CELLULAR AND MOLECULAR CHANGES IMPACTING CANCER PROGRESSION IN NON ALCOHOLIC FATTY LIVER DISEASE

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

May, 2015

Nashville, Tennessee

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ORIGINAL PUBLICATIONS

1. **Mendonsa AM,** Vansaun MN, Ustione A, Piston D, Fingleton B, Lee Gorden. "Host and tumor derived MMP13 regulate tumor cell extravasation and establishment of colorectal cancer metastases in the liver." *Manuscript editorially accepted, Molecular Cancer, December 22, 2014.*

2. **Mendonsa AM[co]**, Vansaun MN[co], Lee Gorden. "Hepatocellular Proliferation Correlates with Inflammatory Cell and Cytokine Changes in a Murine Model of Diet Induced Steatosis."

   PLoS One. 2013 Sep 9;8(9):e73054. doi: 10.1371/journal.pone.0073054

3. Gorden DL, **Mendonsa AM**, Brown A et al. "Biomarkers of NAFLD progression: a lipidomics approach to an epidemic". *Manuscript in submission, Journal of lipid research.*

4. **Mendonsa AM,** Chalfant MC, Gorden DL, Vansaun MN. "Modulation of the leptin receptor mediates tumor growth and migration of pancreatic cancer cells". *Manuscript under review, submitted to PlosOne*

To my beloved family

ACKNOWLEDGEMENTS

There are so many people to thank for getting me to where I am today. I would first like to thank my mentor, Dr. Lee Gorden, for all of his guidance and support over the past five years. Irrespective of the demanding nature of his schedule, juggling between the clinic and lab, and as much as I would like to complain, he has always been there when I've had the need, from struggling to stay awake at my science hour after 48 hours of OR to the immense number of texts from nurses on duty. Lee has given me the chance to pursue whatever I've wanted, from applying for the certificate program in molecular medicine, to the immense number of conference and meetings I've had a chance to attend, and not saying anything when I decide to take time off. Thanks Chief, its been a wonderful 5 years.

I have to make a mention of Dr. Michael VanSaun, without you my training and success with my research would have never been complete. In addition to standing in for Lee whenever needed, you've been a wonderful mentor, from breathing down my back and overlooking everything I did to getting me to think on my own feet and be where I am at now.

One of the perks of being in the lab is having the additional support and scientific guidance of Drs. Barbara Fingleton and Oliver Mcintyre and the rest of the PMM lab. Since we share lab space and have joint lab meetings, they have been an integral part of my scientific training and have always been available to provide a fresh and unbiased perspective to any roadblocks that I have faced over the past five years. I would also like to thank the members of my committee, Drs. Simon Hayward, Richard Peek, Barbara

Fingleton and Alyssa Hasty for all their guidance and support during my graduate career.

I would not have been half as successful with my research if it hadn't been for some amazing lab mates and colleagues. You made coming to lab enjoyable and motivating. I would like to thank Dr. Hasty and Dr. Fingleton for the help with the bone marrow transplants and Kathy Carter and Corey Webb as well. I also have to thank Dr. Alessandro Ustione for the taking the confocal images and help with quantification.

I also want to thank all of my friends, those in and outside Nashville. My gratitude also goes to all of my extended family members residing in the United States for not only putting up with my constant visits, but actually encouraging them.

Last but certainly not least, I want to express my undying gratitude to my family. My older brother Riyan, for putting up with my badgering every time I needed something and paying all my phone bills. Franz for always being there when I needed him, my confidant, My little brother, Shaun, in spite of trying to weasel money out of me always knows how to make me smile and my sister Samantha, for her unlimited amount of energy that is so very contagious. It was like I was back at home having you spend the past few summers with me. And finally, Mom and Dad for always believing in me and encouraging me to pursue my studies. You have been unwavering pillars of support over my entire life, I want to say thank you. I would not be where I am today without you.

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
ATF6	Activating transcription factor 6
BCA	Bicinchoninic acid assay
BMI	Body mass index
CCl3	Cleaved caspase 3
CHTN	Cooperative Human Tissue Network
CM	Conditioned media
cP450	Cytochrome P450
CRC	Colorectal cancer
CTACK	Cutaneous T-cell-attracting chemokine
CXCL	CXC chemokine ligand
DAG	Diacylglygerol
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
DEN	Diethylnitrosamine
DMEM	Dulbecco's Modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FBS	Fetal bovine serum

FFA	Free fatty acid
FVII	Factor VII
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H&E	Hematoxylin and eosin
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNF4	Hepatocyte Nuclear Factor 4
HSC	Hepatic stellate cells
IACUC	Institutional animal care and use committee
IGF	Insulin-like growth factor
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
IRE-1	inositol-requiring enzyme 1
IRF	Interferon Regulatory Factor
IRS-1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
KC	Kupffer cell
KRB	Krebs Ringer Buffer
LPC	Lysophosphatidylcholine
MAPK	Mitogen-activated protein kinases
MCP	Monocyte chemotactic protein
MDSC	Myeloid-derived suppressor cells

MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MMPI	Matris metalloproteinase inhibitor
mRNA	Messanger ribonucleic acid
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCI	National cancer institute
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National institute of health
NK cells	Natural killer cells
NOS	Nitric oxide synthase
PAI	Plasminogen activator inhibitor
PCR	Polymerase chain reaction
PERK	PKR-like endoplasmic-reticulum kinase
PF	Platelet factor
PI3K	Phosphatidylinositide 3-kinases
PPARα	Peroxisome proliferator-activated receptor alpha
PRR	Pattern recognition receptors
PTK-7	protein tyrosine kinase 7
RANTES	Regulated on activation normal T-cell expressed and secreted
RIPA	Radioimmunoprecipitation assay

SCF	Stem cell factor
SREBP	Sterol regulatory element-binding transcription factor
STAT3	Signal transducer and activator of transcription 3
STM	septum transversum mesenchyme
TF	Tissue factor
TG	Triglyceride
TGF	Transforming growth factor
TIMP	The matrix metalloproteinase
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
VCAM	Vascular cell adhesion protein
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
vWF	Von Willebrand factor
WHO	World health organization
WT	Wildtype

CHAPTER I

INTRODUCTION

Overview

As the epidemic of obesity continues to expand worldwide, it has become imperative to understand its impact and that of related co-morbidities on the health, morbidity and mortality of affected patients. Non-alcoholic fatty liver disease (NAFLD) is one such obesity-associated co-morbidity, characterized by the accumulation of fat in the liver resulting in impaired liver function, and linked to the increased risk of primary liver cancer. Of note, obesity is clinically an independent risk factor for the development of malignancies including colon and breast cancer that frequently metastasize to the liver. However, the role of NAFLD in metastatic disease has not been adequately investigated. My work, presented herein, was initiated to determine the impact of NAFLD on the establishment of colorectal cancer metastasis to the liver.

Alterations in the liver microenvironment with NAFLD could influence the ability of tumors to spread and metastasize to the liver. The Matrix Metalloproteinases (MMPs) are a family of proteases that can degrade components of the extracellular matrix and are important in normal physiology, yet frequently altered in many disease settings. Comparative analysis of normal and NAFLD livers revealed altered MMP levels in the liver with NAFLD. MMPs have been demonstrated to exhibit both pro and anti-tumorigenic roles and could be important modulators of the liver microenvironment, influencing tumor establishment and progression in the setting of NAFLD.

In this chapter, I set the historic and scientific foundation for understanding the impact of NAFLD on cancer development and progression. Following this, I introduce the obesity epidemic and the implications it has on the development of NAFLD and cancer. I then describe the MMPs and their role in cancer progression and changes to the liver microenvironment, and end with my hypothesis and dissertation goals.

The Obesity Epidemic

Obesity refers to a medical condition that arises from an excess amount of body fat negatively impacting a person's health [1]. Obesity is the result of a substantial increase in caloric intake which exceeds the caloric expenditure, culminating in an accumulation of fat throughout the body [2]. The most common criterion used to define obesity is the body mass index (BMI). BMI is calculated with the help of a mathematical formula and takes into account both a person's height and weight to determine their degree of obesity.

$$\text{BMI} = \frac{\text{weight in kilograms}}{\text{square of height in meters}}$$

The World Health Organization (WHO) and the National Institutes of Health (NIH) [3, 4] have defined normal weight in adults as a BMI between 20 and 25 BMI units, overweight from 25 to 30, and obesity above 30. The BMI however is not considered very accurate as it does not take into account the actual body fat percentage. Use of measures that take into account body fat measured by tissue resistance using dual-energy X-ray absorptiometry would be more accurate, and are beginning to be used more commonly [5, 6]. The origins of obesity can be traced back 30,000 years to our prehistoric ancestors, when the body habitus was considered a sign of wealth and

prosperity [7]. Over the past few decades there has been a tremendous increase in the incidence of obesity both in the United States and worldwide (Figure 1). Obesity was formally recognized as a global epidemic by the World Health Organization in 1997 [8]. Incidence of obesity has doubled in the past 20 years in the United States and over one third (35%) of adults and 17% of youth are considered obese [9]. Of particular relevance to public health is the fact that obesity is increasingly being seen at earlier ages with about 18% of children in the age group of 6-11 in the United States qualifying as obese [10].

The overall increase in the incidence of obesity is a result of several factors. These encompass genetic and environmental alterations that ultimately result in excessive caloric intake and/or a decrease in physical activity [11]. Several genetic variations that influence metabolism and food intake are addressed in "The human obesity gene map" and can contribute to the development of obesity [12, 13]. Some examples include alterations in leptin or serotonin and their receptors that control energy uptake and expenditure [14, 15]. Mutations in genes involved in lipid metabolism including β-oxidation, very low density lipoprotein (VLDL) secretion, and fatty acid synthesis such as SREBP-1c and PPARα [16] can lead to a predisposition to obesity [12, 13].

The ancient Greeks and Egyptians were the first to recognize the dangers of obesity and its association with disease, and recommended diet and exercise to treat it and its side effects [7]. Obesity is now recognized as a major health problem worldwide and roughly 300,000 deaths in the US per year are related to obesity either directly or through obesity-related co-morbidities [9, 11]. Obesity is associated with increased risk of development of a number of serious chronic diseases including high blood pressure,

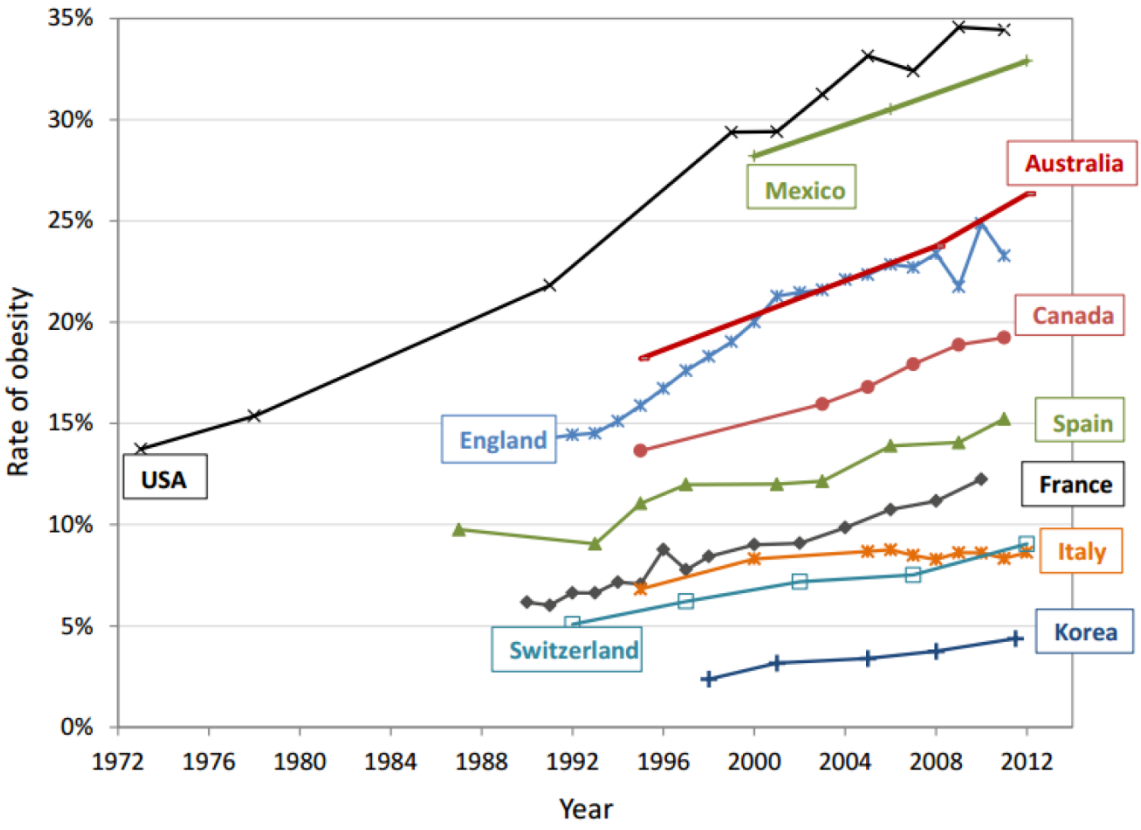


Figure 1: Incidence of obesity worldwide. This graph shows the rate of increase in the number of cases of obesity in several countries across the globe over the past few decades [17].

high cholesterol, type 2 diabetes, coronary heart disease, osteoarthritis, respiratory problems, as well as certain types of cancer. Obesity can also result in accumulation of excessive fat within the liver termed non-alcoholic fatty liver disease (NAFLD) which will be discussed in more detail in the next section [18]. Obesity related co-morbidities are subsequently linked to a reduced life expectancy and premature death. Another major health concern is that with the increased incidence of childhood obesity, which often persists into adulthood, there will be earlier onset of obesity associated chronic illnesses including NAFLD, and increased morbidity and mortality rates in obese patients. Efforts to impart individual and population-based behavior changes leading to healthier eating habits and increased physical activity are being initiated and are anticipated to reduce the incidence of obesity and its related co-morbidities.

Obesity and chronic inflammation

In the setting of obesity, the fat or adipose tissue expands with the increased fat storage and proliferation of the adipocytes [19]. The principal function of adipocytes was believed to be fat and energy storage, and until relatively recently they were considered to play a passive role in the onset of obesity and its related co-morbidities [20]. Evidence demonstrates that the adipocytes actively secrete cytokines and inflammatory mediators as a stress response to excessive accumulation of fat. These findings necessitate a further understanding of how adipocytes and inflammation contribute to the health complications of obesity [20].

With the accumulation of fat, the adipocytes enlarge and undergo molecular and cellular alterations that affect systemic metabolism and trigger an inflammatory response. In the obese state, the adipose tissue produces several pro-inflammatory factors including

tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) [20, 21]. The finding that TNFα is overexpressed in the adipose tissue of obese mice was the first link between obesity, diabetes and chronic inflammation [22]. TNFα can interfere with the insulin signaling pathway and contribute to development of insulin resistance [23]. Obesity induced changes also result in a reduction in adiponectin and an increase in leptin levels that can further contribute to insulin resistance [24]. Other cytokines such as monocyte chemotactic protein 1 (MCP-1), inducible nitric oxide synthase (NOS), transforming growth factor β (TGFβ) , pro-coagulant proteins such as plasminogen activator inhibitor type 1 (PAI-1), tissue factor (TF), and factor VII (FVII) are also increased leading to infiltration of leukocytes into the adipose tissue [20, 25, 26]. Macrophage numbers increase in the obese adipose, contributing to local increases in the production of pro-inflammatory cytokines [27]. Macrophages display a range of phenotypes broadly classified as M1 "inflammatory" or M2 "repair" phenotypes, shifting toward the classical M1 phenotype with increasing adiposity [28]. Direct and paracrine signals from "M1 like" activated macrophages can impair insulin signaling and adipogenesis in adipocytes, leading to development of insulin resistance [25]. Macrophages are also responsible for the removal of necrotic adipocytes and form characteristic crown-like structures to facilitate this process in obese adipose [29]. The expanded systemic influence of the inflammatory response and release of cytokines is responsible for the multi-organ nature of the obesity-related co-morbidities (Figure 2) [25].

