The role of Angiopoietins-Tie2 functional axis in colorectal cancer liver metastasis (CRCLM)

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Abstract

Colorectal cancer (CRC) is the third leading cause of cancer among Canadians, with liver metastases (LM) being the major cause of death. A surgical resection is the only management approach resulting in 5-year overall survival (OS) for one third of the CRCLM patients. Currently, the combination of chemotherapy (chemo) with anti-angiogenic bevacizumab (anti-vascular endothelial growth factor, anti-VEGF, Bev) therapy is approved for use in the first-line treatment of CRCLM patients to improve resection rate and OS. However, the response to this treatment strategy is associated with the diversity of histopathological growth patterns (HGPs) within LM. Three HGPs have been identified: Desmoplastic (DHGP), Pushing (PHGP, uncommon) and Replacement (RHGP). In DHGP, the cancer cells utilize angiogenesis to obtain a vascular supply and respond to chemo plus Bev treatment. However, RHGP tumor cells co-opt pre-existing blood vessels and poorly respond to chemo plus Bev treatment.

Angiopoietins (Ang1 and Ang2) with their receptor Tie2 have been shown to support vessel co-option mechanism and mediate anti-angiogenic drug resistance in other cancer types.

The aim of the present study was to investigate the role of the vascular factors Ang1, Ang2 and Tie2 in regulating the vessel co-option that mediates anti-angiogenic drug resistance in CRCLM with RHGP lesion. To investigate this role, we characterized the vasculature of the DHGP and RHGP based on the expression of Ang1, Ang2 and Tie2 in human CRCLM lesion samples using immunohistochemistry, immunofluorescent and fluorescent in situ hybridization (FISH) methods. Our data showed, for the first time, that the expression of Ang1 was increased in hepatocytes adjacent to tumor cells in chemonaïve and treated (chemo and chemo plus Bev) human CRCLM with RHGP lesions that developed anti-angiogenic drug resistance.

To explore the role of the host Ang1 expression *in-vivo,* Ang1 knockout (KO) mice were injected intra-splenically with metastatic MC-38 colon cancer cells that develop RHGP tumors in control livers. We found a reduction in the metastatic burden, inhibition of mature blood vessel formation and development of angiogenic driven DHGP liver metastasis tumors that respond to treatment in the clinic. Thus, Ang1 deficiency inhibits liver metastasis and impacts RHGP formation *in-vivo.*

It has been reported that upregulation of Ang1-Tie2 signaling activates pathways that are involved in cancer cells survival, proliferation and metastasis. We speculated that the interaction between tumor cells and hepatocytes leads to increase in Ang1 expression in hepatocytes, which may be important for tumor growth and invasion. To study this, primary hepatocytes from adult control (Ang1 wild-type) and Ang1 KO mice were isolated using the two-step collagenase perfusion method. Our *in-vitro* data demonstrated that co-culturing of control primary hepatocytes and MC-38 colon cancer cells using insert significantly induced the expression of Ang1 in the hepatocytes. In addition, conditioned media obtained from control hepatocytes culture increased survival, migration and invasion of MC-38 colon cancer cells. This phenotype was abolished when Ang1 was knocked out in hepatocytes, suggesting that secreted Ang1 from hepatocytes plays a critical role in tumor progression.

Overall, our results provide evidence that high expression of Ang1 in RHGP lesions may maintain vessel co-option formation, tumor growth and metastases. Thus, investigating Ang1-Tie2 pathway as a potential contributor to vessel co-option survival and tumor progression in CRCLMs with RHGP lesions may be a rational next step for an effective therapy.

Résumé

Le cancer colorectal (CRC) est la troisième cause de cancer chez les Canadiens et les métastases hépatiques (LM) sont la principale cause de décès. De plus, pour seulement un tiers des patients la résection chirurgicale est la seule option permettant un survie moyenne jusqu'à cinq ans. Actuellement, la combinaison de chimiothérapie avec un traitement anti-angiogénique avec bevacizumab (anti-VEGF, Bev) est approuvée pour le traitement de première intention des patients atteints de CRCLM. Cela permettra d'améliorer le taux de résection. Cependant, ce traitement stratégique est associée à la diversité des profils de croissance histopathologiques (HGP) présent dans les LM. Trois HGP ont été identifiés: Desmoplastique (DHGP), Pushing (PHGP, peu commun) et Remplacement (RHGP). Pour DHGP, les cellules cancéreuses utilisent l'angiogenèse pour obtenir un apport vasculaire et répondent au traitement chimiothérapie - Bev. Cependant, les cellules tumorales présent dans RHGP cooptent les vaisseaux sanguins préexistants et une faible répondent au traitement par chimiothérapie - Bev.

Les angiopoïétines (Ang1 & Ang2) et leur récepteur, Tie2, supportent le mécanisme de co-option entre vaisseaux et assurent la médiation de la résistance aux médicaments anti-angiogéniques chez d'autres types de cancer.

Le but de la présente étude est d'investiguer le rôle des facteurs vasculaires mentionnés plus haut : Ang1, Ang2 et Tie2 dans la régulation de la co-option des vaisseaux RHGP qui permettent la résistance aux médicaments anti-angiogéniques dans le CRCLM. Pour étudier ce rôle, nous avons d'abords caractérisé le système vasculaire pour DHGP et pour RHGP en pour l'expression de Ang1, Ang2 et Tie2 dans des échantillons de lésions CRCLM humaines à l'aide de méthodes d'immunohistochimie, d'immunofluorescence et d'hybridation in situ fluorescente (FISH). Nos données ont montrées, pour la première fois, que l'expression de Ang1 était

augmentée dans les hépatocytes adjacents aux cellules tumorales dans les lésions non-traités et traitées (chimio et chimio plus Bev) de CRCLM humain RHGP.

Par la suite, nous avons exploré le rôle de l'expression Ang1 *in vivo*, des souris knock-out Ang1 (KO) ont été injectées par voie intra-splénique à des cellules cancéreuses métastatiques du cancer du côlon MC-38 qui développent des tumeurs RHGP dans le foie témoin. Nous avons constaté une réduction de la charge métastatique, une inhibition de la formation de vaisseaux sanguins matures et le développement d'une métastase hépatique de la tumeur angiogénique DHGP qui répond au traitement en clinique. Ainsi, la déficience en Ang1 inhibe les métastases hépatiques et a un impact sur la formation de HGP *in-vivo.*

Précédemment découvert, la régulation de la signalisation Ang1-Tie2 active les voies impliquées dans la survie, la prolifération et les métastases des cellules cancéreuses. Nous avons supposé que l'interaction entre les cellules tumorales et les hépatocytes entraînait une augmentation de l'Ang1 dans les hépatocytes, ce qui pourrait être important pour la croissance et l'invasion de la tumeur. Pour étudier cela, des hépatocytes primaires de contrôle adultes et de souris Ang1 KO ont été isolés à l'aide de la méthode de perfusion de collagénase en deux étapes. Nos données *in-vitro* ont démontré que la co-culture d'hépatocytes primaires de contrôle et de cellules cancéreuses du colon MC-38 à l'aide d'un insert induisait de manière significative l'expression de Ang1 dans les hépatocytes. De plus, les milieux conditionnés obtenus à partir de cultures d'hépatocytes ont augmenté la survie, la migration et l'invasion des cellules cancéreuses du colon MC-38. Cela s’explique puisque ce phénotype a été aboli lorsque l'Ang1 a été neutralisé dans les hépatocytes, ce qui suggère que l'Ang1 sécrétée par les hépatocytes joue un rôle essentiel dans la progression tumorale.

Dans l'ensemble, nos résultats démontrent qu'une expression élevée de Ang1 dans les lésions RHGP peut maintenir la formation de co-option vasculaire, la croissance tumorale et les métastases. Ainsi, étudier la voie Ang1-Tie2 en tant que contributeur potentiel à la survie de la co-option des vaisseaux et à la progression tumorale dans RHGP de CRCLM peut être une prochaine étape pour un traitement efficace.

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First and foremost, I would like to dedicate my first expression of the deepest appreciation and gratitude to my supervisor Dr. Peter Metrakos, M.D, PhD, Department of Surgery, Pathology and Anatomy and Cell Biology, Faculty of Medicine, McGill University. If it were not for Dr. Metrakos' patience, knowledge, and kindness I would not have had such a great PhD's career and experience.

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Lastly, I am humbled with gratitude to Ms. Dana Massaro and Mr. Ken Verdoni for granting me a scholarship to pursue this PhD degree.

Dedication

I dedicate this thesis to the memory of my father (Samir Abdulazim Ibrahim), my mother (Amina Abdulmutallab Al-Sayed), My sister (Al-Shaimaa Samir), and my nephew Adham, who passed away during my PhD journey. I felt that they were always by my side to give me support, confidence and help. I still miss them so much every single day.

This thesis is also dedicated to my kids Salma and Mohamed, my sister Rehab, my brother Abdullah, Galal, my nephews, my niece and all my family who have been extraordinarily tolerant and supportive of my studies.

Loving what you do is a necessity to work hard during nights, weekends, and holidays.

Nisreen

Contribution to original knowledge

The work presented in this thesis offers the first study of the role of Angiopoietins-Tie2 pathway in colorectal cancer liver metastasis (CRCLM). The major contributions to original knowledge are summarized below:

1. This study provides the first characterization about the expression of Ang1, Ang2 and Tie2 in human chemonaïve and treated (chemo and chemo plus Bev) CRCLM lesions.
2. The study demonstrates for the first time the impact of high expression of Ang1 on tumors utilize vessel co-option process for blood supply.
3. This study is also the first to identify high expression of Ang1 in the hepatocytes of the adjacent normal of RHGP tumors in chemonaïve and treated human lesions.
4. It also shows that Ang1 expression is necessary to maintain vessel co-option and RHGP phenotype in CRCLM, and when inhibited favours the formation of angiogenic driven liver metastases DHGP lesions.
5. It demonstrates the first direct evidence that the interaction between cancer cells and hepatocytes increases the expression of Ang1 in the hepatocytes.
6. It displays that high expression of Ang1 in the hepatocytes may play a critical role in tumor progression by inducing cancer cells survival, migration and invasion.

Therefore, Ang1 may be a potential therapeutic target in tumors that utilize vessel co-option mechanism for blood supply in CRCLM. We believe that these results are novel and have important implications for (a) predicting response to anti-angiogenic therapy in the clinic and, (b) for the design of rational strategies aimed at targeting resistance to anti-angiogenic therapy in patients.

Preface & Contribution of Authors

This thesis is presented in accordance with the guidelines for the thesis preparation of the Faculty of Graduate and Postdoctoral Studies of McGill University. The entire thesis was written by me and revised by my supervisor, Dr. Peter Metrakos. My thesis contains four chapters: chapter 1, literature review; Chapter 2, materials and methods; chapter 3, results which are divided into four subchapters with multiple sections for each; and chapter 4, discussion and future directions. Part of chapter 1, 2, 3 and 4 represent accepted paper in a journal of Cancer.

In chapter 3, I performed all the experiments, I created all the figures presented in this thesis. Dr. Anthoula Lazaris and Mrs. Stephanie K. Petrillo were responsible for human samples biobanking and collecting the clinical data. Dr. Laurent Huck and Dr. Rachid Essalmani provided technical assistance in maintaining the Ang1 KO mouse colonies and primary hepatocytes isolation respectively. Dr. Zu-Hua Gao gave suggestion on IHC staining. Dr. Peter Metrakos and Dr. Anthoula Lazaris contributed in designing the experiments, data analysis and comments on the paper. Dr. Peter Metrakos led the project.

Published manuscript not presented in this thesis:

Anthoula Lazaris, Abdellatif Amri, Stephanie K Petrillo, Paublo Zoroquiain, **Nisreen Ibrahim,** Ayat Salman, Zu-Hua Gao, Peter B Vermeulen and Peter Metrakos, "Vascularization of Colorectal Cancer Liver Metastasis: Insight into stratification of patients for anti-angiogenic Therapies". Journal of Pathology Clinical Research. 2018 Jul;4(3):184-192. doi: 10.1002/cjp2.100.

Published abstracts in international conferences and symposiums:

Abstract 1825: Angiopoietin-Tie-2 functional axis in colorectal cancer liver metastasis (CRCLM) provides a new marker for stratification and evaluation of tumor progression. In: Proceedings of the American Association for Cancer Research Annual Meeting 2017; 2017 Apr 1-5; Washington, DC. Philadelphia (PA), USA: AACR; Cancer Res 2017; 77(13 Suppl): doi:10.1158/1538-7445.AM2017-1825. Poster presentation.

Abstract: Angiopoietin-Tie-2 functional axis in colorectal cancer liver metastasis (CRCLM) provides a new marker for stratification and evaluation of tumor progression. Canadian cancer research conference-2017, Vancouver, Canada - Poster presentation.

Abstract 5449: Characterization of clinically relevant mechanism of resistance to angiogenic inhibitors in different growth patterns of human colorectal cancer liver metastases (CRCLM) by studying the angiopoietins-Tie2 mechanisms. In: Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA), USA: AACR; Cancer Res 2018;78(13 Suppl). Poster presentation.

Nisreen S. Ibrahim. Angiopoietin-Tie-2 functional axis in colorectal cancer liver metastasis (CRCLM) provides a new marker for stratification and evaluation of tumor progression. Annual Cancer Research Program Research Day. May 23th, 2017. Montreal, Canada Poster presentation.

Nisreen S. Ibrahim. Angiopoietin-Tie-2 functional axis in colorectal cancer liver metastasis (CRCLM) provides a new marker for stratification and evaluation of tumor progression. Annual

Anatomy & Cell Biology Departmental Retreat. July 6-7, 2017. Montreal, Canada Poster presentation.

Nisreen S. Ibrahim. Angiopoietin-Tie-2 functional axis in colorectal cancer liver metastasis (CRCLM) provides a new marker for stratification and evaluation of tumor progression. Annual Cancer Research Program Research Day. Montreal, Canada 2018. Poster presentation.

Oral presentations:

Nisreen S. Ibrahim. Angiopoietin1 maintains vessel co-option and mediates resistance to anti-angiogenic drug in colorectal cancer liver metastasis. Annual Cancer Research Program Research Day. Montreal, Canada, 2019. Oral presentation.

Nisreen S. Ibrahim. Angiopoietin1 maintains vessel co-option and mediates resistance to anti-angiogenic drug in colorectal cancer liver metastasis. Liver Metastasis Research Network Meeting. Valencia, Spain, 2019. Oral presentation.

List of Figures and Tables

List of Abbreviations

CRC	Colorectal cancer
CRCLM	Colorectal cancer liver metastasis
FAP	Familial adenomatous polyposis
HNPCC	Hereditary non-polyposis colorectal carcinoma
APC	Adenomatous polyposis coli
PTEN	Phosphatase and tensin homolog
CIN	Chromosomal instability
MSI	High-frequency microsatellite instability
CIMP	CpG island methylator phenotype
TNM	Tumor, node, metastasis staging system
5-FU	5-fluorouracil
VEGF	Vascular endothelial growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal–regulated kinases
EMT	Epithelial-to-mesenchymal transition
NPCs	Non-parenchymal cells
LSEC	Liver sinusoidal endothelial cells
KC	Kupffer cells
NK	Natural killer
HepSC	Hepatic stellate cells
BM	Bone marrow
ECM	Extracellular matrix

OS	Overall survival
FLR	Future liver remnant
FDA	Food and Drug Administration
HGPs	Histological growth patterns
DHGPs	Desmoplastic histological growth patterns
RHGPs	Replacement histological growth patterns
PHGPs	Pushing histological growth patterns
ARP2/3	Actin-related protein 2/3
EC	Endothelial cell
PDGF	Platelet-derived growth factor
Angs	Angiopoietins
HGF	Hepatocyte growth factor
EGF	Epidermal growth factor
VEGFR	Vascular endothelial growth factor receptor
CECs	Circulating endothelial cells
EPCs	Endothelial progenitor cells
IFP	Interstitial fluid pressure
FGFs	Fibroblast growth factors
TGFs	Transforming growth factors
PlGF	Placental growth factor
G-CSF	Granulocyte colony-stimulating factor
MDSC	Myeloid-derived suppressor cells
EPC	Endothelial progenitor cells

CAF	Cancer-associated fibroblasts
Tie	**T**yrosine kinase with **i**mmunoglobulin and **e**pidermal growth factor homology domains
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
RTK	Receptor tyrosine kinase
PI3K	Phosphatidylinositol 3-kinase
SMC	Smooth muscle cells
AKT	Protein kinase B
eNOS	Endothelial nitric oxide synthase
BAD	BCL2 associated agonist of cell death
FOXO1	Forkhead transcription factor
Erk	Extracellular signal–regulated kinase
Rac1	Ras-related C3 botulinum toxin substrate 1
FAK	Focal adhesion kinase
Dok-R	Docking protein 2
Rac1	Ras-related C3 botulinum toxin substrate 1
PDGFR-P	Platelet-derived growth factor phosphorylated
ICAM-1	Intercellular adhesion molecule-1
ICAM-2	Intercellular adhesion molecule-2
VE	Vascular endothelial
TNF-alpha	Tumor necrosis factor-alpha
ECM	Extracellular matrix

VE-PTP	Vascular endothelial protein tyrosine phosphatase
SCC	Squamous cell carcinoma
TEMs	Tie2 expressing monocytes
PDGFR-b	Platelet-derived growth factor receptor b
TKIs	Tyrosine kinase inhibitors
H&E	Hematoxylin and Eosin
FFPE	Formalin-fixed paraffin-embedded
BSA	Bovine serum albumin
CT	Central of tumor
PT	Periphery of tumor
ADN	Adjacent normal liver
DN	Distal normal
rtTA	Reverse tetracycline trans-activator
KO	Knockout
Dox	Doxycycline
FBS	Fetal bovine serum
DMEM	Dulbecco's modified eagle medium
RIPA	Radio-immunoprecipitation assay
PVDF	Polyvinylidene difluoride
TBS-T	Tris-buffered saline with Tween-20
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
HSA	Hepatocyte specific antigen
MVD	Microvessel density

1. Chapter 1: Literature Review

1.1. Overview of colorectal cancer (CRC).

Cancers derived from colon and rectum parts located at the final part of the human digestive tract, are called colorectal cancers (CRCs) (Scott and Wang 2015). CRC is the second leading cause of cancer death in the western world (Garcia-Alfonso, Ferrer et al. 2015). It is the third most common cause of death in women and the fourth in men worldwide (Garcia-Alfonso, Ferrer et al. 2015). CRC results from the progressive accumulation of genetic and epigenetic changes that lead to the transformation of normal colonic epithelium to colonic adenocarcinoma (Bogaert and Prenen 2014). There are several risk factors associated with the development of CRC that result in significant differences in the global incidence of this type of cancer. These factors can be divided into environmental or molecular (Scott and Wang 2015).

Sporadic CRC represents 70-80% of all CRC cases and is caused by chromosomal instability (CIN), high-frequency microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). CIN mechanism causes alterations in chromosome number and structure including gains or losses of chromosomal segments, chromosomal rearrangements, and loss of heterozygosity (LOH) resulting in gene copy number variations (CNVs). These alterations influence the expression of tumor associated genes and may activate pathways that are important for CRC initiation and progression (Nguyen and Duong 2018).

MSI is caused by inhibition of DNA mismatch repair system resulting in a failure in the correction of the insertion or the deletion of repeating units during DNA replication. However, CIMP is caused by hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes resulting in genes inactivation (Vacante, Borzì et al. 2018).

The most common mutated genes that contribute to CRC tumorigenesis are; proto-oncogene GTPase (KRAS), proto-oncogene serine/threonine kinase (BRAF), and tumor suppressor genes,

such as adenomatous polyposis coli (APC), phosphatase and tensin homolog (PTEN) and tumor protein p53 (*TP53*). These genetics and epigenetic alterations can be used as biomarkers for CRC diagnosis, progression, and response to treatment as you can see in (Table 1.1) (Vacante, Borzì et al. 2018).

Biomarker	
BRAF mutations	Specific phenotype and metastasis; resistance to anti-EGFR mAb
KRAS mutations	Heterogeneity of CRC; resistance to anti-EGFR mAb
MSI	Resistance to 5-FU
APC mutations	Poorer overall survival
Micro-RNA	Early detection of CRC, prognostic stratification and therapy-response prediction
PIK3CA mutations	Poor prognosis and particular clinico-pathological characteristics; resistance to anti-EGFR mAb
Loss of *PTEN*	High tendency to develop metastasis; Resistance to anti-EGFR mAb
TP53 expression	Poor prognosis
Loss of *NDST4*	Adverse prognosis; molecular predictor of metastasis
Loss of *18qLOH*	Poor prognosis
IGFR-1R	High levels in metastastic CRC; poor overall survival

Table 1.1, Examples of genetics and epigenetic alterations biomarkers in CRC. Reprinted with permission from (Vacante, Borzì et al. 2018).

The incidence of sporadic CRC is increased by environmental factors including ulcerative colitis, obesity, low consumption of fruit and vegetables, consumption of red and processed meat, alcohol consumption, diabetes, a sedentary lifestyle and smoking (Garcia-Alfonso, Ferrer et al. 2015, Scott and Wang 2015, Recio-Boiles, Waheed et al. 2019).

CRC patients with family history represents 15-20% of the disease. The most common hereditary conditions are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal carcinoma (HNPCC, Lynch syndrome) (Scott and Wang 2015). FAP accounts for less than 1% of colorectal cancer patients and is caused by a germline mutation of one allele of APC gene. Alternately, HNPCC displays 3 to 4% of colorectal cancer cases and is caused by a

deficiency in one of a few DNA mismatch repair genes. Other rare inherited syndromes of colorectal cancer, which account for 5% of all colorectal cancer cases include Cowden syndrome, juvenile polyposis, or Peutz–Jeghers syndrome (Scott and Wang 2015).

1.1.1. Staging of CRC

There are four stages of colorectal cancers that describe the extent of penetration of the cancer and determine treatment strategies. The tool that is used to describe the CRC pathologic stage is the tumor, node, metastasis (TNM) staging system that include tumor size (T), lymph node involvement (N) and presence or absence of distant metastasis (M) (Scott and Wang 2015, Recio-Boiles, Waheed et al. 2019). In stage 0, cancer cells are developed on the inner layer of the intestinal wall; stage I, cancer cells are grown in the muscular layer; stage II, cancers are grown and broken the muscular layer but are bounded to the adjacent soft tissues (Figure 1.1). Cancers are spread through the muscular layer to the lymph nodes are termed as stage III but, cancers metastasize to distant organ sites are considered as stage IV (Figure 1.1) (Scott and Wang 2015).

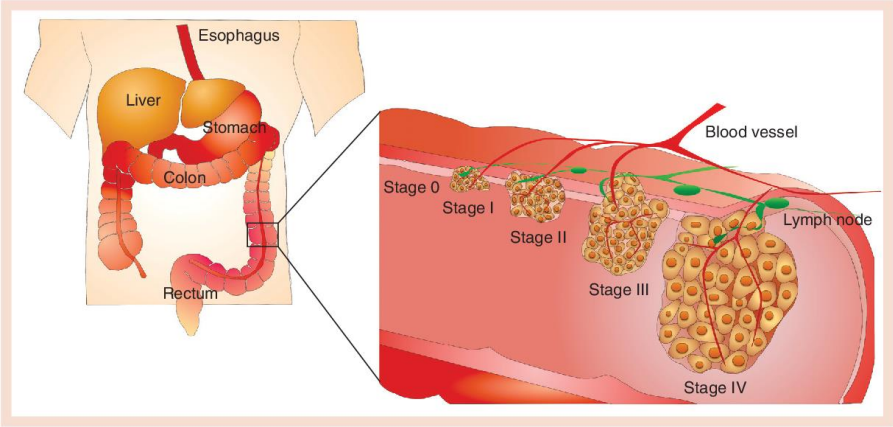

Figure 1.1, Schematic for colorectal cancer pathologic staging. Reprinted with permission from journal of future medicine, (Cisterna, Kamaly et al. 2016).

1.1.2. Screening of CRC

Screening of colorectal cancer include colonoscopy for histological examination of biopsies, fecal occult blood, fecal DNA tests and fecal immunochemical tests. CT abdomen and pelvis provides cost-effective imaging study for initial assessment of the CRC staging T, N and M (Recio-Boiles, Waheed et al. 2019). In addition to screening, clinical features that should increase suspicion for CRC are fatigue and anemia resulting from lesions in the right-sided colon, while signs of left-sided cancers consist of stool with visible red blood and bowel dysfunction (Scott and Wang 2015).

1.1.3. Treatment of CRC

Standard treatments consist of surgical resection to remove the tumor lesion, chemotherapy to kill cancer cells, and radiation therapy to destroy tumor tissue. Chemotherapy, radiation, or both can be a form of neoadjuvant and/or adjuvant treatment. Neoadjuvant treatment is used pre-operatively to improve surgery outcome however, adjuvant treatment is used post-operatively to eradicate residual microscopic disease and prevent tumor recurrence (Al-Sukhni and Gallinger 2010).

1.1.3.1. Surgical resection

Surgical resection is a curative treatment for early-stage CRC patients. It cures over 90% of patients in stage I and over 80% of patients in stage II of the disease. In stage III, the presence of nodal involvement indicates 60% of disease recurrence and is of critical importance for selecting patients for adjuvant treatments. Post-operative chemotherapy treatment for these

patients reduces the recurrence rate to 40% and increases overall survival to 60%. In stage IV, the five-year survival rate is only 8% for patients (Scott and Wang 2015).

