the membrane protein integrin was able to disrupt the differentiation of HUVEC-C3 and tubule formation in the co-culture. The cell surface markers possessed by HUVEC-C3 and the reciprocal partners on HepG2, which mediated EC morphogenesis and tubular formation, are the ultimate targets to understand liver cancer-induced angiogenesis. Recognizing the critical role of liver cancer cells as physical supports to EC differentiation would bring new perspectives in liver cancer-induced angiogenesis.

Although it is not known whether this mechanism is distinct to only HCC angiogenesis or not, the significance of tumor cells as physical supports to tumor angiogenesis should not be overlooked. Importantly, many cell lines that were tested such as the human embryonic kidney HEK293 and human neuroblastoma SK-N-SH (Table 3-1), have the ability to induce EC morphogenesis, showing that this EC differentiation observed in the co-culture model may not be unique to only liver cells. The differences between the normal and tumor tubular networks induced by the participating cells should be taken into account (Figure 3-2). It should also be noted that the kidney, brain and liver cells, which were able to induce EC morphogenesis, are those highly vascularized *in vivo* [200]. However, the tubular networks formed in cancer cell

lines (i.e. liver cancer cell, HepG2) and normal cell lines (i.e. normal liver cell, L0-2) exhibit great differences in terms of their structures and orderliness, (i.e. chaotic networks with dead ends were found in the liver cancer cells-EC co-culture, compared to orderly networks in the normal liver cells-EC co-culture). An invaluable technique is presented here which allows further understanding of angiogenesis from a new context.

The co-culture model provides an insight for the examination of physical cues from tumor cells. To investigate tumor-associated blood vessels, the participation of cancer cells is necessary. Matrigel does not provide a platform for the association of cancer cells and may not be representative of the tumor microenvironment. Furthermore, differentiation induced by the matrigel is not specific, where non-ECs such as HepG2 and MDA-MB-231 cells [201] are also able to form tubule-like networks.

In the fibroblast-endothelial co-culture, a fibrin scaffold is assembled by fibronectin secreted from fibroblast cells [192]. It is similar to the matrigel, where the ECM scaffold serves to promote endothelial morphogenesis. However, unlike the fibroblast-endothelial co-culture, cell-cell communication was essential for endothelial morphogenesis in the HepG2-endothelial co-culture. There also exists a possibility that fibroblast is non-epithelial origin and any differences between fibroblasts and cancer cells in promoting angiogenesis can be a result of cell type difference as well.

In the field of angiogenesis, researchers have focused on VEGF as the prime target and neglected the importance of the tumor microenvironment (signaling cues from tumor, stromal and ECM) in contributing to tumor angiogenesis. The tumor microenvironment is increasingly recognized as a prospective target for cancer-oriented therapeutics and tumor-endothelial contact-induced tumor angiogenesis is now being presented as a new strategy in anti-angiogenesis therapies.

In summary, the co-culture model is able to reflect the microenvironment where ECs and liver cancer cells co-exist, resulting in the reprogramming of various signal transduction pathways, and inducing tubule formations resembling that of the tumor vasculatures. Furthermore, to successfully inhibit tumor growth, chemotherapeutic drugs that target multiple cell types simultaneously are necessary. The co-culture model provides an excellent platform for monitoring real-time apoptosis and inhibition of neovascularization in HCC. New therapeutic strategies for HCC can be identified by targeting both tumor and ECs simultaneously.

CHAPTER 4 Establishing a 3D Co-Culture Platform for Drug Screening

4.1 Introduction

Liver cancer or hepatocellular carcinoma (HCC) is one of the most common cancers. HCC is the third leading cause of cancer death worldwide [7, 8], with more than 500,000 people affected. Currently, there is only one drug, sorafenib, approved for the treatment of HCC. The identification of new anticancer drugs is vital for treating HCC. 2D cultures, 3D cultures [94] and experimental mouse models [95] have been used as models for identifying these drugs. However, 2D models often fail to recapitulate the 3D environment *in vivo* and cells cultured in 2D easily lose their gene functions [96], while animal models are costly and the results may not be easily interpreted [95]. Utilization of 3D cultures helps to bridge the gap between animal models and 2D cell assays, improves the drug discovery success rate and minimizes the cost before performing pre-clinical trials [96, 97].

3D cell culture systems have gained wide popularity in drug screening and tissue engineering in recent years [96, 97, 159]. Cells cultured in 2D and 3D differ in various cell behaviors, including cell morphology, differentiation capacity, replicative ability, cell signaling etc. [96, 202]. Under *in vivo* conditions, cells inhabit a complex and multi-component environment, which makes it increasingly necessary to shift from the conventional, simplified 2D culturing of cells on petri dishes to the more complex 3D spheroidal culture systems.

Many 3D liver cancer spheroids have been previously reported [94, 202, 203], where 3D spheroids are able to enhance HCC characteristics, resulting in gene expressions and metabolic profiles that are similar to the hepatic tissue [202]. In this study, to effectively

evaluate drug targets of HCC, a 3D co-culture spheroid model consisting of a human HCC cell line, HepG2, and HUVEC were used to mimic a vascular tumor of the liver. A key step in liver cancer progression and metastasis is the establishment of a vascular network within the tumor. Co-culturing of endothelial-tumor cells reprograms signaling of both cell types [204] which may increase the aggressiveness of these tumor spheroids. Hence, identification of candidates that target both angiogenesis and tumor signaling, such as sorafenib, would advance the treatment of HCC.

The 3D co-culture system consists of HUVEC-C3 cells which have a caspase 3 sensor [156] capable of detecting apoptosis within cells. Under normal conditions in a living cell, FRET can occur whereby fluorescence from the CFP donor is transferred to the YFP acceptor. In such a scenario, the cell appears green by FRET imaging. If apoptosis is triggered within the cell and caspase-3 is activated, the DEVD linkage between YFP and CYP is cleaved and the cell will appear blue by FRET imaging [98]. This allows us to identify whether HUVEC-C3 are undergoing cell apoptosis, and if drugs prevented EC differentiation through inhibiting angiogenesis or by triggering cell death.

On co-culturing the two cell lines, the intrinsic angiogenic properties of the liver cancer cells are able to induce EC differentiation, where HUVECs elongate and branch out to form vasculatures within the liver tumor. Multicellular spheroids of tumor cells with ECs facilitate closer intercellular host cell-tumor interaction and signaling, allowing for the formation of vascular networks that mimic the *in vivo* solid tumor. This co-culture system relies on the inherent angiogenic potential expressed by the multicellular spheroids and does not require the addition of any external angiogenic factors. Furthermore, a HCC microenvironment is recapitulated through the 3D co-

culture spheroid, where a hypoxic core with true gradient penetration of drugs is achieved.

Sorafenib (the only approved liver cancer drug), sunitinib (an FDA approved anti-angiogenesis drug for RCC and imatinib-resistant GIST) and axitinib (an approved anti-angiogenesis drug for RCC) were tested in the 3D co-culture system. Their effects on tumor growth, EC differentiation and viability were quantified in the co-culture system, and the minimum effective dosages of the drugs were determined from the 3D co-culture system, which were found to be similar to the plasma concentration of the drugs used *in vivo*. Screening for liver cancer drugs targeting both anti-angiogenesis (HUVEC) and anti-tumor growth (HepG2) can be performed concurrently within this co-culture system without the interference of exogenous factors.

4.2 Objectives and Significance of Research

4.2.1 Objective 1: Establish a 3D Co-Culture Model for Physiological Relevance

In the 2D model, liver cancer cells could induce EC differentiation. To enhance the physiological relevance of the co-culture model as a HCC tumor model, the 2D model was developed into a 3D model. The 3D model is able to recapitulate the HCC microenvironment, with a hypoxic core and true gradient penetration of oxygen, nutrients and waste. The generation of a hypoxic core promotes angiogenesis as hypoxia upregulates HIF, which in turn trigger the upregulation of VEGF and promote new blood vessels formation.

4.2.2 Objective 2: Develop the Co-Culture 3D Model into a Dual Drug Screening Platform in a Single Step

The 3D co-culture may reflect the interplay of endothelial cells and liver cancer cells in HCC tumor. True gradient penetration of drugs is achieved in the 3D co-culture, and is relevant for analysis of drug responses. Furthermore, a FRET-based caspase-3 biosensor is expressed within the HUVEC-C3 cells, which allows for detection of real time apoptosis of ECs. Dual analysis of anti-tumor or/and anti-angiogenesis agents can be identified in a single step. In addition, VDAs and AIAs are easily distinguished through the system, which can be administered at different stages of cancer.

4.3 Results

4.3.1 3D Co-Culture of HepG2-DsRed and HUVEC-C3 Induced Vascular-Like Network Formation in a Liver Cancer Spheroidal Model

I have established the differentiation of HUVEC-C3 cells with network formations comparable to those plated on the Matrigel when co-cultured with HepG2-DsRed in a 2D platform [204]. Now, the co-culture model was further developed into a 3D co-culture spheroidal model to better capture the tumor complexity under *in vivo* conditions, where cells exist in a 3D environment with direct contact between adjacent cells. In a compact tumor spheroid, the interior exists as a necrotic and hypoxic core (Figure 4-1A). Due to a lack of oxygen and nutrients, vessel growth is initiated by the hypoxic core,

Figure 4-1. 3D co-culture of HepG2-DsRed and HUVEC-C3 cells induces HUVEC-C3 differentiation similar to an *in vivo* tumor vasculatures. (A) Differential zones within a 3D spheroid due to oxygen, nutrients, CO₂ and waste gradients. (B) 3D co-culture of HepG2-DsRed (red) and HUVEC-C3 (green) result in differentiation of HUVEC-C3 cells, and tubular networks formed within the 3D co-culture. Images of Z-stack confocal images (top) and wide-field microscopy (bottom) of co-culture spheroids. Z-stack confocal images were captured and projected into images with average intensities using ImageJ software. (C) Vascular networks within a liver xenograft tumor model of a mouse.

where pro-angiogenic factors and ECM proteins are secreted by cells, assisting in the establishment of a vascular network [205, 206]. By developing a 3D co-culture model, a more relevant *in vitro* tumor model consisting of a HCC microenvironment could be constructed. The 3D co-culture spheroid can be utilized for *in vitro* studies in a 3D HCC microenvironment, or for analyzing pharmacological responses of anti-cancer drugs with true gradient penetration of drugs. Liver cancer cells (HepG2-DsRed) possessed angiogenic properties that induced HUVEC-C3 cells to differentiate and elongate in the spheroidal tumor (Figure 4-1B). Furthermore, Figure 4-1C shows the pattern of HCC generated blood vessels networks grown in the flank of a mouse, similar to tumor vasculatures observed in the 3D co-culture spheroids (Figure 4-1C). The 3D co-culture tumor vasculatures were also similar to those observed in *in vivo* orthotopic models of breast cancer MDA-MB-435 and prostate tumor PC-3 in BALB/c nude mice as reported, by Yang *et al.* [207].