Many molecular signaling pathways have been linked with obesity related inflammation and metabolic function. Nutrients and metabolites have resulted in the activation of pattern recognition receptors (PRR) of the innate immune system such as

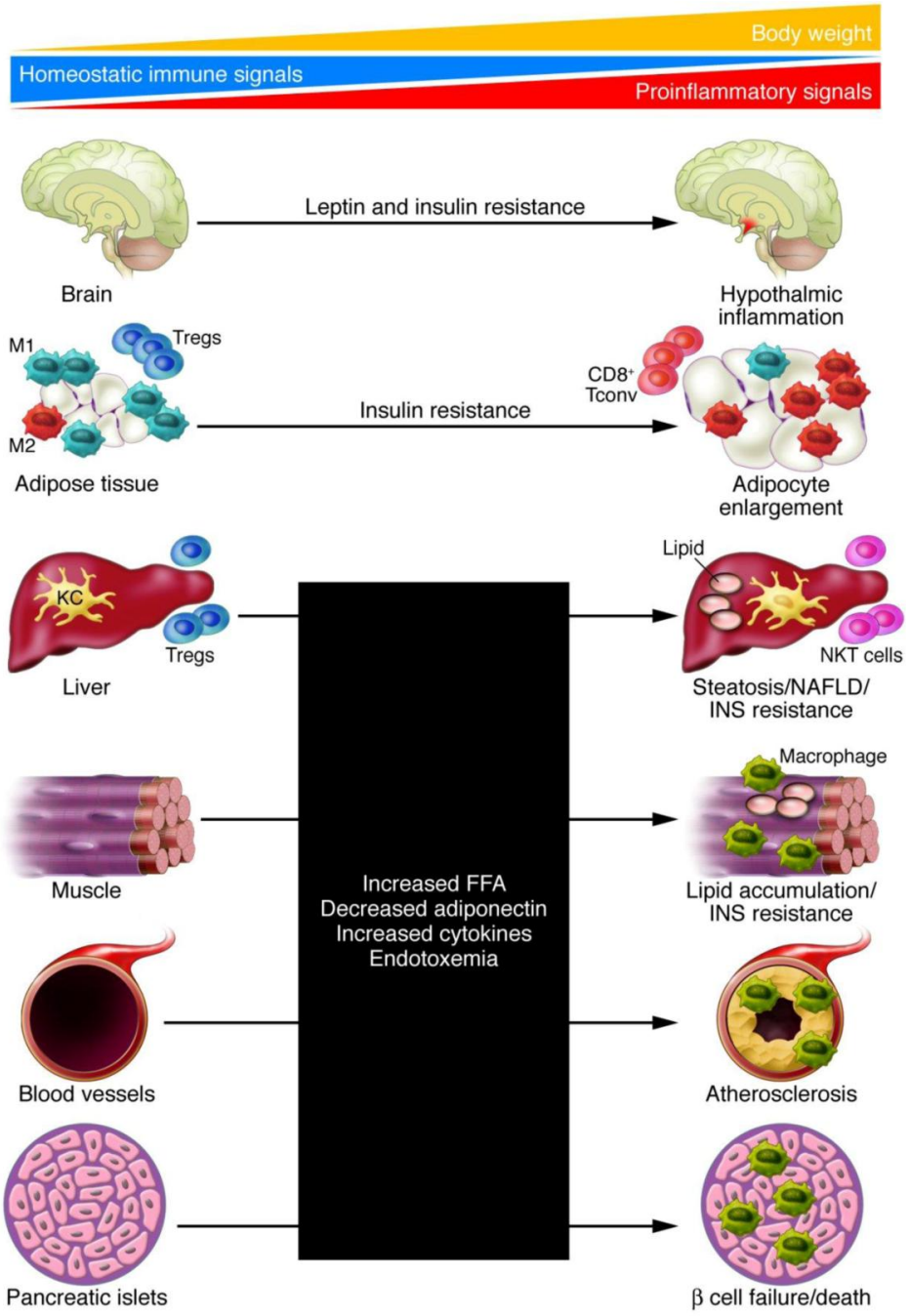


Figure 2: Multi-organ effects of obesity related inflammation. Obesity triggers inflammatory pathways in the brain and adipose tissue that dysregulate physiological responses that maintain insulin and leptin sensitivity. Over time, ectopic lipid accumulation in muscle, liver, and blood vessels activates tissue leukocytes, contributes to organ-specific disease, and exacerbates systemic insulin resistance. Cellular and cytokine mediated inflammation in pancreatic islets accelerates the progression toward diabetes. FFA, free fatty acids; T regs, regulatory T cells; Tconv, conventional T cells; NKT cells; natural killer T cells; INS, insulin [25].

the cytokine or Toll-like receptor (TLR) pathways [30, 31]. These lead to activation of several downstream effectors including c-jun N-terminal kinase (JNK), protein kinase R (PKR) and IKK which activate metabolic responses and result in insulin resistance by inactivation of the insulin receptor IRS-1 [26]. In addition, JNK and IKK/NF-κB pathways are strongly correlated with ER stress in multiple metabolically active tissues leading to activation of ER stress signaling components that include inositol-requiring enzyme 1 (IRE-1), PKR-like endoplasmic-reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [21, 32]. Activation of PRRs alternately induce an inflammatory response through activation of transcription factors AP-1, NF-κB, and IRF. These factors increase the gene expression of inflammatory mediators, which in turn lead to receptor activation, thus leading to a positive feedback loop of inflammation [26, 33]. Unlike the classical inflammatory response, obesity produces a chronic low grade activation of the innate immune system that over time affects the steady state measures of metabolic homeostasis. In addition, the multi-organ pathophysiology of obesity-induced inflammation presents a challenge to researchers attempting to tease out disease mechanisms in complex metabolic systems [25]. It is thus clear that inflammation plays an important role in the downward health spiral of obesity. Understanding the mechanisms underlying chronic inflammation in the setting of obesity might lead to methods through which pharmacologic manipulation could reverse the adverse effects of obesity on an individual's health.

Obesity and cancer

Epidemiological studies show that obesity is associated with an increased risk of development of several types of cancer, including cancers of the colon, endometrium,

postmenopausal breast, kidney, esophagus, pancreas, gallbladder, liver, and hematological malignancies [34, 35]. In addition to risk of development of cancer, obesity can also be an indicator of worse prognosis leading to poorer treatment outcome and increased cancer-related mortality [36, 37]. Figure 3 shows the increased risk in cancer related mortality in both men and women with obesity.

Several potential mechanisms can explain the link between obesity and the increased risk of development of certain cancers. Changes in obesity-related hormones, growth factors, cytokines and adipokines, alterations in metabolism, hyperinsulinemia, modulation of energy balance and calorie restriction could impact cancer initiation and progression. Comorbidities such as diabetes and NAFLD are also associated with increased risk of development of cancer [35, 38–40].

Obesity as discussed earlier leads to a state of chronic inflammation, with increase in levels of circulating cytokines such as TNFα and IL-6. Increase in TNFα has been linked to the development of skin, liver, and colon cancer, possibly through NF-κB induced gene transcription [41]. High circulating levels of IL-6 are associated with the development of Kaposi sarcoma, multiple myeloma, colon cancer and Hodgkin's lymphoma and have been shown to promote cell growth and inhibit apoptosis [42]. Park et al. show that increased IL-6 and TNFα, as a result of diet-induced obesity, lead to enhanced tumorigenesis in the liver via induction of STAT3 [43].

Changes in adipokine levels, particularly leptin and adiponectin have been associated with cancer development. Increase in leptin levels with obesity can induce cancer progression by activation of PI3K, MAPK, and STAT3 pathways [44, 45]. Adiponectin, which can function as an anti-cancer mediator by decreasing IGF and

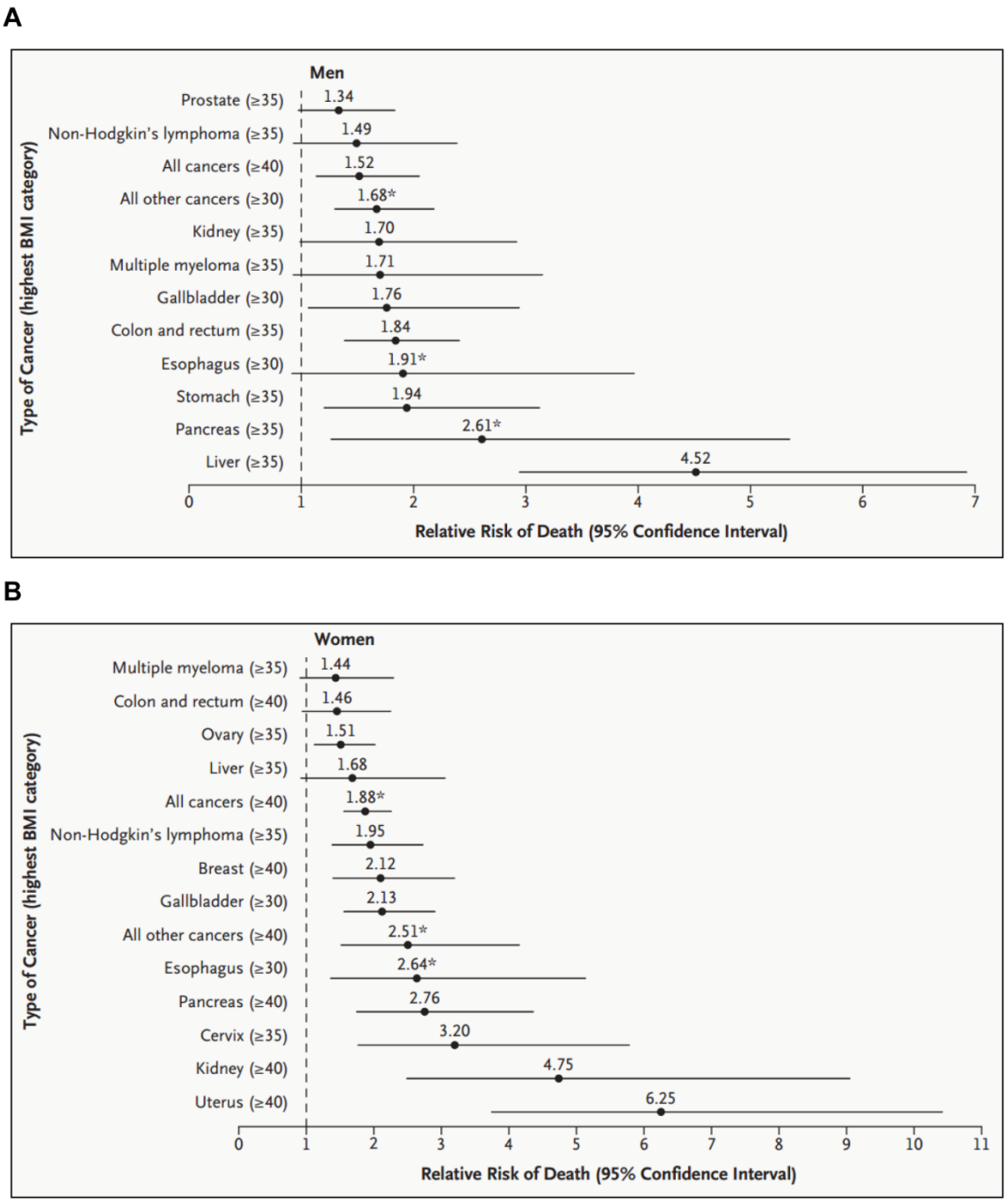


Figure 3: Risk of mortality from cancer with obesity. Summary of mortality from cancer according to body mass index (BMI) for (A) men and (B) women in the United States in the cancer prevention study, 1982 through 1998 [36].

mTOR signaling and inhibiting NF-κB, is decreased with progression of obesity [46]. Hyperinsulinemia may contribute to cancer development through the growth-promoting effect of elevated levels of insulin. Prolonged obesity leads to insulin resistance and hyperinsulinemia and increased IGF signaling. Insulin and IGF-1 signal through the Akt/PI3K/mTOR cascade and promote cell growth and proliferation, thus facilitating cancer growth and progression [47, 48].

Several other factors, including genetic alterations, sex hormones and obesity-related hypoxia, can also play a role in obesity related tumorigenesis [48]. Understanding the mechanisms by which obesity and related co-morbidities influence cancer initiation and progression is necessary to impact treatment of obese cancer patients.

Obesity and colorectal cancer

Colorectal cancer is the third most commonly diagnosed cancer and the third most common cause of cancer related deaths in the US [49]. Epidemiological studies have shown that in the US, obesity increases the risk of colon cancer by 1.5-2 fold with obesity-associated colon cancer accounting for 35% of total incidence [50].

Metastases are responsible for a majority of cancer related deaths. Although tumor cells could theoretically go anywhere in the body, the liver is the most common site of metastasis for colon cancer with about 60% of patients developing liver metastases [51]. This could in part be explained by the venous drainage of the colon and upper rectum that goes through the portal vein and drains directly into the liver. More complex molecular mechanisms influencing tumor cell adhesion to the endothelial cells and extravasation from the vasculature may contribute as well. Although primary colon

cancer has a good prognosis when resected with clear margins and negative lymph nodes, the diagnosis of liver metastases portends a poor outcome [52].

Properties of both the primary tumor and the microenvironment of the distant organ site are important for the establishment of metastases. NAFLD represents an altered microenvironment of the liver, which was demonstrated to preferentially support colorectal metastases [53]. Accumulation of fat in the liver and recruitment of inflammatory cells are hallmarks of this altered liver microenvironment which could contribute to the establishment of metastases in the liver. Before going into detail on the changes in the liver microenvironment, I will first describe the steps involved in the progression of cancer and establishment of metastasis and how it pertains to colon cancer.

The metastatic cascade

The high rate of cancer mortality is most commonly due to the development of metastases at distant organ sites and represents about 90% of cancer related deaths [54]. The development of clinically detectable metastases is the result of a multi-step process commonly referred to as the metastatic cascade (Figure 4). It involves a complex series of sequential biological events starting with the exit of tumor cells from the primary tumor site. This involves local invasion of individual tumor cells into the surrounding tissue and through the basement membrane and their intravasation into the vasculature. The tumor cells need to survive in the circulation and be transported to the distant organ site. Once tumor cells arrive at the distant organ site, they arrest in the vasculature and extravasate from the vasculature into the metastatic organ site. Establishment of clinically detectable metastases requires the tumor cells to adapt, survive and grow in the secondary microenvironment at the distant organ site [55].

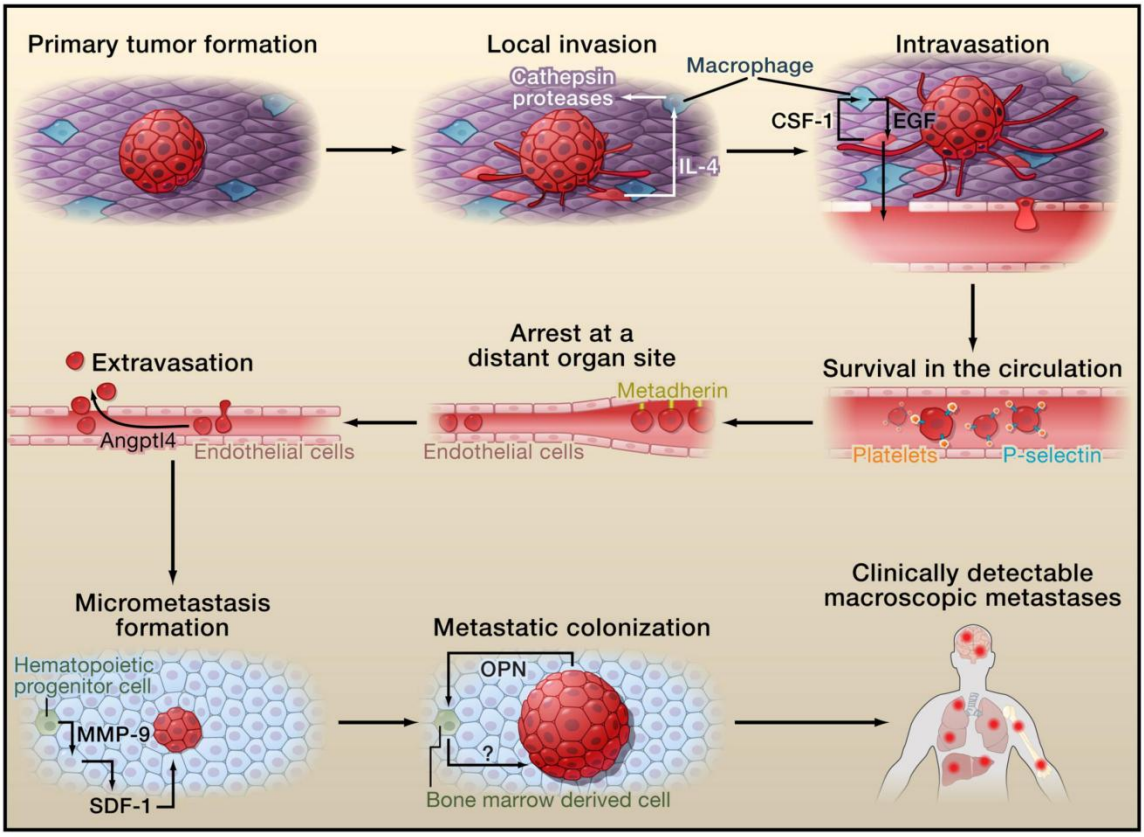


Figure 4: The Metastasis Cascade. CSF-1, colony stimulating factor-1; IL-4, Interleukin-4; Angptl4, Angiopoietin-like 4; SDF-1, stromal cell-derived factor-1; MMP9, Matrix Metalloproteinase 9; OPN, Osteopontin [55].