1.1.3.2. Chemotherapy

In the clinic, most chemotherapy drugs are used to target rapidly proliferating cells. The most common chemotherapy in CRC treatment is 5-Fluorouracil (5-FU, anti-metabolite). In cancer cells, 5-FU is converted to several active metabolites that interfere with RNA and DNA syntheses inhibiting DNA metabolism and causing cell death (Al-Sukhni and Gallinger 2010). 5-FU reduces tumor burden by increasing cytotoxic cell death causing tumor cell debris, characterized by apoptotic cells, necrotic cells, and cell fragments (Chang, Bhasin et al. 2019). 5-FU and leucovorin (Folinic acid, cell anti-toxic) can be used in combination with DNA damage drugs oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) to improve recurrence and overall survival rates of resected CRC. Patients with high-frequency microsatellite instability (MSI) develop resistance to 5-FU (Table 1.1) (Vacante, Borzì et al. 2018). Non-specific modes of chemotherapy action are often accompanied by severe host toxicity as a result of damage to normal host cells (Al-Sukhni and Gallinger 2010).

1.1.3.3. Target therapies

A great deal of effort has been invested to develop cancer specific targets such as oncogenes and altered elements of cell signaling pathways. Target therapies such as monoclonal antibodies bevacizumab (anti-VEGF), cetuximab (anti-epidermal growth factor receptor, anti-EGFR), panitumumab (anti-EGFR) or radiation can be used depending on the tumor profile (Veenstra and Krauss 2018, Scott (2015)).

Vascular endothelial growth factor (VEGF) is an endothelial specific mitogen, an angiogenesis inducer, and its receptors are primarily localized on endothelial cells. The VEGF signaling pathway has been established as the major regulator of angiogenesis (Ferrara, Gerber et al. 2003). Seven different ligands have been identified in the VEGF family in which VEGF-A (also called VEGF) has emerged as the main player in angiogenesis. These different ligands bind to three different VEGF receptors, known as VEGFR-1, -2 and -3, causing phosphorylation of the VEGFR resulting in activation of downstream signaling pathways. These pathways include the phosphoinositide 3-kinase (PI3K-Akt/mTORC2) pathway, mitogen-activated protein kinase (Raf-MEK-MAPK) pathway, and focal adhesion kinase (FAK) pathway, involved in endothelial cells permeability, proliferation, and motility (Figure 1.2) (Kong, Kim et al. 2017, Guo, Murdoch et al. 2018). Bevacizumab (also known as Avastin, Genentech) is a humanized antibody that binds and neutralizes the activity of all human VEGF inhibiting the proliferation of new blood vessels and hence tumor growth (Al-Sukhni and Gallinger 2010). It exhibits the expected pharmacokinetic properties of monoclonal Abs with serum half-life of 17-21 days in humans (Lin, Nguyen et al. 1999).

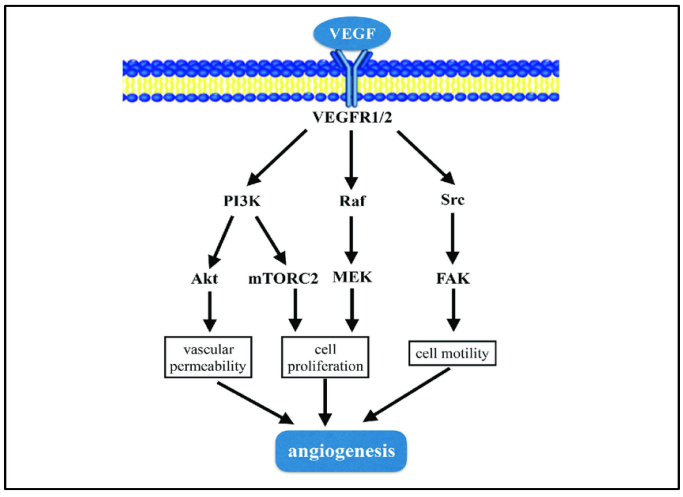

Figure 1.2, VEGF signaling pathway and vessel angiogenesis. Reprinted with permission from (Guo, Murdoch et al. 2018).

Activation of epidermal growth factor receptor (EGFR) stimulates several intracellular signalling pathways including Ras-Raf-MEK-ERK and PI3K-AKT-mTOR, which ultimately promote cellular proliferation and decrease apoptosis. Cetuximab and panitumumab bind to the extracellular domain of EGFR to suppress downstream signalling, and as a result inhibit tumor proliferation and metastasis. All CRC patients with KRAS and NRAS wild type respond to the (anti-EGFR) antibodies cetuximab and panitumumab however, some of patients with mutated KRAS and NRAS do not respond to the same treatment (Table 1.1) (Veenstra and Krauss 2018). KRAS and NRAS mutations result in constitutive activation of the Ras-Raf-MEK-ERK signalling pathway, which is independent of EGFR activation by ligand binding (Benvenuti, Sartore-Bianchi et al. 2007).

1.2. Failure of treatment and tumor recurrence:

About fifty percent of patients that undergo curative resection for CRC and adjuvant therapies will develop tumor recurrence within 3-5 years of surgery (Seo, Lim et al. 2013). CRC recurrences are classified as local recurrence, including intraluminal anastomotic, pelvic, and nodal or systemic recurrence, which is divided into haematogenous metastasis, distant lymph node metastasis and peritoneal seeding groups (Figure 1.3) (Seo, Lim et al. 2013).

1.2.1. Local recurrence

Rectal cancer accounts for approximately one third of colorectal cancers and can be potentially cured. A local recurrence remains a serious problem in rectal cancer due to the presence of the pelvic bone that causes limited access to the rectum and negative resection margins achievement. Despite radical surgical treatment, in addition to chemotherapy and radiation

therapies, 6–12% of rectal cancer patients will experience a local recurrence causing disabling symptoms difficult to treat. These symptoms carry significant morbidity in the form of severe pain, bleeding, discharge, and poor quality of life (Barugel, Vargas et al. 2009). Local recurrence of colon cancer includes intraluminal anastomotic recurrence or regional recurrence in the adjacent mesentery, peritoneum or retroperitoneum (Seo, Lim et al. 2013).

1.2.2. Systemic recurrence

CRC systemically metastasize to vital organs such as the liver and the lung, and less vital organs such as the bone and the peritoneum (Ferrario and Basik 2010). Colon and rectal cancers spread using four paths: through the portal vein, metastasizing to the liver and then to the systemic circulation; through mesothelial spread to the peritoneal surfaces; and through lymphatic spread with progressive local nodal growth (Figure 1.3) (Ferrario and Basik 2010). However, rectal cancer disseminates directly into the systemic circulation (Figure 1.3) (Ferrario and Basik 2010). The destination of both lymphatic and hematogenous spread for the colon, and the lymphatic drainage of the upper part of the rectum is the liver (Figure 1.3).

1.3. CRC metastatic cascade

For CRC to metastasize, a sequence of five steps must successfully take place (Figure 1.4). CRC cells acquire properties that promote invasion, migration, and interactions with host-derived cells in the microenvironment. In the metastasis process, CRC primary tumor cells undergo an epithelial-to-mesenchymal transition (EMT) process to facilitate detachment from surrounding cells, intravasate into the circulation, extravasate into the liver parenchyma, and finally form colonization (Clark, Ma et al. 2016). The “seed and soil” hypothesis was proposed to explain the

pattern of metastatic spread in cancer. The seed presents tumor cells that favourably grow in the microenvironment of select organs or the soil.

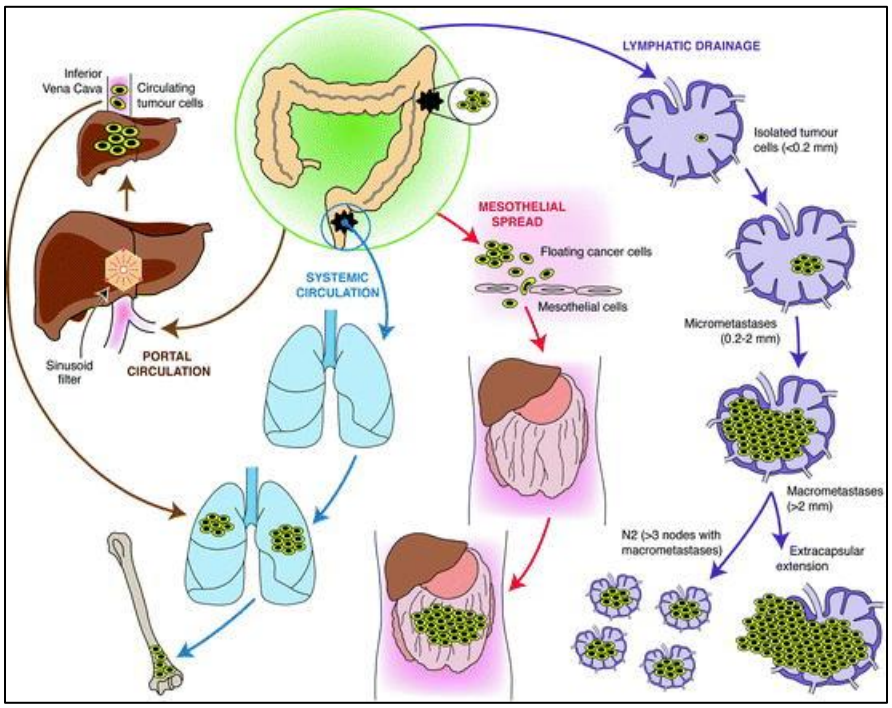

Figure 1.3, Colorectal cancer mechanisms and patterns of spread to metastatic sites. Reprinted with permission from Springer Nature, (Ferrario and Basik 2010).

1.3.1. Liver metastases process

Liver plays an important role in body biosynthesis, metabolism, clearance, and host defense. At the cellular level, the liver microenvironment comprises unique cellular populations including 70% of the parenchymal hepatocytes and cholangiocytes, responsible for the glandular, metabolic, and detoxifying functions. The remaining 30% of cells contain the non-parenchymal cells (NPCs) including liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HepSC), Kupffer cells (KC), dendritic cells, natural killer (NK) liver associated lymphocytes, portal fibroblasts as well as bone marrow (BM)-derived immune cells that are recruited to the liver in

response to inflammatory signals (Vidal-Vanaclocha 2008, Clark, Ma et al. 2016). These cells are important locally for the liver microcirculation, extracellular matrix (ECM) composition and liver tissue regeneration, as well as systemically for blood filtration, molecular scavenging and immune response (Vidal-Vanaclocha 2008, Clark, Ma et al. 2016).

Liver parenchyma possess two different vascular structures; large vessels (such as portal vessels that are lined by a continuous ECs lying on a basement membrane), and liver sinusoids that are lined by fenestrated and discontinuous ECs that have no basement membrane (space of Disse) (Figure 1.4), allowing space for the plasma to interact with the hepatocytes and hepatic stellate cells (liver pericytes) (Vollmar and Menger 2009).

The liver is the second most commonly involved organ by cancer metastasis, after the lymph nodes. The distinctive biology of the liver renders it susceptible to metastases. Architectural and hemodynamic features facilitate direct access of circulating tumor cells, regenerative capabilities that promote the formation of tumor blood vessel and stroma, and regional immune suppression that can allow for tumor cell survival and growth (Clark, Ma et al. 2016).

CRC circulating tumor cells enter the liver via either the portal vein or hepatic artery, and trap in the hepatic sinusoids, where they encounter the sinusoidal immune system, which includes natural killer cells, Kupffer cells, and hepatic endothelial cells (Figure 1.4). Four interrelated phases occur within the liver to promote the metastatic process: (i) the tumor-infiltrating microvascular phase, (ii) the interlobular pre-angiogenic micro-metastasis phase, (iii) the angiogenic micro-metastasis phase, and (iv) the growth phase (Clark, Ma et al. 2016). During these phases, cancer cells undergo cytolysis and phagocytosis by immune surveillance cells natural killer and Kupffer cells (Figure 1.4). Alternatively, the surviving cancer cells adhere to the liver

microcirculation to extravasate and invade into the liver parenchyma causing endothelial cells and hepatocytes apoptosis (Figure 1.4) (Ferrario and Basik 2010, Clark, Ma et al. 2016).

Cancer cells can be confined to the space of Disse and to Glisson's capsule, which rich in extracellular matrix, allow initial survival and proliferation (Figure 1.4) (Ferrario and Basik 2010, Clark, Ma et al. 2016). In later stages, the cancer cells interact with the microenvironment, which provides a substrate for cancer cells migration and infiltration, forming a ''clinical'' macro-metastasis (Figure 1.4) (Ferrario and Basik 2010, Clark, Ma et al. 2016).

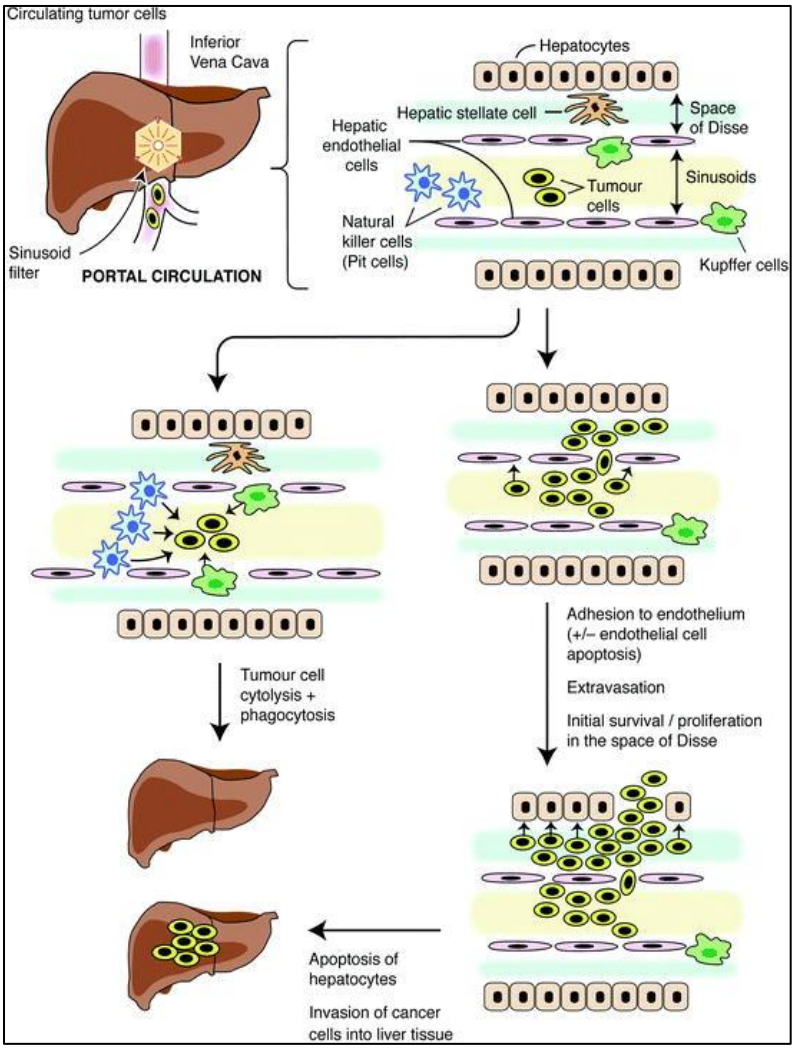

Figure 1.4, The metastatic CRC cells colonization process in the liver. Reprinted with permission from Springer Nature, (Ferrario and Basik 2010).

1.4. Colorectal cancer liver metastasis (CRCLM)

1.4.1. Overview and management

The liver is the major site of metastases for almost 60% of patients with stage IV CRC and is a major cause of cancer related death (Bird, Mangnall et al. 2006). Approximately 25% of CRC patients have detectable liver metastases at the time of diagnosis and a further 25% of patients expected to develop metastases within a two-year period after initial curative resection of their primary tumor. Early diagnosis of liver metastases from colorectal cancer is essential for effective treatment as patient's overall survival is correlated with tumor progression (Bird, Mangnall et al. 2006).

Currently, patients with hepatic metastases on diagnosis fall into three categories: resectable patients with or without neoadjuvant therapy; potentially resectable patients if the tumors burden could be downsized under certain conditions; and non-resectable patients who will be treated with chemotherapy and other therapies (Bird, Mangnall et al. 2006).

1.4.1.1. Surgical resection and chemotherapy

At present, a surgical resection is the only management approach; resulting in 5-year overall survival for one third of the CRCLM patients, only 10 - 30% of the patients are suitable for a radical procedure (E Pavlidis, Symeonidis et al. 2011). Most CRCLM patients are not eligible for surgical resection because of the size, location or number of lesions, as well as anatomical limitations (Kanat 2016). It has been shown that the resectable patients with CRCLM are better off than the unresectable patients. Therefore, converting unresectable patients to resectability should be the aim for all patients. The liver of the resectable patient should be evaluated and an assessment of the future liver remnant (FLR) should be made. Adequate size and function of FLR

are important to support the patient after resection operation (Bird, Mangnall et al. 2006). Improvement in surgical procedure of subtotal hepatectomy elevate the overall survival and become the treatment of choice. The advancement of the chemotherapy regimes has also been essential for these improvements (Bird, Mangnall et al. 2006).

In advanced CRCLM cases, current treatment strategies can be applied such as down-staging chemotherapy, two-stage resections and portal vein embolization, which result in increasing the recectability rate by 15% (E Pavlidis, Symeonidis et al. 2011). In certain group of CRCLM patients, chemotherapy is used as a conversion therapy in a first-line treatment to downsize tumor burden as much as possible before resection (E Pavlidis, Symeonidis et al. 2011). In two-stage resections, the first resection removes the highest number of tumors while, the second resection eradicates noncurative intervention and the remaining tumor metastases after a period of liver regeneration (Adam, Laurent et al. 2000). Portal vein embolization (i.e. blocking) procedure is used to increase the regenerative capacity of the liver before resection to prevent hypertrophy and increase functional ability of the remaining liver (E Pavlidis, Symeonidis et al. 2011).

In patients with initially unresectable CRCLM, the combination of the standard doublet 5-fluorouracil (5-FU) plus either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) offers conversion resectability rates of between 9 and 33% (E Pavlidis, Symeonidis et al. 2011). On the other hand, the combination of intensified triplet chemotherapy regimen of 5-FU, FOLFOX and FOLFIRI (FOLFOXIRI) increases response and resectability rates in many studies (E Pavlidis, Symeonidis et al. 2011).

1.4.1.2. Anti-angiogenic therapy

The administration of anti-angiogenic targeted therapies, such as bevacizumab, aflibercept, and regorafenib, with chemotherapy may lead to improved response rates of CRCLM and increase the proportion of patients eligible for surgical resection (E Pavlidis, Symeonidis et al. 2011). Bevacizumab was approved for the first time as an anti-angiogenic drug for the treatment of metastatic colorectal cancer by the Food and Drug Administration (FDA) in 2004. The addition of bevacizumab to neoadjuvant chemotherapy results in a higher impact on pathological response rate, with a break of 6–8 weeks before surgery to increase liver regeneration and healing ability after resection (Yoo, Lopez-Soler et al. 2006). This drug has a favorable safety profile and may protect against the sinusoidal dilation that is induced in the liver by certain cytotoxic agents (E Pavlidis, Symeonidis et al. 2011). Despite the benefit achieved with the addition of bevacizumab to chemotherapy however, the overall survival is measured in terms of only months (Frentzas, Simoneau et al. 2016). The mechanisms that limit the curative efficacy of anti-angiogenic therapy (including bevacizumab) for CRCLM patients are still poorly understood.

1.4.2. CRCLM histological growth patterns (HGPs)

CRCLM grow in distinct histological growth patterns that are associated with treatment outcome. Three distinctive morphological HGPs were described in CRCLM: desmoplastic (DHGP), replacement (RHGP) and pushing (PHGP) (Paku and Lapis 1993, Vermeulen, Colpaert et al. 2001). In DHGP, the cancer cells are separated from the normal liver parenchyma by a capsule of desmoplastic stroma (ring) (Figure 1.5). DHGP lesions are characterized by high hypoxia-driven angiogenesis, including increased fibrin deposition at the tumor-liver interface and high rates of endothelial cell proliferation. Furthermore, desmoplastic stroma is characteristically

associated with an intense lymphocytic and tumor cell nests infiltration (Vermeulen, Colpaert et al. 2001).

In PHGP and RHGP, the cancer cells are in close contact with normal liver parenchyma (Figure 1.5). In PHGP, the expanding population of cancer cells push the normal liver parenchyma away without invading them. In addition, PHGP cancer cells utilize angiogenesis to obtain a vascular supply. However, in RHGP, the cancer cells infiltrate the liver parenchyma, replace hepatocytes and co-opt pre-existing sinusoidal vessels leading to incorporation of sinusoidal vessels into the tumor causing little perturbation of the liver architecture (Figure 1.5) (Paku and Lapis 1993, Vermeulen, Colpaert et al. 2001). The uncommon PHGP lesions are not as well described and may possibly represent a transition lesion between RHGP and DHGP (Frentzas, Simoneau et al. 2016).

Recently, our group has shown that these distinct HGPs have a different response to combination therapy. The patients with DHGP who received chemotherapy plus bevacizumab prior to resection had a significantly better pathologic response and OS than patients with RHGP. However, there was no difference in OS when DHGP and RHGP patients were treated with chemotherapy alone. Thus, addition of bevacizumab was beneficial only in patients with DHGP. In addition, our clinical data revealed that bevacizumab could negatively affect outcomes in patients with RHGP who developed bevacizumab resistance (Frentzas, Simoneau et al. 2016). Currently, no clinically validated predictive biomarkers are available to identify the patients who will have benefit from anti-angiogenic therapies.

In a single metastasis, only one growth pattern is usually present. Moreover, only the dominant pattern was considered for further analysis in few cases (Vermeulen, Colpaert et al. 2001).

It has been suggested that the tissue of origin might impact the mode of vascularization, as CRCLM can adopt any of the three growth patterns. However, breast cancer liver metastases have only a replacement growth pattern (Kuczynski, Vermeulen et al. 2019).

Recently, our group has shown that vessel co-option could be suppressed through inhibition of cancer cell motility by knocking down actin-related protein 2/3 (ARP2/3) in mouse models. Furthermore, only chemotherapy can suppress vessel co-option by killing the co-opted vessels or inhibiting cancer cell motility (Frentzas, Simoneau et al. 2016). The mechanism that explains what drives different growth patterns in liver metastasis that confer tumor with co-opted vessels versus newly formed vessels is poorly understood.

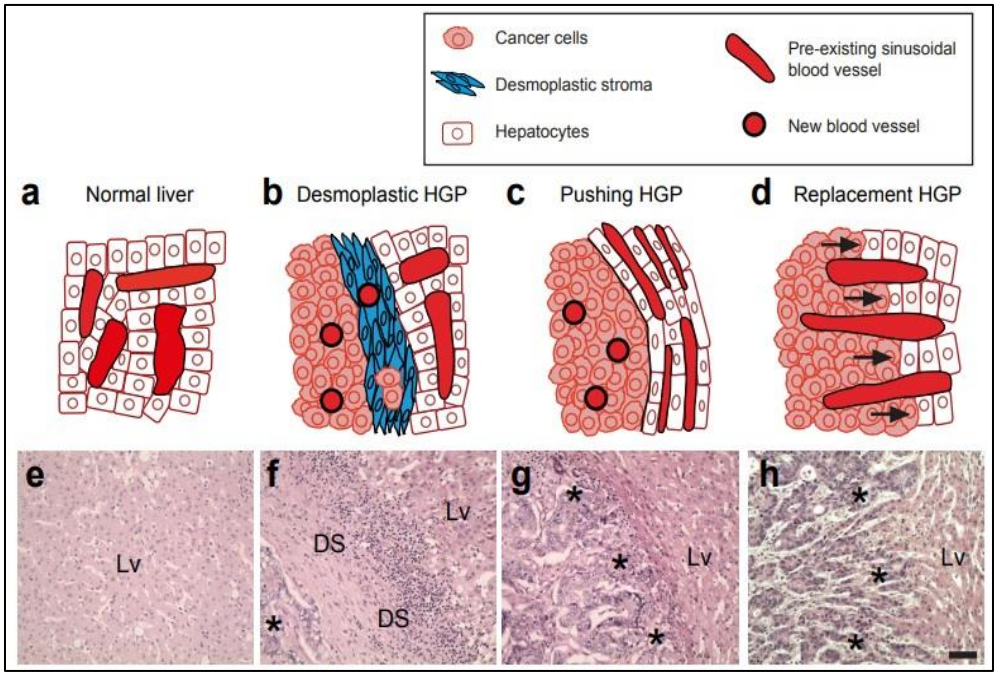

Figure 1.5, Diagrams and H&E staining demonstrate (a, e) the structure of normal liver or the structure of the tumor–normal liver interface in human CRCLM with (b, f) a desmoplastic, (c, g) pushing or (d, h) replacement HGP. Reprinted with permission from Springer Nature, (Frentzas, Simoneau et al. 2016).

1.5. Angiogenesis and angiogenic switch

In vertebrates, two major mechanisms of blood vessel formation exist during embryogenesis; vasculogenesis wherein endothelial cell (EC) differentiates from endothelial progenitor cells; and angiogenesis wherein EC sprouts or intussuscepts from pre-existing blood vessels (Coultas, Chawengsaksophak et al. 2005). The process of generating new blood vessels from pre-existing ones can be divided into two phases: 1) tube formation, in which the ECs respond to growth factors to proliferate, migrate and generate the new sprout, and 2) vessel maturation, in which the new vessels are stabilized by adding basement membrane and recruiting mural cells (pericytes and smooth muscle cells) (Coultas, Chawengsaksophak et al. 2005).