4.3.2 3D Co-Culture of Endothelial Cells with Cancer Cells or with Stromal Cells

The behavior of tumor cells is controlled by their intrinsic properties and the microenvironment, which consists of resident ECs, smooth muscle cells and fibroblasts. Co-cultures of fibroblast and smooth muscle cells with ECs were performed to study the differences in vascularization for normal angiogenesis versus tumor angiogenesis. HUVEC-C3 cells showed a highly differentiated morphology when co-cultured with NIH/3T3 fibroblasts (Figure 4-2A and C). Confocal microscope images were taken to capture tube formation at different layers (Figure 4-2C) due to the highly dense tubular networks formed. Captured images showed that HUVEC-C3 cells differentiated into an orderly network formation in 3D spheroids. Each layer within the spheroid is

systematically filled with sprouting ECs, which were stable even after 48 h of observation. This is consistent with previously established EC-fibroblast model, where mesenchymal cells, such as fibroblasts, secrete high levels of ECM constituents which promotes angiogenesis and vessel stabilization [191, 208]. Co-culture of fibroblasts and ECs has been widely performed and has already been established as a suitable model for studying angiogenesis, thus it will not be a focus in this study.

Co-cultivation of ECs with vascular smooth muscle cells (VSMCs) did not trigger HUVEC-C3 elongation and branching. Instead, ECs migrated to one side of the spheroid even though both cell types were thoroughly mixed at the start of co-culturing (Figure 4-2A). As VSMCs play a supportive role in maintaining blood vessels, they do not have the ability to induce HUVEC-C3 differentiation, driving both types of cells to accumulate and proliferate in two different compartments. Korff *et al.* previously indicated that the co-cultivation of smooth muscle cells (SMCs) and ECs in 3D spheroids generated spheroids with an organization of a surface layer of ECs and a multilayered core of SMCs [93]. The SMCs regulated EC quiescence and abrogated VEGF responsiveness, explaining for the undifferentiated HUVEC-C3 cells visualized in the system.

The differentiation abilities of HUVEC-C3 was studied when co-cultured with other cancer cell types. Angiogenic induction of HUVEC-C3 was not observed in other cancer cell lines, such as breast cancer MCF-7 and MDA-MB-231 cells (Figure 4-2A). HUVEC-C3 cells did not differentiate at all, and, by day two, the HUVEC-C3 cells had proliferated and formed a cluster within the co-cultured spheroids. This is consistent with previous results in the 2D tumor-ECs co-cultures. Only certain cell lines, such as HepG2 liver cancer cells, could induce the differentiation of HUVEC-C3 cells (Figure 4-2A and B). There were remarkable differences between the vascular networks formed

Figure 4-2. Co-culture of tumor and normal cell lines with HUVEC-C3 cells. (A) HepG2 liver cancer cells were able to induce differentiation of HUVEC-C3 cells with tubular network formations (white arrows) whereas MCF-7 and MDA-MB-231 breast cancer cells were unable to induce differentiation of HUVEC-C3 cells (yellow arrows). Cell clusters were formed in co-culture spheroids of MCF-7 and MDA-MB-231 cells. In normal cell lines cultured with HUVEC-C3 cells, NIH-3T3 fibroblasts induced HUVEC-C3 differentiation. In contrast, the co-culture with VSMC, HUVEC-C3 and VSMC were forced to different compartments with no

apparent HUVEC-C3 differentiation. (B) Differentiation of HUVEC-C3 cells (green) in co-culture with HepG2-DsRed (red) or (C) NIH-3T3 cells (colorless). Phase images (top left) and merged images (bottom left) with average intensities from Z-stacks at different intervals (right).

in the HUVEC-C3/HepG2 liver cancer co-culture and the HUVEC-C3/fibroblast NIH-3T3 co-culture. HUVEC-C3 formed chaotic and disorderly networks within the liver cancer spheroids, whereas HUVEC-C3 formed orderly and symmetrical networks within the 3D spheroids of NIH-3T3 cells. In addition, dead vascular ends with abnormal branching were also observed in the endothelial-liver cancer co-culture. These characteristics signified tumor angiogenesis and are not observed in regular structures produced by the endothelial-fibroblast co-culture.

Overall, 3D co-culture of ECs with the host stroma cells has shown that fibroblasts can induce physiological angiogenesis and that muscle cells (VSMCs) act as supportive structures. Furthermore, 3D co-culture of ECs with cancer cells does not always trigger angiogenesis due to intrinsic differences between cancer cell lines. Liver cancer cells have the innate ability to induce angiogenesis, whereas breast cancer cell lines do not induce vascular formation [200].

4.3.3 Growth Factors, Inhibitors and Drugs Modulate HUVEC-C3 Differentiation in 3D Co-Culture Spheroids

To study the importance of growth factors and the ECM within the tumor microenvironment of the 3D co-culture spheroid, VEGF, bFGF and fibronectin were added to further induce angiogenesis. Angiogenesis inhibitors (sorafenib and SU5416) and a lack of serum (to simulate growth factor withdrawal) conditions were also applied to the 3D co-culture system. The addition of fibronectin showed enhanced network

Figure 4-3. The addition of exogenous factors affect tubular network formation. HUVEC-C3 cells (green) elongated and formed networks when co-cultured with HepG2-DsRed cells (red). (A) HUVEC-C3 cells differentiated under bFGF, VEGF and fibronectin treatment (white arrows), and they remained undifferentiated (yellow) when treated with sorafenib, SU5416 and no serum medium. (B) Sorafenib, SU5416 and no serum conditions affect the integrity of the spheroids (outline by white dotted circle). Sorafenib and no serum conditions caused a decrease

in the spheroid size, while cells protruded out of the spheroids under SU5416 and no serum treatment. (C) Percentage of differentiated cells as visualized in A and B. Data represents mean $\pm$ SEM of three independent experiments, $n = 3-5$ spheroids, $*p < 0.05$ vs. control (Student's *t*-test).

formations in the 3D spheroids, and there was an increase of 44% in HUVEC-C3 differentiation (Figure 4-3A and C), whereas bFGF and VEGF did not significantly enhance network formations (Figure 4-3C). Tubular networks that formed in the presence of bFGF and VEGF were similar to the control spheroid (Figure 4-3A and C). As fibronectin secreted by fibroblast cells could trigger network formations and considering ECM proteins such as fibronectin were absent in the EC-liver cancer co-culture spheroids, the addition of fibronectin most probably induced further tubular formation. Contrarily, bFGF and VEGF which were factors mainly secreted by tumor cells were present in high levels. The addition of more growth factors was insignificant to the additional formation of vascular networks. Conversely, sorafenib and SU5416 interrupted the spheroid integrity (Figure 4-3B), and fewer than 50% of differentiated HUVEC-C3 cells were visualized (Figure 4-3C). In addition, when no serum medium was applied to simulate growth factor withdrawal in the co-culture spheroids, only 15% of HUVEC-C3 cells underwent differentiation compared to control. Confocal images of the co-culture spheroids revealed undifferentiated HUVEC-C3 cells suspended within the spheroids, and there were few network branches observed when compared to the control. These phenomena were also observed in 2D co-cultures with HUVEC-C3 and HepG2-DsRed cells [204].

4.3.4 Determining Cell Viability with Diameter and Fluorescence Intensity from the 3D Co-Culture Spheroid

To utilize the 3D spheroids as a drug screening platform, the fluorescence intensity emitted from HepG2-DsRed cells was measured and compared that intensity with the cell density per spheroid. I found strong correlations for both the fluorescence intensity from HepG2-DsRed cells at an emission wavelength of 586 nm ($R^2=0.9978$) and the diameter of the spheroid ($R^2= 0.9829$) when plotted against the density of HepG2-DsRed cells per spheroid (Figure 4-4B). Both methods could be used for determining the density of cells in the co-culture spheroid.

However, because the structure of a spheroid is sometimes uneven, the diameter is not an accurate measurement for the density; as a result, the fluorescence intensity should serve as an effective measurement scale for the density. HepG2-DsRed cells alone was compared with the same density of HepG2-DsRed cells used in the co-culture and determined that the fluorescence intensity from HUVEC-C3 cells did not contribute to the fluorescence emitted by HepG2-DsRed. Therefore, the fluorescence intensity could be used to determine the number of HepG2-DsRed liver cancer cells within the spheroid. Furthermore, when the density of cells cultured within the co-culture spheroids increases, fewer HUVEC-C3 cells appeared to differentiate and there was a reduction in vascular networks (Figure 4-4A). This could be due to the need for serum to induce angiogenesis at the initial stages of development, as demonstrated by absence of angiogenic networks under the lack of serum conditions in Figure 4-3A. Shen *et al.* have demonstrated that the presence of VEGF in a 3D collagen gel is important for promoting vascularization of the engineered tissue [206]. Once the vascular networks are established, these networks can then carry a blood supply to the proliferating tumor, and continue the establishment of more vessels. Therefore, subsequent experiments

were performed with densities of 1,500 HepG2-DsRed cells and 150 HUVEC-C3 cells (a ratio of 10:1 cells) to ensure adequate penetration of serum for initial vascular establishment.