Each of these steps is a complex biological event involving the activation of multiple molecular pathways within both carcinoma cells and the surrounding stromal cells [56]. Metastasis is thus a highly inefficient process and only select cells are capable of completing all the steps to develop clinically evident metastases [57]. Even though tumor cells can potentially grow at any secondary site in the body, many cancers have a propensity to metastasize to specific organs. This observation gave rise to the seed and soil hypothesis proposed by Stephen Paget in 1889 [58] in which he likens the cancer cell to a seed and the site of distant metastasis as fertile soil. Several factors could contribute to this phenomenon including the establishment of a pre-metastatic niche resulting from factors secreted by the primary tumor prior to the arrival of circulating tumor cells [59]. Alternately, a passive process that results in mechanical trapping of tumor cells within the distant vasculature bed based on the size of the capillaries, could also contribute to metastatic site selectivity. Colorectal cancer most frequently metastasizes to the liver, and this could be a combination of both proposed mechanisms. Trapping of colorectal carcinoma cells in the liver is dictated by direction of blood flow from the colon through the portal vein that drains the mesenteric circulation directly into the liver [60]. The vasculature of the liver consists of fenestrated sinusoids that are highly permeable in normal livers and could thus provide only a minor impediment to extravasating tumor cells. Additionally, entry of colorectal carcinoma cells into the hepatic microvasculature can initiate a proinflammatory cascade that results in Kupffer cells being triggered to secrete chemokines that upregulate various vascular adhesion receptors, thereby enabling adhesion of tumor cells in the microvasculature of the liver [61]. Factors expressed by the tumor cells as well as stromal cells including various members of the MMP family

including MMP9, MMP2 and MT1-MMP, selectins and their ligands, integrins and other molecular factors may facilitate tumor cell adhesion to and extravasation from the vasculature and mediate organ specific dissemination and adhesion of tumor cells [56, 62, 63].

The Liver Microenvironment

As the epidemics of obesity and its associated diabetes and metabolic syndrome continue to grow, so has the incidence of NAFLD. NAFLD is a progressive disorder initiated by the accumulation of fat in the liver in the absence of excess alcohol consumption or viral infection. Development of NAFLD can lead to complications of decreased liver function, chronic inflammation and increased risk for development of liver cancer. Before delving into the pathophysiology of the liver in the setting of NAFLD, I will first introduce the normal anatomy and physiology of the liver. I will then discuss how alterations in the liver microenvironment with NAFLD increase the risk of development of liver cancer.

Anatomy and physiology of the liver

The liver is one of the largest organs in the human body and is essential for normal physiology. It synthesizes and secretes bile, fatty acids, proteins and cholesterol while it absorbs and stores nutrients, vitamins and fats, and is critical for maintenance of glucose balance in the body. Hepatic function is required for the breakdown of toxic substances and aids in the excretion of bilirubin, cholesterol and drugs. The liver is essential for survival and any disease affecting its normal function can be life threatening.

The liver is divided into two principle lobes in humans, a large right lobe and a smaller left lobe that are separated by the falciform ligament. The lobes of the liver are made up of numerous microscopic hexagonal functional units called lobules (Figure 5). In addition to the hepatocytes, each lobule consists of a central vein that surrounded by six hepatic portal veins and hepatic arteries. The liver has a dual blood supply. The hepatic artery is a source of oxygenated blood that comes from the lungs. Additionally, the portal vein supplies blood rich in absorbed nutrients from the gastrointestinal tract. Branches of the hepatic portal vein, hepatic artery and the bile duct are typically distributed across the liver in close proximity to each other and are collectively referred to as the portal triad. Additionally, the liver contains sinusoids, which are small fenestrated capillary like tubes that extend from the portal veins and arteries to meet the central vein like spokes on a wheel and facilitate exchange of blood and nutrients to the hepatocytes lining the sinusoids.

Hepatocytes are the epithelial component of the liver and perform most of the liver's physiologic functions (Figure 6). The sinusoids are partly lined with Kupffer cells, the resident liver macrophages that phagocytose aging blood cells and bacteria as well as toxic substances. When Kupffer cells are activated, they can secrete several inflammatory cytokines which are important for the innate immune response. The hepatic stellate cells (HSCs) are pericytes found in the perisinusoidal space of the liver, also known as the space of Disse. They store fat and vitamin A in normal liver. HSCs become activated to a myofibroblastic phenotype during liver injury. Activation of stellate cells leads to changes in their gene expression profiles and synthesis of various extracellular matrix components known to facilitate the development of fibrosis and cirrhosis [66–68].

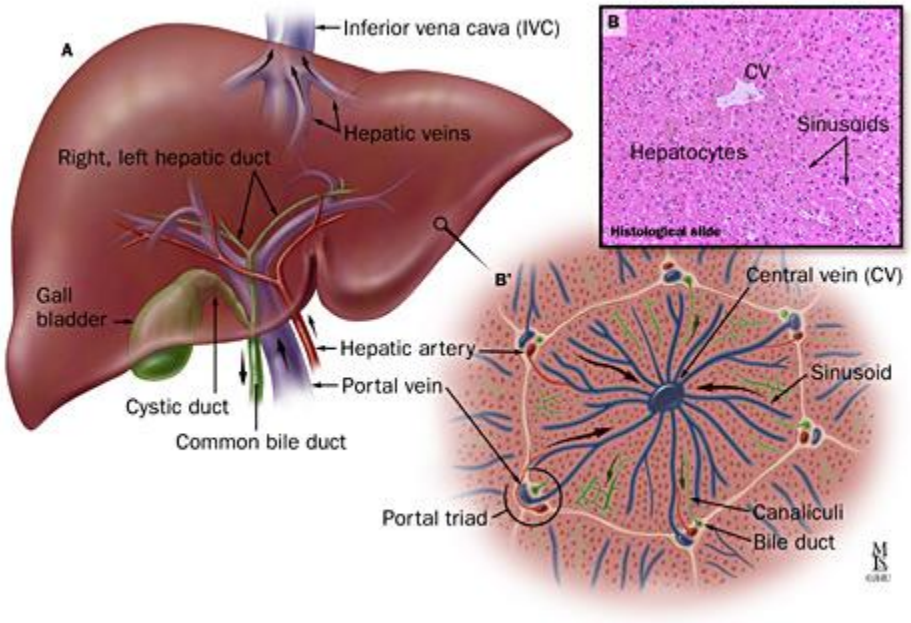


Figure 5: Anatomy of the liver. (A) Normal gross anatomy of the liver. (B) Histology of the liver and (B') histological view of a lobule of the liver [64].

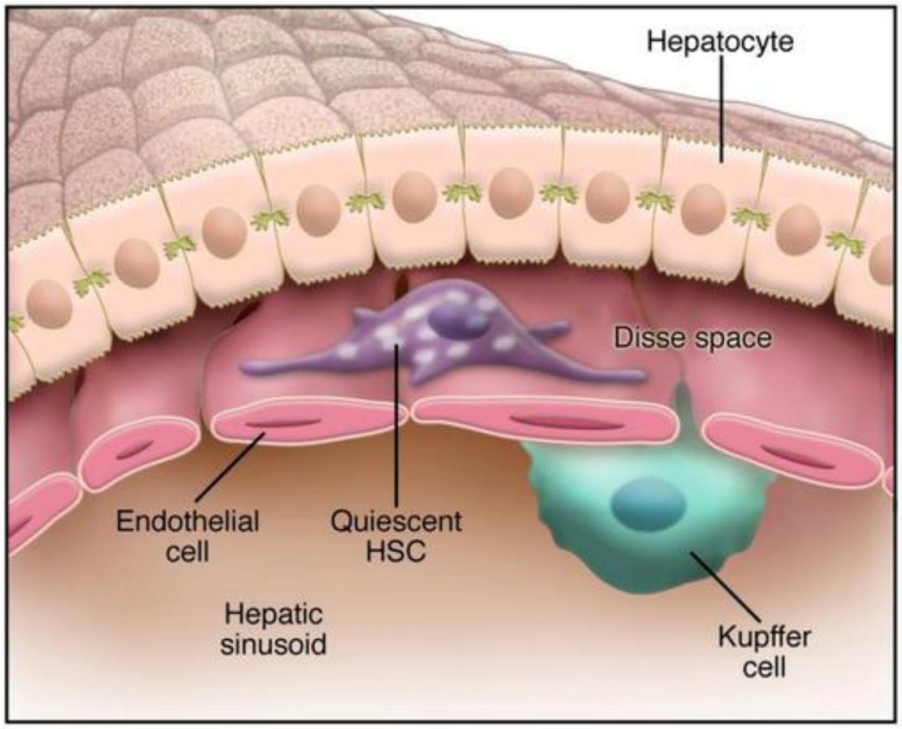


Figure 6: Cellular composition of the liver sinusoid. HSC, hepatic stellate cell [65].

The liver performs many vital metabolic functions. It is responsible for the production of fundamental protein components of the blood plasma and storage of essential nutrients, vitamins, and minerals obtained from the blood [66, 67]. The hepatocytes secrete bile into the bile canaliculi draining into the bile ducts forming a branched network called the biliary tree that carries bile through the liver and into the gall bladder. The bile acids are important for digestion and play a key role in intestinal absorption of fats by emulsification of lipids in the food. Besides its digestive function, bile also serves as the route of excretion for bilirubin, a byproduct of breakdown of hemoglobin from aging red blood cells [69].

In addition to its metabolic and digestive functions, the liver also plays an important role in blood detoxification. When blood passes through the gastrointestinal tract it absorbs toxic substances and microbes that need to be eliminated before the blood is circulated to the rest of the body. These are either filtered when the blood passes through the liver, excreted in the bile or neutralized by the members of the cytochrome P450 enzyme superfamily. The members of the cytochrome P450 superfamily are hemoproteins that reside on the mitochondrial or ER membranes in hepatocytes. These are terminal oxidase enzymes in the electron transfer chain and catalyze the metabolism of a large number of xenobiotics, such as pharmaceuticals, and toxic endogenous compounds. The enzymes function either by directly neutralizing certain chemicals, enhancing the water solubility of toxins that enables excretion through the kidneys, or by converting the toxin to a more chemically active form that facilitates further neutralization reactions. These include sulfation and glucuronidation, glutathione conjugation, methylation, amino acid conjugation, and acetylation. [70–72]. Additionally,

the liver plays an important role in immunity by capturing and digesting bacteria, fungi, parasites, and cellular debris that are phagocytosed by the resident Kupffer cells [66, 67].

The liver plays a critical role in maintaining normal blood glucose levels by balancing the uptake and storage of glucose via glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis. Glucose homeostasis is further regulated by levels of glucose transporters on the hepatocytes which facilitates the final step in the transport of glucose between the liver and the bloodstream [66, 67].

The liver has a central role in lipid metabolism and is instrumental in the regulation of fatty acid and cholesterol levels in the body. The hepatocytes are a hub for de novo fatty acid and cholesterol synthesis, and further facilitates lipid circulation through regulation of lipoprotein synthesis. Fatty acids are converted to energy rich triacylglycerols and along with cholesterol packaged into VLDL for export to other tissues. Excess cholesterol from non-hepatic cells is then brought back to the liver by high density lipoproteins (HDL) that are rich in cholesterol and have low triacylglycerol content. The cholesterol is then either converted to bile salts in the liver or excreted in the bile [73, 74]. Disruption of lipid homeostasis can eventually lead to the accumulation of lipid droplets in the hepatocytes and result in hepatic steatosis.

Non alcoholic fatty liver disease (NAFLD)

NAFLD has become the most common cause of chronic liver disease in the United States and several developed countries worldwide. Some estimates suggest that NAFLD may be present in 17-33% of the US population and that 33% of these patients have a significant component of non-alcoholic steatohepatitis, NASH [75]. Currently 4-10% of liver transplants in the United States are performed for end stage liver disease due

to NAFLD [76] and fatty liver disease is likely to become the leading indication for liver transplant in the next 10 years.

The umbrella term "NAFLD" was first used in 1986 [77] and broadly describes a wide spectrum of liver diseases ranging from simple fatty liver or steatosis to non-alcoholic steatohepatitis (NASH), which may progress to fibrosis and cirrhosis (Figure 7). Though historically cirrhosis is associated with increased risk of development of hepatocellular carcinoma (HCC), a primary cancer of the liver, there is an increasing incidence of cases with steatosis or NASH alone in association with the development of HCC [78]. Simple steatosis results from the accumulation of fat droplets in the hepatocytes. It results in a unique liver microenvironment typified by accumulation of triglycerides and other glycerophospholipids within the cytoplasm of hepatocytes in the liver [78, 79]. Steatosis generally represents a non-progressive disease condition with minimal treatment requirements and low risk of development of end stage liver disease. Transition from steatosis to steatohepatitis is believed to occur when the capacity of hepatocytes to store fat is overwhelmed by continued uptake, local synthesis or impaired breakdown of fatty acids. This leads to lipotoxicity causing cell death and resulting in inflammation [80, 81]. NASH is characterized by the infiltration of inflammatory cells in the liver in the setting of steatosis that results from hepatocyte ballooning and cell death. Untreated, NASH can progress to endstage liver disease characterized by fibrosis and cirrhosis. Factors such as mitochondrial injury, stellate cell activation and microvascular injury can amplify the pathogenic process that lead to the development of fibrosis or cirrhosis through deposition of collagen and scar tissue [82]. Equally ominous is the recognition that NASH and progressive liver fibrosis increase the risk for liver cancer. In

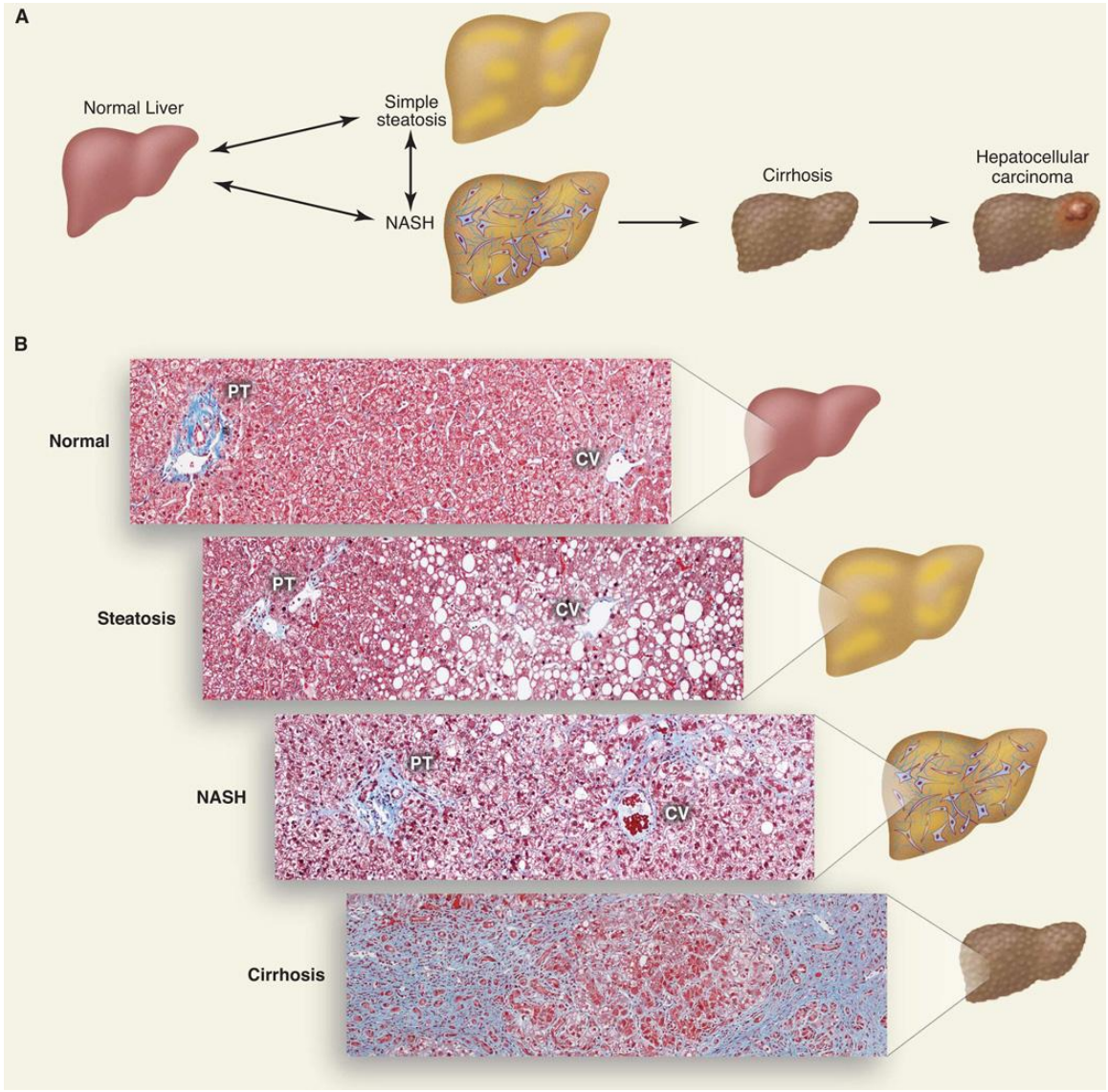


Figure 7: Progression of non-alcoholic fatty liver disease (NAFLD). NASH, non-alcoholic steatohepatitis [78].