In adult tissues, angiogenesis is involved in essential physiological processes, such as wound healing and regeneration of the endometrial lining during the menstrual cycle. Vasculogenesis and angiogenesis are regulated by a few growth factors such as VEGF, angiopoietin and ephrin families (Coultas, Chawengsaksophak et al. 2005). Angiogenesis is tightly controlled by angiogenic stimulators (pro-angiogenic factors) and inhibitor molecules (anti-angiogenic factors) (Coultas, Chawengsaksophak et al. 2005). Numerous pro-angiogenic factors and their receptors have been identified, including VEGF, platelet-derived growth factor (PDGF), angiopoietins (Angs), hepatocyte growth factor (HGF), and epidermal growth factor (EGF) (Kong, Kim et al. 2017).

Angiogenic switch is an imbalance between pro- and anti-angiogenic factors resulting in pathologic angiogenesis, which contributes to the pathophysiology of many diseases such as cancer (Hanahan and Folkman 1996). Angiogenic switch in tumors is regulated by a balance of pro- and anti-angiogenic factors whereby the shift in this balance towards the pro-angiogenic state promotes active angiogenesis and tumor development (Hanahan and Folkman 1996). Pro and anti-

angiogenic factors are derived from cancer cells, stroma cells, endothelial cells, blood, and the extracellular matrix (Carmeliet and Jain 2000).

Pro-angiogenic factors, such as VEGF and PDGF in cancer microenvironment activate tissue ECs, circulating endothelial cells (CECs), and endothelial progenitor cells (EPCs) from the bone marrow to generate new blood vessels (Gasparini, Longo et al. 2005). These activated ECs secrete numerous enzymes to breakdown the extracellular matrix for ECs invasion, proliferation, and migration (Gasparini, Longo et al. 2005).

1.6. Tumor vessels formation mechanism

Tumor vessels develop from pre-existing vessels by angiogenesis via sprouting or intussusception, in which interstitial tissue columns are inserted causing splitting the lumen of pre-existing vessel in two (Carmeliet and Jain 2000). In addition, vasculogenesis from circulating endothelial progenitor cells derived from bone marrow can contribute to tumor vessel development. Moreover, tumor cells can co-opt pre-existing vessels for a vascular supply. Tumor cells can also mimic endothelial cells and form vessel lumen by vasculogenic mimicry mechanism (Carmeliet and Jain 2000).

Tumor vessels are structurally and functionally different from normal vessels. They are highly disorganized, tortuous, and dilated with uneven diameter, excessive branching and shunts (Carmeliet and Jain 2000). These abnormal vessels have poor stability due to defects in pericyte coverage, causing changes in tumor blood flow, increasing tumor interstitial fluid pressure (IFP) and leading to a reduction in the efficacy of chemotherapy (Goel, Duda et al. 2011).

1.7. Anti-angiogenic drug categories

Anti-angiogenic drug is an efficient means of anticancer treatment for several reasons; the target cells ECs are in direct contact with the blood facilitating drug delivery; ECs are genetically stable and less likely to accumulate mutations that promote drug resistance; eradicating a few of ECs kills many tumor cells; and body ECs are generally quiescent, limiting the side effect of the drug (van Beijnum, Nowak-Sliwinska et al. 2015).

In preclinical and clinical studies, many of angiogenesis inhibitors and strategies are currently being used (Kerbel and Folkman 2002). There are two categories of angiogenesis inhibitors based on their mechanism, direct and indirect. Direct angiogenesis inhibitors are usually endogenous and prevent ECs from proliferation and migration in response to pro-angiogenic proteins such as VEGF, fibroblast growth factor (FGF) and cyclin-D1. In mice, tumors treated with direct angiogenesis inhibitors therapy did not develop drug resistance (Kerbel and Folkman 2002). Although there are a wide number of endogenous angiogenesis inhibitors, VEGF was considered as an attractive target. Indirect angiogenesis inhibitors target a growth factor that regulate angiogenesis or block its receptor (Kerbel and Folkman 2002).

Anti-VEGF agents can be divided into two groups based on their molecular structure. The first group consists of antibodies and recombinant proteins that can prevent the interaction between VEGF ligand and receptor such as aflibercept and bevacizumab (Figure 1.6) (Gasparini, Longo et al. 2005). The second group is tyrosine kinase inhibitors (TKIs), which inhibit the activity of the EC receptor tyrosine kinases, preventing downstream signaling activation important for EC proliferation, survival, migration and interaction, for examples Sorafenib and Sunitinib (Figure 1.6) (Gasparini, Longo et al. 2005).

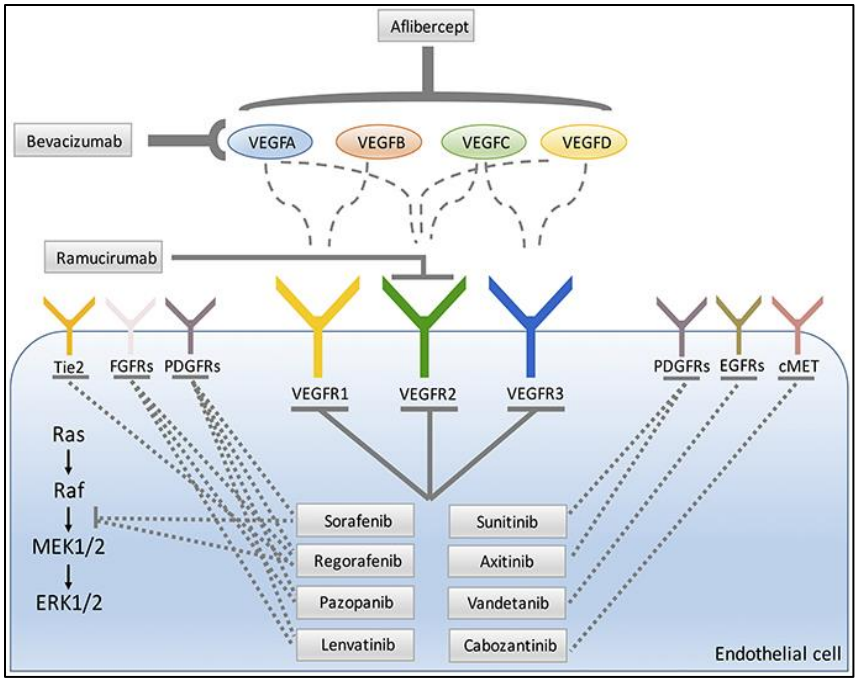

Figure 1.6, Molecular targets of anti-VEGF drugs approved for cancer patients' therapy. Reprinted with permission from (Comunanza and Bussolino 2017).

1.8. Mechanisms of resistance to anti-angiogenic therapy

Anti-angiogenic therapy is one of the promising therapeutic approaches for several types of solid tumors. Tumor vessels are heterogenous; some vessels can be sensitive to anti-angiogenic treatment while others eventually become resistant. Tumors can employ a variety of strategies to receive nutrients and oxygen as well as escape from anti-angiogenic treatment. The principal mechanisms of anti-angiogenic treatment resistance can be either acquired resistance during the course of the treatment or pre-existing intrinsic resistance (Comunanza and Bussolino 2017). Most of the resistance mechanisms to anti-angiogenic therapy are not genetic (van Beijnum, Nowak-Sliwinska et al. 2015).

1.8.1. Acquired mechanisms of resistance

In this mechanism the tumor and host microenvironment allow to escape from anti-angiogenic by promoting alternative pathways for angiogenesis such as:

1.8.1.1. Upregulation of alternative angiogenic factors

Numerous growth factors can substitute VEGF for sustaining tumor angiogenesis and EC activation including; angiopoietins (Angs), fibroblast growth factors (FGFs), transforming growth factors (TGFs), HGF, and placental growth factor (PlGF). These growth factors bind to transmembrane receptors expressed on EC and activate the downstream signalling pathways that are important for angiogenesis (van Beijnum, Nowak-Sliwinska et al. 2015).

1.8.1.2. Recruitment of bone marrow derived cells

Target tumor EC with anti-angiogenic therapy leads to increased expression of pro-angiogenic factors such as PlGF, VEGF, Ang1, and FGFs, and granulocyte colony-stimulating factor (G-CSF), that stimulate the recruitment of bone marrow derived cells into the tumor environment (van Beijnum, Nowak-Sliwinska et al. 2015). These myeloid cells including monocytes, macrophages, myeloid-derived suppressor cells (MDSC), endothelial progenitor cells (EPC), and cancer-associated fibroblasts (CAF), can play a main role in anti-angiogenic drug resistance induction (van Beijnum, Nowak-Sliwinska et al. 2015).

1.8.1.3. Local stromal cells

Local stromal cells like pericytes and CAF, which may be released from bone marrow, can resist anti-angiogenic treatment (van Beijnum, Nowak-Sliwinska et al. 2015). Pericytes interact with EC to stabilize the newly formed endothelial tubes, as well as regulate endothelial proliferation and differentiation (van Beijnum, Nowak-Sliwinska et al. 2015). It has been shown that increased vascular pericyte coverage and CAF resulted in protection of tumor EC from anti-angiogenic therapy. Tumor cells secrete growth factors to activate CAF that can impact cancer cell function, regulate angiogenesis and induce anti-angiogenic resistance (van Beijnum, Nowak-Sliwinska et al. 2015).

1.8.1.4. Endothelial cell heterogeneity

Tumor associated ECs differ from normal ECs in structure, gene expression profile and response to treatment. Tumor EC may display mesenchymal properties that enhance angiogenesis and may determine the sensitivity to anti-angiogenic treatment (van Beijnum, Nowak-Sliwinska et al. 2015).

1.8.1.5. Selection of more invasive tumor cells

Treatment with anti-angiogenic drugs reduces blood perfusion and metabolites' exchanges which increase hypoxia. Hypoxia can induce selection of more aggressive cancer cells for invasion and metastases. Furthermore, hypoxia can maintain cancer stem cells to promote tumor growth and invasion. Hypoxia can also induce autophagy, a cytoprotective mechanism that leads to treatment resistance (Giuliano and Pages 2013, Comunanza and Bussolino 2017).

1.8.2. Intrinsic mechanisms of resistance

Tumor cells may escape the anti-angiogenic treatment by adopting different growth patterns in highly vascularized organs, such as lungs, brain, and liver. Vasculogenic mimicry and vessel co-option can be generated to escape anti-angiogenic treatment (Giuliano and Pages 2013, van Beijnum, Nowak-Sliwinska et al. 2015, Comunanza and Bussolino 2017).

1.8.2.1. Vasculogenic mimicry

Tumor cells can mimic ECs and form vascular-like structures that can transport blood and contribute to oxygenation of the tumor. In preclinical studies, there is evidence for increasing vasculogenic mimicry after anti-angiogenic treatment (van Beijnum, Nowak-Sliwinska et al. 2015). The most important regulatory mechanism of vasculogenic mimicry are hypoxia-related pathways such as hypoxia-inducible factor 1-alpha (HIF-1α). Under hypoxia, sunitinib (VEGFR2 inhibitor) was able to transform tumor cells into endothelial-like cells and form vasculogenic mimicry. In addition, melanoma cells positive for the blood vessel marker cluster of differentiation 31 (CD31) had the ability to incorporate with vascular lumens and might lead to anti-VEGF therapy resistance (Ma, Pradeep et al. 2018).

1.8.2.2. Vessel co-option

Vessel co-option is known as a non-angiogenic process whereby tumor cells hijack pre-existing blood vessels for blood supply. It could be a major barrier to the efficacy of anti-angiogenic drugs in cancer patients. In this mechanism, cancer cells infiltrate the tissue space between pre-existing vessels, and incorporate pre-existing vessels from surrounding tissue into the tumor instead of inducing new vessel growth. Co-opted vessels have been observed in

histopathological specimens in a wide range of cancer types mainly in well-vascularized organs such as lung, liver, brain, skin, and lymph node cancers (Kuczynski, Vermeulen et al. 2019).

In glioblastoma and melanoma brain metastases, treatment with anti-angiogenic drugs induced vessel co-option formation, resulting in anti-angiogenic drug resistance. In addition, treatment with sunitinib and sorafenib (VEGFR inhibitor) was associated with vessel co-option induction and drug resistance in lung metastasis and hepatocellular carcinoma (HCC) respectively (Ma, Pradeep et al. 2018).

It has been shown that tumors with co-opted vessels exhibited up-regulation of Ang2 that initiated an apoptotic cascade, followed by regression of the vessels in the absence of VEGF and angiogenesis in the presence of VEGF. The intense autocrine expression of Ang2 by the tumor ECs enhanced a paracrine stabilization or survival signal via Ang1 expression by normal tissues (Holash, Maisonpierre et al. 1999). The co-opted vessels are usually supported by pericytes for stabilization and increasing anti-angiogenic drug resistance (Qian, Tan et al. 2016). Thus, Angs-Tie2 system is important for vessel co-option mechanism and anti-angiogenic drug resistance.

1.9. Angiopoietins -Tie system overview

In addition to VEGFs and their receptors, the angiopoietins and **T**yrosine kinase with **i**mmunoglobulin and **e**pidermal growth factor homology domains (Tie1-Tie2) receptors describe the second endothelial specific receptor tyrosine kinase (RTK) signaling pathway (Saharinen, Jeltsch et al. 2015). Four secreted vascular growth factors, Ang1, Ang2, & Ang4 (which is the human orthologue of mouse Ang3), have been identified as ligands for the receptor tyrosine kinase Tie2, while Tie1 is an orphan receptor (Salajegheh 2016, Saharinen, Eklund et al. 2017).

Ang1 and Agn2 have been the focus of most studies. Ang3 and Ang4 are not yet fully understood (Saharinen, Eklund et al. 2017). Ang1 and Ang4 are involved in vessel maturation, stabilisation and quiescence after angiogenic processes, while Ang2 and Ang3 promote vascular regression, cell death, and inflammation (Salajegheh 2016).

The Ang-Tie pathway plays a key role in remodeling and maturation of cardiac, blood vascular, and lymphatic vessel development. It maintains vascular homeostasis, which is a fine balance between stabilization and angiogenesis. Blood vessel stabilization requires the coordination of cell-cell adhesion and cell survival. However, angiogenesis relies on the inhibition of cell-cell adhesion as well as induction of endothelial cells migration and proliferation (Fukuhara, Sako et al. 2009). This system regulates pathological endothelial inflammation and vascular leak in various diseases as well as tumor angiogenesis, lymph-angiogenesis, and metastasis (Saharinen, Jeltsch et al. 2015).

1.9.1. Angiopoietins-Tie system biological functions

1.9.1.1. Tie1 and Tie2

Tie1 and Tie2 receptors are found exclusively on the cell surface of endothelial cells (Salajegheh 2016, Saharinen, Eklund et al. 2017). Subsequent studies revealed that Tie2 is also expressed in hematopoietic stem cells (Yano, Iwama et al. 1997), certain monocytes (De Palma, Venneri et al. 2005), neutrophils (Sturn, Feistritzer et al. 2005), eosinophils (Feistritzer, Mosheimer et al. 2004), thyroid follicular cells (Ramsden, Cocks et al. 2001), dorsal root ganglion cells (Kosacka, Figiel et al. 2005), as well as tumor cells including colorectal adenocarcinomas (Nakayama, Hatachi et al. 2005), schwannomas (Nakayama, Inaba et al. 2007), gliomas (Lee, Xu et al. 2006), prostate (Caine, Blann et al. 2003), and non-small cell lung and ovarian cancers

(Takahama, Tsutsumi et al. 1999). Phosphorylation of the receptor tyrosine kinase Tie2 by ligand binding activates phosphatidylinositol 3-kinase (PI3-kinase) and more downstream intracellular pathways that are important for ECs survival, proliferation and migration (Figure 1.7) (Cascone and Heymach 2012). It has been shown that knockout Tie2 in mice is embryonic lethal at day 9.5 due to cardiac and vascular defects (Dumont, Gradwohl et al. 1994). In adult mice, the expression of Tie2 in the quiescent ECs is essential for their integrity and survival (Wong, Haroon et al. 1997). Tie2-deficient mice also display severely impaired definitive hematopoiesis, suggesting that Tie2 mediated signaling is essential for the development of hematopoietic stem cells (Takakura, Huang et al. 1998).

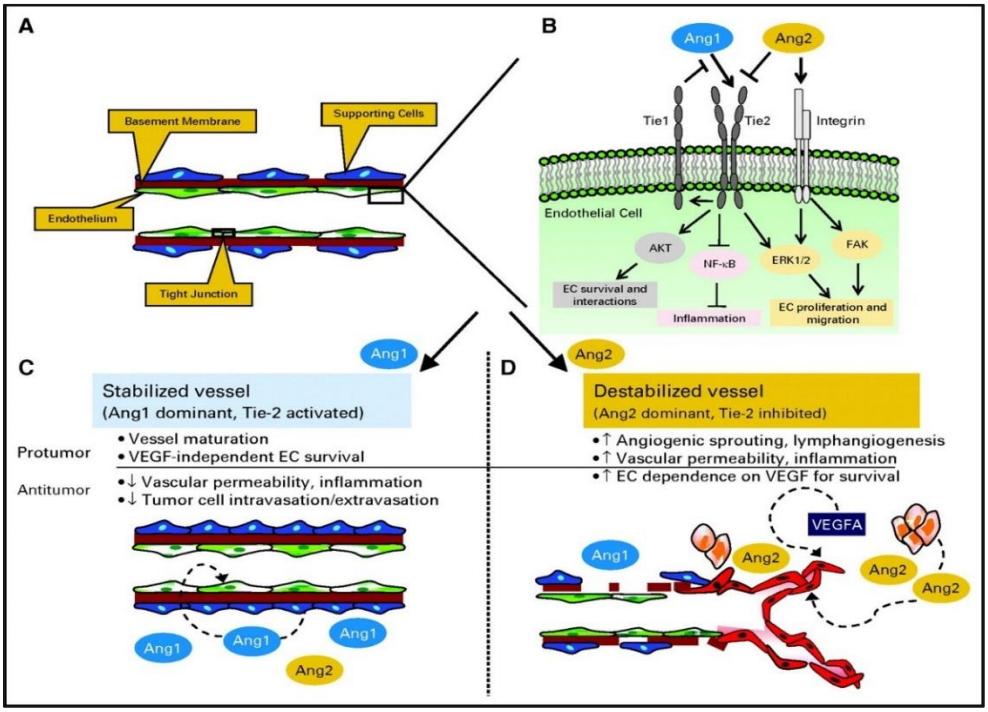

Figure 1. 7, Schematic model represents the role of Angs-Tie system in vascular homeostasis and tumor angiogenesis. (A) Nascent tumor blood vessel structure at quiescent state. (B) Ang1-Tie2 interaction and its inhibitors Ang2 and Tie1. During tumor progression (C) Ang1 stabilizes vessels. However, (D) Ang2 destabilizes vessels in the presence of VEGF-A. Reprinted with permission from Springer Nature, (Cascone and Heymach 2012).

The function of the Tie1 receptor is not yet completely understood, it can heterodimerize with Tie2 to enhance signal transduction (Fagiani and Christofori 2013). Tie1 gene-targeted mouse embryos was lethal at E13.5 due to hemorrhages and impaired endothelial integrity (Puri, Rossant et al. 1995). Tie1 is not required in early angiogenic processes during vasculogenesis, but it plays an important role during the late phases of embryonic angiogenesis, especially in the developing kidney and the brain (Partanen, Puri et al. 1996). Tie1 deletion from the endothelium of adult mice decreased sprouting angiogenesis in the postnatal retinal vasculature, but had little outcome on mature vessels in adult tissues (D'Amico, Korhonen et al. 2014).

Tie1 is also important for lymphatic vascular development. Tie1 mutant embryos were associated with lymphatic vascular abnormalities at E13.5–14.5 and during postnatal (D'Amico, Korhonen et al. 2010, Shen, Shang et al. 2014). The deletion of both Tie1 and Tie2 caused more severe outcomes in stability of the mature vasculature than single gene deletion, and genetic analysis has revealed an absolute requirement for both Tie1 and Tie2 in endothelial cells during late organogenesis and in adult tissues (Puri, Partanen et al. 1999).

1.9.1.2. Ang1

Ang1 is expressed in many adult tissues, smooth muscle cells (SMC) and pericytes that cover and support ECs for Tie2 paracrine signaling (Davis, Aldrich et al. 1996, Sundberg, Kowanetz et al. 2002, Jain 2003). It is also expressed by osteoblasts (Horner, Bord et al. 2001), fibroblasts (Stacker, Runting et al. 2000), hepatic stellate cells (Taura, De et al. 2008), and tumor cells (Huang, Bhat et al. 2010).

Ang1 functions as Tie2 agonist that induces phosphorylation of Tie2 and activates its downstream signaling pathways (Huang, Bhat et al. 2010). These signaling pathways promote ECs

migration (Witzenbichler, Maisonpierre et al. 1998, Jones, Master et al. 1999), sprouting (Kim, Kim et al. 2000), tube formation (Hayes, Huang et al. 1999), and survival (Figure 1.7) (Jones, Master et al. 1999, Kwak, So et al. 1999, Papapetropoulos, Garcia-Cardena et al. 1999).

Ang1-Tie2 signaling activates PI3-kinase and subsequently promotes phosphorylation of protein kinase B (AKT), which is crucial in cell survival through the induction of the endothelial nitric oxide synthase (eNOS). PI3-kinase can induce high expression of survival factors and inhibit pro-apoptotic factors such as caspase-9 and BCL2 associated agonist of cell death (BAD). In addition, activation of AKT inhibits phosphorylation of forkhead transcription factor (FOXO1), which is a transcription factor for Ang2 expression. Thus, Ang1 induces a negative feedback loop on production of Ang2 by endothelial cells. Ang1 also activates extracellular signal–regulated kinase (Erk) pathway for EC proliferation, as well as focal adhesion kinase (FAK), docking protein 2 (Dok-R) and ras-related C3 botulinum toxin substrate 1 (Rac1) pathways for EC migration (Figure 1.7) (Fukuhara, Sako et al. 2009).

VEGF is a proinflammatory cytokine that induces the expression of adhesion molecules on ECs to facilitate the binding of leukocytes to the ECs and induction of inflammation. Ang1-Tie2 signaling counteracts VEGF function via several mechanisms. Activation of Ang 1 - Tie2 signaling mediates vessel maturation by enhancing platelet-derived growth factor phosphorylated (PDGFR-P)-dependent pericyte recruitment, inhibiting inflammation and preventing VEGF-induced vessel permeability (Gamble, Drew et al. 2000, Kim, Ryu et al. 2002, Huang, Bae et al. 2009). It has been reported that Ang1 inhibits VEGF-induced expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin adhesion molecules that are required for leukocyte- EC attachment (Kim, Moon et al. 2001).

In contacting ECs, Ang1 interacted with Tie2 forming a receptor complex and bridged Tie2 molecules from opposing endothelial cells in EC-EC junctions (Fukuhara, Sako et al. 2008). It has been reported that Ang1 maintained vascular integrity and reduced vascular leakage by strengthening the junctional complexes platelet EC adhesion molecule-1 (PECAM-1) and VE-cadherin. Furthermore, Ang1 suppressed the tumor necrosis factor-alpha (TNF-alpha) stimuli which induced the transmigration of leukocytes from the blood into the tissues to promote inflammation and vessel permeability (Gamble, Drew et al. 2000). Thus, Ang1 acts as an anti-inflammatory factor and counteracts VEGF-induced inflammation.

Ang1 stimulates the expression of collagen IV, a major component of basement membranes (BMs), which plays a vital role in blood vessels structure and function (Kalluri 2003, Eklund and Saharinen 2013). Endothelial cell junctions facilitate the interaction with the surrounding cells allowing passage of plasma and cellular material into the cells. Ang1 regulates interactions between ECs and the extracellular matrix (ECM). Ang1 binds to the ECM promotes trans-association of Ang1-Tie2 complex to cell-ECM attachments. Vascular endothelial protein tyrosine phosphatase (VE-PTP) associates with Ang1-Tie2 signaling causing dephosphorylation of Tie2 and inhibition of the signaling (Eklund and Saharinen 2013).

It has been reported that deletion of Ang1 in mice was embryonic lethal by day 12.5 or postnatal, due to vascular abnormalities comparable to Tie2 knocked down mice, i.e., with dilated vessels and a decreased complexity of the vascular network (Suri, Jones et al. 1996, Fukuhara, Sako et al. 2009). Another mouse study has analyzed the effect of deleting Ang1 at each embryonic day. Embryo at E10.5 showed vascular abnormalities in several organs, remarkably in the liver and kidney. A greater number as well as dilated vessels were detected in the liver, whereas glomerular basement membrane and microvasculature beds of the glomeruli have displayed

abnormalities in the kidney. Thus, reorganisation and maturation of the vessels are associated with Ang1 during development and deleting of it in mice, causing vascular deficiencies, poor patterning and death (Jeansson, Gawlik et al. 2011).

Deletion of Ang1 after E13.5 had no impact on normal vascular homeostasis and was not required under normal conditions, but it was necessary to control pathological angiogenic responses and fibrosis after injury or during microvascular stress (Salajegheh 2016).