Figure 4-4. Different densities of spheroids correlate with their diameters and fluorescence intensities. (A) Images of spheroids containing 1:10 of HUVEC-C3 (green):HepG2-DsRed (red) cells. 2500, 5000, 7500 and 10000 HepG2-DsRed cells were seeded with their respective numbers of HUVEC-C3 cells for each spheroid. (B) The diameters and fluorescence intensities

were measured for each spheroid density and plotted into a graph, data represents mean $\pm$ SEM of three independent experiments, n = 3-5 spheroids.

4.3.5 The 3D Co-Culture Model as a Drug Screening Platform for Liver Cancer

With the development of a 3D co-culture spheroid, the model was tested with anti-angiogenic drugs available in the market. The following three drugs were chosen: (1) sorafenib, the only approved systemic therapeutic drug for advanced HCC that has exhibited both anti-tumor and anti-angiogenesis efficacies [22, 209]; (2) sunitinib, an oral multi-targeted receptor tyrosine kinase for platelet-derived growth factor (PDGF) and VEGF [210], which are important for tumor angiogenesis and tumor proliferation; and (3) axitinib, an anti-angiogenic drug that is under pre-clinical trials for HCC [211].

All three drugs prevented vascular formation at high concentrations, among which sunitinib displayed a strong anti-angiogenic effect by reducing the vascular networks by 35% at a low concentration of 10 nM (Figure 4-5 and Figure 4-6A), and 1 μ M of sunitinib further reduced the network formation by 87%. Although axitinib had a lower anti-angiogenic effect than sunitinib, it still reduced vascular formations by 53% at 1 μ M and reduced networks formation by ~90% at 5 μ M compared to control (Figure 4-6A). In summary, both anti-angiogenesis-based drugs were able to effectively target vascular formation, and sunitinib displayed greater efficacy at a lower concentration in the 3D co-culture platform.

In contrast, sorafenib was ineffective in inhibiting angiogenesis at a lower concentration and only decreased 66% of network formation (Figure 4-6A), with 12.1% apoptotic rates in HUVEC-C3 at a concentration of 20 μ M (Figure 4-6C). However, it effectively reduced the viability of HepG2-DsRed cells by 40% at this concentration

Figure 4-5. Assessing effects of sorafenib, sunitinib and axitinib on the 3D co-culture spheroid. The co-culture spheroids were treated with sorafenib, sunitinib and axitinib for two days. All three drugs prevented network formation and reduced differentiation of HUVEC-C3 cells. Sunitinib and axitinib induced HUVEC-C3 apoptosis (blue cells, red arrows). Sunitinib also resulted in HepG2-DsRed cell death, and loss of red fluorescence was observed. All drugs were used at a concentration of 20 μ M.

(Figure 4-6B). As an anticancer drug, sorafenib is capable of inhibiting the VEGFR/PDGFR signaling cascade that is vital for angiogenesis and blocking the RAF kinase in the RAF/MEK/ERK signaling pathway, which regulates cell division and proliferation [22]. Both properties allowed for simultaneous targeting of angiogenesis and tumor growth, which is observed in the 3D co-culture system (Figure 4-6A and B).

Sunitinib, although effective as an anti-angiogenic drug at low concentrations, did not significantly reduce tumor growth in the 3D co-culture model. However, at a concentration of 10 μ M, there is a significant drop in the cell viability to 11% (Figure 4-6B). Upon closer examination, it was discovered that the spheroid was disrupted and

Figure 4-6. Quantification of the co-culture spheroids under two days of treatment of sorafenib, sunitinib and axitinib. (A) The percentages of networks formed in the co-culture spheroids were quantified and normalized to control. (B) Cell viability of HepG2-DsRed cells when treated with the three inhibitors at different concentrations. Cell viabilities were quantified by measuring the red fluorescence intensities released by HepG2-DsRed cells in co-culture and normalized to control. (C) Apoptotic rates of HUVEC-C3 were quantified by counting the number of blue fluorescence cells over the total number of HUVEC-C3 cells. Data shown are means $\pm$ SEM of three independent experiments $n = 3-5$ spheroids. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs. control.

most HepG2-DsRed cells appeared to be dead with no signal emitted by the red fluorescent protein (Figure 4-5). HUVEC-C3 cells were undergoing apoptosis with cells that appeared blue in contrast to the green color when alive (Figure 4-5), with high apoptotic rates of 73.0% at 20 μ M (Figure 4-6). This is consistent with sunitinib triggering apoptotic events in ECs [212] and in xenograft models of HCC [213]. Sunitinib is effective as an anti-angiogenesis agent at pharmacologically relevant concentrations (~ 100 nM), but it does not kill tumor cells at this concentration. However, if high concentrations, such as 20 or 40 μ M of sunitinib, are used *in vivo*, there might be side effects, as observed with the high percentage of HepG2-DsRed cell death in the co-culture model (Figure 4-5 and Figure 4-6B). High toxic events have previously been observed in pre-clinical trials of HCC with the use of sunitinib [214]. These results emphasized the importance of using the 3D co-culture system to assess the *in vivo* toxicity of the tested compounds.

Although axitinib significantly reduced 91% of network formation at 5 μ M, a non-significant decrease of 11% of tumor growth was observed compared to control. Axitinib was able to trigger apoptosis in ECs [69], and HUVEC-C3 cells were observed to change from their initial green color to blue color when undergoing apoptosis with an

apoptotic rate of 18.6% at 5 μ M (Figure 4-6C). Herein, the function of axitinib as an effective anti-angiogenic drug is demonstrated, which works by inducing apoptosis of ECs instead of as an anti-liver cancer agent. The combined use of anti-cancer drugs with an anti-angiogenic drug, axitinib, to simultaneously target both tumor and vascular cells could be a valuable approach for treating HCC [67, 200, 215].

Here, the 3D co-culture spheroid has effectively modeled a HCC microenvironment, with a hypoxic core and true gradient penetration of drugs. The effective drug concentrations of sorafenib, sunitinib and axitinib determined in the 3D co-culture system are similar to human plasma concentrations. The 3D co-culture may serve as a useful predictor for *in vivo* drug efficacy.

4.3.6 Upregulation of Signaling Factors in the 3D Co-Culture Model

To determine the differences between 2D and 3D cultures, cells in 2D and 3D cultures were collected and analyzed for the expressions of signaling proteins. The levels of phosphorylated p70s6k and Akt were much higher in cells in 3D co-culture compared to in 2D co-culture and HepG2-DsRed monoculture (Figure 4-7A). Akt/mTor signaling is not only a central regulatory pathway regulating cell proliferation, growth and survival, but also plays a crucial role in the induction of angiogenesis in HCC [216], while phosphorylation of p70s6k upregulates protein synthesis. The high levels of p70s6k and Akt expressed may be due to close interactions between both liver cancer and ECs, triggering both ECs differentiation, protein synthesis and the growth of cancer cells in 3D co-culture. There was high proliferation observed at the periphery of the 3D spheroids, where cells were closely packed and much tighter than the center of the spheroid (Figure 4-7B). A necrotic core was also generated in the 3D co-culture, whereas these events were not observed in the 2D co-culture. In a 2D co-culture, cells

were packed within 2D petri dishes and had limited area for growth, while the real *in vivo* situation was not reflected.

A very high level of ERK phosphorylation was observed when HepG2-DsRed cells were grown in a monoculture, which was lowered when co-cultured with HUVEC-C3 cells in either 2D or 3D (Figure 4-7). This high level of ERK activation may allow sorafenib to elicit its dual inhibitory effect on liver cancer cells through the MEK/ERK pathway and inhibit both tumor proliferation and angiogenesis. In addition, VEGF expression is also upregulated in the 3D co-culture, signifying more VEGF secretion from HepG2-DsRed cells in 3D compared to the 2D co-culture. High VEGF expression was also observed in IHC analyses performed on both xenograft tumor and 3D co-culture (Figure 4-7C). The physiological relevance of the 3D co-culture and its similarity to *in vivo* xenograft tumor is demonstrated by the similarities in vasculatures formation (Figure 4-1), and the protein expressions through western blotting and IHC analysis (Figure 4-7A and C).

Figure 4-7. Analysis of different gene expressions in 2D, 3D co-culture and xenograft tumor. (A) Western blot analysis of 2D HepG2-DsRed monoculture, 2D and 3D co-culture. (B) H&E staining of xenograft tumor and 3D co-culture model. Cells were packed much tightly at

the rim of the 3D co-culture (proliferation zone) compared to the center of the spheroid (hypoxic core). (C) IHC staining for VEGF protein expression in xenograft tumor and 3D co-culture. High VEGF protein expression was observed (brown staining).

4.4 Discussion

3D co-culture of tumor-endothelial models has been studied previously. Timmins *et al.* previously established a hanging-drop multicellular spheroid consisting of tumor cells with ECs, and showed that vascular networks in these tumor-EC spheroids can be established [203]. However, no further studies have reported the use of these spheroids for drug screening with biosensors. Anand *et al.* previously employed a caspase biosensor to allow for longitudinal detection of apoptosis in 3D breast tumor model [159]. A FRET sensor for caspase-3 is now utilized in the co-culture to detect apoptosis of ECs [98, 156], as well as red fluorescence protein to identify liver cancer cells. This approach allows for real-time detection of apoptosis in ECs and dual screening of anti-angiogenesis and anti-tumor drugs in a single step. The similarities of the 3D co-culture model to an *in vivo* xenograft tumor were also demonstrated, which expresses upregulated gene expression and has stronger cellular functions and activities [202] in contrast to 2D cultures.