fact, about 25% of patients affected by NASH can develop cirrhosis with the subsequent risk of HCC [76]. Moreover, there is an increasing number of HCCs developing in non-cirrhotic NASH and steatotic livers [83, 84]. Clinically NAFLD is distinct from other liver diseases including viral hepatitis and ethanol abuse that may also be characterized by accumulation of fat in the hepatocytes.Assessment of disease severity and monitoring of NAFLD disease progression is a major challenge. Currently, a liver biopsy is still considered the gold standard. This procedure is invasive and can put the patient at potential risk for severe complications [85]. Available serum biomarkers and imaging techniques lack sensitivity and specificity to distinguish steatosis from NASH and cannot stage the presence and extent of liver fibrosis [80, 86]. There is thus a need for better non-invasive tools and biomarkers to identify the progression of disease in NAFLD as well as to determine the risk of disease progression, and/or response to therapy [87]. Several studies have been carried out to identify serum markers of inflammation, apoptosis, oxidative stress, and fibrosis to distinguish the different stages of NAFLD [88–92]. Although some markers can distinguish between early and late stage disease, there is still a need for more robust markers. Imaging techniques such as elastography, magnetic resonance elastography and acoustic radiation force imaging are becoming more established and can distinguish fibrosis in a variety of chronic liver conditions in addition to NAFLD [93–95], however there is still a need to be able to distinguish steatosis from steatohepatitis. Biomarkers for early detection of NASH and of possible indicators of progression to cirrhosis could establish novel therapeutic approaches and thus prevent progression to cirrhosis. Assessment of changes in the lipid

profiles in the blood or urine could identify a lipid signature that would be able to distinguish steatosis from NASH [96].

NAFLD and cancer

While major advances have been made in understanding the physiological characteristics of NAFLD, the pathogenesis of this progressive condition and the exact mechanism behind the development of HCC with NAFLD remains unclear [97]. Figure 8 highlights some of the proposed mechanisms of NAFLD related HCC. Accumulation of excessive fat in the liver can initiate liver hepatocyte damage, which when followed by inflammation and cycles of necrosis and regeneration could potentiate the induction of HCC. Hepatic inflammation and injury in NASH are effective in activating HSCs. Activation of HSCs during liver regeneration can also promote cirrhosis by production and deposition of type I collagen-rich scar tissue and production of MMP13 [98]. This results in an environment that promotes hepatocyte proliferation and is permissive to genetic modulations leading to neoplastic transformation [43, 99]. Increase in obesity related pro-inflammatory cytokines including TNFα, IL-6 and leptin, as well as a reduction in the amount of adiponectin could favor tumorigenesis within the liver [100]. HCC has extraordinary heterogeneity of genomic aberrations, and more than 90% of HCC develops in the setting of chronic inflammation as displayed in NASH. Such evidence highlights the critical role of hepatic inflammation in NAFLD associated hepatocarcinogenesis [98, 101].

The importance of the steatotic change in the liver microenvironment for the establishment and growth of primary tumors is recognized, however little is known about its effect on establishment of metastasis. Study of the tumor/stromal interactions in this

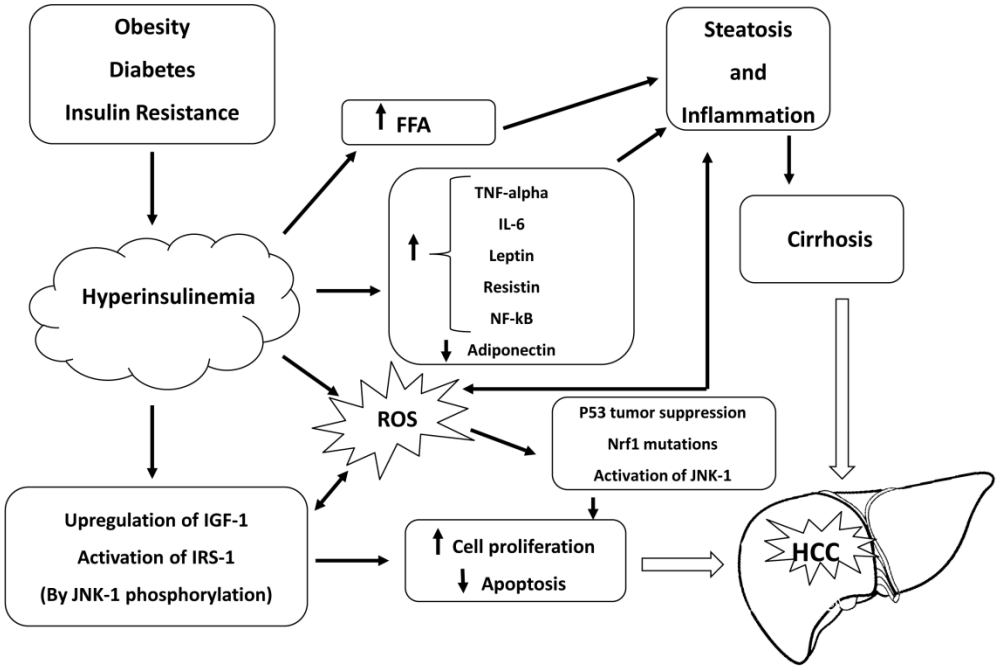


Figure 8: Proposed pathogenesis of hepatocellular carcinoma (HCC) in non-alcoholic fatty liver disease (NAFLD). IGF-1, Insulin-like growth factor; IRS-1, Insulin receptor substrate 1; JNK-1, c-Jun N-terminal kinase; FFA, free fatty acids; ROS, reactive oxygen species; TNF α, tumor necrosis factor- α; IL-6, interleukin-6; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf1, nuclear respiratory factor-1 [100].

unique liver microenvironment is important as a greater percentage of the population increasingly suffers from NAFLD. Changes in inflammatory mediators and cytokines as well as other important metabolic regulators have been implicated in the progression of NAFLD and liver fibrosis, and may be important in tumorigenesis [102]. A better understanding of the tumor and microenvironment interplay for the progression of primary and metastatic tumors in the setting of NAFLD, may prove valuable for understanding the development and devising treatment of these diseases.

Matrix Metalloproteinases

Matrix Metalloproteases (MMP) were first described by Jerome Gross and Charles Lapiere in 1962 when they identified the collagenase activity of MMP1 during tadpole tail morphogenesis [103]. Since then, many other MMPs have been identified and characterized for their activity. The MMPs are a subfamily of zinc dependent endopeptidases that together are collectively capable of cleaving all components of the extracellular matrix (ECM). They include 23 members in humans and were originally classified as collagenases, stromelysins, gelatinases, matrilysins, membrane type MMPs and others based on their ability to cleave specific types of ECM components [104]. As newer MMPs began to be identified, and it became clear that their substrates were not limited to ECM components, MMPs were assigned a sequential numbering system. Another means to classify MMPs is according to their structure. The MMPs all share a similar domain structure that typically consists of a pro-domain, a catalytic domain, a variable length hinge region and a C-terminal hemopexin domain. The catalytic domain is the enzymatic domain that is conserved amongst MMPs and contains the zinc-binding domain that is essential for function. Based on their domain structures, MMPs are

classified into 8 groups as shown in Figure 9 [105]. MMPs are initially synthesized as inactive zymogens with the pro domain that must be removed prior to enzymatic activity. The pro peptide is part of the "cysteine switch" that contains a conserved cysteine residue that prevents access to the activation site by interacting with the zinc in the active site, maintaining the enzyme in an inactive form [106]. The catalytic domain of all MMPs is similar and has a shallow cleft on the front that forms a grove that runs across the catalytic domain forming the active site [107]. The active site contains two zinc ions and one or two calcium ions. One zinc ion is involved in the catalytic process and coordinates with three histidine residues that are conserved among all the MMPs [108]. The catalytic domain is connected to the C-terminal hemopexin domain though a hinge region of variable length and structure. The hemopexin domain has structural homology to the serum protein hemopexin and is highly conserved in all MMPs except MMP7 and MMP26, which lack this domain. The hemopexin domain has been shown to play a functional role in substrate binding and interaction with the tissue inhibitors of metalloproteinases (TIMPs), the endogenous MMP inhibitors [109]. The hemopexin domain has also been shown to be important for cell migration and invasion [110]. Correia et al. showed that the hemopexin domain of MMP3 simulates mammary epithelial invasion and branching though interaction with the intracellular chaperone heat-shock protein 90β [111]. In addition to these basic domains, members of the MMP family are further classified based on additional structural and functional domains they have incorporated.

MMPs are highly regulated and their expression as well as their activity is controlled at multiple levels. In order to maintain normal physiological function, MMPs

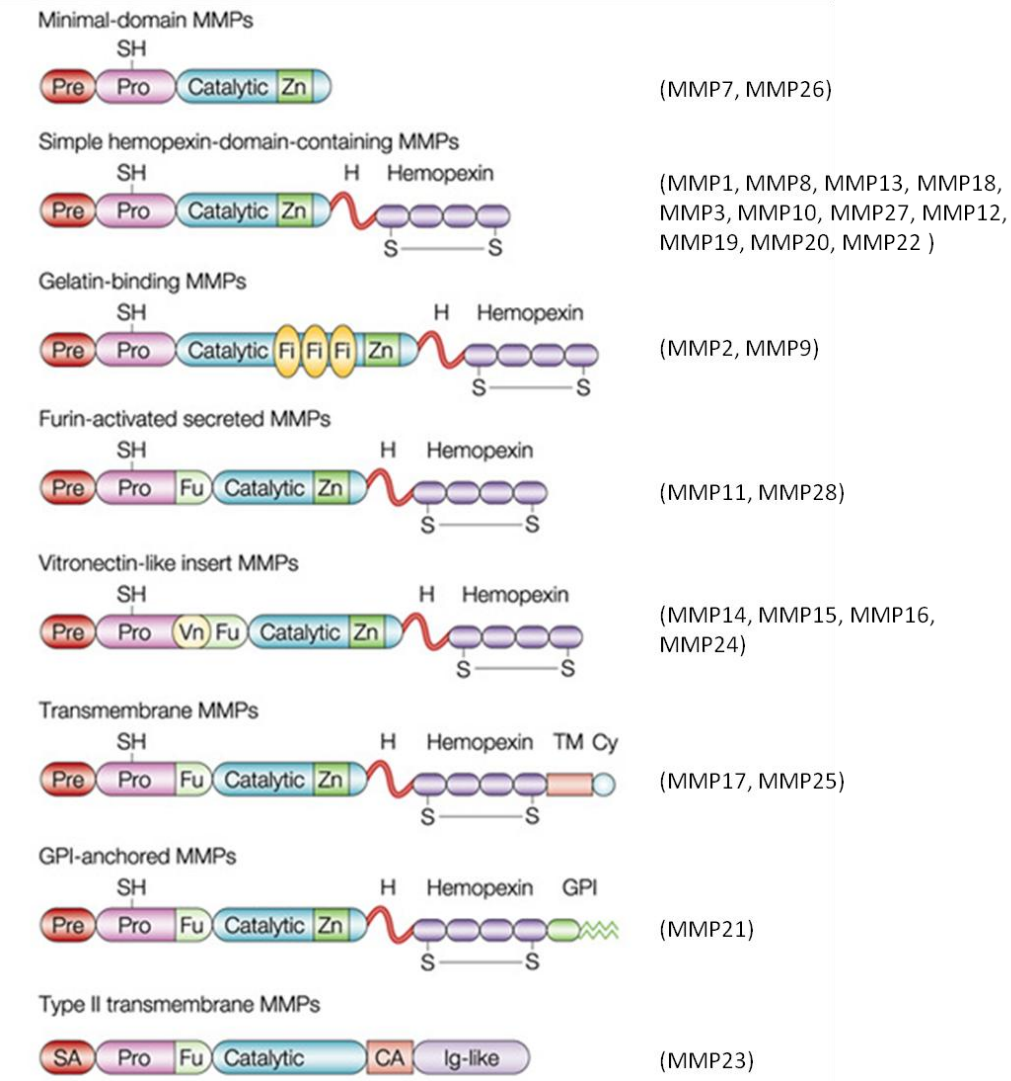


Figure 9: Classification of MMPs based on domain structure. MMPs are divided into eight groups, five of which are secreted and three are membrane bound. Pre, signal sequence; Pro, propeptide with a free zinc-ligating thiol (SH) group; Fu, furin-susceptible site; Zn, zinc-binding site; Fi, fibronectin domain; H, hinge region; TM, transmembrane domain; C, cytoplasmic tail; GPI, glycophosphatidyl inositol-anchoring domain; (CA) cysteine array and (Ig) immunoglobulin like domains [104, 105].

have to be expressed in the right cell type and maintain the correct pericellular location in required amounts to elicit relevant physiological activity. They are tightly regulated at the transcriptional and post-transcriptional levels [112]. In addition, the proteolytic activity is controlled by multiple mechanisms. They are secreted as inactive zymogens, and activation requires proteolytic removal of the propeptide by either other activated MMPs or serine proteases. Endogenous inhibitors include the TIMP family members, that reversibly inhibit MMPs by binding to them, thus preventing MMP interaction with their substrates. Other endogenous inhibitors of MMPs include α2-macroglobulin and thrombospondin that bind to MMPs and facilitate scavenger receptor mediated endocytosis and clearance of MMPs [104, 105].

MMPs in cancer

MMPs have long been recognized to be associated with almost every type of human cancer, correlating with late stage disease, increased migration and invasion and poor prognosis [104]. Historically, the ability of MMPs to cleave major components of the ECM including collagen, gelatin, proteoglycans, laminin and fibronectin surrounding the tumors has been considered to be their principle role in processes of tumor invasion and metastasis [113]. In recent years, the biologic functions of metalloproteinases have expanded to include roles in cell growth and differentiation, apoptosis, migration, tumor angiogenesis, and immune surveillance as a result of their ability to cleave and alter the biological activity of potent regulatory molecules [104, 114]. MMPs have now been shown to be involved at almost all stages of tumor progression starting with malignant transformation and tumor initiation, cellular proliferation, cell intravasation as well as

extravasation from the vasculature, and culminating in the establishment of distant metastases [115].

MMPs can cleave substrates that are responsible for regulating cell death, including Fas ligand (FasL). Deactivation of such regulators help tumor cells circumvent apoptosis [116, 117]. MMP mediated proteolysis of ECM has been shown to initiate cytokine signaling through release or activation of growth factors such as IGF, EGF, PTK7 or TGFβ that are either anchored in the matrix or inactive and contribute to cell proliferation, invasion, and survival [118–121]. Specifically, MMP release of TGFβ can stimulate alpha smooth muscle actin and collagen secretion from activated HSCs upon liver injury [122, 123]. Additionally, the reciprocal interaction between adhesion proteins such as E cadherin and MMP expression have been shown disrupt junction integrity and lead to an increase in the invasive ability of tumor cells [124]. MMPs can thus be viewed as mediators of cell: cell communication or host: tumor interaction that further modulate tumor progression.

Apart from their anti-tumorigenic effect, MMPs have also been shown to exert protective functions as well. For example, elevated MMP12 and MMP9 were shown to decrease tumor vascularity and growth of endothelial cells leading to decreased tumors and metastases in the lung and skin respectively [125, 126]. Increased MMP8 was shown to decrease tumor growth as well as establishment of metastasis with breast cancer cell lines [127, 128]. Mice without MMP3 expression, showed enhanced tumor growth rates and increased metastasis in squamous cell carcinoma (SCC) suggesting that MMP3 was protective in SCC tumorigenesis [129]. Thus, the findings that MMPs can have a protective role in cancer progression have put to rest the general belief that upregulation

of MMPs by either tumor or host cells benefit tumor development at both the primary and secondary site [130].