In mice, conditional overexpression of Ang1 in the liver resulted in disorganization of lymphatic and blood vessel architecture. This disorganization was characterized by an increase in hepatic vein diameter, instances of turbulent venous flow, the proliferation and dilatation of hepatic lymphatic vessels, and enlarged spleens and kidneys. Thus, overexpression of Ang1 in the liver resulted in vascular remodelling that might contribute to the portal hypertension progression (Haninec, Voskas et al. 2006). In another study, overexpression of Ang1 in mouse skin resulted in larger, more numerous, and more highly branched vessels, suggesting that Ang1 can promote angiogenesis (Suri, McClain et al. 1998). In addition, acute administration of Ang1 to adult mice protected adult vasculature from leaking that causes serious problem in many diseases, in contrast to the potentially lethal actions of VEGF and inflammatory agents. Therefore, overexpression of Ang1 *in-vivo* experiments suggested that Ang1 maintains vessel quiescence and inhibits vessel leakage, which could be beneficial treatment for many diseases (Thurston, Rudge et al. 2000).

1.9.1.3. Ang2

Ang2 is mainly expressed in tissues undergoing vascular remodelling, such as placenta, ovary and uterus (Huang, Bhat et al. 2010). It is synthesized by endothelial cells and stored in Weibel-Palade bodies within the cytoplasm until secretion by stimuli for Tie2 autocrine signaling

(Fiedler, Scharpfenecker et al. 2004). It is also secreted by parenchymal cells of the extravascular tissue and some malignant cells for Tie2 paracrine signaling (Salajegheh 2016).

Ang2 functions as an Ang1 antagonist that prevents Ang1-mediated endothelial stabilization. Ang2 responses to pro-inflammatory stimuli, pro-angiogenic cytokines (eg, VEGF) and promotes angiogenesis in tumors by pericytes detachment and blood vessels destabilization. However, in the absence of VEGF, Ang2 promotes endothelial cell apoptosis and consequent blood vessel regression (Figure 1.7) (Huang, Bhat et al. 2010).

Ang2 can act as receptor agonist and induce Tie2 phosphorylation based on the type of stimulation, cell type, and confluence (Eroglu, Stein et al. 2013). The agonistic versus antagonistic function of Ang2 was determined by the occurrence of Tie1 receptor. The cleavage of Tie1 receptor in inflamed endothelium changed the Ang2 activity from agonistic to antagonistic. In Tie1-deficient mice, the agonistic activity of Ang2 was absent (Akwii, Sajib et al. 2019). In addition, VE-PTP can regulate the agonistic and antagonistic function of Ang2 on the Tie2 receptor. *In-vivo,* inhibition of VE-PTP changed Ang2 into an agonist for Tie2 activation in blood endothelium (Souma, Thomson et al. 2018).

Ang2 has proinflammatory actions, Ang1 reduces the actions of Ang2 and other inflammatory factors but, Tie2 may promote both types of actions (Salajegheh 2016).

In mice, overexpression of Ang2 was embryonic lethal and caused severe cardiovascular defects similar to Tie2 and Ang1 knockout mice outcomes (Maisonpierre, Suri et al. 1997). However, Ang2 null mice revealed lymphatic vessel defects, abnormal outgrowth of retinal vasculature, defective response to inflammatory stimuli, and death within 2 weeks after birth (Gale, Thurston et al. 2002, Fiedler, Reiss et al. 2006).

Conditional deletion of both Ang1 and Ang2 in mice resulted in subcutaneous edema in the embryos at E12.5 and eye lymphatic defects more severe than in the Ang2 single knockout mice and similar to those observed in conditional Tie2 deletion, suggesting the importance of Tie2 ligand cooperation in the lymphatic vessel (Thomson, Heinen et al. 2014).

Angs-Tie system can cross talk with multiple integrin heterodimers that are expressed on endothelial cells. Both Ang1 and Ang2 directly binds with different integrin subunits in the presence or absence of Tie2 receptor (Saharinen, Jeltsch et al. 2015).

Ang2 can bind with integrins expressed on endothelial cells with lesser affinity, in comparison to its binding to Tie2, inducing angiogenesis in a Tie2-independent manner. In Tie2-negative ECs (Tie2lo) mice, Ang2 interacted with EC integrins resulting in phosphorylation of the integrin adaptor protein FAK, consequently RAC1 activation, migration, and sprouting angiogenesis of ECs. Blockade of Ang2 affected integrin signaling and prevented FAK phosphorylation and sprouting angiogenesis of Tie2lo ECs (Felcht, Luck et al. 2012).

Ang1 can bind with αvβ5-integrin in the retinal astrocytes and stimulate fibronectin accumulation and radial distribution along the sprouting endothelial cells, resulting in induction of guided angiogenesis in the retina. Thus, Ang1-αvβ5-integrin interaction promotes angiogenesis (Lee, Kim et al. 2013). The integrin dependent functions of angiopoietins should be considered when designing target therapy for cancer or other human diseases.

Ang1 and Ang2 are normally present in blood serum and are generally found at equilibrium in a healthy individual. The blood serum ratio of Ang1 to Ang2 is quite low but elevates in inflammation, vascular regression, tumors and some diseases (Salajegheh 2016). Ang2

can be used as a biomarker of endothelial cell activation in many diseases, such as sepsis, which remains a significant health problem in children (Giuliano, Lahni et al. 2007). The concentration of plasma Ang2 was found significantly higher in patients with colon cancer stage III compared to stage II (Engin, Ustundag et al. 2012).

1.9.2. Angiopoietins-Tie system in cancer

Angiogenesis, one of hallmarks of cancer, is a necessary process for tumor growth. During tumor angiogenesis, first the small tumor combines with an existing blood vessel for nourishment; second, Ang2 expression stimulates tumor vessel regression; then the tumor becomes avascular and undergoes apoptosis causing up-regulation of VEGF expression that promotes angiogenesis at the tumor external margin. Thus activation of Ang2-Tie2 axis in the presence of VEGF is necessary for tumor development and metastasis (Scholz, Plate et al. 2015).

It has been shown that Ang2 is highly expressed in the mouse and human tumor vasculature. Ang2 can activate tumor EC and recruit myeloid cells into the tumor region. These tumor myeloid cells are pro-angiogenic and could be a potential mechanism for anti-angiogenic therapy resistance. Blockade of Ang2 in mouse tumor models did not inhibit the recruitment of pro-angiogenic myeloid cells but promoted the association of these myeloid cells with the tumor vasculature. However, blocking the EC– myeloid cells interaction inhibited the tumor neo-vascularization restoration and therefore induced anti-angiogenic therapy action (Scholz, Plate et al. 2015).

In a liver metastasis mouse model, inhibition of host Ang2 induced granulocyte colony-stimulating factor (G-CSF) expression, larger number of recruited neutrophils and Tie2-expressing monocytes (TEM) recruitment which promoted angiogenesis and tumor growth. The metastatic

colonies formed in the livers of knocked out Ang2 mice were larger and had greater vascular density with more pericyte coverage compared to metastatic colonies in the wild type mice (Im, Tapmeier et al. 2013).

In some tumors, upregulation of Ang2 counteracted anti-angiogenic therapy targeting VEGF. Double inhibition of VEGF and Ang2 in mouse models promoted the effectiveness of anti-angiogenic therapy in those tumors with up regulated Ang2 (Rigamonti, Kadioglu et al. 2014).

In nude mice, overexpression of Ang2 in human colorectal cancer cell line HT29 was markedly increased tumor growth rate, vessel count, and proliferation. However, overexpression of Ang1 in HT29 cell line was accompanied with fewer tumor vessels, consistent with the known stabilizing role of Ang1 (Ahmad, Liu et al. 2001).

There is a discrepancy about the role of Ang1 expression in cancer. The aberrant overexpression of Ang1 remains controversial and is dependent on the type of cancer cells. Overexpression of Ang1 in breast and colorectal cancer cells delayed xenograft tumor growth (Syed, Young et al. 2001, Qian, Tan et al. 2016). In contrast, overexpression of Ang1 in glioblastoma cells resulted in extensive vasculature and accelerated tumor growth (Machein, Knedla et al. 2004). Expression of Ang1 stimulated vessel stabilization which promoted vascular normalization and perfusion, consequently improving the potency of chemotherapy in prostate xenograft (Chakroborty, Sarkar et al. 2011). Overexpression of Ang1 in human squamous cell carcinoma (SCC) was associated with enhanced Tie2 phosphorylation levels and inhibition of tumor growth *in-vivo* (Hawighorst, Skobe et al. 2002). Furthermore, VEGF blockage in tumor induced Ang1 expression that inhibited tumor hypoxia, reduced vessel ablation, and prevented vessel regression (Saharinen, Eklund et al. 2011).

Inhibition of Ang1 expression in HeLa cells reduced tumor angiogenesis, increased tumor cell apoptosis, decreased tumor necrosis and reduced tumor growth (Winston, Ming et al. 2001). Therefore, the expression of Ang1 may promote or inhibit tumor growth according to tumor types and the experimental designs. Despite the research on Ang1 functions, the role of Ang1 in the tumor-associated angiogenesis is still unclear and a direct relationship with tumorigenesis has yet to be proven.

1.9.3. Tie2-expressing monocytes / macrophages (TEMs)

Tie2 is expressed by certain hematopoietic progenitors and subset of circulating monocytes. Tie2 expressing monocytes (TEMs) localize in both perivascular and hypoxic regions of different mouse and human tumors. Macrophages are innate leukocytes which play critical roles in immunological responses and tissue regeneration after injury. Macrophages may polarize into either pro-inflammatory phenotype (M1-like macrophages) or anti-inflammatory phenotype (M2-like macrophages). TEMs are considered proangiogenic and associated with newly formed vessels in mouse tumors. TEMs can display an M2-like macrophages polarization which are known to supress inflammation and induce tissue remodeling and angiogenesis. Co-injection of mouse or human TEMs with cancer cells into mice provoke marked vascularization of formed tumor, suggesting the important role of TEMs in regulating angiogenesis (Guo, Buranych et al. 2013, Lee, Jeong et al. 2019).

It has been shown that Ang2 interacted with Tie2 on TEMs inducing Tie2 activation and upregulation of various tumor promoting factor expressions. Blockade of Ang2 inhibited the activation of Tie2 in TEMs and thereby impairing their role in tumor vasculature (Kiss and Saharinen 2018). Using a Tie2 kinase inhibitor rebastinib reduced pro-tumoral TEMs infiltration,

tumor growth and metastasis in an orthotopic metastatic mouse mammary carcinoma (Harney, Karagiannis et al. 2017). Currently, TEM can be used as a diagnostic marker for angiogenesis in many tumors including hepatocellular carcinoma in the liver (Guo, Buranych et al. 2013).

1.9.4. Angiopoietins in Metastasis

Ang2 has been shown to promote tumor metastasis in numerous studies. Inhibition of Ang2 using Ang2 blocking antibody supressed tumor growth and metastases in MMTV-PyMT mice, which develop mammary tumors metastasizing in lungs (Mazzieri, Pucci et al. 2011). However, Ang2-deficient mice increased metastatic growth of mouse colon carcinoma cells in the liver and tumor angiogenesis (Im, Tapmeier et al. 2013).

Transgenic mice overexpressing endothelial Ang2 decreased endothelial barrier integrity and increased the metastatic burden of B16 melanoma cells in the lungs after their intravenous administration in mice. Additionally, wild type mice treated with adenoviral expressing Ang2 showed increased lymph node and lung metastasis in tumor xenografts. Ang2 blocking antibodies abolished the effect of the Ang2 overexpression by reducing lymph node and lung metastasis as well as decreasing tumor cell metastasis to the lungs after intravenous injection (Holopainen, Saharinen et al. 2012).

Expression of Ang2 has been associated with vessel destabilization and pericyte loss. Inhibition of platelet-derived growth factor receptor b (PDGFR-b) activity using imatinib therapy induced pericyte depletion, inhibited tumor growth, elevated Ang2 expression and increased lung metastasis. Combination of imatinib with Ang2 blockage reduced both metastasis and tumor growth, suggesting that Ang2 promoted metastasis in the tumor with destabilizing vasculature (Keskin, Kim et al. 2015).

It has been reported that Ang1 deficient mice increased lung metastasis without affecting primary tumor growth. The results suggested that Ang1 was required to reduce the attachment and extravasation of tumor cells via pulmonary capillaries (Michael, Orebrand et al. 2017).

Therefore, Ang1 and Ang2 mediated effects on tumors and in vascular vessels of various tissues are complex and we need to carefully evaluate the context-dependent role of Ang1 and Ang2 in each tumor type.

1.9.5. Targeting of the Angs–Tie2 pathway in clinical development

Targeting the Angs-tie2 system can be a perfect candidate for anticancer therapy. In recent years, two strategies are currently used to inhibit this system including, traps (epitopes) targeting Ang1 or Ang2, and tyrosine kinase inhibitors (TKIs) for Tie2 inhibition (Table 1.2) (Huang, Bhat et al. 2010, Saharinen, Eklund et al. 2017).

The peptide– Fc fusion protein (AMG386/trebaninib) which blocks the binding of both Ang1 and Ang2 to Tie2, has struggled in three Phase III advanced ovarian cancer trials (Saharinen, Eklund et al. 2017).

Three human Ang2-targeting monoclonal antibodies MEDI3617, LY3127804, and nesvacumab have been used to neutralize the interaction of Ang2 with Tie2. These antibodies are still in active clinical development in phase I and II clinical trials. Some of these antibodies are tested in combination with chemotherapy and others in combination with VEGF- targeting anti-angiogenic drugs including sunitinib, sorafenib, bevacizumab and aflibercept in cancer patient trails (Table 1.2) (Saharinen, Eklund et al. 2017).

The bispecific Ang2–VEGF targeted antibodies; RG7716 and vanucizumab (RG7221) are potentially promising drugs. Vanucizumab is being evaluated in combination with antibodies

against the inhibitory programmed cell death protein 1 (PD1) immune checkpoint molecule in separate phase I studies (Table 1.2) (Saharinen, Eklund et al. 2017).

Although many studies have reported the efficacy of blocking Ang2 in mouse tumors that is further improved when combined with anti-VEGF drugs or with immune checkpoint treatment, the function of Ang2 as a target, prognostic, or predictive biomarker in human cancer remains to be examined.

Currently, there are a few small-molecules of TKI drugs that inhibit Tie2 in phase I trial (Table 1.2). Some of them also inhibit VEGFR2. Regorafenib, which is a multi-targeted TKI that inhibits Tie2, VEGFRs and some other kinases, is the only drug that achieved regulatory approval as a salvage therapy for gastrointestinal stromal tumors and previously treated metastatic CRC. The contribution of Tie2 inhibition to the efficacy of regorafenib is difficult to evaluate as TKIs are multi-targeted drugs (Saharinen, Eklund et al. 2017).

There is no available drug for targeting only Ang1 in the clinic. In preclinical studies, excess Ang1 expression reduced the efficacy of anti-VEGF and anti-Ang2. Concurrent Ang1 inhibition can be a "doubled-edged sword", with the potential to promote either pro- or antitumor effects. Ang1 inhibition can allow for the regression of existing tumor blood vessels or can remove an endogenous gatekeeper of endothelial barrier function which hinders tumor metastasis to distant organs (Cascone and Heymach 2012). The addition of Ang1 inhibitor pre/post-surgical treatments against micro-metastatic disease is now an area of investigation.

Angiopoietins can be either pro- or antitumorigenic, based on the tumor type, and the best targeting strategy remains unclear. Targeting of Angs-Tie2 pathway is a promising approach in some tumor types as the existing clinical data is encouraging and the current anti-angiogenic

therapies are under serious consideration. It will be essential for antiangiogenic therapies to advance and move beyond VEGF-targeted therapies by using other important pathway inhibitors.

Compound	Description	Phase	Indication	Co-treatment	Refs
ANG1-targeted and ANG2-targeted biologicals					
Trebananib (AMG386)	A peptide–Fc fusion protein (peptibody) that acts by binding both ANG1 and ANG2, thereby preventing their interaction with TIE2	III	Ovarian cancer	Paclitaxel	155,156, 297
		II	RCC	Sunitinib	188
		I	Solid tumours	Bevacizumab, motesanib	298
		Preclinical	Human xenografts	Bevacizumab	71
Nesvacumab (REGN910, SAR307746)	Fully human monoclonal antibody against ANG2 that blocks the binding of ANG2 to TIE2	I	Solid tumours	Aflibercept	299
		Preclinical	Human xenografts	Aflibercept	50
MEDI3617	Fully human monoclonal antibody against ANG2 that blocks the binding of ANG2 to TIE2	I	Solid tumours	Chemotherapy	–
		I	Melanoma	Tremelimumab	–
		Preclinical	Human xenograft and mouse glioma models	Cediranib	66,80, 300
		Preclinical	Cardiac transplantation	None	115
Vanucizumab (RG7221)	Bispecific monoclonal antibody. One arm binds ANG2 and the other binds VEGF (design based on bevacizumab and developed using CrossMab technology)	I	Solid tumours	Atezolizumab	–
		Preclinical	Orthotopic and syngeneic mouse tumours, human cell-line and patient-derived xenografts, transgenic mammary and pancreatic tumour models	Chemotherapy, PD1 antibody	68, 79,306
RG7716	Bispecific antibody targeting both ANG2 and VEGF (developed using CrossMab technology)	II	wAMD and DMO	None	–
		Preclinical	Spontaneous mouse CNV model, laser-induced CNV in non-human primates	None	301
REGN910-3	Fully human antibody against ANG2	I–II	wAMD and DMO	Aflibercept	–
AKB-9778	Competitive VE-PTP inhibitor	II	DMO	Ranibizumab	157,158
		Preclinical	Mouse models of choroidal neovascularization and ischaemic retinopathy	Aflibercept	81
LY3127804	Humanized monoclonal antibody against ANG2	I	Solid tumours	Ramucirumab	–
TIE2 TKIs					
Regorafenib	A small-molecule multi-kinase inhibitor of TIE2, VEGFRs, c-KIT, PDGFRβ, FGFR1, RET, RAF1, BRAF and p38 MAPKs	Approved	GIST and previously treated mCRC	None	302
Rebastinib	A TIE2 kinase inhibitor that also inhibits VEGFR1 and BCR–ABL	I	Breast cancer	Antitubulin therapy	–
Altiratinib (DCC-2701)	A small-molecule inhibitor of MET, TIE2, VEGFR2, TRKA, TRKB and TRKC	I	Advanced-stage solid tumours	None	–
		Preclinical	PyMT mammary tumour model and human xenografts	Bevacizumab	303
ARRY-614	Small-molecule inhibitor of p38 MAPKs and TIE2	I	MDS	None	304

ANG, angiopoietin; CNV, choroidal neovascularization; DMO, diabetic macular oedema; FGFR1, fibroblast growth factor receptor 1; GIST, gastrointestinal stromal tumour; MAPK, mitogen-activated protein kinase; mCRC, metastatic colorectal cancer; MDS, myelodysplastic syndrome; PD1, programmed cell death protein 1; PDGFRβ, platelet-derived growth factor receptor-β; PyMT, polyoma virus middle T antigen; RCC, renal cell carcinoma; TKI, tyrosine kinase inhibitor; TRKA, tyrosine kinase receptor A (also known as high-affinity nerve growth factor receptor); TRKB, tyrosine kinase receptor B (also known as BDNF/NT3 growth factors receptor); TRKC, tyrosine kinase receptor C (also known as NT3 growth factor receptor); VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; VE-PTP, vascular endothelial protein tyrosine phosphatase; wAMD, wet age-related macular degeneration.

Table 1.2, Drugs target Angs-Tie2 system in clinical development. Reprinted with permission from Springer Nature, (Saharinen, Eklund et al. 2017).

Rationale for the study

Our recent data demonstrated that bevacizumab, which is approved for treating CRCLM in combination with chemotherapy, failed to show significant benefits in patients with RHGP. Furthermore, resistance to anti-angiogenic therapy, that was observed in patients with RHGP lesions was found to be mediated by vessel co-option (Frentzas, Simoneau et al. 2016). Evidences have shown Angiopoietins-Tie2 pathway is essential to maintain vessel co-option and resistance to anti-angiogenic therapy. Therefore, it is reasonable to speculate that Angiopoietins-Tie2 pathway may play a role in regulating the vascular angiogenesis and vessel stabilization in CRCLM. A better understanding of Angiopoietins-Tie2 pathway in CRCLM may be of great clinical importance and may lead to the identification of potentially new drug targets, especially for the RHGP lesions which are resistant to anti-angiogenic therapy.

Hypothesis:

The Angiopoietins-Tie2 pathway regulates the vascular type of colorectal cancer liver metastasis (CRCLM).

Aims of the study:

Aim1: Characterize the vasculature of the different HGPs; DHGP and RHGP in human CRCLM tissues using vascular markers (Angiopoietin 1, Angiopoietin 2, and Tie2).

Aim 2: Evaluate the function of the host Ang1 expression in the HGP formation and tumor growth using mouse models *(in-vivo).*

Aim 3: Investigate the impact of knocking out Ang1 expression in mouse hepatocytes on mouse colon cancer cells (*in-vitro)*.

2. Chapter 2: Materials and Methods

2.1. Clinical data

This study included a total of 43 lesions from 43 patients. A prior written informed consent was obtained from all the subjects to participate in this study under a protocol approved by McGill University Health Centre Institutional Review Board (IRB). Clinical data was collected for each patient through the hospital database and medical records including demographics, primary and metastatic disease characteristics, relevant laboratory results, chemotherapy and co-morbidities. The median age of diagnosis was 63 (range 31-81) years. Rectal cancer accounted for 34% of the cases. Approximately two thirds (64%) of the patients had synchronous liver disease. The patients from whom these specimens were collected, were either chemonaïve (no preoperative therapy) or treated preoperatively with a combination of chemotherapy (chemo) and bevacizumab (Bev). Twenty-three lesions (11 DHGP & 12 RHGP) were chemonaïve, 8 lesions (3 DHGP & 5 RHGP) received chemo and 10 lesions (5 DHGP & 5 RHGP) received chemo plus Bev, with an average of 7 cycles (Range 3-28). Estimated 1 and 3-year OS was 100% and 82.6% respectively. Twenty-seven (54%) of patients had recurrence to the liver, estimated 1-year and 3-year disease free survival (DFS) is 49.9% and 44.4% respectively (26.5 months mean follow up duration).

2.2. Hematoxylin and Eosin (H&E) staining

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut into sections 4 μm in thickness using a microtome. Sections were deparaffinized, rehydrated, stained and dehydrated according to the H&E staining optimized protocol. Slides were scanned at 40X magnification using the Aperio AT Turbo system (total magnification of 400X) and viewed using the Aperio ImageScope software program. Stained sections were prepared from all cases for an initial histopathological assessment with a pathologist.

2.3. Immunohistochemical (IHC) staining

FFPE human resected CRCLM and mouse liver with metastatic tumor blocks were used for this study. Serial sections 4 μm thick were cut from each FFPE blocks, mounted on charged glass slides (Superfrost Plus; Fisher Scientific), baked at 65^0C for 1 hr and then stored at 4^0 C until use. For the IHC, sections were rehydrated and exposed to heat-induced epitope retrieval for 20 minutes in a citrate buffer (10 mmol/L citric acid, pH 6.0) using a steamer. Sections were incubated with peroxidase block (Dako) for 20 minutes, followed by incubation in blocking buffer (5% goat serum and 2.5% *Bovine Serum Albumin* (BSA) in 1% Phosphate Buffered Saline with Tween 20 (PBS-T)) for 1 hour at room temperature. Sections were then incubated overnight at 4°C with primary antibody diluted in blocking buffer.

Primary antibodies used include: Ang1 (1:1500, ab102015, Abcam), Ang2 (1:200, ab153934, Abcam), Tie2 (1:1250, PA5-28582, Invitrogen), human CD31 (1:20, M0823, Dako), mouse CD31 (1:50, DIA-310, Optistan), CD45 (1:500, MA5-13197, Dako Cytomation) and Hepatocyte Specific Antigen (1:1500, HSA, SC58693, Santa Cruz). The secondary antibodies were the Dako EnVision plus System- HRP Labelled Polymer Anti-Rabbit (Code K4003) and Anti-Mouse (Code K4007) which were used according to the manufacture's protocol. The sections were counterstained by hematoxylin, rehydrated and mounted by Permount mounting media (SP15-500, Fisher Chemical).

2.4. Scoring of IHC staining

All IHC slides were scanned at 40X magnification using the Aperio AT Turbo system (total magnification of 400X). Images were viewed using the Aperio ImageScope ver.11.2.0.780 software program (Aperio Technologies Inc., Vista, CA) for scoring analysis and assessment of

signals. The positivity [Total number of positive pixels divided by total number of pixels: (NTotal – Nn)/(NTotal)] was chosen to represent the positive staining.

The positivity was assessed with the ImageScope software (algorithm: positive pixel count V9). We randomly selected and averaged three areas for each of four different regions that were representing the central of tumor (CT), periphery of tumor (PT), adjacent normal liver (ADN) and distal normal (DN) (Figure 2.1) (Lazaris, Amri et al. 2018). For each region the algorithm was used to calculate the positivity of the averaged three areas. The average number of each region was exported to an excel file for statistical analysis.