A more realistic HCC microenvironment consisting of a hypoxic core is generated within the 3D co-culture, and true gradient penetration of drugs is achieved, when compared to the 2D model. Hypoxia or lack of oxygen leads to the stabilization of hypoxia-inducible transcription factors (HIFs), which in turn trigger the upregulation of VEGF and promote new blood vessels formation. In the 2D co-culture system [204], EC differentiation could be induced as long as physical interactions existed between ECs and tumor cells. Direct cell-cell communication between ECs and tumor cells was essential for vascular formation. However, the 2D co-culture system could not reflect the true physiological condition in which cells exist as 3D but not 2D. 2D co-cultures of tumor cells with ECs have demonstrated reprogramming of important signaling pathways [161]; however the factors are upregulated even more in 3D models.

Furthermore, the importance of VEGF was reflected in the 3D co-culture and xenograft tumor as opposed to the supporting role of VEGF in inducing EC differentiation in the 2D co-culture system. Physical interactions were the key inducer for EC differentiation in the 2D co-culture due to excessive VEGF present in the serum, and VEGF inhibitor did not elicit a significant reduction in EC differentiation [204]. The physiological relevance of the 3D co-culture allows for functional screening of anti-angiogenic drugs compared to 2D co-culture.

A FRET-based caspase 3 sensor was expressed by HUVEC-C3 cells, which allows for real time detection of apoptosis. This is crucial in identifying angiogenesis inhibiting agents (AIAs) or vascular disruptive agents (VDAs). AIAs elicit their effect by preventing neo-angiogenesis, whereas VDAs trigger the death of existing blood vessels [53, 217]. AIAs are important during tumor development where neo-angiogenesis occurs, whereas VDAs can target blood vessels that are already established within the tumor, damaging vascularized tumors. The 3D co-culture system can effectively identify both VDAs and AIAs in the administration of suitable drugs at different stages of tumor development. While all three drugs demonstrated their abilities to inhibit EC differentiation, sunitinib and axitinib triggered apoptosis in HUVEC-C3 cells, while sorafenib did not induce apoptosis in HUVEC-C3 cells. Drug inhibition to angiogenesis via the induction of apoptosis or through the quiescence of ECs can be demonstrated by the 3D co-culture system.

All three drugs prevented angiogenesis at their pharmacologically relevant dosages; 20 μ M sorafenib reduced 66% of networks, 100 nM of sunitinib reduced 47% of networks and 1 μ M of axitinib reduced 53% of networks (Figure 4-6A). It has been reported that sorafenib has a steady state concentration of 15-20 μ M [218] and sunitinib at a steady state concentration of ~100 nM [212], while axitinib was kept to a plasma

concentration of 100-400 nM for RCC treatment [68, 215]. Because effective concentrations of the drugs found in the 3D co-culture system are similar to plasma concentrations used in patients, the 3D co-culture may serve as a useful predictor of *in vivo* drug efficacy.

The 3D co-culture system has successfully demonstrated differences in three multi-tyrosine receptor kinase inhibitors. Sorafenib functions as an anti-angiogenesis and anti-liver cancer drug, simultaneously preventing vascularization and killing liver cancer cells. Sunitinib serves as an anti-angiogenesis drug at low concentrations, triggering apoptotic cell death of ECs, while killing liver cancer cells at high dosages. Axitinib acts as an anti-angiogenic drug, effectively reducing vascular networks, but is unable to kill liver cancer cells. There is a need to discover drugs that can both simultaneously inhibit network formations and kill cancer cells for effective therapeutic treatment of liver cancer [10], and the 3D co-culture model allows for efficient pursuit of such therapeutic agents.

Here, biochemical properties were compared between the xenograft, 2D co-culture and 3D co-culture. Western blot was utilized to compare protein levels in cell lysate of 2D and 3D co-culture, however the contribution of each cell line to the protein measured cannot be determined. As the ratio of HUVEC cells in the 3D co-culture (1:10 HepG2:HUVEC) were less than that in the 2D co-culture (1:2 HepG2:HUVEC), an increase in the protein levels of the 3D co-culture compared to the 2D co-culture could be visualized as an increase in signaling proteins from liver cancer cells. Immunocytochemistry would be a more relevant method to determining the contribution of individual cell line. The 3D model is a useful *in vitro* system for drug screening. However, further investigations on the biochemical properties of the cell system would enhance the understanding and suitability for its applications.

Here, a short testing duration and other limitations may exist in the current system for drug screening, such as the poor metabolic capacity of P450 enzymes in the HepG2 cells. Other cell lines can be added into the 3D spheroidal system, to build a much more representative liver cancer tumor. However, here, only the interplay between liver cancer and ECs are studied to reflect tumor angiogenesis as the primary study subject.

A limitation exists in the current model, wherein vessels formed are not stabilized, and the networks would decline and diminish after three days. This is due to the absence of pericytes, such as fibroblast and VSMC, to support differentiated ECs. Similar to the 2D co-culture system [204] and the Matrigel assay, tubular networks are disrupted and starts to regress after culturing for a period of time. Example periods of time are five days in the 2D co-culture system, three days in the 3D co-culture and a day in the Matrigel assay. Fibroblasts have been reported as a major component of the cancer stroma, and are important in cancer angiogenesis, invasion and metastasis. In the future, a combination of fibroblasts within the 3D co-culture could be used to stabilize the vascular network structures and better mimic the *in vivo* environment. The combined use of tumor and stromal cells in 3D would be especially useful for future drug discovery applications.

CHAPTER 5 Autophagy in Metastatic Breast Cancer Cell Lines

5.1 Introduction

In this thesis, another important component of metastasis was studied. Autophagy, as an adaptation to adverse conditions in the tumor microenvironment was studied with regards to metastatic breast cancer and its dissemination of distant metastases. During metastasis, tumor cells face unfavorable environmental stress while travelling within the blood circulation and upon arriving at a new site. Stresses such as fluidic shear stress (within the blood circulatory system), lack of cell/ECM attachment (anoikis), and nutrient deprivation (after extravasation and arriving at a new microenvironment without sufficient nutrients and growth factor) can trigger autophagy. To overcome these stresses, cells undergo autophagy to remove damaged organelles, and counteract the oxidative damage experienced during circulation. Autophagy also assists cells to scavenge for energy and promote their survival when cells do not receive sufficient nutrient supply from the surrounding environment after arriving at distant sites and preparing for colonization.

Our group has previously injected metastatic breast cancer MDA-MB-231 cells expressing the C3 sensor (231-C3) through the tail vein of a mouse model and isolated a lung micrometastasis (231-M1). 231-M1 cells were re-injected into the tail vein of a mouse model and a lung macrometastasis (231-M1A) was isolated (Figure 5-1). All three cell lines, 231-C3, 231-M1 and 231-M1A, were originally derived from the triple negative breast cancer MDA-MB-231 cell line, yet they exhibited different metastatic capabilities [219]. 231-M1A was found to be much aggressive and metastatic, forming large macrometastases *in vivo*, followed by 231-M1 which formed micrometastasis and subsequently 231-C3. Furthermore, 231-M1A was found to be less apoptotic and more

resistant to fluidic shear stress under circulation due to higher MnSOD levels than 231-M1 and 231-C3 [219]. The association between MnSOD and the autophagic capabilities of these three cell lines were further explored. The autophagic, antioxidant and metastatic capabilities were studied between these three cell lines.

Figure 5-1. Schematic diagram of the steps to generate 231-C3 cells with increased metastatic ability to form metastases in nude mice. Figure modified and adapted from reference [219] with permission from the author, Dr. Fu Afu.

5.2 Objectives and Significance of Research

5.2.1 Objective 1: Understanding Autophagic Capabilities of Metastatic Cells

During metastasis, cells travel through the bloodstream and are exposed to various forms of stresses. In order to survive, cells undergo autophagy as a survival adaptation mechanism. Cells with stronger autophagic capabilities would most probably survive and colonize in distant organs, forming metastases. Our group has previously isolated a series of MDA-MB-231 triple negative breast cancer cells with enhanced metastatic potential from metastases derived from mice's lungs. To understand the correlation of autophagy and metastasis, these cells were studied to understand their autophagic capabilities.

5.2.2 Objective 2: Characterize Vacuoles Visualized under Serum Starvation

While performing starvation experiments to induce autophagy, large abnormal vacuoles were observed in the metastatic breast cancer cells. The vacuoles observed in these cells were larger in size ($>2\ \mu\text{m}$) compared to typical autophagic vacuoles with diameters of $0.5\ \mu\text{m} - 1.5\ \mu\text{m}$ [220]. The vacuoles were studied in detail in order to understand their functions in autophagy.

5.2.3 Objective 3: Identify Role of MnSOD in Autophagy

Upon characterizing the vacuoles, I found that they might be related to mitophagy (the selective degradation of mitochondria by autophagy). As our group has previously shown that MnSOD is upregulated in the more metastatic cell lines, the MnSOD protein,

which is present in the mitochondria, was studied to understand its role in ROS generation, the formation of vacuoles and autophagy.