The protective features of select MMPs, including their function in development, wound healing and normal physiology, could explain why clinical trials using MMP inhibitors have failed [131]. The MMP inhibitor trials used broad spectrum MMP inhibitors targeting the majority of MMPs, good or bad, which led to an overall adverse outcome. Current understanding of pro and anti-tumorigenic roles of select MMPs, in combination with selective targeting of individual MMPs in a particular disease setting, could circumvent some of the issues encountered by these trials. Thus, further understanding of the roles of the individual MMPs and how they function in a particular microenvironment and their role in normal physiology could be important for the future use of MMPs as potential drug targets [131, 132].

MMPs in liver development and disease

MMPs play an important role during embryonic development, being required for remodeling of the ECM and cell migration. During embryologic liver development, an epithelial liver bud consisting of liver progenitors called hepatoblasts forms as an outgrowth of the endoderm. The hepatoblasts then invade into the neighboring septum transversum mesenchyme (STM) at embryonic day 9 where they intermingle with mesenchymal and endothelial cells. Several MMPs including MMP2, MMP11, MMP14, MMP15, MMP16, MMP17 and MMP19 are expressed during liver development. MMP2 and MMP14 activity is important for transit of hepatoblasts from an epithelial state to a migratory phenotype and enable them to invade the STM [133]. Liver development then proceeds through proliferation of the hepatoblasts and their differentiation into

hepatocytes or biliary cells. MMP1, MMP13, MMP2, MMP7, and MMP9 are expressed in the early developing human liver, although their function is still unknown [134].

The normal adult liver has a basal expression level of most MMPs and TIMPs [135, 136]. Upon liver injury, changes in MMP levels are frequently observed. For instance, MMP13, MMP2, MMP9, MT1-MMP, MMP3 and MMP10 were all increased after experimental CCl_4 exposure in rats with the highest expression changes coinciding with induction of inflammatory cytokines [137]. Expression of MMPs is altered during tissue regeneration. For example, following partial hepatectomy there is rapid induction of MMP2 and MMP9 [138]. This suggests that MMPs play an important role in hepatic tissue repair post injury. Immuno-histochemistry revealed MMP9 localization in the scar areas that contained active fibrogenesis [139]. Further, bile duct ligation studies also showed that MMP2 and MMP9 were elevated throughout the fibrotic process [140]. During the process of liver injury, HSCs, the liver resident stromal fibroblasts, become activated and undergo morphological and functional changes with increased expression of αSMA. Further, elevated MMP9 has been shown to be important in activation of HSCs when stimulated with IL1α, suggesting the importance of MMPs in the fibrogenic process [141]. Progression of fibrosis results from changes either in the deposition or removal of the ECM. By modulating the levels of MMPs, there may be hope for the reversal of cirrhosis, characterized by extensive fibrosis, and regarded as the end-stage of liver disease [142]. Adenoviral-mediated delivery of MMP1 promoted the resolution of thioacetamide induced fibrotic liver tissue [143]. Further, Murphy et al. show that inhibition of MMP activity decreases apoptosis of HSCs and leads to persistence of liver fibrosis [144]. Thus, MMPs play dual roles and have both adverse and beneficial

outcomes on liver fibrosis depending on the timing of their expression and activation [145].

Chemokines play an important role in shaping this inflammatory response and MMPs have been shown to be important in regulation of chemokine activity [146]. MMPs can directly cleave these chemokines or their precursors resulting in their activation or inactivation. MMPs can additionally lead to the physical release of chemokines and growth factors that are bound to the ECM or to other substrates, and facilitate their release by proteolytic cleavage of the ECM [147]. NAFLD results in an inflammatory microenvironment in the liver. Changes in MMP levels might be responsible for maintaining a prolonged state of chronic inflammation in NAFLD by activation of cytokines and inflammatory mediators that could potentiate inflammatory cell infiltration [148]. Studies using mouse models of diet induced obesity show that MMP12 and MMP13 are elevated in the livers and adipose tissue of mice [149]. The recognized role of MMPs in inflammatory processes and their ability to alter chemokine levels could thus impact tumor development and progression in the liver microenvironment affected by steatosis and steatohepatitis [150].

Matrix metalloproteinase 13

MMP13 is a member of the collagenase subfamily of MMPs, along with MMP1 and MMP8. *MMP13* was originally cloned from a breast carcinoma and identified as collagenase-3 based on presence of specific residues (Tyr-214, Asp-235, and Gly-237), fundamental for collagenase specificity and the ability to degrade type I fibrillar collagen [151]. The human *MMP13* gene shares 84% homology to murine *Mmp13*, which is the primary collagenase in mice. Normal physiological expression of human MMP13 is

limited to the hypertrophic chondrocytes and osteoblasts in the developing fetal bone where it is required for restructuring the collagen matrix for bone mineralization [152, 153]. There is little or no expression of *MMP13* in normal human adult tissue with undetectable *MMP13* mRNA levels in normal liver, placenta, ovary, uterus, prostate, parotid gland, or even breast tissues [151, 154]. However, *MMP13* is re-expressed in diseases where there is a need for tissue repair and remodeling of the ECM. *MMP13* expression is elevated in several pathological conditions including arthritis, chronic cutaneous ulcers, intestinal ulcerations, chronically inflamed periodontal tissue, and in atherosclerotic plaques and aortic aneurysms that are characterized by inflammation and remodeling of collagenous ECM [154].

MMP13 expression has been observed in invasive malignant tumors either within the tumor cells or the surrounding stroma, including breast carcinomas, where it is associated with metastasis to the bone, as well as invasion and metastasis in melanoma and basal cell carcinomas [155–157]. MMP13 is associated with squamous cell carcinomas (SCCs) of the head and neck and vulva [158], transitional cell carcinoma of the urinary bladder [159] and in colorectal cancer where it is associated with poor prognosis in patients with the presence of liver metastases [160, 161].

Importantly, MMP13 has also been associated with liver disease. Uchinami and colleagues demonstrated that MMP13 contributes to progression of fibrotic liver disease [162]. They showed that mRNA expression levels of inflammatory mediators, such as TNFα, were suppressed in livers of mice genetically deficient in *Mmp13*. They noted that upregulation of fibrogenic factors including TGFβ1, was also significantly suppressed in livers of *Mmp13* deficient mice as compared to wildtype mice. Functionally MMP13

contributed to fibrogenesis by mediating the initial inflammation of the liver in cholestatic livers induced by bile duct ligation in mice [162]. Other investigators found MMP13 to be active in the reabsorption of scar tissue in the liver, reflecting the activity of this protease in the setting of liver injury [163]. MMP13 was shown to be elevated in the liver tissue and epididymal fat of mice with diet induce obesity and NAFLD [149].

These reports suggest that MMP13 could play an important role in altering the liver microenvironment with progression of NAFLD. Since MMP13 expression is limited in normal tissues and elevated in the setting of disease, MMP13 could be a realistic target for treatment of several types of cancers.

Summary and Dissertation Goals

The obesity epidemic has led to an increase in the incidence of NAFLD. As a greater percentage of the population lives with fatty liver disease, the impact of this steatotic microenvironment on progression of liver disease, development of primary liver cancers such as HCC and cholangiocarcinoma, and the establishment of metastatic tumors in the liver must be better understood. Metastasis represents the final stage in cancer progression and is the most significant cause of cancer related mortality. The liver is a common site of metastasis of several epithelial cancers and we have previously demonstrated an increase in the incidence of colon cancer metastasis to the liver in the setting of steatosis [53].

Prolonged steatosis results in a state of chronic inflammation that leads to several changes in the liver microenvironment that could explain the differences in tumor establishment and metastasis observed in patients with NAFLD. The extracellular matrix plays an important role in cell-cell interactions and changes in the matrix components

have been shown to affect all stages of tumor progression. MMPs can degrade components of the ECM and have been shown to influence the ability of tumor cells to migrate and invade into tissues and affect the ability to form metastases. MMP13, collagenase 3, is a member of the MMP family that becomes elevated in the setting of NAFLD and could be integral to some of the changes seen in the steatotic liver. MMPs can be produced by both tumor and stromal cells and reports suggest that MMP13 is produced by activated stellate cells, Kupffer cells and other subpopulations of inflammatory cells; all of which could potentiate the increased level of MMP13 observed in the setting of steatosis. Activation of Kupffer cells can also lead to changes in gene expression and upregulation of pro-inflammatory cytokines that can further recruit inflammatory and bone marrow derived cells, ultimately impacting adjacent cell populations and sustaining NASH.

The goals of this dissertation were to better understand the steatotic liver microenvironment and identify cellular and molecular factors altered in the setting of steatosis and steatohepatitis that impact the establishment of metastases in the liver. Many studies have demonstrated that alterations in specific genetic factors, as well as stromal-tumor interactions, are important in the establishment of carcinoma metastases. The molecular mechanisms governing the growth and proliferation of tumors in the setting of steatosis and steatohepatitis are only beginning to be elucidated. I **hypothesize** that molecular and cellular changes in the steatotic liver relative to the normal liver contribute to a more permissive microenvironment for tumor growth and establishment of metastases in the liver. My thesis work examines how changes in the liver microenvironment, as the result of hepatic steatosis and steatohepatitis, affect the

processes of tumor cell establishment and growth in the liver. In chapter III, I explore the changes in inflammatory cell population and cytokine production in the steatotic liver microenvironment and how this effects hepatocellular proliferation. Chapter IV evaluates the role of MMP13, an MMP elevated in the setting of steatosis, on tumor cell extravasation and establishment of metastases in the steatotic liver microenvironment. My results show that both tumor and stromal derived MMP13 play a role in establishment of liver metastases. This suggests that inhibiting MMP13 could be a possible target to suppress cancer metastasis to the liver.

CHAPTER II

MATERIALS AND METHODS

Ethics Statement

All animal experimental procedures and protocols were approved by the Vanderbilt University Medical Center IACUC protocol #M/09/216 and performed according to institutional ethical guidelines for animal care and use. Human de-identified tissue samples were collected under National Cancer Institute (NCI) Best practices and Cooperative Human Tissue Network (CHTN) standard operating procedures.

Patient Samples

Liver biopsies from consented patients undergoing bariatric surgery were collected and flash frozen for protein or RNA collection and further de-identified in accordance with a protocol approved by Vanderbilt's Internal Review Board (VUMC IRB#120829). A portion of the sample was sent for histology and reviewed by a Vanderbilt University pathologist and classified as either Normal (<5% Steatosis), Steatosis, Steatohepatitis or NAFLD related cirrhosis. Additional de-identified formalin-fixed, paraffin-embedded liver tissue sections from normal and steatotic patients, and patients with colorectal cancer metastasis to the liver were obtained with permission from the Vanderbilt Translational Pathology Shared Resource.

Mice

C57bl/6J male mice were obtained from Jackson Research Laboratories (Bar Harbor, ME) and a breeding pair of B6-*Mmp13*-/- mice was obtained (Laboratory of Dr. Zena Werb, UCSF). The *Mmp13*-/- mice were originally generated by crossing mice homozygous for the floxed *Mmp13* allele ($Mmp13^{fl/fl}$) with mice carrying the *Cre* recombinase driven by the β-actin promoter [164]. *Mmp13*-/- mice have no observable liver phenotype. Mice were housed in a level 6 animal facility at Vanderbilt University. Once mice were 8 weeks old, they were fed either regular chow, a 13.5% fat diet (RD, 5001, LabDiet: 13.5% calories from fat, 58% from carbohydrates, and 28.5% from protein) or a 42% fat "high fat/western-style" diet (HF, TD.88137, Harlan Teklad (North America): 42% calories from fat, 42.7% from carbohydrates, and 15.2% from protein) *ad libitum* for 3 months at which time point we have shown that wildtype mice develop prominent steatosis (Figure 10) [53].

Acquisition of Liver Inflammatory Cells

At the end of 3 months, mice were sacrificed and weighed. To isolate liver cells from both normal and steatotic mice, mice were anesthetized and an incision was made in the abdomen cavity. Hepatic tissue was dually perfused, first through the heart and then though the portal vein with heparinized Krebs Ringer Buffer (KRB) (154mM NaCl, 5.6mM KCl, 5.5mM Glucose , 20.1mM HEPES, 25mM $NaHCO_3$, pH7.4) to remove any intravascular blood cells. The liver was then perfused with 2.5mls warm heparinized KRB (37°C) containing collagenase IV (500U/ml), DNase I (1500 U/ml), $CaCl_2$ (2.5mM)

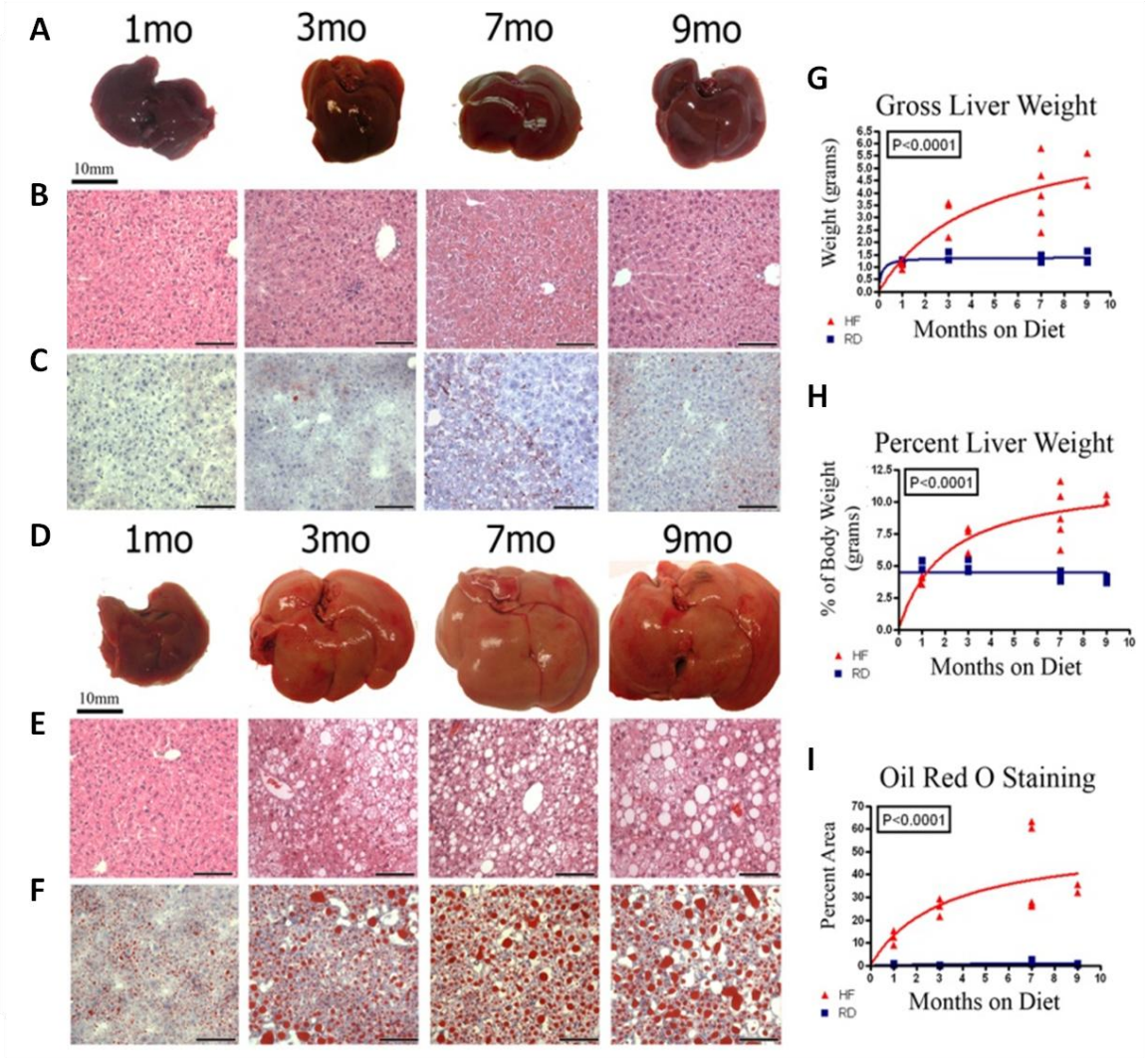


Figure 10: Characterization of the mouse model of diet induced steatosis. Mice fed regular diet (RD: A-C) of 13.5% fat or high fat diet (HF: D-F) consisting of 42% fat through a time course of 1 to 9 months (mo). Gross liver images show that livers of mice on the regular diet remain dark, red, free of lipid accumulation, and show little change in size over time (A). Mice on high fat diet show an increase in gross size along with yellowish appearance characteristic of steatosis over time (D). Accumulation of fat and development of steatosis can be seen in livers of mice on high fat diet as seen by vacuolation in hematoxylin and eosin stain (H&E) (E) and more definitively by oil red O (ORO) staining of lipids (F, red droplets) in mice on a high fat diet. Mice on regular diet do not show such changes (B, C). Gross liver weight (G), the percent liver weight as compared to total body weight (H), and the percent area of ORO staining (I) in livers of mice fed regular diet and mice fed the high fat diet are plotted on the right. Scale bars are 10mm for all gross liver images and 100μm for H&E and ORO images [53].

and $MgCl_2$ (2mM) through the portal vein. Post perfusion, livers were removed and weighed. For flow cytometric analysis, 1 gram of liver tissue was transferred to a MACS C tube, processed on cycle A of the gentle MACS dissociator in 5ml of the KRB collagenase solution. The resulting tissue suspension was incubated at 37°C for 30 min on a MACSmix tube rotator. Following enzymatic digestion, the C tube was put back on the MACS dissociator and processed with liver cycle 02. The suspension was passed through a 40 mm filter and 20 ml of ice cold PEB (Phosphate buffered saline pH7.2, 2mM EDTA, 0.5%BSA; Miltentyi Biotec) with DNase I (1500 U/ml) was added to the filtrate and centrifuged at 30g for 6 minutes to pellet out the hepatocytes. The supernatant was collected and centrifuged at 300g for 10 minutes to pellet the inflammatory cells, which were subsequently processed for flow cytometry. The remaining tissue samples of each liver were either fixed in buffered formalin, frozen in OCT compound, or homogenized in RIPA buffer (10mM Tris pH 7.5, 150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton) with addition of a complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN) for protein analysis. Additionally, endpoint heparinized blood samples were collected prior to perfusion and centrifuged at 2000xg for 20 minutes at 4^oC to collect plasma for cytokine array analysis. All samples not immediately used were stored at −80°C.