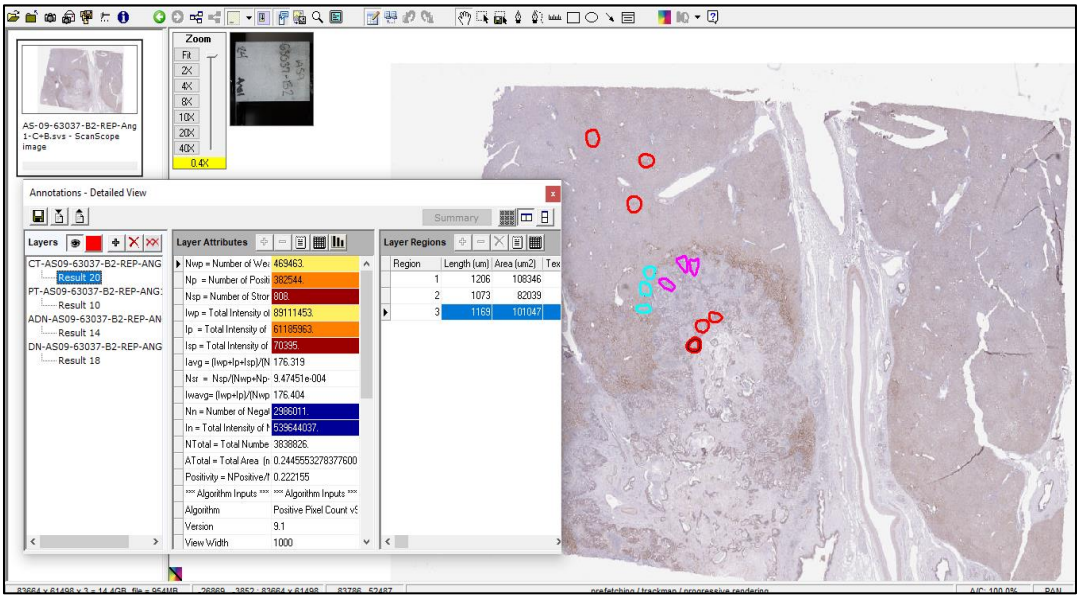

Figure 2.1, Aperio Image Scope software showed the stained slide scan with the annotation and analysis box displaying the results of the selected colored areas. Bottom red circles represent- center of the tumor area, pink circles represent- periphery of the tumor area, Turquoise circles represent- adjacent normal of the tumor area and top red circles represent- distal normal of the tumor area.

2.5. Immunofluorescent (IF) staining

FFPE tissue blocks were cut into sections 4 μm in thickness. Sections were rehydrated and exposed to heat-induced epitope retrieval for 20 minutes in a citrate buffer (10 mmol/L citric acid, pH 6.0) using a steamer. Sections were incubated with peroxidase block (Dako) for 20 minutes, followed by incubation in blocking buffer (5% goat serum and 2.5% BSA in 1% PBS-T) for 1 hour at room temperature. Sections were then incubated overnight at 4°C with primary antibody diluted in blocking buffer. Sections were then washed with 1% PBS-T and incubated with secondary antibody diluted in blocking buffer for 2 hours at room temperature.

The secondary antibodies were a goat anti-rabbit Alexa Fluor 488, and goat anti-rabbit Alexa Fluor 594 conjugated antibodies (1:1000, Molecular Probes, Life Technology). The secondary antibody was removed by washing with 1% PBS-T and subsequently incubated with a 1:1000 dilution of DAPI (Thermo Fisher Scientific, D1306) in PBS for 10 minutes at room temperature. 1-2 drops of ProLong® Gold Antifade Mountant (Thermo Fisher Scientific, P36934) were added to each section prior to mounting under cover glasses. Slides were visualized using Carl Zeiss LSM 700 and Zen software (Zeiss international, Germany).

2.6. Fluorescent in Situ Hybridization (FISH)

The RNAscope assay was performed manually using RNAscope® Multiplex Fluorescent Reagent Kit v2 according to the manufacturer's instructions. Briefly, the FFPE tissue sections were deparaffinized and pre-treated with hydrogen peroxide. Sections were incubated in RNAscope® 1X Target Retrieval Reagent for 15 minutes at 99°C and treated with RNAscope® Protease Plus for 30 minutes at 40°C in the HybEZ oven sequentially. Samples were incubated with RNAscope® Probe- Hs-ANGPT1 482901 for 2 hours at 40°C. After three steps of amplification, RNAscope®

HRP-C1 was added on to the sections that were incubated after that with cyanine-3 for 30 minutes. RNA in-situ hybridization was followed by IF protein staining for the pan-cytokeratin (CK20) (1:250, ab76126, Abcam). Slides were mounted using mounting media (Fluoromount G 4958-02, Invitrogen) and confocal microscopy was performed using Carl Zeiss LSM 700 and Zen software (Zeiss international, Germany).

2.7. Generation of Ang1 knocked out (KO) mice using ROSA-rtTA/ tet O-Cre system

The mice were maintained according to an approved protocol from animal care committee in McGill University Health Center (MUHC, Montreal, Quebec, Canada) and were conducted in accordance with guidelines established by the Canadian Council on Animal Care.

In ROSA-rtTA/ Tet O-Cre transgenic mice, the coding sequence for reverse tetracycline trans-activator (rtTA) was targeted into the ubiquitously expressed *ROSA26* locus. *Cre* recombinase expresses under the control of a tetracycline-responsive promoter element Tet O-Cre. In the presence of tetracycline, or one of its analogs such as doxycycline (Dox), rtTA binds to Tet O-sequence and activates the transcription of Cre. Activated Cre recognizes and binds with two directly repeated DNA fragment sequences called *loxP* and mediates site-specific deletion of DNA sequences between the two *loxP* sites to generate conditional knockout mice (Figure 2.2) (Kim, Kim et al. 2018).

Inducible Ang1 KO mice were obtained from Dr. S. Quaggin (Jeansson, Gawlik et al. 2011). Briefly, the ROSA-rtTA/ Tet O-Cre transgenic system was used to generate inducible whole-body knockout of Ang1 upon administration of Dox (Bioshop, Canada) in drinking water. A BAC recombineering approach was used to generate a floxed Ang1 allele, with loxP sites

inserted around exon 1. Cre-mediated excision of the floxed allele is predicted to generate a null Ang1 allele through frameshift (Figure 2.2).

Floxed Ang1 males (129 background) were crossed with ROSA-rtTA (C57BL/6) females or with Tet-O-Cre (C57BL/6) females. These F1 mixed mice (129/C57BL/6) were then interbred for three generations to generate the experimental Ang1$^{fl/fl}$/Cre/Rosa-rtTA mice in a C57BL/6 background. The animals were kept on Dox in drinking water (2mg/ml) for 4 weeks followed by one week on regular water (Jeansson, Gawlik et al. 2011).

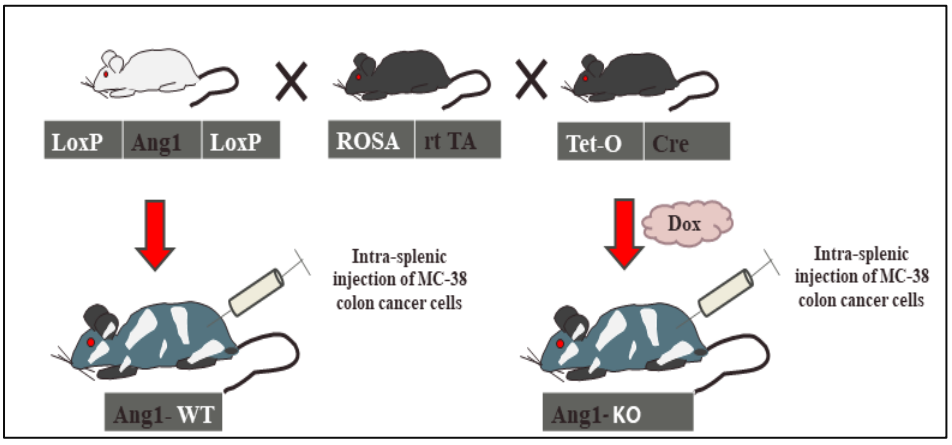

Figure 2.2, Schematic represents conditional Ang1 KO mice generation using ROSA-rtTA/ tet O-Cre system and generation of liver metastases by intra-splenic injection of MC-38 cells into control and Ang1 KO mice.

2.8. Cell culture, mouse experiments and metastasis induction

MC-38 mouse colon cancer cells form RHGP metastatic lesions in the liver of C57BL/6 mice (Lazaris, Amri et al. 2018). MC-38 cells were grown in *Dulbecco's* Modified Eagle Medium (DMEM) medium (Invitrogen) supplemented with 10% heat-inactivated *Fetal Bovine Serum* (FBS) (Hyclone, Logan) and 1% Penicillin-Streptomycin solution (450-201-EL, Wisent

Bioproduct). Liver metastases in control (not treated with Dox) and Ang1 KO mice were generated by intra-splenic injection of 50 µl of DMEM media containing $2x10^5$ viable MC-38 cells, followed by splenectomy 3 min after injection (Ham, Wang et al. 2015). Control females (n = 10) and Ang1 KO females (n=9) were used at 9-11 weeks of age in these experiments. Mice were killed 14–20 days post cells injection when animals in the control group became moribund.

Visible metastases on the surface of the liver were enumerated and sized prior to fixation. Sections of the liver were also fixed in 10% buffered neutral formalin, and paraffin embedded.

Mice were genotyped by PCR using the following primer pairs; Ang1 flox (For 5′-CAATGCCAGAGGTTCTTGTGAA, Rev 5′-TCAAAGCAACATATCATGTGCA, Ang1 wild-type (wt) 233 bp, flox 328 bp), Ang1 del (For 5′-CAATGCCAGAGGTTCTTGTGAA, Rev 5′-TGTGAGCAAAACCCCTT TC, 481 bp), ROSA-rtTA (For 5′-GAGTTCTCTGCTGCCTCCTG, Rev 5′-AGCTCTAATGCGCTGTTAAT), general Cre allele (For 5′-ATGTCCAATTTACTGAC CG, Rev 5′-CGCCGCATAACCAAGTGAA, 673 bp) from (Invitrogen).

Experimental procedures were conducted in compliance with Canadian Committee on Animal Care guidelines in McGill University Health Center (MUHC, Montreal, Quebec, Canada).

2.9. Intrahepatic implantation of tumor fragments

To examine the ability of RHGP tumor to convert into DHGP in the liver. Liver metastases from female control mice (n=3) were used to do intrahepatic implantation of RHGP tumor fragments into female Ang1 KO mice (n=3) and control mice (n=3).

Tumors obtained from control mice dissected into small pieces approximately 2x2x2 mm fragments, washed in PBS and then placed in (DMEM) medium (Invitrogen) supplemented with 10% heat-inactivated *Fetal Bovine Serum* (FBS) and 1% Penicillin-Streptomycin solution. The

mice were anesthetized, and analgesia was administered pre-operatively. The anesthetized mouse was placed on its back and the front side sterilized with Chlorhexidine.

A transverse bilateral subcostal skin incision was made exposing the area under the ribs (2 cm long closed with 3 wound clips). An incision was made in the peritoneal wall, above the liver. Gently the liver was exposed with forceps and stabilized with cotton-tipped applicators. An incision (3 mm in length and depth) was made on the surface of the liver using a sterile No. 10 scalpel blade. Surgicel and gentle pressure was immediately applied to the incision site to achieve hemostasis. Tumor fragments kept in Media containing antibiotics were placed into the liver incision. A small piece of surgicel was applied over the liver incision to prevent displacement of the tumor fragment and ensure continued hemostasis.

The peritoneal wall was sutured, and the skin incision closed with wound clips. Post-operative analgesia was administered once a day for three days. Staples are removed 10 days following the injection.

2.10. Hepatocyte isolation and culture conditions

Hepatocytes were isolated from adult Ang1 KO female mice using the two-step collagenase perfusion method as previously described (Essalmani, Susan-Resiga et al. 2011). Briefly, under anesthesia with 2% isoflurane inhalation, the peritoneal cavity was opened, and the liver was perfused in-situ via the portal vein for 50 ml/10 min at 37°C with calcium-free HEPES buffer I (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES, pH 7.6) containing 0.19 mg/ml EGTA and for 50 ml/10 min, then followed by perfusion with calcium-supplemented HEPES buffer II (4.7 mM $CaCl_2$, 66.7 mM NaCl, 6.7 mM KCl, 100 mM HEPES, pH 7.4) containing 0.5 mg/ml collagenase type V (Sigma Aldrich).

To separate undigested tissue pieces, the suspended hepatocytes were passed through a 70 mm nylon filter into 50 ml Falcon tubes. The cell suspensions were centrifuged twice at 50g for 5 min at 4ºC, and the cell pellet was resuspended in Williams' medium E supplemented with 10% *Fetal Bovine Serum* (Invitrogen) and 1% antibiotic-antimycotic mixture (Gibco, 15240-096). Cells were used only if cell viability was above 80% as assessed by trypan blue exclusion. Cells (1-1.5x10^6) were seeded in Corning cell bind surface polystyrene 6-well plates (Costar, 3335).

After allowing for cell attachment for 2 hours, cells were cultured in high glucose (25mM) DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic mixture overnight. The cells were then cultured in serum free media with/without MC-38 cells in contact (ratio 5 to 1) or using inserts (Falcon; 353090) so the cells do not contact each other (Figure 2.3). After 24hrs, the conditioned media from the different conditions were collected and frozen. These different conditioned media were then added to the MC-38 cells, incubated for 24hrs and then the cells were harvested and analyzed.

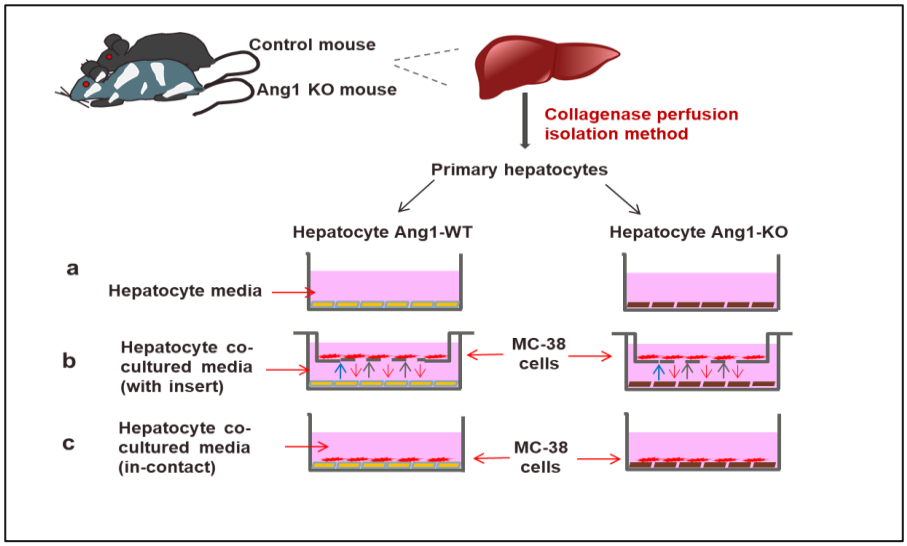

Figure 2.3, Schematics of different culture methods of isolated primary hepatocytes in serum free media. a) Hepatocytes cultured in serum free media, b) Hepatocytes co-cultured with MC-38 cells using insert, c) Hepatocytes co-cultured in close contact with MC-38 cells.

2.11. Western blot

Primary hepatocyte cells lysate was prepared in ice-cold Radio-Immunoprecipitation Assay (RIPA) lysis buffer. (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris, pH 8.0). Protein content was quantitated with the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher, 23227) following the manufacturer's guide. Protein was resolved by 10% sodium dodecyl sulphate polyacrylamide gels and electrophoresis was carried out using running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3) for 90 mins at 100V.

The protein then transferred onto polyvinylidene difluoride (PVDF) membrane by electrophoresis using transfer buffer (48 mM Tris, 39 mM glycine and 20% methanol, pH 9.2) at constant 100V for 2 hr. After transfer, the blots were blocked with 5% non-fat milk dissolved in the tris-buffered saline with Tween-20 (TBS-T) buffer (145 mM NaCl, 20 mM Tris-base, 0.5% Tween 20, pH 7.5) for 1 h at room temperature with shaking. The Ang1 primary antibody and Anti-GAPDH (1:2000, Abcam, ab9485) which acts as a loading control, were hybridized overnight at 4 °C. The blots were then washed with TBS-T and then the secondary antibody was added (1:5000, Rabbit IgG HRP; Bio-Rad, 170-6515) and incubated for one hour at room temperature.

The protein blot was then visualized using the Pierce enhanced chemiluminescence ECL Western Blotting Substrate (Thermo, 32106) for detection of horseradish peroxidase (HRP) enzyme activity and digital image acquisition using the Image-Quant LAS 400 camera system. We used ImageLab (Biorad) software to measure the band intensity. Both blots were normalized to their respective GAPDH signals. The normalized signals confirmed inhibition of Ang1 protein expression in the isolated Ang1 KO hepatocytes compared to the control hepatocytes and increasing of Ang1 protein expression in the control hepatocytes co-cultured with MC-38 cells

using insert compared to the control hepatocytes cultured alone.

2.12. MTT cell proliferation and viability assay

MTT cell proliferation assay was used to quantify cell proliferation and viability. The assay is based on the conversion of water soluble MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to an insoluble formazan product.

MTT is broken down to formazan, which has a purple color, by the mitochondrial dehydrogenase succinate-tetrazolium reductase, which is present in viable cells with active metabolism. Dead cells lose this ability and do not show signal. Thus, the formed color represents the proportional number of viable cells (Berridge, Herst et al. 2005).

Cell viability was assessed using an MTT assay (Abcam, ab211091) kit according to the manufacture's protocol. Briefly, MC-38 cells were seeded in 96-well plates at a density of 1×10^3 cells/well and cultured in 200µl DMEM supplemented with 10% FBS. Following overnight starvation in the different hepatocyte conditioned media (see Figure 2.3). Conditioned media were discarded, and serum free media were added as well as MTT reagent. The plate was incubated at 37ºC for 3 hours then MTT solvent was added, and the plate was agitated for 15 minutes. The absorbance was measured in an ELISA microplate reader at 562 nm (Tecan infinite M200 pro, Switzerland). Control conditions consisted of MC-38 cells alone which had an equal volume of serum-free medium.

Data are expressed as the mean of a minimum of three independent experiments performed in triplicate. The cell survival equation was determined by; averaging the triplicate reading for each sample, subtracting the serum free medium reading for the assay reading to obtain the corrected reading. The survival equation used was: % of cell survival = (100 x (control – sample)) / control.

2.13. Scratch cell migration assay

A monolayer of MC-38 cells was grown to 70% confluence in a 6-well plate, and at experimental time zero a scratch was made in each well using a pipette tip.

The cells were cultured in the different conditioned media (Figure 2.3) and imaged at time zero and again 24 hours later. A measurement was taken for how much the denuded area had filled in the 24 hours period using a light microscope a Nikon eclipse TS2 microscope (Nikon, USA).

2.14. Boyden chamber invasion assay

In vitro invasion assay was measured using a Matrigel invasion chamber with 8µm pores (Corning, 354483). Briefly, MC-38 cells (1×10^5) were cultured in different conditioned media and placed into upper individual Boyden chamber (Figure 2.4). The DMEM medium containing 10% FBS was placed in the lower chamber to facilitate chemotaxis (Figure 2.4).

Invasion assays were run for 24 hours, the cells passed through the Matrigel membrane and attached to the bottom of the Boyden chamber membrane. Non- invasive cells stay in the upper chamber of the Boyden chamber membrane and removed by cotton swab (Figure 2.4).

The invaded cells were stained with 0.1% crystal violet in 20% methanol. Representative images of invaded cells were taken using a light microscope a Nikon eclipse TS2 microscope (Nikon, USA).

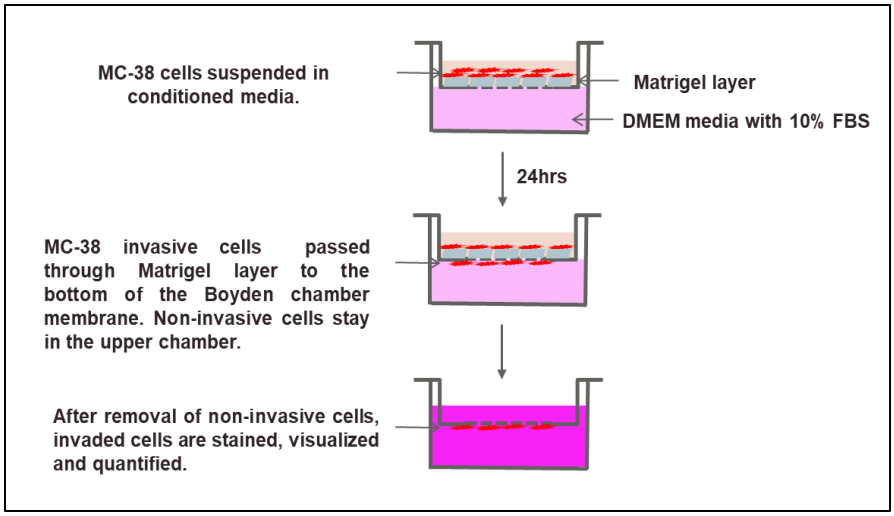

Figure 2.4, Schematic shows the principle of Boyden chamber invasion assay.

2.15. Statistical analysis

Statistical analysis was performed with a two-tailed Fisher's exact test or a two-tailed Student's t-test using GraphPad Prism 6 and Microsoft Excel. Data are presented as a standard error of the mean +/− SEM. P-values < 0.05 were considered significant.

3. Chapter 3: Results

3.1. Expression of vascular factors Ang1, Ang2 and Tie2 in chemonaïve CRCLM human lesion samples.

3.1.1. Expression of Ang1 in chemonaïve RHGP and DHGP lesions.

To assess the vascular marker Ang1 expression in chemonaïve CRCLM, IHC analysis was performed on twenty-three lesions of CRCLM with DHGP (n=11) and RHGP (n=12).

In chemonaïve RHGP lesions, we observed higher levels of Ang1 expression in the cytoplasm of hepatocytes adjacent to the tumor compared to the cytoplasm of tumor epithelial cells and to the cytoplasm of hepatocytes at the distal normal of the liver (i.e., hepatocytes distant from tumor cells) (Figure 3.1 A, B, C). Positive staining for Ang1 expression was also found in the blood vessel walls, as expected and thus served as an internal positive control (Figure 3.1 B). To our knowledge, this is the first time that Ang1 expression has been demonstrated in hepatocytes.

However, in DHGP lesion, Ang1 was shown to be expressed by the cytoplasm of tumor epithelial cells but, the cytoplasm of hepatocytes adjacent to the desmoplastic ring did not exhibit increased expression of Ang1 compared to distal normal liver and as was observed in the RHGP lesion (Figure 3.1 D, E).

To further quantitate these findings to confirm their significance, the positivity of the IHC staining was quantified in three areas for each of four different regions that were representing the CT-Center of the tumor, PT-Periphery of the tumor, ADN-Adjacent normal, and DN-Distal normal by digital analysis using the algorithm of Aperio ImageScope software (Figure 2.1). The average number of the positivity for each region was exported to an excel file for statistical analysis.

In RHGP lesions, the expression of Ang1 in adjacent normal hepatocytes was significantly higher compared to its distal normal and adjacent normal hepatocytes of DHGP lesions (P-value < 0.005) (Figure 3.1 G). In addition. The expression of Ang1 was significantly up-regulated in the tumor regions (CT and PT) of RHGP lesions compared to tumor regions (CT and PT) of DHGP lesions (P-value < 0.05) (Figure 3.1 G). Higher levels of Ang1 expression was detected in the

periphery of the tumor compared to the center tumor region however, this could be due to the infiltrating hepatocytes that expressed Ang1. No significant difference was found between the expression of Ang1 in distal normal hepatocytes of RHGP and DHGP lesions (Figure 3.1 C, F, G).

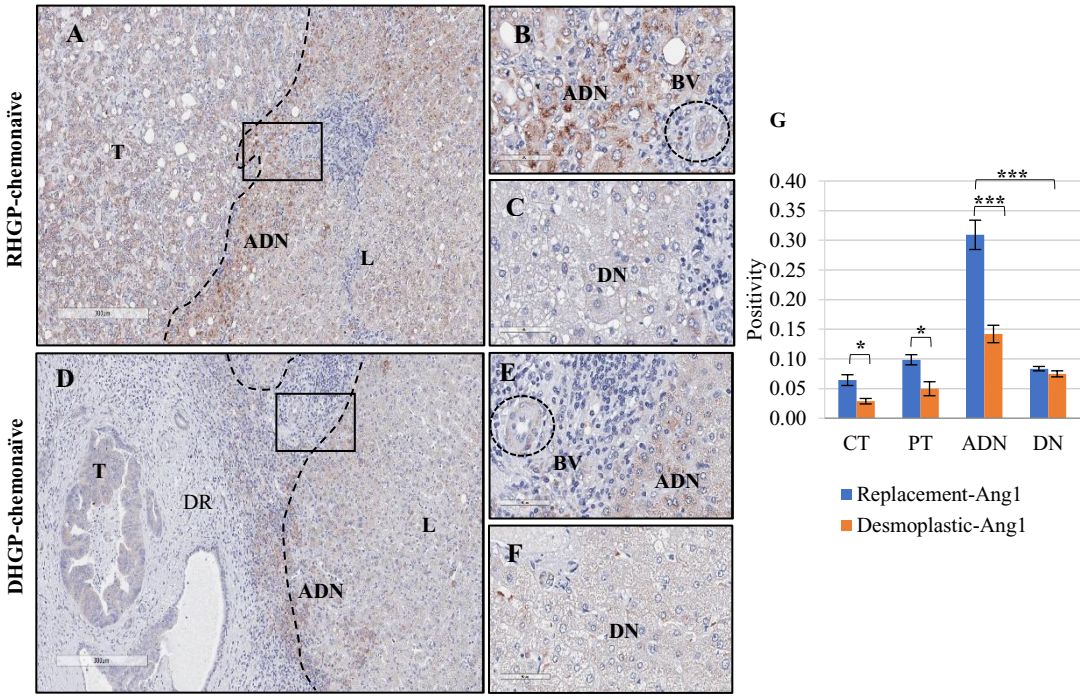

Figure 3.1, IHC staining of Ang1 in RHGP and DHGP chemonaïve lesions (A-F). Positivity representing positive pixels staining quantified with Aperio software (G). T-tumor, L-liver, BV-Blood vessel, CT-Center of the tumor, PT-Periphery of the tumor, ADN-Adjacent normal, DN-Distal normal, DHGP ring-DR, dashed-lines represent the border between tumor region and livers, data are represented as the mean +/− SEM, and * significant P-value < 0.05.