5.3 Results

5.3.1 231-M1A, 231-M1B Displayed Large Vacuoles with High Autophagy Levels Followed by 231-M1 and 231-C3

When the 231-C3, 231-M1, 231-M1A and 231-M1B were cultured in 10% serum medium for 4 days without any change of medium, large vacuoles could be visualized in 231-M1A and 231-M1B. 231-M1 formed smaller vacuoles compared to 231-M1A and 231-M1B, while no vacuoles were observed in the 231-C3 cells (Figure 5-2). These vacuoles may be a consequence of the autophagy process, or self-eating of the cell, when cells are under stress or facing nutrient deprivation. To confirm that cells were experiencing autophagy, western blot analysis was performed to check the expression

Figure 5-2. 231-M1A and 231-M1B displayed large vacuoles and high autophagy levels under starvation. (A) After the 231-series were cultured in 10% serum medium for 4 days without changing medium, vacuoles (white arrows) were observed in 231-M1A and 231-M1B followed by 231-M1. (B) The percentages of cells with vacuoles are counted. (C, D) Western blots of autophagy marker LC3B after culturing for 1 day and 4 days respectively.

of autophagy markers, LC3B-II. LC3B-II, the membrane protein on autophagosomes, was upregulated when 231-M1A and 231-M1B cells were cultured for 4 days without any medium change (Figure 5-2). This indicated that 231-M1A and 231-M1B cells, which had larger vacuoles, were also experiencing higher autophagy levels compared with 231-M1, and 231-C3. As 231-M1A and 231-M1B had similar metastatic capabilities and were derived from the same parental cell line, 231-M1, subsequent experiments were performed with 231-M1A.

5.3.2 Starvation of 231-Series under Different Conditions

To identify the conditions that had triggered the vacuoles formation, different starvation methods was tested to induce autophagy. At 48 h, no serum medium triggered large vacuoles formation in the 231-series, while EBSS did not (Figure 5-3). Even when the cells were cultured for longer periods under EBSS conditions, no formation of vacuoles was visualized. The vacuoles were formed due to a lack of growth factors in the serum-free medium. However, at 48 h, all 231-series were forming large vacuoles of similar sizes (Figure 5-3). A quick analysis of western blot revealed no differences in terms of autophagosome marker, LC3B-II, at 48 h after starvation with serum-free medium. Therefore, the next step was to determine an optimal time point that can reveal the differences in vacuole formation and their autophagic potential.

Figure 5-3. Only serum free medium and not EBSS induced in the formation of large vacuoles in the 231-series. (A) Cells were starved in EBSS or serum free medium for 48 h. No formation of vacuoles were observed in EBSS. (B) Western blot analysis of 231-series at 48 h after serum free starvation.

A series of time points were performed and an optimal time point was determined at 30 h after starvation with serum-free medium (Figure 5-4A). When 231-M1A cells were cultured in serum-free medium for 30 h, the formation of outsized vacuoles were observed, while 231-C3 cells did not form any autophagic vacuoles yet (Figure 5-4A). More vacuoles were again visualized in 231-M1 and 231-M1A compared to 231-C3,

with vacuoles observed in 231-M1A greater in diameter than 231-M1 cells (Figure 5-4). Autophagy levels were measured by western blots and the conversion of LC3B-II was greater for 231-M1A, followed by 231-M1 and then 231-C3. Lack of growth factors was determined to be the contributing factor to autophagy and the formation of vacuoles, and 30 h post starvation with serum-free medium was determined as the optimal time point to reveal the differential autophagic capabilities of the 231-series.

Figure 5-4. Optimizing the starvation period to differentiate the autophagic abilities in the 231-series. Cells were starved for 30 h under no serum medium conditions before collecting for western blot analysis. (A) 231-C3 formed the least number of vacuoles, followed by a large percentage of small vacuoles in 231-M1 and large vacuoles in 231-M1A. (B) Percentage of cells with autophagic vacuoles are counted from five independent experiments, data are means $\pm$ SEM, *** $p < 0.005$ vs 231-C3. (C) Western blot analysis of autophagy markers at 30 h, and (D) 48 h after starvation.

5.3.3 Vacuoles or Autophagic Vacuoles?

The appearance of the large vacuoles may not be associated with autophagy as autophagosomes are generally small and between 0.5 – 1.5 μm [220]. Therefore, to confirm that the vacuoles were indeed induced by autophagy, the protein expression of an autophagy related protein (ATG7), which is responsible for the formation of autophagosomes, was silenced. Silencing of the key autophagy protein ATG7 prevents the conversion of LC3-I into LC3-II and therefore, the formation of autophagosomes.

Figure 5-5. Silencing of ATG7 in 231-M1A cells reduced autophagosome formation. (A) Less autophagic vacuoles are visualized in cells with knockdown of ATG7. The vacuoles also appeared to be smaller in size. (B) Quantification of autophagic vacuoles in siNeg and siATG7 cells. Percentage of cells with autophagic vacuoles were counted from three independent experiments and data are means $\pm$ SEM. (C) Western blot analysis of ATG7 and LC3B-II at 72 h post-transfection and 30 h after starvation. (D) Normalized ratio of LC3B-II/ β -actin.

An ATG7 specific siRNA (siATG7) and a negative control of scrambled siRNA (siNeg) was transfected into 231-M1A before serum-free starvation was performed. Silencing ATG7 expression significantly reduced the formation of large vacuoles, with a marked decrease in LC3B-II expression levels (Figure 5-5). These results confirmed that the vacuoles are of autophagic origins and are formed when autophagy is induced.

5.3.4 Identify Stage which Autophagic Vacuole Represents in the Autophagy Process

To identify which step the autophagic vacuoles represent in the autophagy process, various inhibitors targeting different stages of autophagy were used. Utilizing autophagy inhibitors at different stages of autophagy affected the formation of autophagic vacuoles. PI3K inhibitors were used to inhibit the initial step of autophagy, where PI3K is essential for the sequestration of autophagic cargo and the formation of autophagosomes. When LY294002 and wortmannin were used in serum free conditions, formation of autophagic vacuoles were decreased to 1% and 20% respectively (Figure 5-6A and B). By inhibiting the sequestration process in autophagy, the next steps of autophagy were inhibited, therefore reducing the formation of the large autophagic vacuoles.

Conversely, inhibition of the last steps of autophagy with lysosomal degradation inhibitors, CQ and Baf-A1, increased the formation of autophagic vacuoles from 16% to 51% (Figure 5-6C and D). CQ and Baf-A1 inhibits degradation of autophagosomes, as fusion of lysosome to the autophagosome is prevented. Inhibition of autophagosomal degradation leads to an increase in the vacuole formation as autophagosomes are continuously deposited into the large vacuoles without degradation of the cargo.

Figure 5-6. Starvation with autophagy inhibitors affected the formation of vacuoles. (A) 231-M1A cells were incubated with LY294002 (10 μ M) or wortmannin (20 nM) with starvation medium for 30 h. (B) Percentage of cells with autophagic vacuoles were counted from three independent experiments and data are means $\pm$ SD. * $p < 0.05$ and *** $p < 0.001$ vs control. (C) 231-M1A cells were incubated with CQ (10 μ M) or Baf-A1 (200 nM) with starvation medium for 27 h. (D) Percentage of cells with autophagic vacuoles were counted from three independent experiments and data are means $\pm$ SD. *** $p < 0.001$ vs control.

5.3.5 Dissection of Autophagic Vacuoles

To identify the localization of the autophagic vacuoles, LC3B immunostaining for autophagosomes was performed on 231-M1A cells at different time points. The LC3B antibody stains both the pre-activated form of LC3B-I localized throughout the

cytoplasm and the autophagosome marker, LC3B-II. Upon conversion of LC3B-I into LC3B-II, LC3B-II is integrated into the lipid membrane of autophagosomes, creating puncta-like appearances under microscopy images due to the small sizes of autophagosomes.

Figure 5-7. Autophagic vacuoles are marked by LC3B staining and upregulation. (A) LC3B staining (red) of 231-M1A cells (green) at 0, 24 and 48 h after starvation. White arrows point to LC3B staining visualized as punctate or surrounding the rims of autophagic vacuoles. The nucleus is stained blue. (B) Western blot analysis of LC3B at different time points after starvation.

At 0 h, no LC3B puncta formation were detected, with only a light background staining of LC3B visualized. Autophagosome formation could be visualized by LC3B puncta at 24 h, with the LC3B punctate co-localizing in vacuoles, which displayed an absence of GFP signal (Figure 5-7). By 48 h, the vacuoles have enlarged to ~10 μm , with LC3B staining observed at the rim of the vacuoles. Smaller punctate stained with LC3B are also visualized around the large vacuoles. These results coincide with the LC3B western blot analysis, where LC3B-II elevated at 24 h and even higher at 48 h. The large vacuoles observed marked for autophagosome membrane marker LC3B-II, and also stained for smaller autophagosomes. Although large vacuoles are generated when autophagy is induced, they do not represent typical autophagosomes of 0.5 – 1.5 μm .

While performing immunostaining of the autophagic vacuoles, there were noticeable small stained LC3B puncta marked within the vacuoles (Figure 5-8A). Enlarged images of the vacuoles showed small puncta (Figure 5-8B) within the vacuoles, and electron microscopic examination of the autophagic vacuoles from 100 nm thick cross sections revealed that autophagosomes were found within the large autophagic vacuoles. The vacuoles had sizes ranging between 2 to 10 μm (white arrowheads in Figure 5-8C), with autophagosomes (black arrows in middle right pane) located near the vacuoles. Furthermore, within the large vacuoles, double- membraned autophagosomes could be visualized (bottom panes, Figure 5-8). There exists the possibility that multiple autophagosomes were engulfed into an expanding vacuole compartment within the 231-series, generating a large abnormal autophagic vacuole. The vacuoles signified stronger autophagic capabilities within the cell, which may be a survival mechanism triggered under stressful conditions such as nutrients deprivation.

Figure 5-8. Autophagosomes and other substances were visualized in the large vacuoles.