Histology

Formalin-fixed, paraffin-embedded tissue samples from both mouse and human samples were cut at 6μm on a Leica microtome and dried. For histology, sections were rehydrated with xylenes and a decreasing ethanol series and then stained with Mayer's Hematoxylin (Sigma) and Eosin. For immunohistochemistry, hydrated sections were boiled in a citric

acid solution (10 mM trisodium salt dihydrate pH 6.0, 0.5% Tween-20) for 8 minutes to unmask antigen. OCT embedded frozen tissue sections were cut at 8 μm on a Microm HM550 cryostat, air dried and fixed in ice cold acetone for 10 minutes. Slides were stained with primary antibodies at 4^{o}C overnight directed against: Ki67 (Abcam, ab15580), HNF4α (Santa Cruz, sc-655), CD68 (eBioscience, 12-0689-71), CD3 (BioLegend, 300415), CD8 (BioLegend, 301008), CD56 (BioLegend, 318327), MMP13 (Santa Cruz sc-12363), Cleaved caspase 3 (cell signaling D175) or vWF (Dako A0082). For diaminodenzidine, sections were labeled with appropriate species specific biotinylated secondary antibody (Vector Labs, Burlingane, CA), processed with a Vectastain kit (Vector Labs) and developed in chromogen solution (0.1M Tris-HCl pH 7.4, 1.125mM diaminobenzidine, 0.01% H_2O_2), counterstained with Mayer's Hematoxylin Solution (Sigma), dehydrated with ethanols and mounted with permount. Slides were imaged with a Q Imaging Micropublisher color digital camera mounted to a Zeiss Axioplan 2 microscope using MetaMorph software for acquisition. For immunofluorescence, sections were labeled with anti-rabbit Alexa Fluor (594) conjugated secondary antibody and counterstained with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride). Fluorescent images were acquired with a Hamamatsu Orca ER CCD camera mounted to a Zeiss Axioplan 2 Microscope using MetaMorph software acquisition (Molecular Devices, Dowington, PA). The number of total cells, the number of hepatocytes and the number of proliferating cells were determined using nuclear markers by quantifying the number of DAPI positive, HNF4α positive and Ki67 positive nuclei respectively. This was done by thresholding images to a preset background level through imageJ software and measuring the total number of particles over 75 pixels, to

eliminate background and select only nuclei. Average number of cells from five random 20x fields per sample were obtained. Quantification of immunohistochemical (IHC) staining in was carried out by setting a threshold to a fixed intensity and calculating the percent thresholded area with metamorph software analysis. For IHC analysis, the average thresholded area from five random 10x fields per sample were obtained.

Flow Cytometry

Pelleted inflammatory cells from digested livers were resuspended in PEB. CD45 or CD11b positive cells were isolated using positive selection with magnetic beads. 20 μl of respective magnetic beads (Miltenyi Biotec) were incubated with the pelleted cells for 20 minutes on ice, inverting the tubes every 5 minutes. Cells were spun down at 300g, resuspended in PEB, filtered through 0.3 μm mesh and magnetically isolated via LS columns (Miltenyi Biotec) according to manufacturer's instructions. Total number of cells, isolated by CD45 magnetic beads, was determined per gram of liver tissue with a BioRad TC10 automated cell counter. CD45 isolated cells were resuspended in 250 μl of staining solution containing lineage specific markers. Markers used for mouse T-cell subpopulations staining were CD3ε (488), CD4 (A700), CD8 (PE), CD25 (APC) and CD62L (e-Fluor 450). Granulocytes were stained with F4/80 (APC), GR1 (PE), Ly6C (e-Fluor 450). Dendritic cells were identified with monoclonal antibodies to CD11c (e-Flour 450) and B-cells were revealed as CD19 (APC) positive. 7-AAD was used as a vital stain. All fluorophore-conjugated monoclonal antibodies were obtained from BD Pharmingen. Cells were labeled for 20 minutes at room temperature with constant agitation on a Nutator. Post staining, cells were washed twice with 1ml PEB and resuspended in a final volume of 250 μl. Flow cytometry data acquisition was performed

on a 3-laser BD LSRII at the VMC Flow Cytometry Shared Resource. Five mice per group were used for flow cytometric analysis. FlowJo software was used for data and statistical analysis of flow cytometric results. Figure 11 depicts the gating stategy used for the analysis. Additionally, magnetically isolated CD11b positive cells were spun at 300g and resuspended in RPMI media and 150,000 cells per well were cultured for 24 hours before isolating conditioned media for cytokine analysis.

Cytokine Arrays

Liver tissue lysates from normal and steatotic mice were quantified using the BCA assay (Pierce). 100 micrograms of protein lysate was added to each array (n=3). Plasma collected from both normal and steatotic mice was diluted 1:10 in blocking buffer and 100 μl of sample was used for cytokine array analysis (n=3). Additionally, 100μl of pooled conditioned media collected from CD11b positive cells isolated from both normal (n=3) and steatotic mice (n=3) was run for cytokine profile analysis. Cytokine array analysis was carried out using RayBiotech Mouse cytokine arrays (AAM-CYT-G3) as directed by their protocol. Briefly, the array surface was first blocked and then incubated with sample overnight. Arrays were washed and subsequently incubated with fluorescently tagged secondary antibody. Stained slides were scanned using a GenePix 4000B Microarray Scanner at the Vanderbilt VANTAGE Core. Densitometric analysis was then performed using GenePix Pro Acquisition and Analysis Software. Background was subtracted and data were normalized against positive controls included on each array. All data directly compared were derived from the same batch of arrays.

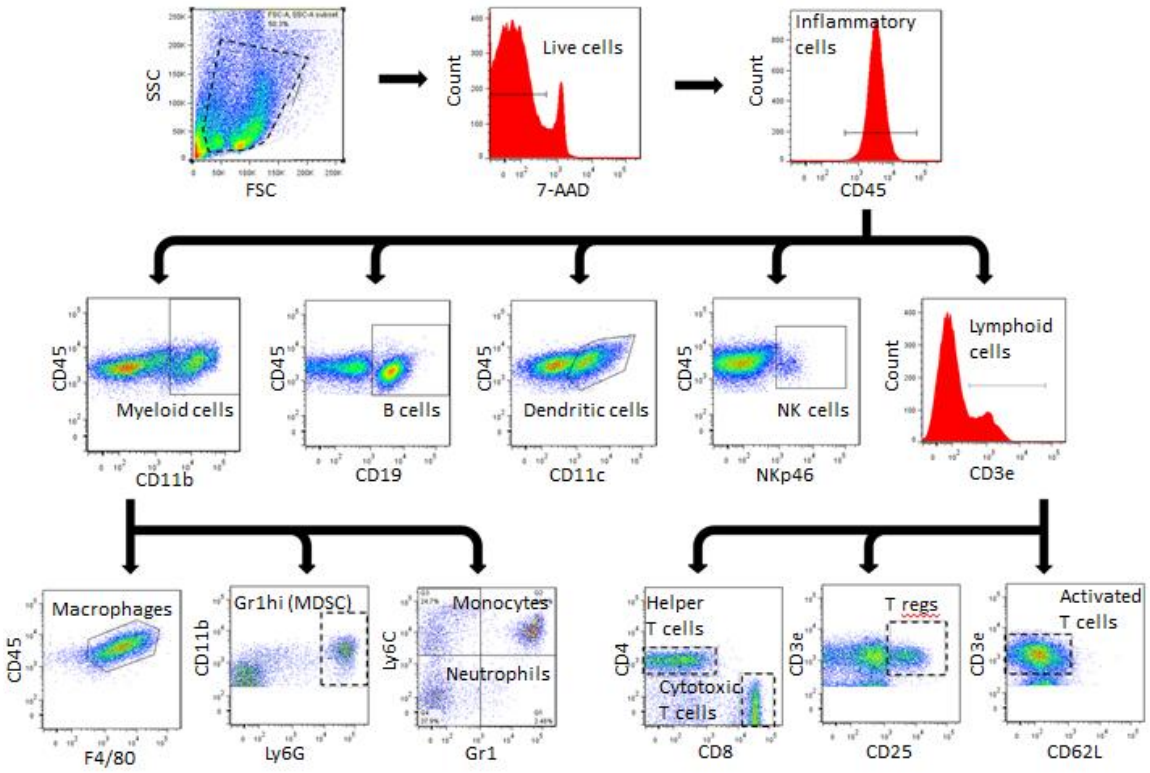


Figure 11: Gating strategy for flow cytometry analysis of inflammatory cells in the liver. Inflammatory cells were gated for based on forward and side scatter distribution. Cells within this region, negative for 7-AAD were identified as live cells. Among the live cells, CD45 positive inflammatory cells were identified by positive staining on the APC-Cy 7 channel. CD45 positive cells were then classified as CD11b positive myeloid cells, CD3 positive lymphocytes, CD19 positive B cells, CD11c positive dendritic cells and NKp46 for Natural killer cells. CD11b myeloid cells were further stained with F4/80, Gr1 and Ly6C to identify myeloid cell sub populations and CD3 positive lymphoid cells were stained with CD4, CD8, CD62L and CD25 for lymphoid cell subpopulations.

Cell Lines

HepG2 human hepatocellular carcinoma cells were obtained from American Tissue Culture Collection (ATCC, Rockville, MD, USA). MC38 murine colon cancer cells lines, syngeneic to C57Bl/6 background were provided by Dr. Steven Libutti, National Cancer Institute. HCT116 human colorectal carcinoma cell line was obtained from ATCC (CCL-247). Cell lines were grown in culture conditions of 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) in Dulbecco's Modified Eagle Media (Gibco BRL, Carlsbad, CA) at 37°C and 5% CO_2, and harvested at 75% confluence for experimental studies. HEPT mouse hepatocyte cells were isolated from the immortomouse expressing the temperature sensitive SV40 gene encoding large T antigen [165]. The HEPT cells are grown in DMEM with 10% FBS, P/S, L-glutamine and ITS (insulin, transferrin, selenium, Gibco 41400045), 1U/ml IFN-γ and incubated at 32°C in 5% CO_2. Prior to experimental analysis, cells were split and transferred to 37^oC in 5% CO_2 to deactivate SV40 for a minimum of 24hours.

MTT Assay

Proliferation of cells was determined using the MTT assay. Briefly, $1x10^5$ cells were plated in each well of a 96 well plate and allowed to attach overnight. Cells were then serum starved for 24 hours. For determination of effect of cytokines on proliferation different concentrations of Leptin, CXCL1, CXCL2 and CXCL16 (100 ng/ml, 25 ng/ml and 1 ng/ml) in serum free media were added to each well. Serum free conditions and 10% FBS were used as controls. For effect of loss of MMP13 on proliferation, 24 hours post serum starvation, media was replaced with DMEM containing 10% FBS. After 24

hours, 20 µl of MTT reagent (5 mg/ml, Sigma) was added to each well and returned to the incubator for 2 hours after which the media was aspirated from each well and the remaining MTT formazan crystals dissolved in 100 µl of isopropanol. Absorbance at 570 nm was read using a Victor3 V 1420 Multilabel Plate Counter. Experiments were carried out in triplicate with 5 replicates per plate.

Western Blot Analysis

Protein lysates were prepared with RIPA lysis buffer. Protein concentration was determined by the Pierce BCA protein assay (Thermo Scientific). 30 µg of protein was loaded into each well and separated on a 10% SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane, subsequently blocked with 3% milk, and then incubated with anti MMP13 antibody (Abcam, ab39012) overnight at 4^{o}C. The blots were washed and then incubated with secondary antibody (IR700 conjugated donkey anti rabbit IgG) for 1 hour at room temperature. Blots were washed and then imaged with Licor Odessy scanner.

qRT-PCR

RNA was extracted using a combined Qiazol extraction and subsequent Qiagen RNeasy mini kit. One microgram of total RNA was reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems) and real-time PCR was performed using specific primers for mouse MMPs with GAPDH as control (Qiagen, QT00116116, QT00107751, QT00110012, QT00113540, QT00108815, QT00115521, QT00099729, QT01658692, QT00098945, QT01064308). Real-time PCR was performed with the iQ SYBR green supermix kit (Bio-Rad) according to the manufacturer's instructions and

measured via a CFX96 real time PCR detection system (Bio-Rad). Experiments were done in triplicate with three replicates per sample. Fold-change was determined relative to normal wildtype samples and calculated using GAPDH levels as a reference.

Experimental Liver Metastasis

Experimental liver metastasis was carried out as previously described (Figure 12) [53]. Briefly, five month old wildtype or *Mmp13* knockout mice that had been on diet for 3 months were injected with 5×10^5 MC38 parental or *Mmp13* knockdown cells into the spleen and allowed to perfuse to the liver for 3 minutes before splenectomy. Mice were sacrificed at 14 days post-injection and the burden of metastatic liver tumors were compared between the different groups. At the time of sacrifice the mice were weighed, the livers were removed and weighed, and the livers were processed for histology as described above. Graphical representation of metastatic burden was calculated using GraphPad software to compare the total liver weights, as well as the percentage of liver weight relative to the total animal weight. Further, in-depth quantitative analysis of tumor burden was assessed on the left lateral lobe of the liver. The fixed liver was cut sagittally into four parts and paraffin-embedded, sectioned and stained for H&E. Slides were scanned at 20X using an Ariol® SL-50 scanner. Ariol software was used to quantify the size and number of tumors per section.

Tumor Cell Extravasation

To determine the number of tumor cells extravasating in the liver, we utilized the methodology previously described by Martin et al. [166], with slight modifications (Figure 13,14). Briefly, mice were injected intrasplenically with $1x10^6$ cell tracker red

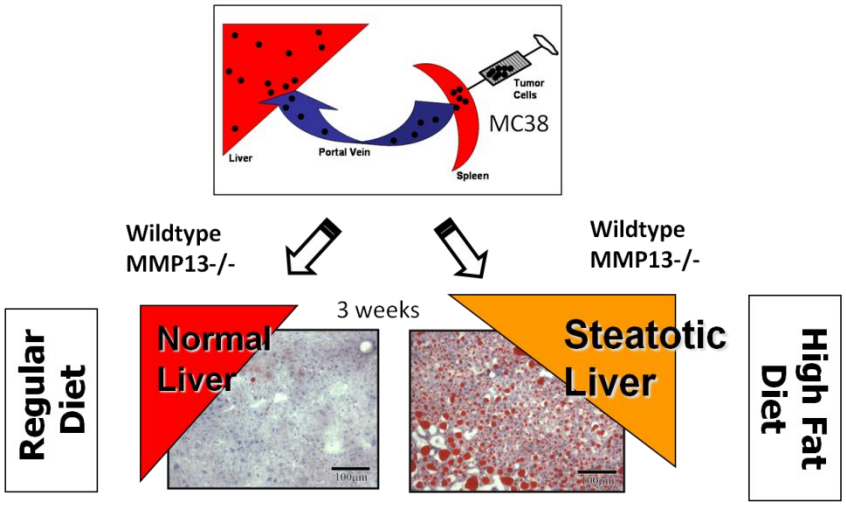


Figure 12: Splenic injection model of experimental liver metastasis. Tumor cells are injected via the spleen and populate the liver through the portal venous route.