3.1.2. Expression of Ang1 in hepatocytes at the adjacent normal in chemonaïve RHGP lesions.

Since Ang1 is a secreted protein, we therefore performed a quantitative Fluorescent in Situ Hybridization (FISH) double staining using a probe specific for Ang1 mRNA followed by

immunofluorescent staining for a cancer cell marker CK20 protein. The location of Ang1 gene expression was assessed using microscopic imaging.

In RHGP lesions, a significant of Ang1 mRNA expression was highly detected in the hepatocytes (yellow signal) at the interface region compared to the tumor regions (CT and PT) (Figure 3.2). Tumor region is shown by tumor marker CK20 protein expression (green signal) that co-localized with low signal of Ang1 gene expression (yellow signal) (Figure 3.2). Therefore, high expression of Ang1 gene at the adjacent normal of RHGP lesions using FISH staining validated the data obtained from Ang1 IHC staining of RHGP lesions.

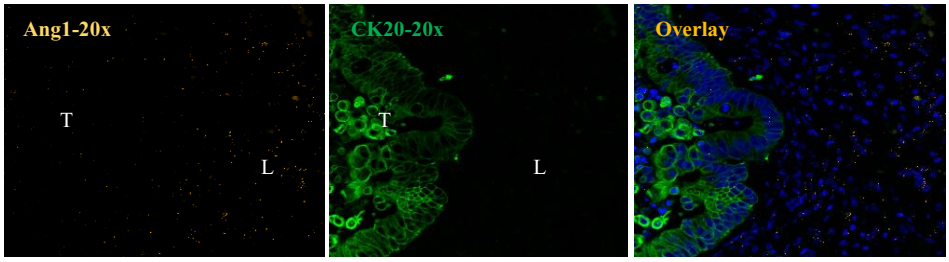

Figure 3.2, Fluorescent in Situ Hybridization (FISH) double staining indicates higher Ang1 mRNA (yellow signal) in hepatocyte region compared to tumor region stained by tumor marker CK20 protein (green signal). T-Tumor, L-Liver.

Furthermore, immunofluorescent co-staining of the hepatocyte specific antigen marker (HSA) and Ang1 proteins demonstrated co-localization of the HSA (green signal) and Ang1 (red signal) staining in the cytoplasm of hepatocytes adjacent to tumor region (Figure 3.3 A). Ang1 (red signal) was also highly expressed adjacent to the blood vessels marker (CD31) (green signal) near the tumor region thus, more mature blood vessels were formed at the adjacent normal of RHGP lesions (Figure 3.3 B).

It has been reported that Ang1 can stimulate the expression of αSMA1 via activation of hepatic Stellate Cells (HSCs) (Novo, Cannito et al. 2007, Kang, Gores et al. 2011). In the present study, *α*SMA1 positive cells were detected in perivascular region and associated with high expression of Ang1 at the adjacent normal of RHGP lesions (Figure C).

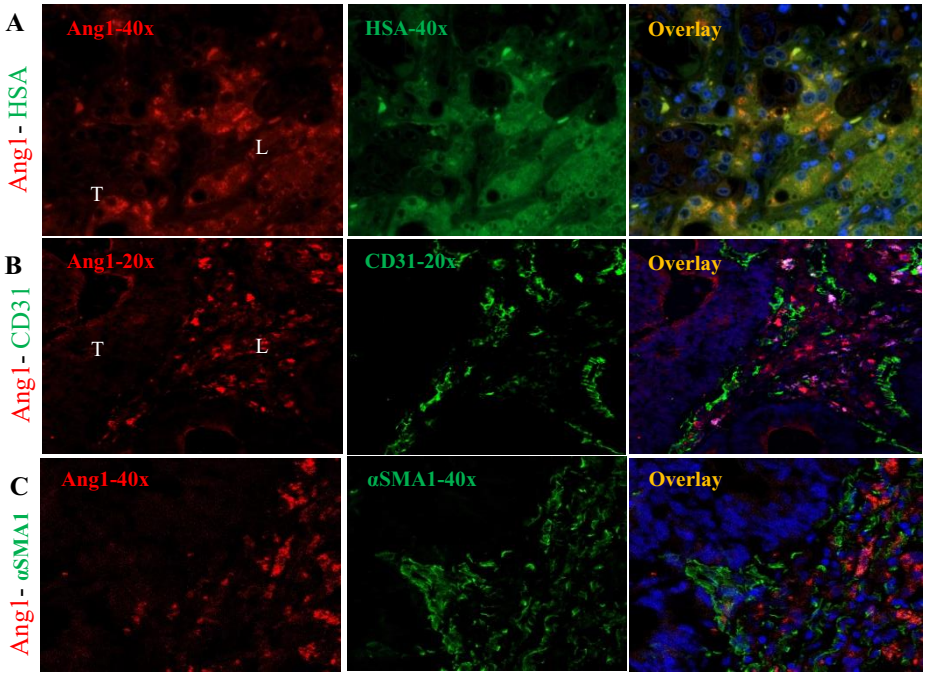

Figure 3.3, Immunofluorescent staining showed (A) co-localization of Ang1 (red signal) and HSA hepatocyte marker (green signal), (B) high expression of Ang1 (red signal) adjacent to CD31 blood vessel marker (green signal) and (C) high expression of Ang1 (red signal) and αSMA1 (green signal) at the adjacent normal in RHGP chemonaïve lesions. T- tumor, L-liver.

3.1.3. Expression of Ang2 and Tie2 in chemonaïve RHGP and DHGP lesions.

Ang2 was observed expressed in the cytoplasm of tumor epithelial cells at the periphery and center regions in the RHGP and DHGP lesions (Figure 3.4 A, B). Quantification of the positivity of the Ang2 IHC staining by Aperio ImageScope software showed that the expression of Ang2 in the center region of the RHGP lesions was significantly higher than its periphery region

and to the center region of the DHGP lesions (P-value < 0.005) (Figure 3.4 C). No significant difference was found between the expression of Ang2 in the adjacent normal and distal normal hepatocytes of RHGP and DHGP lesions (Figure 3.4 C).

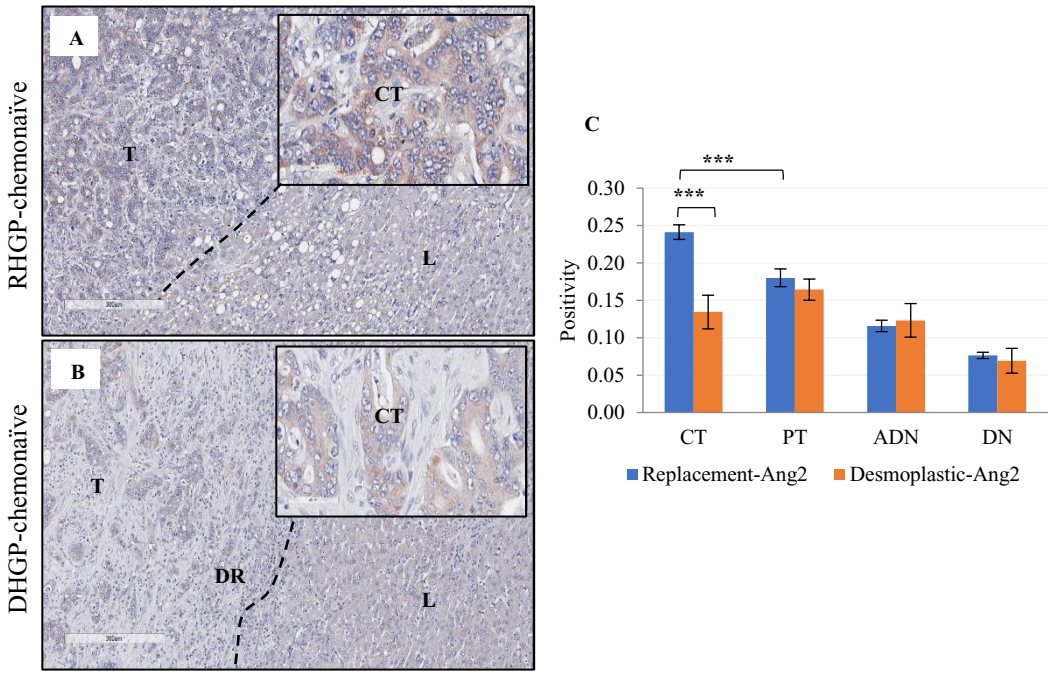

Figure 3.4, IHC staining of Ang2 in RHGP and DHGP chemonaïve lesions (A-B). Positivity representing positive pixels staining quantified with Aperio software (C). T- tumor, L-liver, CT-Center of the tumor, PT-Periphery of the tumor, ADN-Adjacent normal, DN-Distal normal, DHGP ring-DR, dashed-lines represent the border between tumor region and livers, data are represented as the mean +/− SEM, and * significant P-value < 0.05.

Tie2 was expressed in the ECs, and cytoplasm of tumor epithelial cells at the periphery and center regions of the RHGP and DHGP lesions (Figure 3.5 A, B). In digital analysis, the positivity of Tie2 expression was significantly elevated in the center of the tumor, periphery of the tumor, and adjacent normal regions of RHGP lesions compared to tumor regions (CT, PT) and adjacent normal region of DHGP lesions (P-value < 0.005) (Figure 3.5 C). No significant difference was found between the expression of Tie2 in distal normal hepatocytes of RHGP and

DHGP lesions (Figure 3.5 C). Thus, Ang2 and Tie2 are expressed in the tumor regions (CT, PT) and ECs without expression in the hepatocytes.

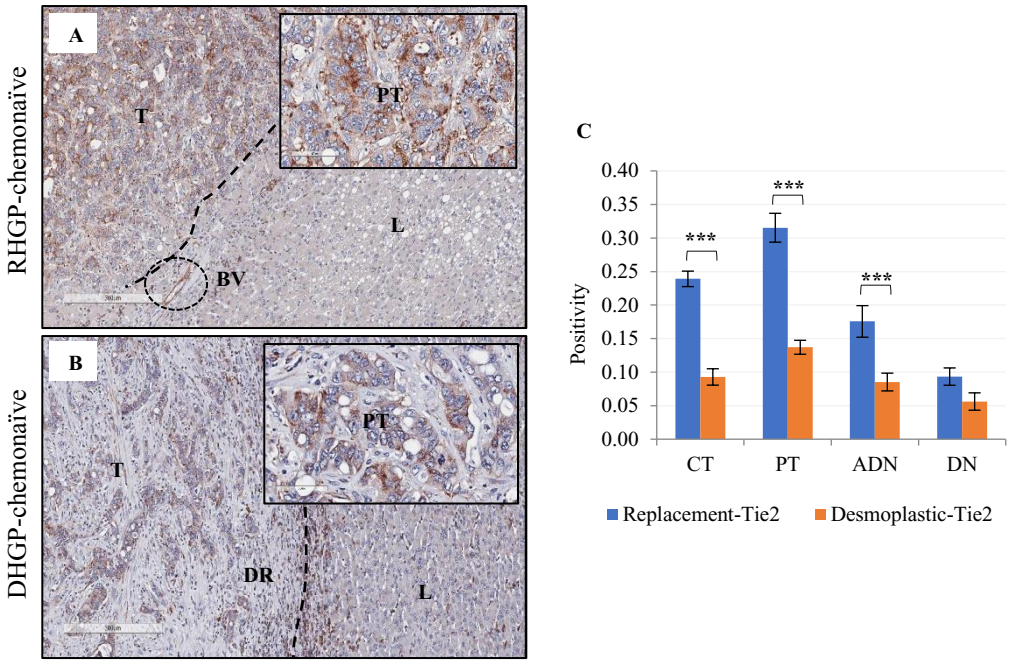

Figure 3.5, IHC staining of Tie2 in RHGP and DHGP chemonaïve lesions (A-B). Positivity representing positive pixels staining quantified with Aperio software (C). T-tumor, BV-Blood vessel, CT-Center of the tumor, PT-Periphery of the tumor, ADN-Adjacent normal, DN-Distal normal, DHGP ring-DR, dashed-line represents the border between tumor region and livers, data are represented as the mean +/− SEM, and * significant P-value < 0.05.

3.1.4. Expression of Tie2 in leukocytes in chemonaïve RHGP and DHGP lesions.

Another unique feature observed during our staining was the expression of Tie2 in different cell types. Tie2 was highly expressed in blood vessels, tumor epithelial cells and immune cells in the RHGP and DHGP lesions. The expression of Tie2 was high in leukocytes which were positive for CD45, and were abundant around the DHGP ring but, randomly distributed in the

adjacent normal of RHGP lesions (Figure 3.6 B). The positivity of leukocytes expressing Tie2 was included in the Tie2 IHC staining quantification.

Immunofluorescent co-staining was performed to confirm the co-localization between Tie2 and CD45 (Figure 3.6 C). These cells could be lymphocytes or/and macrophages more analysis for these cells are currently performed to characterize them.

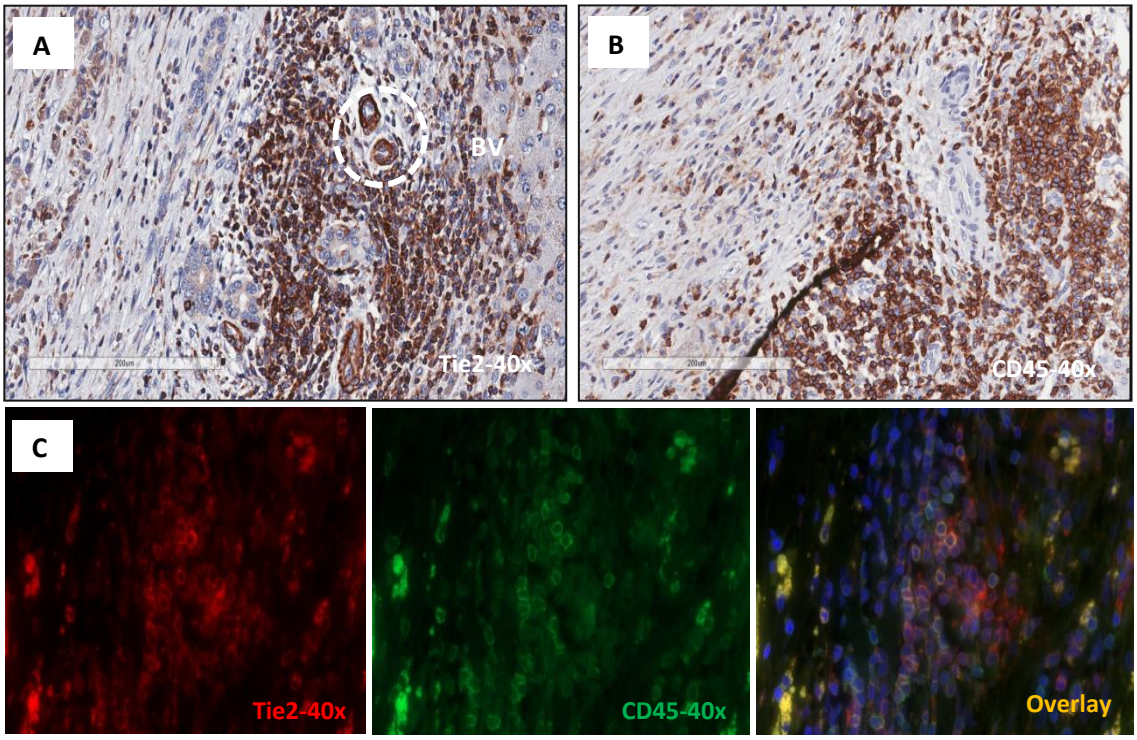

Figure 3.6, IHC staining for (A) Tie2 and (B) CD45 in serial sections of human chemonaïve DHGP lesions. (C) Immunofluorescent co-staining of Tie2 and CD45 in DHGP ring. BV-blood vessel.

3.2. Expression of Ang1, Ang2 and Tie2 in treated (chemo and chemo plus Bev) CRCLM human lesion samples.

3.2.1. Expression of Ang1, Ang2 and Tie2 in treated (chemo and chemo plus Bev) RHGP human lesion samples.

It has been proposed that anti-VEGF (Bev) treatment normalizes (stabilizes) tumor blood vessel structure by activating Ang1-Tie2 signaling. Ang1 promotes vessel normalization in the tumor microenvironment by increasing pericyte coverage, reducing vascular leakiness and interstitial fluid pressure IFP resulting in improved blood flow and tumor perfusion in brain tumors (Winkler, Kozin et al. 2004).

Patients with RHGP lesions did not respond to the addition of anti-angiogenic treatment which could negatively affect outcomes of the patients who developed anti-angiogenic drug resistance. In order to evaluate the impact of chemo and chemo plus Bev treatments on the expression of Ang1, Ang2 and Tie2, we determined the expression of these vascular factors after treatment in RHGP lesions using IHC staining and digital analysis.

We identified the expression of Ang1 in CRCLM after treatment with chemo and chemo plus Bev. We used 10 RHGP lesions that were treated with Chemo (n=5) and chemo plus Bev (n=5) For IHC staining. Using Aperio ImageScope software, the positivity of Ang1 expression was found still high at the adjacent normal of RHGP lesions treated with chemo and chemo plus Bev, with no significant difference whether the samples were chemonaïve or treated (Figure 3.7). However, the positivity of Ang1 was significantly up-regulated in the distal normal of the liver of RHGP lesions treated with chemo and chemo plus Bev compared to RHGP chemonaïve lesions ($P < 0.05$) (Figure 3.7). Furthermore, comparison of chemonaïve and chemo RHGP lesions showed that the expression of Ang1 was significantly increased in the center of the tumor after treatment (P-value < 0.005) (Figure 3.7).

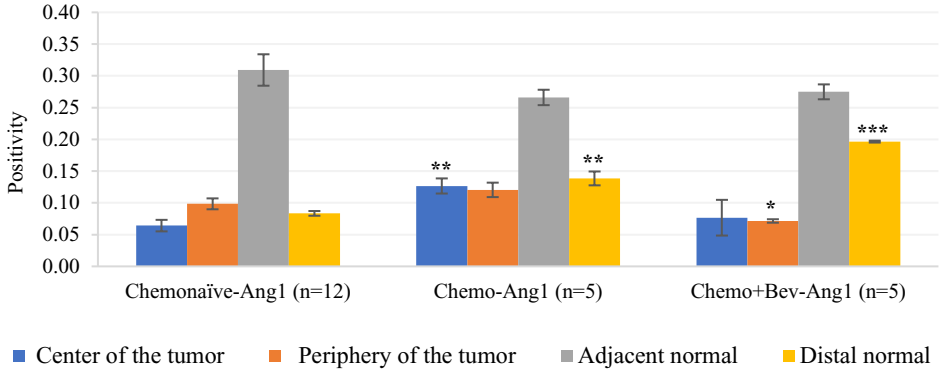

Figure 3.7, Quantification of Ang1 staining in RHGP chemonaïve, chemo and chemo plus Bev lesions. Positivity representing positive pixels staining of Ang1 quantified with Aperio software. Data are represented as the mean +/− SEM, and * significant P-value < 0.05.

We also quantified the expression of Ang2 and Tie2 in RHGP after treatment with chemo and chemo plus Bev using Aperio ImageScope software.

The positivity of Ang2 expression was significantly down regulated in the RHGP tumor regions (CT and PT), adjacent normal and distal normal of the liver of chemo treated RHGP lesions compared to chemonaïve RHGP lesions (P-value <0.05) (Figure 3.8 A). However, the positivity of Ang2 expression was significantly down regulated in the periphery of the tumor and adjacent normal of RHGP lesions treated with chemo plus Bev compared to RHGP chemonaïve lesions (P-value <0.05) (Figure 3.8 A).

The positivity of Tie2 expression was significantly induced in tumor regions (CT, PT) of chemo plus Bev RHGP lesions compared to RHGP chemonaïve lesions (P-value < 0.05) (Figure 3.8 B). Therefore, the expressions of Ang1 in the host liver and Tie2 in tumor regions (CT, PT) were significantly increased after chemo plus Bev treatment in RHGP tumor lesions.

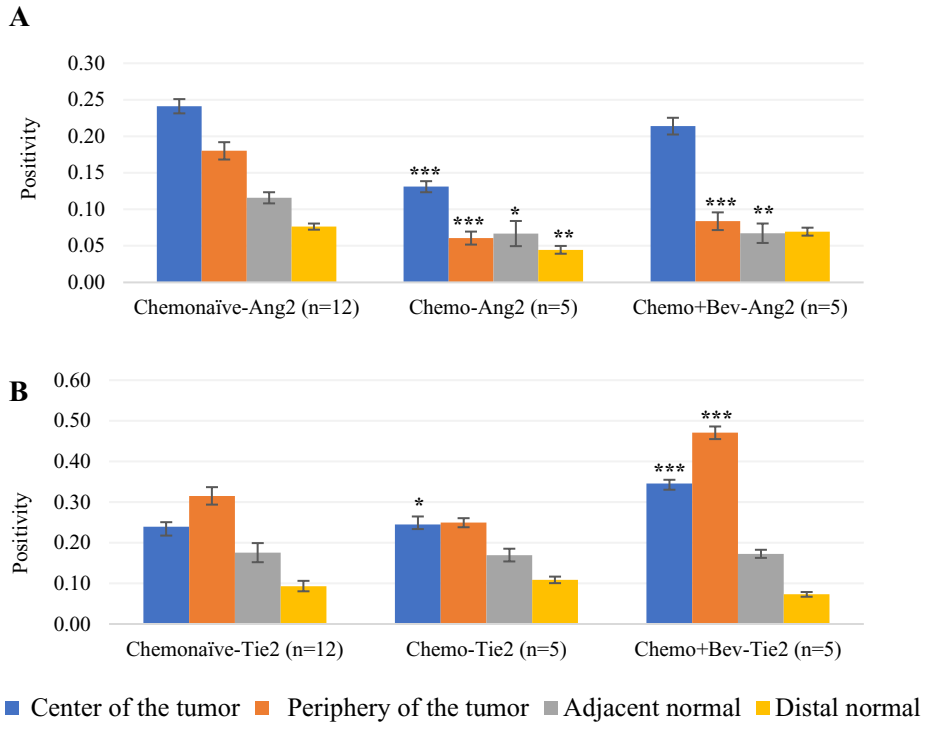

Figure 3.8, Quantification of Ang2 (A) and Tie2 (B) staining in RHGP chemonaïve, chemo and chemo plus Bev lesions. Positivity representing positive pixels staining of Ang1 quantified with Aperio software. Data are represented as the mean +/− SEM, and * significant P-value < 0.05.

3.2.2. Expression of Ang1, Ang2 and Tie2 in treated (chemo and chemo plus Bev) DHGP human lesion samples.

Despite the tumors with DHGP responded to chemo plus Bev treatment, we measured the expression of Ang1, Ang2 and Tie2 in DHGP cancer cells remaining after treatment with chemo (n=3) and chemo plus Bev (n=5) using Aperio ImageScope software. It is important to note that the chemo plus Bev DHGP lesions had very few to no viable tumor cells. The remaining DHGP tumor cells were found only in the periphery region attached to desmoplastic ring.

The positivity of Ang1 expression was significantly increased in distal normal of chemo treated DHGP lesions compared to chemonaïve DHGP lesions (P-value < 0.05) (Figure 3.9 A). In addition, the positivity of Ang1 expression was significantly induced in adjacent normal, distal normal and tumor region remaining after chemo plus Bev treatment in DHGP lesions compared to chemonaïve DHGP lesions (P-value < 0.05) (Figure 3.9 A). Thus, Ang1 is important for surviving of DHGP tumor cells remaining after treatment with chemo plus Bev.

The positivity of Ang2 expression was significantly increased only in the center of the tumor chemo treated DHGP lesions and periphery region of the tumor treated with chemo plus Bev in DHGP lesions compared to chemonaïve DHGP lesions (P-value < 0.05) (Figure 3.9 B).

The positivity of Tie2 expression was significantly up-regulated after chemo treatment in all regions of DHGP lesions compared to chemonaïve DHGP lesions (P-value < 0.05) (Figure 3.9 C). However, the positivity of Tie2 expression was significantly up-regulated at the periphery region of the remaining tumor after treatment with chemo plus Bev in DHGP lesions compared to chemonaïve DHGP lesions (P-value < 0.05) (Figure 3.9 C).