(A) Staining of the LC3B marker displayed small stained puncta within the autophagic vacuoles. (B) Enlarged images of boxes 1, 2 and 3 from (A). White arrows point to the LC3B puncta within the autophagic vacuoles. (C) TEM images of normal and starved cells. White arrowheads point to autophagic vacuoles. Black arrows point to autolysosomes. Black arrowheads point to autophagosomes and proteins/organelles within the vacuoles. Red arrowhead points to double membrane of autophagosome within the large vacuole.

Another autophagosomal marker, p62 was stained to check the localization of autophagosomes relative to the vacuoles. p62 acts as a specific substrate in autophagy, where it brings ubiquitinated proteins to the autophagosome. It binds to LC3B to facilitate specific cargo degradation and is localized within autophagosomes, and may act as a marker for autophagosomes. Therefore, p62 immunostaining and western blot was performed to check for the localization of autophagosomes. A mild background stain of p62 was observed in 231-series under normal conditions; however, p62 proteins were localized in small vacuoles without GFP proteins when starved after 24 h (Figure 5-9, white arrows). The small vacuoles without GFP proteins were autophagosomes. In addition, p62 staining was most intense in 231-M1A cells (Figure 5-9). This indicated that the onset of autophagy and the formation of autophagosomes were induced much rapidly in 231-M1A cells, followed by 231-M1 and 231-C3. Furthermore, p62 autophagosomal proteins was found within the vacuoles at the onset of autophagy, indicating the vacuoles contains autophagosomal cargo proteins within.

Figure 5-9. The p62 substrate marks autophagic vacuoles, where p62 is accumulated in autophagosomes during autophagy. 231-C3, 231-M1 and 231-M1A were starved for 24 h before staining with p62 antibody (red) and Hoechst 33342 (blue). All cells contained the FRET sensor (green) in the cytoplasm. White arrows point to autophagosomes with the co-localization of p62 antibody and vacuoles without the FRET sensor.

Lysotracker and mitotracker staining was performed to locate the presence of lysosomes and mitochondria in the starved cell. Interestingly, it was possible to capture moving FRET proteins present within the large vacuoles of live cells (Figure 5-10).

Furthermore, the mitochondria was also present within the vacuole (Figure 5-10B), and may have fused into the large vacuole during starvation (yellow arrow). The mitochondria may have been sequestered by autophagosomes (mitophagy), and brought into the vacuoles before degradation of the organelle. LysoTracker staining also revealed that lysosomes were surrounding the large autophagic vacuoles. They contain digestive enzymes that can fuse with the large vacuoles to digest proteins within.

Figure 5-10. Cytoplasmic proteins and the mitochondria are present in the vacuoles. Cells were stained with (A) 500 nM of LysoTracker® Red or (B) 500 nM of MitoTracker® Red after starvation for 48 h. Within the autophagic vacuoles, cytoplasmic proteins containing the FRET C3 sensor (green, white arrows) could be visualized. Lysosomes were located outside the large vacuoles or found in smaller vacuoles. Mitochondria were also present in the vacuole (red arrow).

A possibility may be inferred from the above results. Proteins may be sequestered or transferred into the large autophagic vacuoles before fusion with lysosomes for degradation, explaining the presence of LC3B staining surrounding the large vacuole (Figure 5-7), the presence of autophagosomes, cytoplasmic proteins and organelles within the large vacuoles (Figure 5-8 and Figure 5-10), and also the existence of lysosomes around the large vacuoles. The proteins and organelles were not yet degraded within the vacuoles, as the FRET sensor was still functional, and intact mitochondria were stained by mitotracker, which was dependent on the presence of a positive mitochondria membrane potential. Therefore, the autophagosome may have fused with

into the large vacuole. Autophagosomal fusion with vacuoles (MVBs) to form amphisomes have been reported in many studies [221], where MVBs play an important role in the autophagy pathway, and facilitate the fusion of lysosomes and amphisomes (mentioned in chapter 1.5.3). The large autophagic vacuole observed during nutrient deprivation may be an amphisome after fusing with autophagosomes.

5.3.6 Measuring Autophagy Flux for Complete Autophagy Process

In the starvation assay with 231-series, a possibility exist that autophagy is dysfunctional where lysosomes are unable to fuse with the autophagosomes resulting in the accumulation of p62 proteins and appearance of autophagic vacuoles, therefore autophagic flux was measured to check for the completion of the autophagy process.

The autophagy flux or the autophagic degradation activity in a cell is important to ensure that the whole autophagy process is complete, as autophagy is a multistep process. The mere detection of the number of autophagosomes and the presence of LC3B is insufficient to evaluate the entire autophagy system. In any autophagy assay, an increase in LC3B-II levels may represent an increase in autophagosome or an impairment in the downstream degradation process. It is therefore important to measure autophagic flux to understand whether the entire autophagy process is functional. A method to measure autophagic flux is through the measurement of LC3 turnover rates and p62 degradation levels (Chapter 1.5.5).

The level of LC3B-II was measured in the presence and absence of a lysosomal inhibitor, CQ under starvation conditions. When autophagy is induced, LC3B-II levels will increase due to the rapid conversion of LC3B-I into LC3B-II to form autophagosomes. In the presence of CQ, fusion of lysosomes with autophagosomes is inhibited, thereby preventing the degradation of LC3B-II proteins through the

autophagic pathway and leading to its accumulation. However, if autophagy was dysfunctional in the first place, LC3B-II would not accumulate in the presence of CQ.

First, the basal autophagy levels under normal conditions with fresh medium was compared by adding CQ for 3 h before collecting the cells. The levels of both LC3B-II and p62 cargo proteins increased in the presence of CQ, as the transition of both proteins through the autophagic pathway was blocked, whereas both LC3B-II and p62 protein levels are lower (representing normal autophagic flux) during the absence of a lysosomal inhibitor, (Figure 5-11A). Furthermore, at basal levels, autophagic flux occurred strongest in 231-M1A, with both proteins upregulated the strongest in the presence of CQ, followed by 231-M1 and 231- C3 (Figure 5-11A). Under basal levels, the autophagic capability of 231-M1A was strongest, with the highest autophagic flux, followed by 231-M1 and finally 231-C3.

Figure 5-11. LC3B turnover assay for measuring autophagy flux. (A) Basal autophagic levels in 231-C3, 231-M1 and 231-M1A. Addition of lysosomal degradation inhibitor, CQ, for 3 h result in accumulation of LC3B and p62 proteins. 231-M1A shows strongest autophagic

flux. (B) CQ leads to the accumulation of LC3B-II and p62 levels, demonstrating normal autophagic flux under starvation conditions.

Similar measurements on autophagic flux was also performed when cells were starved in serum-free medium for 48 h. The levels of LC3B-II and p62 increased in the presence of CQ, indicating normal autophagic flux during starvation (Figure 5-11B). The results indicated that the appearance of the autophagic vacuoles during starvation was due to the induction of autophagy and not because of an impairment in the degradation process.

Another measurement of autophagic flux is through the degradation of selective substrates such as p62 proteins. During autophagy, p62 protein acts as an adaptor protein to bring ubiquitinated proteins into the autophagosome. The ubiquitinated proteins are then degraded within autolysosomes and released as reusable macromolecules into the cytosol. With the onset of autophagy, p62 levels should decrease during the process, while an accumulation of p62 would represent a dysfunction or an impairment in the autophagy process.

However, normal autophagic flux has already been demonstrated by the upregulation of LC3B-II levels in the presence of CQ during starvation (Figure 5-11B). During prolonged starvation, a feedback loop may be triggered to enhance the synthesis of p62 protein levels to bring p62 levels back to basal levels [222]. Henceforth, the upregulation of p62 may be due to an increase in p62 mRNA levels and not due to an impairment in the autophagy process. Thus, PCR was conducted to measure the mRNA levels of p62. Figure 5-12 shows the upregulation of p62 mRNA levels during starvation at 24 h and 48 h, while a mild decrease in the β -actin mRNA levels. The upregulation of p62 mRNA

levels may have contributed to the synthesis of p62 proteins, therefore contributing to an accumulation of p62 proteins observed during starvation.

Figure 5-12. p62 protein levels are accumulated during starvation. (A) Western blot analysis at different time points of starvation with 231-C3, 231-M1 and 231-M1A. p62 levels increased strongest for 231-M1A. (B) p62 mRNA levels in 231-M1A cells at 24 h and 48 h.

5.3.7 *In Vivo* Autophagy Rates Correlates with Metastatic Capabilities of Tumor

As autophagy is a method for cells to adapt to various stresses during the metastasis process, more metastatic cells would most probably possess higher autophagic levels compared to less metastatic cells. The autophagy levels of C3-derived metastases and M1-derived metastases were measured to check the correlation of metastasis to autophagy levels. Immunohistochemistry (IHC) of both LC3B and p62 protein levels were performed on the C3-derived metastases and M1-derived metastases. A comparison of the LC3B levels showed higher numbers of LC3B positive puncta in M1-derived metastases than C3-derived metastases (Figure 5-13). M1-derived metastases

were undergoing autophagy much stronger than the C3-derived metastases as visualized by the brown dot like punta stain.

Figure 5-13. 231-M1-derived metastasis revealed cells undergoing autophagy compared to 231-C3-derived metastasis. IHC staining for LC3B autophagosome marker in 231-C3-derived metastasis (left) and 231-M1-derived metastasis (right). More positive puncta (brown dot-like staining) marking for autophagosomes are observed in the 231-M1-derived metastases.

The p62 intensities were also stronger in the M1-derived metastases than the C3-derived metastases. Furthermore, immune cells marked by the smaller nuclear stains was visualized around the M1-derived metastasis (Figure 5-14). This may be due to the protective role of p62 in inducing antioxidant response by targeting the transcription factor NRF2 and NF- κ B to activate downstream pro-inflammatory responses, thus directing the immune cells to the tumor site. Under *in vivo* conditions, stronger autophagy levels were also induced in the more metastatic tumor, M1-derived metastasis compared to the less metastatic tumor, C3-derived metastasis. This correlates well with *in vitro* analyses of LC3B and p62 western blot. LC3B-II was upregulated with more autophagosome formation in 231-M1A cells, followed by 231-M1 cells and

finally 231-C3 cells, and a higher accumulation of p62 protein in 231-M1A cells, followed by 231-M1 cells and finally 231-C3 cells.