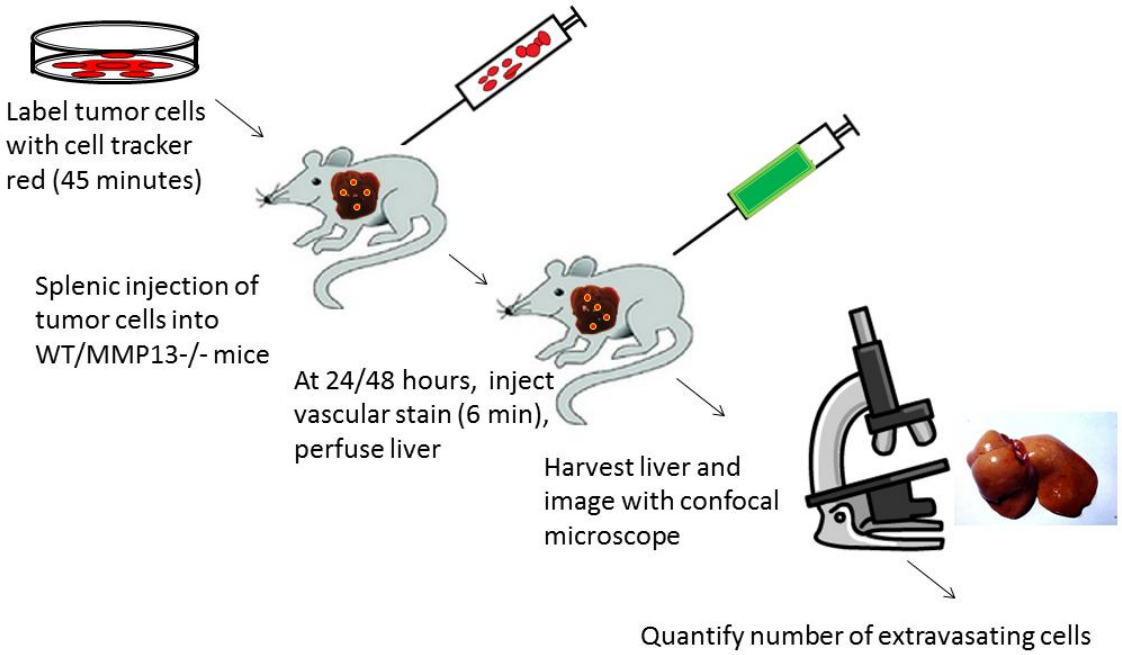


Figure 13: Schematic demonstrating use of whole organ confocal microscopy determine tumor cell extravasation. Cell tracker red labeled MC38 cells are injected into wildtype or *Mmp13*-/- mice. 24 or 48 hours post injection vasculature is labeled with 488-tagged tomato lectin followed by dual perfusion of the liver. Whole liver is then harvested and imaged with confocal microscope to identify individual tumor cells in the vasculature.

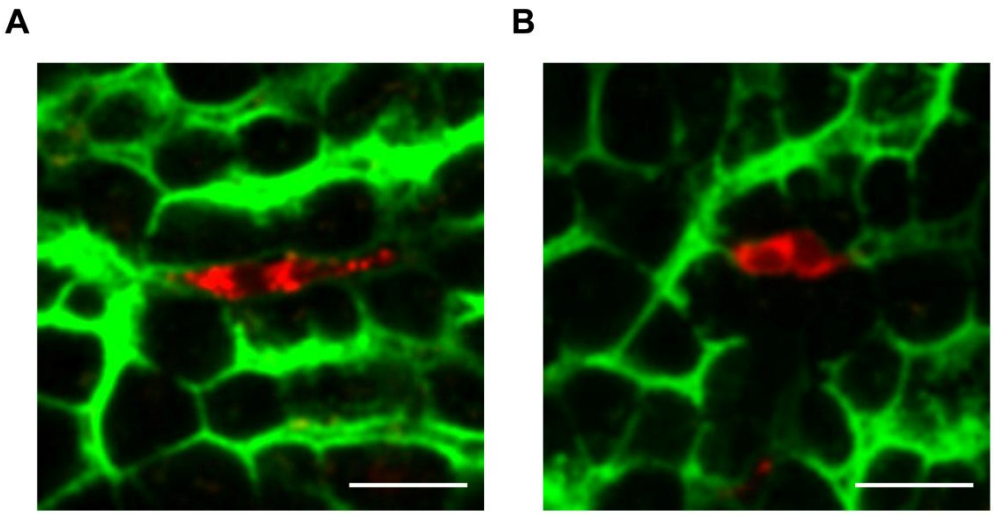


Figure 14: Classification of tumor cells as within or extravasating from the vasculature. 2-Dimentional images showing MC38 tumor cells labeled in red (A) that are completely within the liver vasculature labeled with 488- tagged tomato lectin (green) or (B) that are extravasating outside the vasculature. Scale bar represents 25 microns.

(Life Technologies) labeled MC38 tumor cells. At 24 and 48 hours post injection, mice were anesthetized using isoflurane and placed on mechanical ventilation at a rate of 60 breaths/ minute through surgical tracheostomy. 100 µl of 0.5 mg/ml 488-tomato lectin vascular label (Vector Laboratories, DL-1174) was injected into the spleen and allowed to circulate throughout the body for 6 minutes. Next, the abdominal aorta was cut to provide outflow and livers were perfused using gravity pressure through the heart and spleen. After the effluent had cleared, the liver was resected en-bloc, placed in a glass bottom dish with #1.5 glass (In Vitro Scientific) and immediately imaged.

All the images were acquired using a LSM780 confocal microscope (Carl Zeiss Inc.) using a Fluar 40X oil objective with NA =1.30 at room temperature. 488nm and 561nm laser lines were used to simultaneously excite fluorescence from the vasculature stain and from the cell tracker loaded tumor cells. The band-pass emission filters for the two channels were set as follows: 499 – 552 nm for the green fluorescence, and 602 – 747 nm for the red fluorescence. The pixel size was set to 0.692 µm and the dwell time was 12.6 µs. Images were acquired every 0.700 µm for every z-stack. The laser power of the laser lines was independently adjusted for each stack, between 0.1% and 3%, to avoid saturated pixels and to maintain a good signal to noise ratio through all the planes. The bit depth of the images was set to 12 bit. The image analysis was performed using Fiji [167]. The tridimensional structure of the vasculature was reconstructed from each z-stack by using the tubeness function [168]. The resulting tridimensional vasculature was overlaid to the red channel to identify a cell as extravasating or not. Percentage of cells extravasating at each time point was determined from over 30 individual tumor cells per mouse (n=3 per group).

Establishment of Stable *Mmp13* Knockdown Cell Lines

Pooled lentiviral particles targeting three different regions of murine *Mmp13* (Open Biosystems: V2LMM_28573, V2LMM_ 34601, V2LMM_37490) and control particles were used to transfect MC38 cells. Control non-target and specific human *MMP13* shRNA lentiviral particles targeting three different regions of human *MMP13* (Santa Cruz Biotechnologies, sc-41559) were used to transfect HCT116 cells. Post-transfection, shRNA-expressing cells were selected with puromycin. Knockdown was confirmed in resulting clones with qRT-PCR and western blot analysis.

Transwell Migration and Invasion Assays

Cell migration and invasion were assessed by a modified Boyden assay using 24 well multiwell inserts (BD) with 8 µm pore PET membrane. *MMP13* knockdown and control MC38 or HCT116 cells ($1x10^5$ cells/chamber) were seeded in DMEM plus 1% FBS directly on the insert membrane (Migration) or resuspended in 100 µl of 1 mg/ml of basement membrane matrix (BD) (Invasion) and seeded on the membrane. DMEM with 10% FBS was added to the lower chamber and cells were allowed to migrate through the filter for 16 h at 37°C in 5% CO_2 for MC38 cells and 48 h for HCT116 cell lines. MC38 cells were allowed to invade for 24 h and HCT116 cells for 72 hours. Cells on the lower surface of the membrane were fixed in 100% methanol, stained with Dapi, and imaged. Experiments were carried out in triplicate with three replicates per experiment and the number of cells migrated/invaded per 10X field was determined from five random fields per well.

Bone Marrow Transplants

WT C57Bl6/J male and *Mmp13*-/- male mice at 8 weeks of age, were the recipients of either WT or *Mmp13*-/- bone marrow. Using a cesium irradiator, the recipient mice were placed in a plastic pie shaped container and rotated at a very slow speed to be exposed to a single lethal dosage of 900 rads from the cesium source. There is no external damage to the mice during this procedure. Mice were injected with donor bone marrow after 4 hours. WT C57Bl6/J female and *Mmp13*-/- female mice, at 6-8 weeks of age, were used as donors for the bone marrow transplant experiments. To harvest the bone marrow, mice were euthanized using CO_2 and their hind legs amputated, and as much muscle and tissue removed as possible from the femur and tibia. The bones were then clipped as close to the tips as possible to retain bone marrow. Using a 5 ml syringe filled with RPMI, 2% FBS and 10U/ml heparin, the bone marrow was flushed into the petri dish and drawn up and down to break up the marrow. Cells collected from all mice were pooled in a conical tube and spun at 1200 rpm for 10 min at 4^oC. The supernatant was aspirated and resuspended in 20 mL filtered RPMI and again spun at 1200 rpm for 10min at 4^oC. Washed cells were resuspended in 1 ml of RPMI and counted, then diluted to 20 million cells/ml. Recipient mice were anesthetized, 4 hours post irradiation, using an isovet and then injected with 100 µl of donor bone marrow cells (2 million cells) via the tail vein. Mice were routinely monitored and maintained on antibiotic water for 3 weeks before putting them on specified diet for 3 months prior to splenic injection with MC38 tumor cells as previously described.

Statistical Considerations

Statistical analysis was performed using GraphPad Prism software. Data was analyzed by using one-way ANOVA or Kruskal-Wallis test followed by Newman-Keuls Multiple Comparison Test or Dunns test between all sample sets. P values are represented by stars where: * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

CHAPTER III

HEPATOCELLULAR PROLIFERATION CORRELATES WITH INFLAMMATORY CELL AND CYTOKINE CHANGES IN A MURINE MODEL OF NONALCOHOLIC FATTY LIVER DISEASE

The contents of this chapter have been published:

Mendonsa AM, VanSaun MN, Gorden DL (2013): PLoS ONE 8(9): e73054. doi:10.1371/journal.pone.0073054 [169]

Introduction

Nonalcoholic fatty liver disease (NAFLD) is commonly associated with obesity, the metabolic syndrome and type II diabetes mellitus and thus its significance parallels that of the epidemic rise of these diseases in this country and much of the world [170]. NAFLD can present as a spectrum of pathology ranging from benign steatosis, defined by triglycerides and other glycerophospholipids within hepatocytes of the liver and progress, to non-alcoholic steatohepatitis (NASH) characterized by the development of concomitant inflammation in the liver. Steatohepatitis is a unique liver microenvironment typified by accumulation of triglycerides, characteristic pathologic findings such as Mallory bodies as well as the infiltration of inflammatory cells as the disease progresses to steatohepatitis [78, 79]. Over time, this can progress to end-stage liver disease with fibrosis and cirrhosis. Some estimates suggest that NAFLD may be present in 17-33% of the U.S. population and that 33% of these patients have a significant component of NASH [75]. Currently 4-10% of liver transplants in the U.S. are performed for end stage liver disease due to NASH [76]. Equally ominous, is the increasing recognition that

NASH and progressive liver fibrosis in this setting are risk factors for primary hepatocellular cancer.

Obesity is a recognized independent risk factor for the development of a number of epithelial malignancies including breast, colon and hepatocellular carcinoma (HCC)[100]. As many as 10% of patients with end stage liver disease due to NASH have concomitant hepatocellular carcinomas [100, 171]. In addition, there are increasing reports of HCC developing in the background of NASH, without accompanying cirrhosis [82]. Preclinical studies have shown that hepatic steatosis increases both development of primary hepatocellular cancer growth as well as the seeding of metastatic tumors [43, 53, 100]. In accordance with these reports, previous studies from our lab have shown that there is an increase in the number of metastatic tumor foci in the liver in the setting of steatosis using a mouse model of diet induced steatosis. Mice fed a high fat diet for over 9 months also develop spontaneous premalignant adenomatous tumors [53] .The importance of the steatotic change in the liver microenvironment for the establishment and growth of primary and metastatic tumors is not clearly defined. Obesity-associated alterations in cytokine levels leading to increased levels of reactive oxygen species may evoke proliferative response from the hepatocytes [172]. There is mounting evidence that alterations in inflammatory mediators and cytokines as well as other factors such as insulin resistance, lipotoxicity and other metabolic regulators such as leptin, adiponectin and TNF-α have been implicated in the progression of NAFLD and liver fibrosis, and may also be important in tumorigenesis [102].

The liver is comprised of several resident cell types, which can contribute to recruitment of circulating inflammatory cells [173]. Hepatocytes comprise 60% to 80%

of all liver cells and conduct the metabolic, biosynthetic, detoxification and biliary secretory functions of the liver. During development of steatosis, hepatocytes accumulate lipids and stain positive for triacylglycerides (TG). Accumulation of TGs in hepatocytes leads to generation of lipid metabolites such as lysophosphatidylcholine (LPC) and is associated with endoplasmic reticulum (ER) stress, c-Jun NH(2)-terminal kinase (JNK) activation that leads to lipoapoptosis of hepatocytes [174, 175]. Lipoapoptosis in turn leads to the recruitment of inflammatory cells contributing to the progression to NASH. Subsequently, NASH elicits pathological elements of hepatocellular injury, evident as cellular ballooning, appearance of Mallory bodies and apoptosis which exacerbates NAFLD.

As the epidemic of NAFLD increases, improved understanding of the changes in inflammatory cell populations and concomitant release/activation of cytokines in this unique liver microenvironment is necessary in order to develop strategies that could modulate these for therapeutic benefit. Importantly, studies of murine models that recapitulate human disease are crucial for translational studies to succeed. This study demonstrates significantly increased hepatocyte proliferation, alterations in serum and tissue cytokine levels, as well as local recruitment of inflammatory cell populations in livers in the setting of high fat diet induced steatosis.

Results

Hepatocellular proliferation in steatotic livers

To study the progression of NAFLD, we have used a previously validated mouse model of diet induced steatosis [53]. In this study, prolonged 42% high fat diet led to fibrosis, inflammation and development of dysplastic lesions in the liver. We therefore

wanted to determine whether high fat diet induced hepatic steatosis led to changes in proliferation of cell populations within the steatotic liver. To do so, human and murine liver tissues with and without steatosis were triple stained for the nuclear markers Ki67, HNF4α and DAPI. HNF4α has previously been demonstrated to specifically stain hepatpatocytes in the liver [176]. The overall number of proliferating Ki67 positive cells as a percentage of total number of DAPI positive cells as well as the percentage of co-positive Ki67/HNFα proliferating hepatocytes vs total HNF4α positive hepatocytes were counted and quantified per field. We found a significant increase in the percentage of total Ki67 positive cells as well as the percentage of Ki67 positive hepatocytes in both human and murine steatotic liver samples vs normal liver samples (Figure 15). These results indicate that as NAFLD correlates with an increase in the number of proliferating hepatocytes in the liver.

Inflammatory cell population changes in the murine steatotic liver

As NAFLD progresses to NASH, it is characterized by an influx of circulating inflammatory cells and an alteration in the subpopulations of local inflammatory cells. Studies have shown a significant increase in CD45 positive hematopoietic inflammatory cells in mice with diet-induced steatosis [177]. To profile early changes in inflammatory infiltrates in the steatotic liver, samples were characterized for the effect of high fat diet-induced steatosis on various inflammatory cell populations by flow cytometry analysis. CD45 positive inflammatory cells were isolated from dissociated livers using magnetic beads and a positive selection. Absolute number of CD45 positive cells per gram of liver tissue were counted and demonstrated a significant increase in the steatotic livers versus normal livers ($8.027x10^6 \pm 0.38x10^6$ and $4.84x10^6 \pm 0.32x10^6$ respectively, $p<0.0029$).