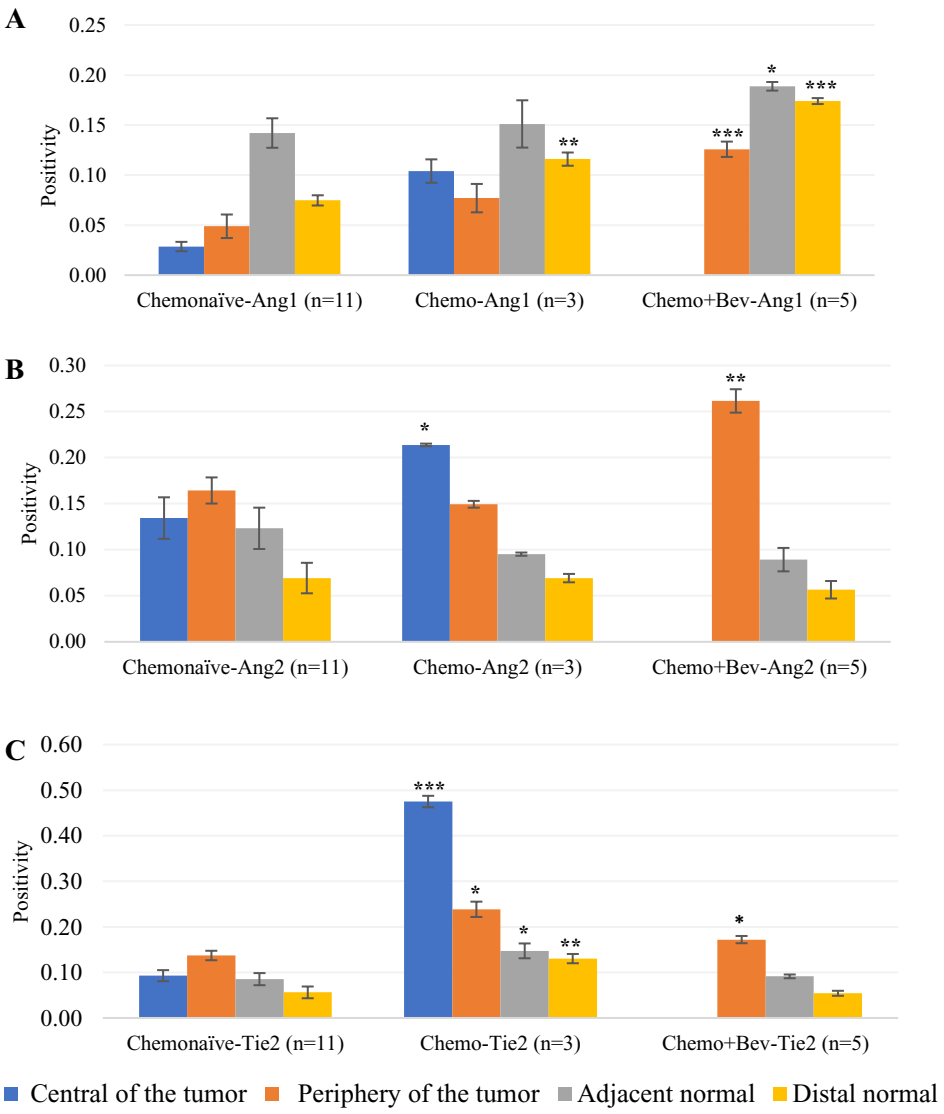

Figure 3. 9, Quantification of Ang1 (A), Ang2 (B) and Tie2 (C) staining in DHGP chemonaïve, chemo and chemo plus Bev lesions. Positivity representing positive pixels staining of Ang1, Ang2 and Tie2 quantified with Aperio software. Data are represented as the mean +/− SEM, and * significant P-value < 0.05.

3.3. The impact of the host Ang1 expression deficiency *in-vivo.*

3.3.1. Ang1 expression deficiency inhibits liver metastasis and impacts HGP *in-vivo.*

Ang1 was shown differentially expressed in the adjacent normal liver tissues of replacement HGPs; we asked whether the host Ang1 expression deficiency could affect tumor growth or maintenance of the HGPs in a mouse liver metastasis model. We used a conditional Ang1 KO mouse model to perform intra-splenic injections of MC-38 colon cancer cells.

In control mice, MC-38 colon cancer cells developed liver metastases that expressed Ang1, Ang2 and Tie2 (Figure 3.10 A, B, C). We also observed a direct correlation between the expression of Ang1 in liver and the size of the nearby tumor i.e., the larger the size of the tumor the more hepatocytes expressed Ang1 (Figure 3.10 D-F).

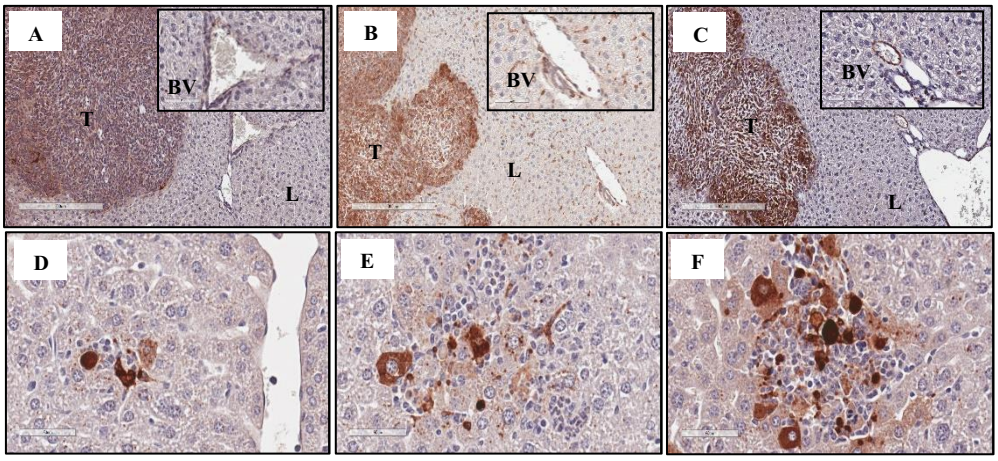

Figure 3.10, IHC staining of (A) Ang1, (B) Ang2 and (C) Tie2 expressions in RHGP mouse tumors developed from MC-38 cells metastasized into control mouse liver. (D-F) Ang1 IHC staining in hepatocytes surrounding tumor cells of control mouse livers. T-Tumor, L-Liver and BV-Blood vessel.

A higher number of control mice (7/10, 70%) developed metastases with multiple larger lesions (Figure 3.11 A, B) compared to Ang1 KO mice (2/9, 22%) that formed metastatic lesions

with smaller size and number (Figure 3.11 A, B). Assessing the HGP using H&E staining showed that all the control mice formed lesions with replacement HGPs (Figure 3.11 C) whereas, the Ang1 KO mice developed mostly lesions with desmoplastic HGPs (Figure 3.11 D) or no lesion.

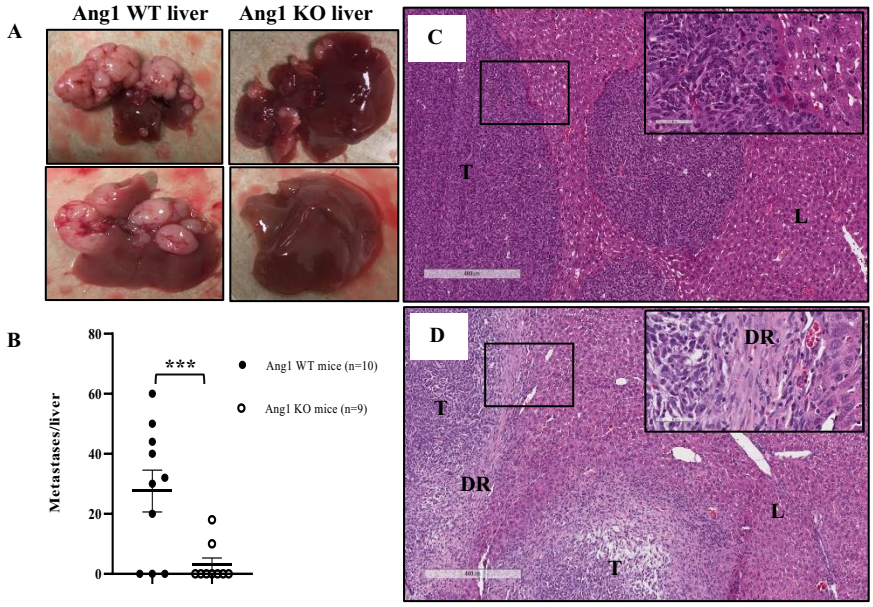

Figure 3.11, Liver metastatic lesions and H&E staining of metastatic MC-38 mouse colon cancer cells. (A) Metastatic foci and (B) number of lesions formed in control and Ang1 KO livers. H&E staining showed (C) RHGP lesions developed in control mouse liver and (D) DHGP lesions formed in Ang1 KO mouse liver. Tumor-T, Liver-L, DHGP ring-DR, data are represented as the mean +/− SEM, and * significant P-value < 0.05.

We asked a question whether tumor with RHGP can be grown and converted to DHGP tumor in Ang1 deficient mouse liver. To answer and validate the change in the HGP formation, intra-hepatic transplantation of RHGP tumor fragments into Ang1 KO mice was performed. Control mice (n=3) were injected intra-splenically with MC-38 colon cancer cells to develop tumor with RHGP lesions. Tumors with RHGP were dissected into small fragments which were transplanted after that into control (n=3) and Ang1 KO (n=3) mouse livers. Control mice (n=2)

developed tumors with larger size (Figure 3.12 A) compared to Ang1 KO mouse (n=1) which formed tumor with smaller size (Figure 3.12 B). H& E staining was performed to assess the HGPs for the mice that developed tumors. Tumor fragments transplanted into control mouse livers grew and developed tumor with RHGP (Figure 3.12 C). However, the tumor fragment that grew in Ang1 KO mouse formed tumor with DHGP (Figure 3.12 D).

Thus, this experiment validated that Ang1 expression in the host liver is important for tumor with RHGP phenotype formation in colorectal cancer liver metastasis mouse models.

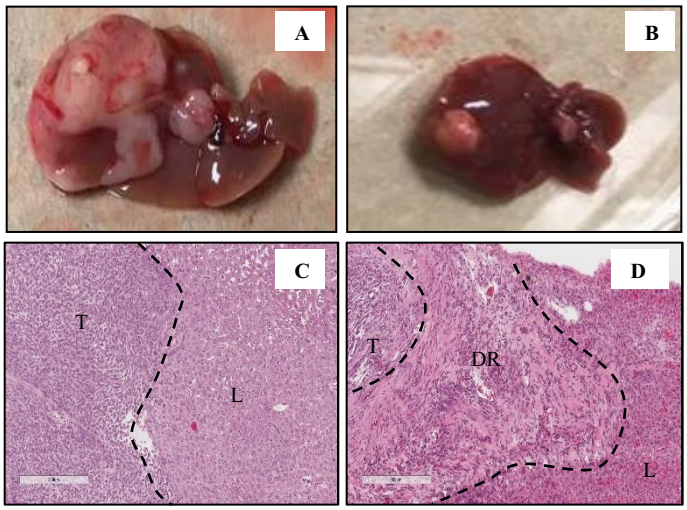

Figure 3.12, Liver lesions and H&E staining of transplanted RHGP tumors. Transplanted RHGP tumor grew in (A) control mouse and (B) Ang1 KO mouse. H&E staining for (C) RHGP tumor developed in control mouse and (D) DHGP tumor developed in Ang1 KO mouse. Dashed-lines represent the border between tumor region and livers. Tumor-T, Liver-L, DHGP ring-DR.

3.3.2. The expression of Ang1 in control and Ang1 KO mice.

The expression of Ang1 in the host liver of control and Ang1 KO mice was assessed by IHC and qPCR (Figure 3.13). In control mice, IHC staining demonstrated that the expression of Ang1 was found high in tumor regions, hepatocytes at the adjacent normal of the tumor and blood

vessels (Figure 3.13 A). However, we observed low levels of Ang1 expression in the liver of the Ang1 KO mice that developed small metastatic lesions (Figure 3.13 B). This expression may be due to incomplete KO of the Ang1 expression and limitation of using Cre and lox system. Mice with higher KO efficacy in the blood vessel walls and the hepatocytes did not develop tumor in the liver. The expression of Ang1 gene in control and Ang1 KO mice was quantified using qPCR. The expression of Ang1 gene was significantly elevated in the control mouse livers injected with MC-38 cells compared to control mouse livers without injection and metastases (P-value < 0.05) (Figure 3.13 C). However, the expression of Ang1 gene in Ang1 KO mouse livers was down regulated compared to control mouse livers without injection and metastases.

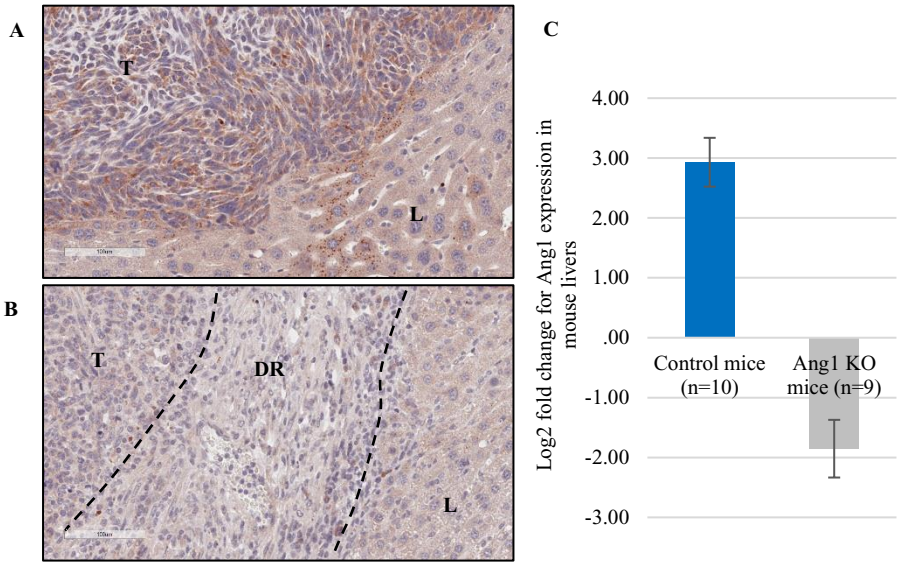

Figure 3.13, Expression of Ang1 in control and Ang1 KO mouse livers. IHC staining of Ang1 in hepatocytes at the adjacent normal of (A) control mouse liver and (B) Ang1 KO mouse liver that developed DHGP tumor. (C) qPCR for expression of Ang1 gene in control and Ang1 KO mouse livers. T-Tumor, L-Liver, DR-DHGP ring, dashed-lines represent the border between tumor region and livers, data are represented as the mean +/− SEM, and * significant P-value < 0.05.

3.3.3 Reduction of Ang1 expression inhibits mature blood vessel formation *in-vivo.*

It is possible that tumor cells metastasize into the liver stimulate Ang1 expression in their environment, which is important for blood vessel formation and growth in the tumor. The number of blood vessels that stained with mature blood vessel marker CD31 in the tumor of the control mice (n=7) was significantly higher than the number of blood vessels in the tumor developed from Ang1 KO mice (n=2) (P-value < 0.05) (Figure 3.14 A, B, C). The blood vessel numbers were manually counted, using a 20x field, 10 regions per lesion and then the average was determined for each group. In our human and mouse liver metastases data, we observed that high expression of Ang1 in the hepatocytes was correlated with disorganization of the liver vasculature (some sinusoidal dilation) (Figure 3.14 D, E) consistent with a previous study of conditional Ang1 overexpression in the liver of mice led to changes in lymphatic and blood vessel architecture (Haninec, Voskas et al. 2006).

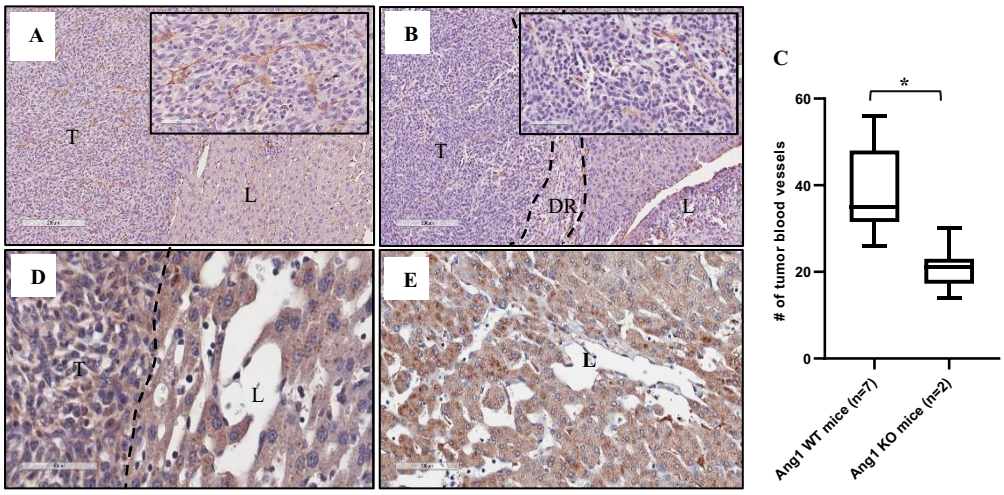

Figure 3.14, IHC staining of CD31 and Ang1 in livers. CD31 IHC (A) in control mouse tumor and (B) in Ang1 KO mouse tumor. (C) Blood vessel counts in control and Ang1 KO mice. Expression of Ang1 in the hepatocytes of (D) mouse and (E) human LM samples. T-Tumor, L -Liver, DHGP ring-DR, dashed-lines represents the border between tumor region and livers, data are represented as the mean +/− SEM, and ***** significant P-value < 0.05.

3.4. The effect of down regulation of Ang1 expression in hepatocytes on colon cancer cells *in-vitro.*

3.4.1. Ang1 expression is upregulated in hepatocyte cells upon co-culture with the colon cancer MC-38 cells *in-vitro.*

To confirm *in-vivo* findings that the interaction between tumor cells and hepatocytes leads to an increase in Ang1 expression in hepatocytes, primary hepatocytes from female Ang1 WT (control) (n=2) and Ang1 KO (n=3) mice were isolated. We first examined the percentage of Ang1 knock-out in hepatocytes harvested from the livers of mice that were induced with Dox. Ang1 KO mouse hepatocytes had approximately 60% reduction of Ang1 expression as shown by qPCR and western blot using ImageLab software (Figure 3.15 A, B). To test whether Ang1 expression in control hepatocytes may be affected by the tumor cells interaction, we co-cultured control and Ang1 KO primary hepatocytes with MC-38 cells using inserts. Strikingly, co-cultured with MC-38 cells strongly increased the expression of Ang1 in the control hepatocytes (lane 3) compared to control hepatocytes cultured alone with only serum free medium (lane 1) (Figure 3.15 C).

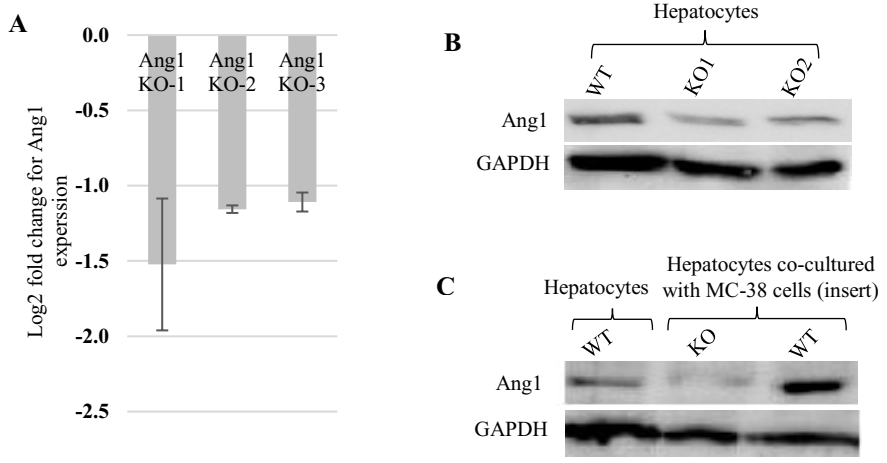

Figure 3.15, Expression of Ang1 in isolated hepatocytes. (A) qPCR of Ang1 mRNA expression in isolated hepatocytes. Western blot of Ang1 protein expression in (B) isolated hepatocytes and (C) hepatocytes cultured in serum free medium alone or co-cultured with MC-38 cells using insert, and data are represented as the mean +/− SEM.

3.4.2. Effect of Ang1 expression in hepatocytes on MC-38 cells viability *in-vitro.*

The effect of Ang1 inhibition in hepatocytes on the viability of MC-38 cells was examined after incubation of MC-38 cells for 24 hrs in conditioned media obtained from Ang1 KO hepatocytes, cultured in serum free media with or without MC-38 cells. MC-38 cells incubated in serum free media served as a control for the experiment. Employment of the MTT assay revealed significant inhibition of MC-38 cell viability cultured in conditioned media obtained from Ang1 KO hepatocytes cultured in direct contact with MC-38 cells compared to conditioned media from Ang1 WT hepatocytes (P-value < 0.05) (Figure 3. 16 A, B). However, no significant decrease in viability was found when MC-38 cells were cultured in conditioned media obtained from Ang1 KO hepatocytes cultured alone or Ang1 KO hepatocytes co-cultured with MC-38 using inserts.

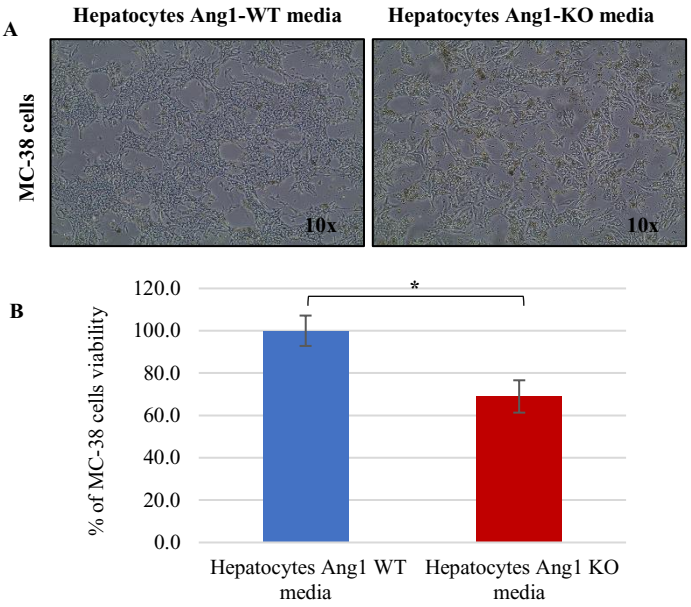

Figure 3.16, MC-38 cell viability. (A) Phase contrast microscopy of MC-38 cells cultured in hepatocyte Ang1 WT and Ang1 KO conditioned media (yellow floating cells represent dead cells). (B) MTT assay for MC-38 cells cultured in control or Ang1 KO hepatocyte media, data are represented as the mean +/− SEM, and * significant P-value < 0.05.

3.4.3. Effect of Ang1 expression in hepatocytes on MC-38 cells migration and invasion *in-vitro.*

We also evaluated the effect of the control and Ang1 KO hepatocytes conditioned media on MC-38 cells invasion using Boyden chamber assay and the ability of cells to migrate using scratch assay. In invasion assays, the MC-38 cells passed through the Matrigel membrane and attached to the bottom of the Boyden chamber membrane were counted. The number of MC-38 cells that invaded the Matrigel and migrated through the pore in the membrane, was markedly lower when MC-38 cells cultured in conditioned media obtained from Ang1 KO hepatocytes (Figure 3.17 A, B). In addition, culturing MC-38 cells with conditioned media obtained from Ang1 KO hepatocytes dramatically reduced their migration abilities (Figure 3. 17 C). However, MC-38 cells cultured in conditioned media obtained from control hepatocytes showed migration and invasion abilities (Figure 3.17 A, B, C).

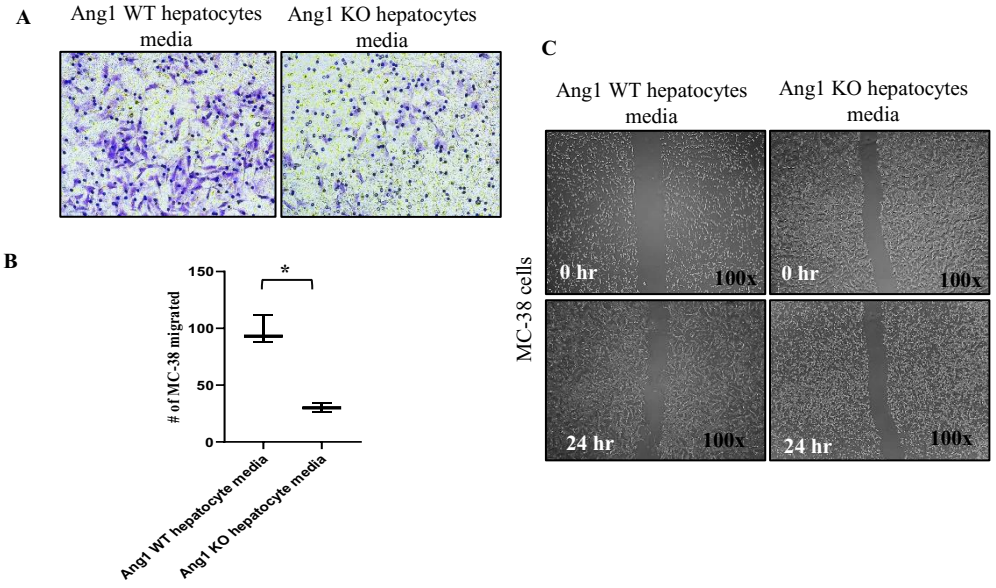

Figure 3.17, Invasion and Migration assays of MC-38 cells cultured with hepatocytes Ang1 WT and Ang1 KO conditioned media, data are represented as the mean +/− SEM, and * significant P-value < 0.05.