Figure 5-14. 231-M1-derived metastasis (right) displayed higher p62 protein levels compared to 231-C3-derived metastasis (left). (A) Tumor tissue with IHC analysis of p62 protein in 231-C3 and 231-M1-derived metastases. More positive puncta (brown dot-like staining) is observed in the 231-M1-derived metastasis. (B) Enlarged image of box up area in (A). 231-M1-derived metastasis appears to be infiltrated by immune cells.

5.3.8 Cells with Stronger Autophagic Capabilities have Reduced Apoptotic Rates

Both apoptosis and autophagy share common pathways and cells may switch between apoptosis and autophagy under stress. Autophagy is a stress adaptation mechanism, which allows cells to avoid cell death by apoptosis. Cells with stronger autophagy abilities are able to adapt to stresses and escape programmed cell death (apoptosis) under adverse conditions. Here, to understand the function of autophagy in cell survival, apoptotic rates of the 231-series were measured under starvation. Both 231-M1 and 231-M1A cells displayed apoptotic rates of 19% and 22% respectively, while 231-C3 cells showed a significant increase in apoptotic rates of 33% (Figure 5-15B). Here, both apoptosis and autophagy are triggered by starvation. As 231-C3 has the lowest autophagic levels, some cells are unable to adapt to the nutrient deprivation and underwent apoptosis. In contrast, 231-M1A has the strongest autophagic levels,

Figure 5-15. More metastatic breast cancer cells revealed lower apoptotic rates compared to less metastatic cells under starvation. (A) 231-C3, 231-M1 and 231-M1A after starvation for 54 h with 0% serum medium. Apoptotic cells are blue, while live cells are green. (B) The percentage of apoptotic cells were counted from four independent experiments and data represent means $\pm$ SD. * $p < 0.05$ vs 231-C3 cells.

therefore, more metastatic cells can undergo autophagy and escape the programmed cell death mechanism. More metastatic breast cancer cells have stronger autophagic capabilities for stress adaptation, and thus escape cell death more easily than less metastatic breast cancer cells.

5.3.9 Autophagic Vacuoles are Influenced by the Presence of Antioxidants During Starvation

Our laboratory has previously reported an upregulation of the mitochondrial MnSOD protein levels in the metastases-derived cell lines of 231-M1 and 231-M1A compared to the parental 231-C3 [219]. During nutrient starvation, ROS is induced and causes oxidative damage to the mitochondria. H_2O_2 is an important mediator in autophagy [145]. The high levels of MnSOD proteins present in 231-M1A may have converted O_2^- into H_2O_2 , thereby triggering autophagy in response to high ROS levels and damaged mitochondria. Mitotracker staining of the mitochondria in Figure 5-10 showed that the mitochondria might have been absorbed into large vacuoles or the presence of mitophagy.

Experiments were conducted to check if ROS was modulating the formation of vacuoles. First, antioxidants were added to determine if ROS was present and modifying the autophagic behaviors of 231-M1A cells. Antioxidant NAC, which scavenges all types of ROS (O_2^- and H_2O_2) successfully reduced the vacuoles from 41% to 21%, while PG, which catalyze the conversion of O_2^- to H_2O_2 , did not prevent vacuole formation, but resulted in more vacuole formation. 50% of 231-M1A cells formed vacuoles after the addition of PG during starvation (Figure 5-16). The formation of vacuoles appeared to be modulated by antioxidants where an increase conversion of H_2O_2 induces more vacuole formation, and reduce formation when O_2^- is removed from the system.

Figure 5-16. Antioxidants NAC and PG resulted in different vacuole formation. (A) 2 mM of NAC or 20 μM of PG was added to the starvation medium before incubated with 231-M1A cells for 30 h. (B) The percentages of cells with vacuoles were counted from three independent experiments. Data represent means $\pm$ SD, * $p < 0.05$ vs control.

MnSOD is the key enzyme to catalyze the conversion of O_2^- to H_2O_2 within the mitochondria. As previous studies have already shown an upregulation of MnSOD levels in the more metastatic 231-M1A compared to 231-M1 and 231-C3 [219], the possibility that rapid conversion of O_2^- to H_2O_2 was generating excessive H_2O_2 , which triggered the formation of vacuoles. Inhibitor (2-ME) and mimetic (MnTBAP) of MnSOD were added into the starvation medium to study the involvement of MnSOD in the formation of autophagic vacuoles during starvation. 2-ME successfully inhibited autophagic vacuoles, while MnTBAP resulted in the formation of more autophagic vacuoles after 30 h of starvation, compared to control (Figure 5-17A and B). Inhibition of the MnSOD function resulted in cells appearing more rounded and decreased the formation of autophagic vacuoles, while enhanced MnSOD function with MnTBAP led to the formation of more autophagic vacuoles, from 40% to 52% (Figure 5-17). Furthermore, western blot analysis revealed that LC3B-II levels were reduced when 2-

ME was added to the starvation medium, indicating a reduction in autophagy (Figure 5-17C). Conversely, when 231-M1A cells were treated with MnTBAP, an increase in LC3B-II levels was observed. These results indicated that MnSOD played a role in the formation of vacuoles and regulated autophagy during starvation.

Figure 5-17. Inhibiting or simulating MnSOD protein in 231-M1A cells leads to variations in vacuole formation during starvation. (A) Addition of 20 μM of MnSOD inhibitor, 2-ME, or 100 μM of MnTBAP, which simulates the function of MnSOD for 30 h during starvation results in reduction or enhanced vacuole formation respectively. (B) The percentages of cells with vacuoles were counted from three independent experiments. Data represent means $\pm$ SD, * $p < 0.05$, ** $p < 0.01$ vs control. (C) Western blot analysis of LC3B markers upon starvation with 2-ME or MnTBAP.

To confirm the role of MnSOD in vacuole formation during starvation, two MnSOD-specific siRNA (siMnSOD-1 and siMnSOD-2) were used to repress its protein expression in 231-M1A cells. Silencing MnSOD expression significantly decreased vacuole formation from 41% in control siNeg to 10% in siMnSOD-1 and negligible formation of vacuoles observed in siMnSOD-2 (Figure 5-18A), similar to the MnSOD inhibitor 2-ME. Western blot analysis showed a decrease in LC3B-II levels when 2-ME or siMnSOD-1 were employed to inhibit MnSOD (Figure 5-17C and Figure 5-18C).

MnSOD was important in modulating the formation of vacuoles, and removal of the protein decreased or totally inhibited the formation of vacuoles.

Figure 5-18. Inhibiting MnSOD protein in 231-M1A cells via MnSOD-specific siRNAs leads to a decrease in vacuole formation during starvation. (A) Silencing MnSOD using two specific siRNA (siMnSOD-1 and si-MnSOD2) reduced the vacuole formation in 231-M1A cells. (B) The percentages of cells with vacuoles were counted from two independent experiments. Data represent means $\pm$ SD, *** $p < 0.001$ vs siNeg. (C) Western blot analysis of MnSOD and LC3B markers upon starvation with 2-ME siNeg or siMnSOD-1.

When both antioxidant NAC and PG were used, NAC could effectively reduce vacuole formation as NAC assisted in the removal of H_2O_2 . On the other hand, PG functions as a MnSOD mimic, and dismutates O_2^- to form H_2O_2 molecules [223]. The accumulated H_2O_2 would therefore enhance the formation of vacuoles during autophagy. MnSOD and MnSOD mimics (PG, MnTBAP) resulted in the formation of vacuoles during autophagy via conversion of H_2O_2 molecules from O_2^- radicals. Whereas, removal of H_2O_2 by NAC, or suppressing MnSOD with siRNA or its inhibitor (2-ME) to inhibit H_2O_2 formation, resulted in a decrease in the formation of vacuoles. H_2O_2

appears to act as an intermediary signaling molecule for the formation of vacuoles and autophagy.

5.4 Discussion

Enhanced autophagic capabilities were observed in the 231-series, with incremental autophagic capabilities from the parental 231-C3 cells to the more metastatic daughter cell lines 231-M1 and 231-M1A. The enhanced autophagic capabilities were displayed during basal level and under starvation. This was also observed *in vivo*, in the isolated lung metastases derived from 231-C3 and 231-M1. M1-derived metastasis displayed higher autophagy levels *in vivo*, compared to the C3-derived metastasis from IHC analysis. These results revealed that more metastatic cells undergo higher autophagy levels in contrast to less metastatic cells both *in vitro* and *in vivo*.

Another phenomenon that have not been observed in other cell types was observed here: the formation of large vacuoles signifying autophagy (self-eating). A series of experiments were performed to verify the role of vacuoles in autophagy, and proteins and mitochondria were found in the vacuoles. Autophagosomes were found within the vacuoles, which may be due to the fusion of the vacuoles and autophagosome. The vacuoles may be of endosomal origins, however further studies have to be conducted to verify its origins. Multivesicular bodies (MVBs) are a kind of late endosome which are involved in sorting of proteins into the MVB to generate intraluminal vesicles [117]. The MVBs may then go on to fuse with an autophagosome to form an amphisome before fusion with lysosomes. MVBs have a close relationship with autophagy and studies have revealed the coordinated action of the MVB pathway and autophagy [224]. The cytoplasmic proteins, mitochondria and autophagosomes identified within the vacuole are evidence of the possibility of the large vacuole functioning as an amphisome. The vacuoles appeared to contain mitochondria, and this may be due to mitophagy. Mitophagy occurs when defective mitochondria are sequestered by autophagosomes through the autophagy pathway [225]. The mitochondria could have been sorted into

the vacuole after sequestration by autophagosomes, therefore, both autophagosomes (Figure 5-8), proteins and the mitochondria (Figure 5-10) were localized within the vacuoles. The vacuoles would most probably continue to fuse with lysosomes (surrounding the vacuoles, Figure 5-10A) to degrade the cargo within, before releasing degraded macromolecules back into the cell. Time-lapse microscopy could be taken to capture the whole autophagy process. More evidences are needed to characterize the vacuoles, and to identify the role of the vacuoles or the existence of mitophagy in the 231-series.

Knockdown of ATG7 or PI3K class I inhibitors (wortmannin and LY294002) prevented the first steps of autophagy, which is the sequestration of proteins into a phagophore. Both approaches displayed a decrease in vacuole formations, but did not completely inhibited the vacuole formation. Zhang *et al.* have previously reported that ATG7 regulated mitochondrial clearance, and could delay, but not completely inhibit of mitophagy [153]. A blockage to the formation of autophagosomes could reduce the number and size of the vacuoles as autophagosomes are unable to fuse with the vacuole, however they could not completely prevent the formation of the vacuole. In contrast, inhibition of lysosomal fusion to autophagosomes by the lysosomal inhibitors, CQ and Baf-A1, resulted in enhanced formation of the vacuole, as lysosomes are unable to fuse with the large vacuole to degrade molecules within. The vacuoles most probably represented autophagy at the autophagosomal stage, with mitochondrial clearance associated with it.

p62 proteins are accumulated due to synthesis of the protein at the onset of starvation. Although p62 protein serves as an important adaptor protein in autophagy, and is degraded during autophagy, the accumulation of p62 protein represents another survival mechanism triggered by the cell here. 231-M1A has the strongest p62 accumulation,

and this is also observed in the M1-derived metastases compared to C3-derived metastases. p62 may function as an indirect activator Nrf2 transcription gene, triggering a battery of detoxifying and antioxidant genes in response to oxidative stress. During the onset of starvation, oxidative levels are elevated immediately [134], therefore, p62 upregulation may function as a feedback response to induce the transcription of antioxidant genes. Thompson *et al.* have demonstrated that p62 is expressed in abundance in malignant breast tissue by p62 mRNA and protein level increase [226], which is also detected in the 231-series. Furthermore, many lines of evidences have shown that p62 is accumulated in metastatic cell lines [227, 228]. p62/SQSTM1 functions as a signaling hub for diverse cellular events, and its function is not only limited to autophagy. More data is needed to address its role in autophagy, tumor initiation, or oxidative protection of the 231-series.

As 231-M1A has the highest levels of MnSOD among the 231-series, the cell would be able to efficiently convert O_2^- to H_2O_2 , reducing apoptosis by preventing mitochondria damage. However, both catalase and glutathione, which catalyze H_2O_2 into water molecules, have similar protein expression levels in the 231-series [219]. This would cause an accumulation of H_2O_2 , after 231-M1A has successfully converted O_2^- into H_2O_2 molecules. H_2O_2 as a signaling molecule in the regulation of a variety of biological processes have been well documented [229-231]. The H_2O_2 molecule may have acted as a signaling molecule to trigger autophagy, resulting in the formation of the large vacuoles.

In summary, understanding the association of autophagy, mitophagy and oxidative levels may bring about new perspectives on the metastatic potential of breast cancer cells. Further studies have to be performed to validate the role of MnSOD in autophagy and metastasis.

CHAPTER 6 Conclusions and Future Directions

Cancer metastasis is an important contributor to cancer deaths worldwide. To understand the mechanisms involved in metastasis, I focused on angiogenesis and autophagy, two essential elements in the dissemination of cancer cells. Angiogenesis is the primary route for the spread of cancer cells to distant organs. Many angiogenesis inhibitors often target only ECs, disrupting angiogenesis; however, tumor cells employ multiple compensatory signaling pathways to establish transformed vasculatures or angiogenesis-independent vascular remodeling processes, allowing for the propagation of cancer. Furthermore, the destruction of blood vessels creates a hypoxic and acidosis environment, which encourages genomic alterations and metastatic capabilities in tumor cells. This further promotes the spread of tumor cells to distant organs when anti-angiogenesis agents are withdrawn after treatment. Therefore, potential effective strategies should aim to target cancer cells through disrupting the tumor microenvironment and the interactions between tumor and stromal cells.

Due to the hyper-vascularity of HCC, I employed HCC tumor cells to study create a HCC microenvironment with tumor angiogenesis. I revealed the importance of tumor contact-induced angiogenesis and the inherent characteristic of certain metastatic cell lines such as lung, kidney and liver to induce tumor angiogenesis. Tumor-endothelial interaction is a key aspect in the tumor microenvironment and play important roles in tumor angiogenesis. Here, I focus on the approaches in which tumor cells directly induce angiogenesis by cell-cell communication. This effectively targets cancer cells and ECs, preventing angiogenesis and disrupting the tumor microenvironment, eventually preventing the spread of distant metastases.

A co-culture model was developed to study the signaling pathways regulating tumor angiogenesis. Inhibitors targeting molecular signaling pathways effectively prevented tumor angiogenesis. Some of the signaling pathways have been identified to play important roles in tumor angiogenesis and small molecules targeting the cellular pathways are currently utilized in tumor therapies. Other signaling pathways identified such as the FAK pathway may play critical roles in both tumor pathogenesis and tumor angiogenesis. Future works involving Wnt/ β -catenin, integrins and Notch signaling pathways may be studied through the co-culture assay, which focus on the direct cell-cell communication of tumor-endothelial interactions. Furthermore, β -catenin is overexpressed in many HCC tumor cells, and may be important in initiating tumor angiogenesis.

I further developed this 2D co-culture assay into a 3D co-culture assay to recapitulate the *in vivo* HCC microenvironment. A hypoxic core with true gradient penetration of drugs and substances is achieved in the 3D model, and is more relevant as an *in vitro* model for studying the HCC microenvironment and for drug screening purposes. The 3D co-culture assay has demonstrated effective therapeutic concentration ranges of agents in inhibiting network formation and/or tumor growth. Dual screening of anti-angiogenesis and anti-tumor agents is reflected in a single assay. Furthermore, VDAs and AIAs can be identified in the 3D co-culture assay, where VDAs induce cell death of ECs, whereas AIAs only prevent endothelial tubule formation. This is useful for employing different vascular targeting agents in different stages of tumors, with AIAs used for small growing tumors establishing a tumor vasculature, and VDAs for large tumors with an already established vascular network.

Another essential element for metastasis was studied. Autophagy has been implicated in the survival of many cancer cells during adverse conditions such as nutrient starvation

and oxidative stress. I discovered the importance of autophagy in the progression of metastasis, and an enhanced autophagic capability was linked to more metastatic cells, where enhanced autophagic capabilities promoted cell survival by reducing apoptosis of more metastatic cell lines under nutrient starvation.

Here, I studied three cell lines derived from lung-metastases, with each progressively metastatic compared to the previous. High basal autophagic levels was observed in the most metastatic cell line, 231-M1A, followed by 231-M1, before the parental 231-C3 cell line. Large autophagic vacuoles were also identified in the three cell lines, signifying their autophagic capabilities. A large number of punctate LC3 was observed in the M1-derived metastasis, suggesting that tumor cells were also exhibiting high autophagic levels *in vivo*, compared to the C3-derived metastasis. More metastatic cells undergo higher autophagy levels in contrast to less metastatic cells both *in vitro* and *in vivo*.

In addition, the large vacuoles were studied. Enhanced expression of the LC3B-II marker signifying for autophagy was observed at the onset of the vacuoles. Knockdown of ATG genes necessarily for autophagy reduced the formation of the vacuoles, whereas treatment of cells with lysosomal inhibitors enhanced the formation of the vacuoles. The large autophagic vacuoles may be a new concept in autophagy, or they may also be a fusion with large endosomes or MVBs, where cytoplasmic proteins and autophagosomes are carried into vacuoles or amphisomes before degradation by the lysosome. Characterizing these vacuoles would assist in the study of the differential autophagic capabilities in the 231-series, and provide insights into the process of autophagy.

I found that the enhanced autophagic capabilities were associated to the upregulation of MnSOD protein in the 231-series. Inhibiting the function of MnSOD or silencing

MnSOD could prevent the formation of autophagic vacuoles. Preliminary studies suggest that ROS production mediated the formation of autophagic vacuoles, and MnSOD played a role in the vacuole formation observed in the 231-series. However, the role of MnSOD in autophagy is not completely understood. Further studies will have to be conducted targeting MnSOD and its role in the mitochondria to confirm its role in autophagy or mitophagy.

Finally, a strong accumulation of the p62 protein was observed in the most metastatic cell line 231-M1A. Clinically, high p62 levels have been associated with enhanced metastatic potential in many tumors including triple negative breast cancer (such as MDA-MB-231) [227, 228], and is also observed here in the 231-series. Future work can be conducted to determine the importance of p62 as a tumor initiator in terms of antioxidant defenses in metastatic cancer cells.

In conclusion, I have discovered new mechanisms in two aspects of metastasis: angiogenesis and autophagy. I explored new mechanisms crucial for cancer angiogenesis and identified an essential component that has been neglected in the field: tumor-endothelial contact-induced angiogenesis. I also revealed that more metastatic cells undergo higher autophagy levels in contrast to less metastatic cells both *in vitro* and *in vivo*, and identified an antioxidant protein, MnSOD as an important player in regulating autophagy. Autophagy in association with mitophagy and antioxidant capabilities in metastatic breast cancer cells was also studied. These new mechanisms would be valuable as therapeutic targets in both angiogenesis and autophagy.

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