4. Chapter 4: Discussion

Histopathological growth patterns of liver metastases have exposed distinct means of vascularization, which correlates with the patient OS and response to anti-angiogenic treatment. In DHGP and PHGP lesions, the cancer cells utilize angiogenesis to obtain a vascular supply however, the cancer cells co-opt pre-existing blood vessels in RHGP lesions. Recently, our group has shown that the patients with RHGP lesions who received chemo plus Bev had a worse pathological response and OS than those with DHGP lesions receiving the same treatment. However, there was no difference in OS when patients with DHGP and RHGP lesions were treated with chemotherapy alone (Frentzas, Simoneau et al. 2016).

Anti-angiogenic treatments seek to block tumor proliferating blood vessels to inhibit tumor growth. Most clinically approved anti-angiogenic drugs target VEGF pathway, which has been identified as a major driver of tumor angiogenesis. However, tumor vessel co-option has been implicated in mediating resistance to anti-angiogenic (anti-VEGF) drug in various tumor types, including breast cancer, glioblastoma and CRCLM (Comunanza and Bussolino 2017). As vessel co-option mechanisms have been reported to support tumor growth, it becomes essential to characterize the vasculature of different tumor types with vessels co-option using different vascular markers.

In our lab, we are trying to understand the mechanisms regulating the HGPs of CRCLMs with a focus on RHGP tumor with vessel co-option, which mediates ant-angiogenic drug resistance. RHGP and DHGP lesions have been stratified based on the expression of vascular markers that are required for vessel formation. We have previously shown that DHGP lesions contain immature blood vessels whereas RHGP lesions contain mature blood vessels, supporting the role of vessels co-option (Lazaris, Amri et al. 2018). We used CD31 staining and Aperio ImageScope software digital analysis to evaluate the microvessel density (MVD) and blood vessel

maturity in DHGP and RHGP. We found that MVD was significantly higher in RHGP lesions compared to DHGP lesions in chemonaïve and treated (chemo and chemo plus Bev) CRCLM samples. In addition, staining of alpha-smooth muscle actin1 (αSMA1), a marker for differentiated vascular mural cells, was determined higher in RHGP lesions compared to DHGP lesions. Both DHGP and RHGP lesions have shown similar levels of VEGF staining, primarily tumor cells. However, a higher number of tumor proliferating blood vessels was found only in chemonaïve DHGP lesions compared to chemonaïve RHGP lesions using IHC co-staining of CD34/Ki67 which presents a marker for proliferating blood vessels. Thus, RHGP lesions were characterized with more mature blood vessels than DHGP lesions, which had more immature blood vessels in chemonaïve CRCLMs (Lazaris, Amri et al. 2018).

In treated (chemo plus Bev) CRCLM lesions, the number of vessels did not change in RHGP lesions. However, there was a dramatic reduction of vessels in the DHGP lesions, indicating the efficacy of anti-angiogenic drug on DHGP lesions compared to RHGP lesions. Therefore, bevacizumab that targets VEGF is effective on DHGP, as has been shown in our patient cohort (Frentzas, Simoneau et al. 2016). Based on this data, we speculate that the lack of anti-angiogenic drug efficacy on RHGP lesions could be due to a low number of immature vessels and high numbers of mature blood vessels, which could induce the effectiveness of chemotherapy alone (Lazaris, Amri et al. 2018).

Vessel co-option in tumors stimulates the expression of Ang1, Ang2 and VEGF to induce angiogenesis and tumor growth. Therefore, Angs-Tie2 pathway plays a role in maintaining the vessel co-option process. Angs-Tie2 pathway represents an attractive therapeutic target as it is not only crucial for vascular homeostasis, but it also provides an important link between angiogenic and inflammatory pathways (Huang, Bhat et al. 2010).

In the current study, we have further investigated other vascular factors involved in vessel co-option. We performed IHC staining on FFPE human DHGP and RHGP lesions to the vascular markers Ang1, Ang2 and Tie2 antibodies. In order to compare our previous vascular characterization using CD31, VEGF and CD34/Ki67 results with the current study results, we used serial sections from the same samples used in our previous paper (Lazaris, Amri et al. 2018).

4.1. RHGP lesion characterized by high expression of Ang1 in the hepatocytes adjacent to the tumor in chemonaïve lesion samples.

In chemonaïve DHGP and RHGP human lesions, our data demonstrated a distinct expression pattern of Ang1, Ang2 and Tie2. We observed significantly higher expression of Ang1 in the hepatocytes adjacent to the tumor in RHGP lesions compared to DHGP lesions using Aperio ImageScope software digital analysis (Figure 3.1 G). Furthermore, a marked infiltration of Ang1-expressing hepatocytes was observed in the tumor region of RHGP compared to DHGP tumor, as shown by co-localization of the hepatocyte marker HSA and Ang1 in immunofluorescent staining. FISH experiments showed that Ang1 mRNA was highly expressed in the adjacent hepatocytes compared to the tumor region of the RHGP lesions. The signal observed for Ang1 expression was therefore due to *de novo* synthesis and not uptake of secreted Ang1. Tie2 expression was found significantly elevated in the adjacent normal (ADN) and tumor regions (CT and PT) of RHGP lesions compared to DHGP lesions, consistent with the role of the strong agonist Ang1 in up-regulating Tie2 expression. Ang2 expression was shown in the tumor regions (CT and PT) and blood vessels in RHGP lesions.

In DHGP chemonaïve lesions, Ang1 was shown to be expressed by the tumor cells (Figure 3.1 D), but the hepatocytes adjacent to the desmoplastic ring did not exhibit increased expression

of Ang1, contrary to what was observed in the RHGP lesions. Ang2 and Tie2 expressions were shown in the tumor regions (CT and PT) and blood vessels in DHGP chemonaïve lesions.

It has been reported that overexpression of Ang1 in the hepatocytes of mice led to sprouting, dilation, and disorganization in lymphatic and blood vessel architecture in the mouse livers (Haninec, Voskas et al. 2006). Overexpression of Ang1 can inhibit or stimulate tumor growth depending on tumor type. A previous study has shown that high expression of Ang1 is associated with a large vascular lumen and highly branched vessels that increase tumor perfusion and growth *in-vivo* (Suri, McClain et al. 1998, Hayes, Huang et al. 2000). Furthermore, high expression of Ang1 caused ECs proliferation and enlargement of pre-existing vessels that might contribute in blood supply maintenance during VEGF blockade (Huang, Bae et al. 2009). In addition, hepatic stellate cells (HSCs), which are known as liver pericytes, are not active in normal livers. They become activated aHSCs, proliferated and expressed αSMA1 in response to inflammation, injuries and mechanical stimulation. Accumulated data suggests that aHSC promote tumor cell migration, growth and survival by increasing the deposition of extracellular matrix. Additionally, the paracrine effect of Ang1 stimulated proliferation, migration of aHSCs and infiltration of mesenchymal cells into the tumor region resulting in stabilization of tumor edge-associated blood vessels (Novo, Cannito et al. 2007, Kang, Gores et al. 2011).

A number of studies have shown that presence of inflammation (leukocytes) is often accompanied with tumor angiogenesis (Grivennikov, Greten et al. 2010). In DHGP, the abundance of leukocytes at the stromal ring and the expression of Tie2, Ang2 and VEGF (Lazaris, Amri et al. 2018) by tumor cells indicate activation of angiogenesis mechanism in this lesion as compared to the RHGP lesion.

Based on our present findings in chemonaïve CRCLMs, overlapping signal between the expression of Ang1, Tie2, CD31 and αSMA1, along with enlarged vessel lumens in the RHGP lesion, suggest a role for high expression of the host Ang1 in stabilizing vessels in CRCLM with RHGP lesions. Accordingly, we postulate that over expression of Ang1 by hepatocytes in the interface region of chemonaïve RHGP lesions may affect blood vessel formation at the edge of the tumor and induce blood vessel stabilization via paracrine effect. Therefore, vascular quiescence maintained by Ang1-Tie2 signaling found in RHGP lesions prevails over destabilization and pro-inflammatory Ang2-Tie2 signaling compared to DHGP lesions.

The next steps in moving closer to using this knowledge for the benefit to the patient is to identify biomarkers, preferably in the blood of patients, which can stratify these patients. Ang1 and Ang2 are normally present in blood serum and are generally found at equilibrium in a healthy individual. Therefore, Ang1 can be used as a biomarker in CRCLM patients to predict response to anti-angiogenic therapy in the clinic. The identification/development of a blood biomarker may therefore be very feasible.

4.2. Expression of Ang1, Ang2 and Tie2 in treated (chemo and chemo plus Bev) RHGP and DHGP CRCLM lesion samples.

In RHGP treated (chemo and chemo plus Bev) human samples, the expression of Ang1 remained high at the adjacent normal region of the tumor, and with no significant difference when compared to the chemonaïve samples as well. However, the expression of Ang1 was significantly increased in the distal normal region of the liver tissue of chemo and chemo plus Bev RHGP lesions compared to RHGP chemonaïve lesions. In addition, the expression of Ang2 was significantly decreased in the tumor regions (CT and PT) of RHGP chemo treated lesions as well

as in periphery region of the RHGP tumor treated with chemo plus Bev. Tie2 expression was significantly induced in the tumor regions (CT and PT) of RHGP treated with chemo plus Bev samples.

In DHGP CRCLM treated human samples, the expression of Ang1 significantly increased in DHGP lesions remaining after treatment with chemo plus Bev and in the distal normal of DHGP chemo treated lesions compared to DHGP chemonaïve lesions. The expression of Tie2 was significantly higher in the tumor regions (CT and PT), adjacent normal and distal normal of the DHGP lesion after treatment with chemotherapy compared to the DHGP chemonaïve lesion. Most of the positive Tie2 cells are Tie2-expressing leukocytes.

It has been observed that the combination of chemotherapy with anti-angiogenic therapy has complex actions depending on the type of the tumor as well as the type, dose and schedule of the chemotherapy treatment (Sennino and McDonald 2012). Tumors treated with chemotherapy do not use *de novo* mechanisms of cancer cell progression, they induce the already existing ones via infiltration of a wide variety of bone marrow- derived cells (BMDCs) and mesenchymal stem cells (MSCs), including proangiogenic and intratumoral macrophages (Karagiannis, Condeelis et al. 2018). Tie2 expressing macrophages/monocytes are an example of BMDCs and are known to promote angiogenesis. These Tie2 expressing macrophages/monocytes were found to be accumulated in the necrotic area of tumors after chemotherapy treatment resulting in revascularization and subsequently a tumor relapse. MSCs are presented by mural cells expressing Ang1 (Kopp, Avecilla et al. 2005, Karagiannis, Condeelis et al. 2018). It has been shown that the expression of Ang1 and Tie2 expressing cells increase after chemotherapy treatment to reconstruct a functional vasculature and tumor relapse (Kopp, Avecilla et al. 2005). Therefore, Tie2 and Ang1 are essential maintenance and repair signaling in adult microvasculature after chemotherapy

treatment. In solid tumors, anti-angiogenic drugs cause vessel regression or retardation of vessel growth, and induce vascular normalization (stabilization) (Jain 2005). Tumors with mature blood vessels were less sensitive to drugs and acquired resistance after administration of angiogenesis inhibition (Ribatti, Nico et al. 2011). Ang1-Tie2 signal acts as a complementary mechanism when tumor vessels are stressed by anti-VEGF. It changes vessels to a less VEGF- dependent state, protects them from destabilization, maintains the blood supply and sustains survival of the tumor cells (Huang, Bae et al. 2009).

Based on the treated (chemo and chemo plus Bev) CRCLMs results, low expression of Ang1 at the periphery of the chemo plus Bev RHGP lesions may correlate with inhibition of immature blood vessel normalization, due to Bev treatment that may be pruning immature blood vessels. In addition, high expression of Ang1 in hepatocytes at distal normal of RHGP treated lesions may be caused by the treatments and may stimulate vessel reconstruction after regression of vascular tissue in response to treatment. Treatment with chemotherapy in DHGP might induce the expression of Tie2-expressing leukocytes that can develop chemotherapy resistance and reconstruct blood vessels and tumor relapse after chemotherapy treatment. Tie2 expression was found significantly elevated in DHGP chemo treated lesions. Our group has shown that chemotherapy was not effective on DHGP (Frentzas, Simoneau et al. 2016). Thus, chemo resistance developed in DHGP lesion might be caused by Tie2- expressing leukocytes that significantly accumulated in this lesion after treatment.

4.3. Mice with deficiency in Ang1 expression developed tumor with DHGP lesions.

Low availability of selective angiopoietin inhibitors limits understanding the effects of individual angiopoietins on the tumor vasculature extensively in loss-of-function experiments. In

a few studies, blocking Tie2 expression in tumor cells has been shown to reduce tumor angiogenesis. However, the specific roles of Ang1 and Ang2 can't be identified (Melani, Stoppacciaro et al. 2004, Jeong, Lee et al. 2005). Neutralizing Ang2 expression using systemic delivery of aptamers or pentaiodides caused inhibition of angiogenesis and tumor growth *in-vivo* (Oliner, Min et al. 2004, Sarraf-Yazdi, Mi et al. 2008). Inhibition of Ang1 in a cancer cell line using antisense RNA resulted in reduced angiogenesis and tumor growth in immunodeficient mice (Shim, Teh et al. 2001). In colon (Colo205) tumors, inhibition of Ang2 induced normalization of blood vessels which was prevented by Ang1 inhibition and resulted in reduced tumor vascularity *in-vivo* (Falcon, Hashizume et al. 2009). In Ang1 deficient mice, primary tumors of mammary and melanoma cells were not affected by global inhibition of Ang1. A significant increase in metastasis to the lung compared to control mice has been shown (Michael, Orebrand et al. 2017). However, the effect of the down-regulation of Ang1 expression in CRCLM is not well known.

To further understand the role of Ang1 in CRCLM with RHGP lesions, Ang1 KO mouse model was used. MC-38 colon cancer cells that generate RHGP lesions were injected intra-splenically into both control and Ang1 KO mice. Our results demonstrated that metastasis, tumor growth and HGPs were strongly influenced by host Ang1 expression. In control mice, we demonstrated that the expression of Ang1 in hepatocytes directly correlated with the presence of tumor cells, suggesting cross-talk between the tumor and hepatocytes. Interestingly, increased expression of Ang1 correlated with a visible dilation of the sinusoid blood vessel, potentially increasing blood flow and modifying the extracellular matrix to allow for the tumor and hepatocytes to migrate. However, knocking-down of Ang1 in mouse livers reduced the number and size of metastases allowed for the formation of desmoplastic lesions. Furthermore, these lesions were characterized by fewer mature blood vessels compared to the replacement lesions that

developed in control mice. The mice with highly efficient Ang1 inhibition did not develop liver metastasis lesions.

It has long been recognized that the Ang1 is important for vascular remodeling. Ang1 is not only important for blood vessels stabilization during the late stage of angiogenesis, but it is also critical in promoting the sprouting of endothelial cells at the early stage of angiogenesis via MAPK and PI3K pathways (Kim, Kim et al. 2000, Hawighorst, Skobe et al. 2002). Silencing of Ang1 in breast cancer cells inhibited migration and tube formation of endothelial cells (Liang, Ge et al. 2018). Therefore, inhibition of Ang1 expression in the liver inhibits mature blood vessel formation and hence tumor growth, as the tumor may not able to initiate angiogenesis from a vasculature stabilized by Ang1 (Liang, Ge et al. 2018). Accordingly, knocking down of Ang1 in the host liver decreased metastases, reduced the formation of tumor with vessel co-option and developed DHGP lesions which can respond to anti-angiogenic treatment in the clinic. Thus, using Ang1 inhibitor in addition to anti-VEGF drug to treat liver metastasis mouse models which develop tumor with RHGP in the liver may be a rational next step.

4.4. Deficiency of Ang1 expression in hepatocytes decreased survival, migration and invasion of colon cancer cells *in-vitro.*

It has been recently reported that upregulation of Ang1-Tie2 signaling activated pathways involved in cancer cells survival, proliferation and metastasis (Ye, Li et al. 2018). In addition, Ang1 has been shown to be a strong anti-apoptotic factor for various cells such as ECs, cardiomyocytes and neurons (Liu, Jiang et al. 2008). Furthermore, induced paracrine activity of Ang1 promoted ECs survival (Kwak, So et al. 1999). *In-vitro*, knockdown of Ang1 expression

decreased the proliferation and migration of breast cancer cells, and it was found that the Ang1 overexpression rescued proliferation and migration of these cancer cells (Liang, Ge et al. 2018).

It is therefore possible that tumor cells that metastasize into the liver stimulate Ang1 expression in their environment, which is important for blood vessel formation and tumor growth. This is supported by our *in-vitro* data, where co-culturing control primary hepatocytes and MC-38 cells using inserts significantly induced the expression of Ang1 in the hepatocytes. In addition, control hepatocyte conditioned media increased survival, migration and invasion of MC-38 cells. This phenotype was abolished when Ang1 was knocked out in hepatocytes, suggesting that secreted Ang1 from hepatocytes plays a critical role in tumor survival, migration and invasion.

Overall, these results provide evidence to suggest that high expression of Ang1 in RHGP lesions may regulate vessel co-option formation, tumor growth and metastases. Thus, target Ang1 in CRCLM with RHGP lesions may be a potential therapy for these patients, in addition to anti-VEGF treatment. By targeting direct mechanisms critical to tumor vessel co-option function and survival, resistance to current anti-angiogenic drugs may be overcome. Clinically, this could lead to significant improvements in overall survival rates for CRCLM patients with RHGP lesions.

Future directions

Activation of Ang1-Tie2 signaling may activate pathways that are involved in cancer cells survival, proliferation and migration like the PI3K/AKT pathway (Ye, Li et al. 2018). Stromal cells secrete a host of tumor promoting growth factors and cytokines that promote tumor progression and drug resistance. One of the vital growth factors in the tumor microenvironment is the hepatocyte growth factor (HGF), a ligand of c-Met receptor (Cascone, Xu et al. 2017). Numerous types of cells, including hepatocytes and endothelial cells express HGF that has been associated with multi-cellular responses, including growth, cytoskeleton, reorganization, and motility (Parikh, Wang et al. 2014). The c-Met receptor is highly expressed in CRC cells and is associated with advanced stage of CRC and its liver metastases (Zeng, Weiser et al. 2008). Moreover, paracrine HGF/ c-Met interactions promoted tumor growth and motility in colorectal tumor (Rasola, Fassetta et al. 2007). HGF can regulate the expression of ARPC3 (actin-related protein 2/3 complex subunit 3) which controls nucleation and the rate of actin polymerization that drives cell motility (Jiang, Martin et al. 2005). Furthermore, we found ARPC3 to be important for vessel co-option development in RHGP lesions (Frentzas, Simoneau et al. 2016). Several strategies targeting the HGF/ c-Met pathway are currently in development to evaluate for efficacy in Phase II and Phase III clinical trials (Parikh, Wang et al. 2014).

It has been reported that Ang1 dramatically stimulated expression of HGF *in-vitro* and this finding was not cell-specific (Kobayashi, DeBusk et al. 2006). Accordingly, we speculate that CRC cancer cells interact with hepatocytes and induce the expression of Ang1. Increased expression of Ang1 in hepatocytes may enhance HGF expression which activates c-Met receptor on CRC cells. Paracrine HGF/ c-Met interactions may enhance ARPC3 expression promoting

tumor cells migration and invasion. Furthermore, stimulation of HGF/ c-Met signaling may induce tumor cells survival and proliferation.

Experimental design and preliminary results: we will elucidate the effects of Ang1 expression in hepatocytes on colorectal cancer cells survival, proliferation and migration via downstream HGF/ c-Met signaling. Primary mouse hepatocytes from control and Ang1 KO mice will be isolated by collagenase perfusion method and cultured as described previously in (Tabaries, Dupuy et al. 2012). First, we will evaluate the impact of the tumor cell secretory factors on the expression of HGF/ c-Met signaling in hepatocytes as follows; MC-38 cells will be cultured, and their condition media will be added into the control and Ang1 KO hepatocytes. Second, MC-38 cells will be co-cultured with the control or Ang1 KO hepatocytes with/without addition of the transcription factor HGF. Finally, we will perform cancer cells proliferation assay using MTT assay and cancer cells migration assay using a wound-healing assay. In addition, cancer cells and hepatocytes in all culture conditions will be harvested for mRNA and protein extractions. The expression of Ang1, HGF and ARPC3 proteins will be determined in all culture conditions by WB and IF.

Preliminary IHC staining of HGF in control and Ang1 KO mouse livers that developed tumors revealed inhibition of HGF expression in Ang1 KO mouse hepatocytes compared to control mouse hepatocytes which showed high expression of HGF (Figure S1). Thus, expression of Ang1 in the hepatocytes impacts the expression of HGF in the mouse hepatocytes. By doing the functional test experiments we will determine if the inhibition in MC-38 cells survival, migration and invasion was due to inhibition of Ang1 signaling or its downstream signaling HGF/ c-Met. We will then stain the human CRCLM lesion samples with HGF as has been previously described for Ang1 staining and we will evaluate the significance of the staining through the digital analysis.

The results of these experiments will guide us to the best therapeutic target for RHGP lesion in CRCLM patients.

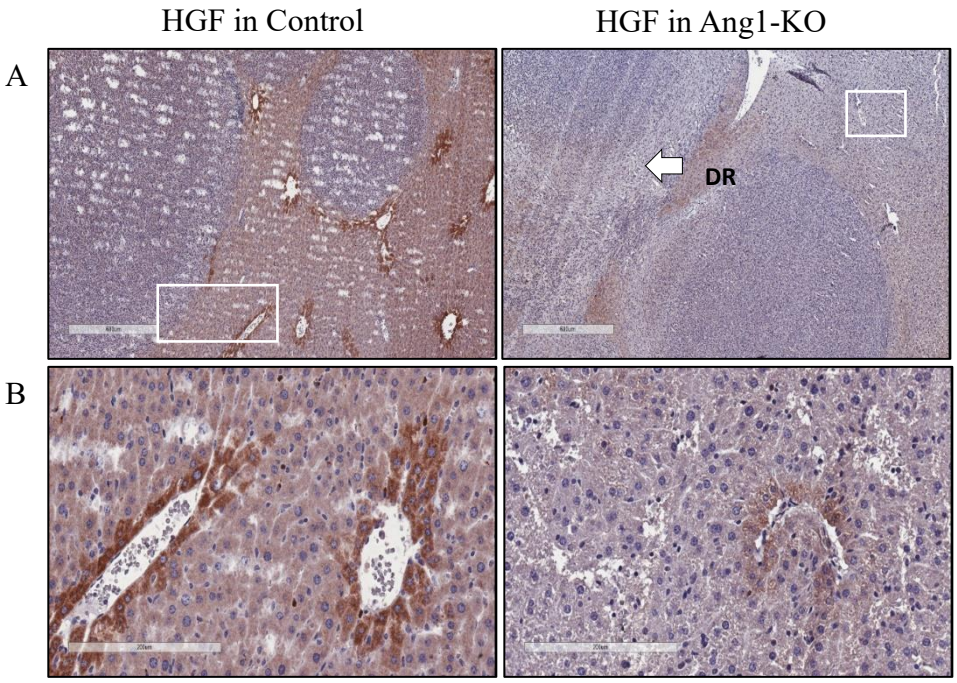

Figure S1, IHC staining of HGF in lesion developed from control and Ang1 KO mouse livers. A) high expression of HGF in control hepatocytes liver. B) Inhibition of HGF expression in hepatocytes of Ang1 KO livers.

Conclusion

Histopathological growth patterns in CRCLM are critical for identifying the type of patient's treatment. Better understanding of the vessel co-option mechanism essential for RHGP survival will help overcome tumor anti-angiogenic drug resistance. In this research study, we characterized the vasculature of the human RHGP and DHGP lesions based on the expression of Ang1, Ang2 and Tie2. Ang1 expression was found up-regulated in the host hepatocytes adjacent to the RHGP tumor in chemonaïve and treated (chemo and chemo plus Bev) human samples. Inhibition of Ang1 expression *in-vivo* reduced tumor metastasis and developed tumor with DHGP that can respond to anti-angiogenic treatment in the clinic. Furthermore, reduction in Ang1 expression in hepatocytes decreased colon cancer cells survival, migration, and invasion *in-vitro.* The function of Ang1 expression identified in this thesis therefore, adds an extra layer of complexity for the role of the host liver cells in RHGP formation and tumor progression. High expression of Ang1 in hepatocytes upon co-culture with colon cancer cells highlights the importance of interaction between cancer cells and host cells in regulating crosstalk between pathways during tumor progression. Since clinical trials targeting Angs-Tie2 system are already under way, extending our knowledge of Ang1 inhibition in health and disease will help minimize side effects of the drug and improve therapeutic strategies.

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