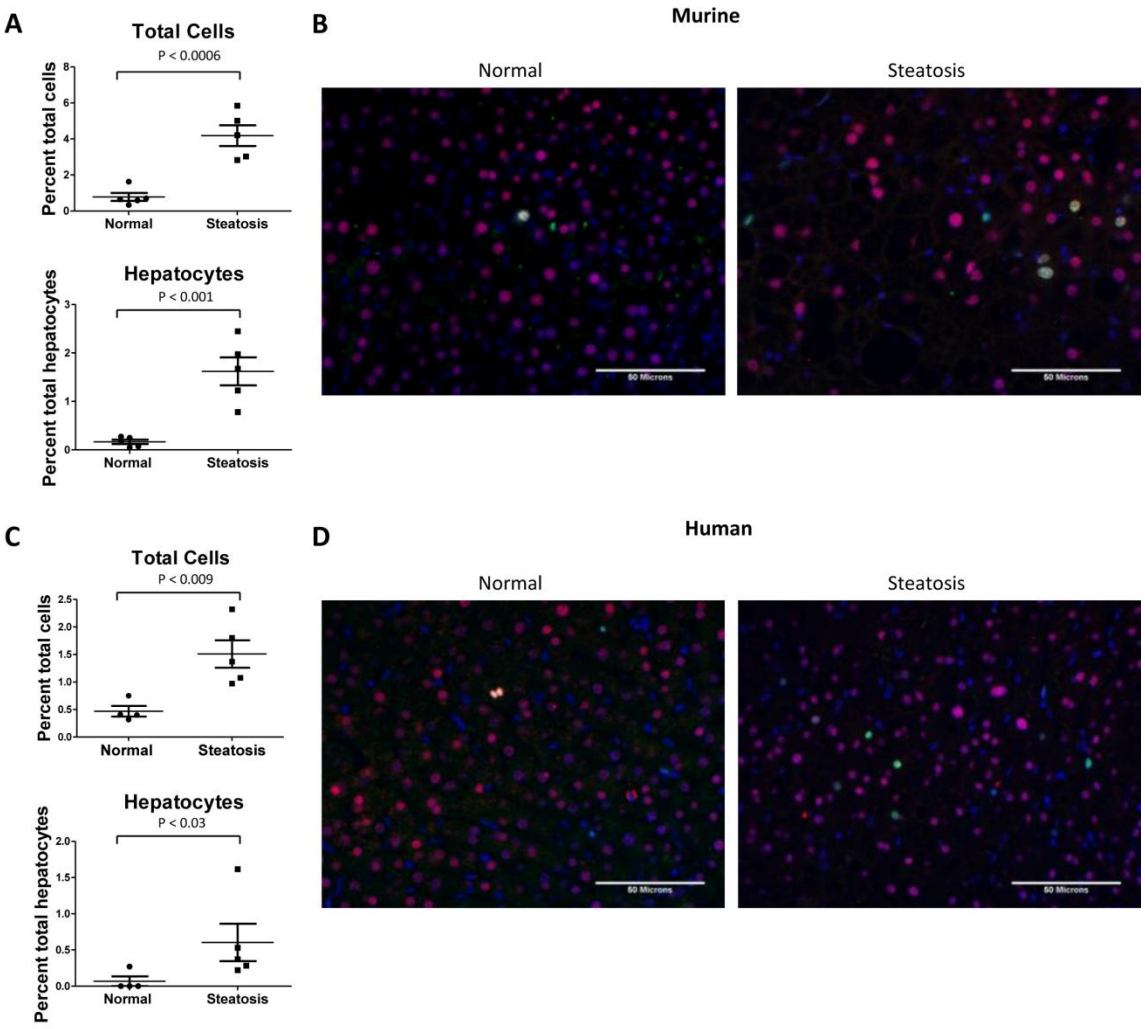


Figure 15: Steatosis results in increased cellular proliferation in the liver. The percentage of total Ki67 positive cells and the percent of Ki67/HNF4α double positive hepatocytes are increased in steatotic livers compared to normal livers in (A, B) murine and (C, D) human samples. Representative images of Ki67 (green), HNF4α (red) and DAPI (blue) immunofluoresence staining in livers of normal and steatotic (B) murine and (D) human liver sections showing increased number of Ki67 positive cells in steatotic livers. Images are taken at 40X and scale bars represent 50 microns.

Figure 16A is a representative scatter plot showing an overall increase in CD45 positive cells in the steatotic compared to normal livers. Changes in inflammatory cell populations were further represented as a percentage of the isolated CD45 positive cells. High fat diet-induced steatosis resulted in an overall increase in the CD45/CD11b positive myeloid cell population and the CD45/CD3ε positive lymphocytic population (Figure 16B). Next, to look at whether there were changes in the individual myeloid cell subpopulations, cells were co-stained with F4/80, Gr1 and Ly6C to look at differences in macrophage, activated monocytes, infiltrating neutrophil and MDSC cell populations after gating for CD11b as shown in Figure 17. No significant difference was observed in the percentage of the subset of F4/80 positive macrophages, however there was a significant decrease in the percentage of $GR1^{hi}$ expressing MDSC's and $Ly6C^{+}Gr1^{+}$ activated monocytes while there was a decrease in the percentage of $Ly6C^{-}Gr1^{+}$ infiltrating neutrophils in the steatotic livers compared to the normal livers.

The lymphocytic cell population was identified from CD45 positive isolated cells by staining for CD3ε and additionally T cell subpopulations were identified with markers for CD4, CD8, CD25 and CD62L (Figure 18). From the overall increase in $CD3\varepsilon^{+}$ cells in steatotic livers, we did not detect any significant differences in percentage of T cell subpopulations except for a slight increase in the total number of CD25 positive regulatory T cells in steatotic livers compared to normal livers ($P=0.05$).

Additionally, CD45 positive cells were stained with CD19, CD11c and NKp46 to look at changes in B cell, dendritic cell and natural killer cell subpopulations respectively between the livers of normal and steatotic mice (Figure 19). There was a significant decrease in B cell subpopulation, an increase in dendritic cell population and no

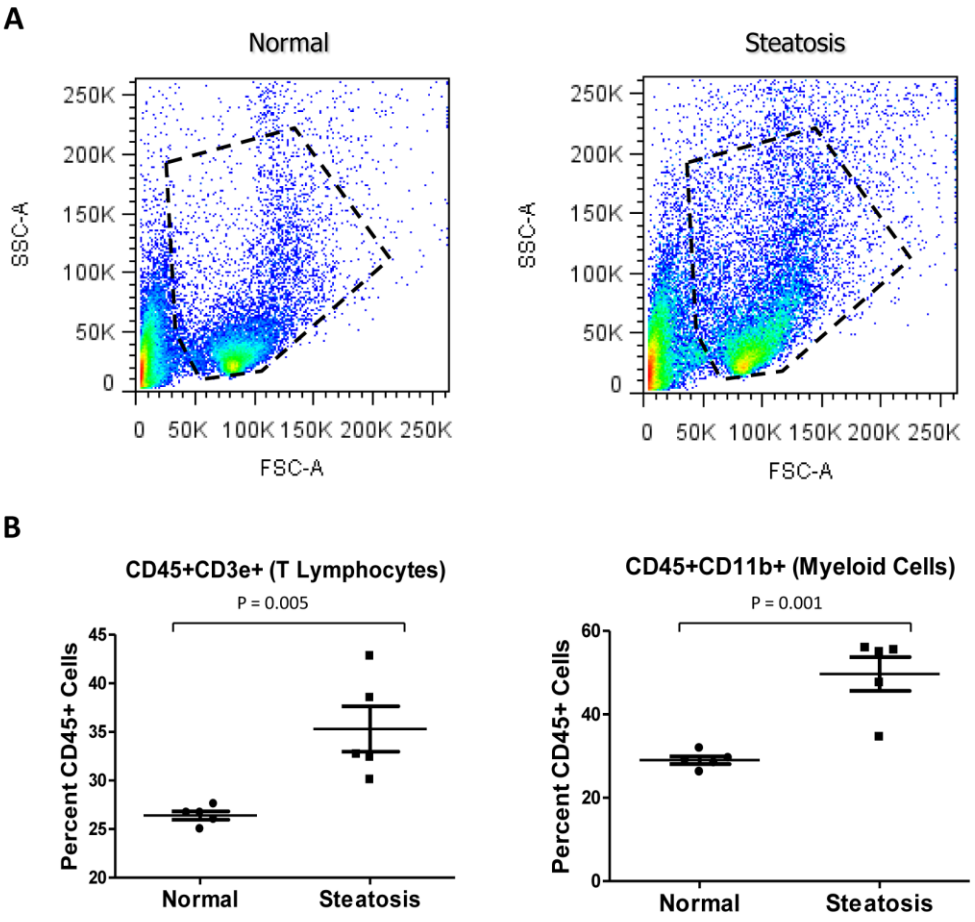


Figure 16: Steatosis results in changes in the inflammatory cell populations in the murine liver. CD45 positive cells were isolated from normal and steatotic digested liver samples by immuno-magnetic beads and then stained for the immune markers CD3 and CD11b before being subjected to flow cytometric analysis. (A) Diagrams are representative scatter plot of flow cytometric analysis for overall CD45 positive inflammatory cells in the livers. (B) CD45 positive cells were then gated for CD3ε positive T lymphocytes or CD11b positive myeloid cells. CD3ε positive and CD11b positive subpopulations were both increased in the steatotic livers versus the control livers. FSC, forward scatter; SSC, side scatter.

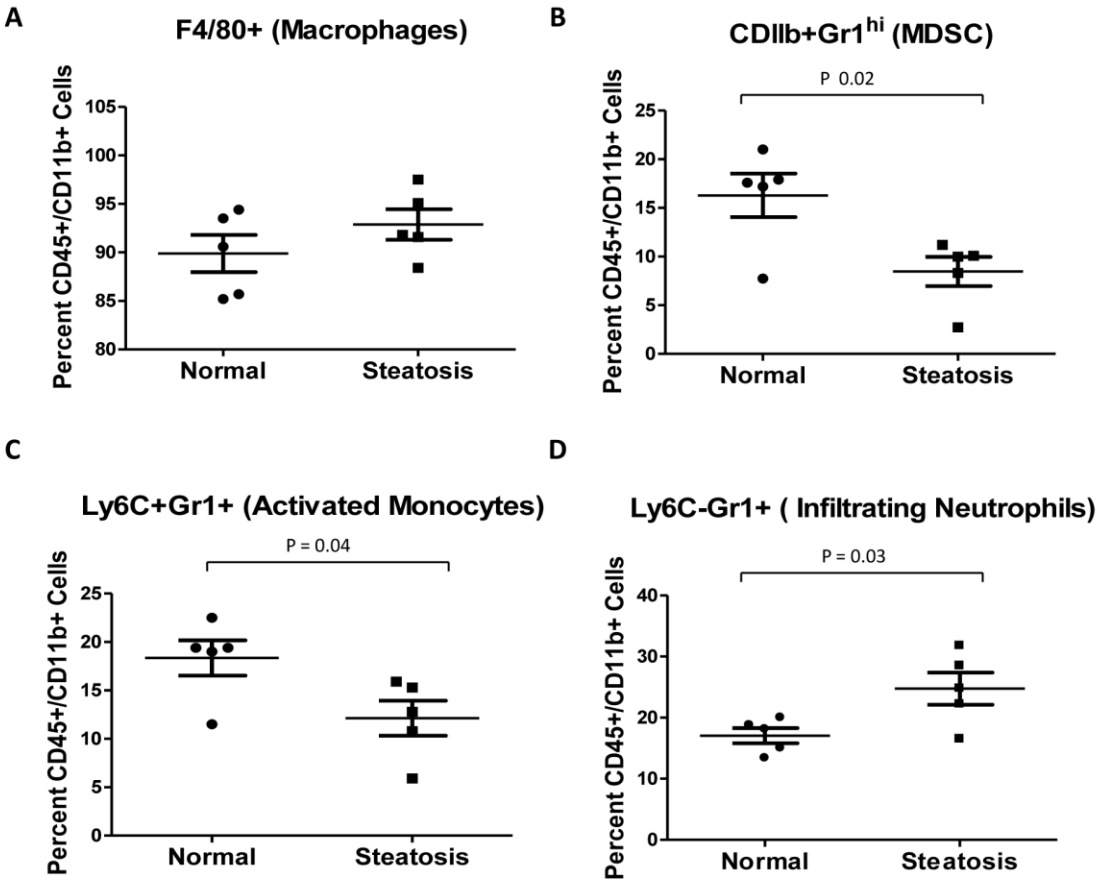


Figure 17: Changes in the myeloid cell sub-populations in normal vs steatotic murine livers. Isolated CD45 positive cells from normal and steatotic livers were stained for myeloid markers, subjected to flow cytometry and gated as a fraction of CD11b positive myeloid cells. (A) CD11b positive cells were analyzed for changes in the total percentage of F4/80 positive macrophages, (B) $Gr1^{hi}$ expressing cells, (C) Ly6C positive Gr1 positive activated monocytes and (D) Ly6C negative Gr1 positive infiltrating neutrophils between normal and steatotic murine livers. Results show a significant decrease in the $GR1^{hi}$ and $Ly6C^{+}Gr1^{+}$ subpopulations and a significant increase in the $Ly6C^{-}Gr1^{+}$ subpopulation of steatotic versus normal livers, while there was no difference in the F4/80 positive subpopulation.

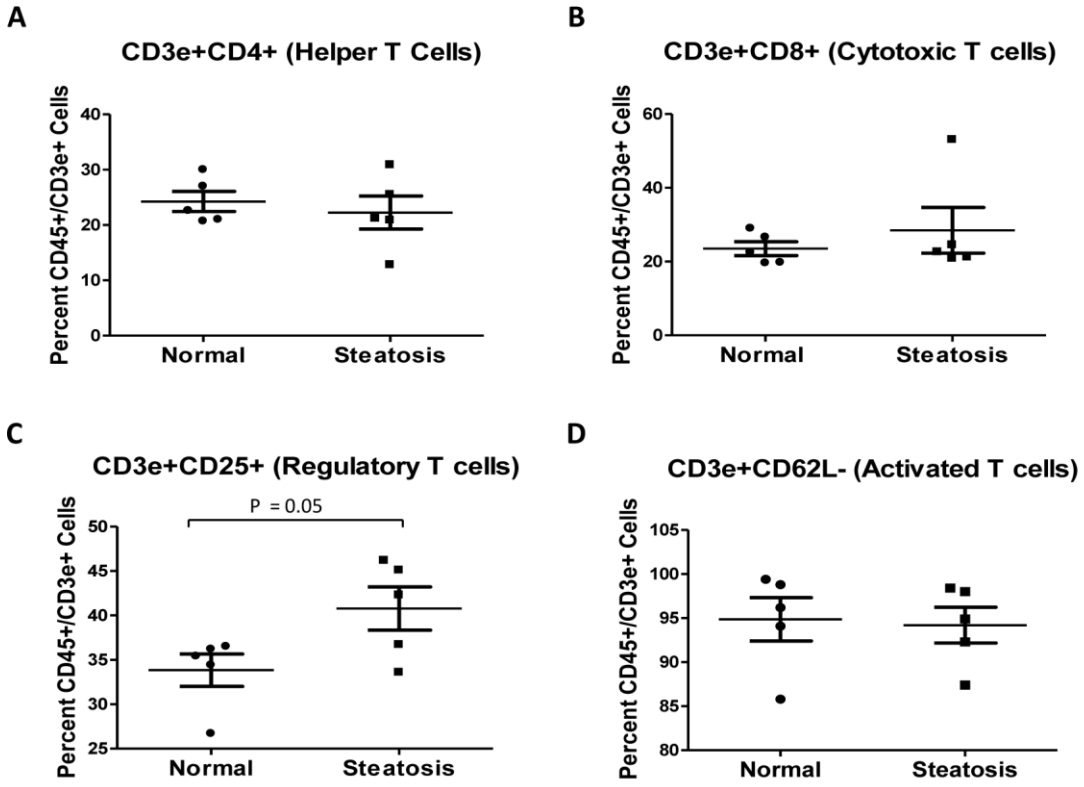


Figure 18: Changes in the lymphoid cell sub-populations in normal vs steatotic murine livers. Isolated CD45 positive cells from normal and steatotic livers were stained for lymphoid lineage markers, subjected to flow cytometry and gated as a fraction of CD3ε positive lymphocytic cells. CD3ε positive cells were analyzed for changes in the percentage of (A) CD4 positive helper T cells, (B) CD8 positive cytotoxic T cells, (C) CD25 positive regulatory T cells, and (D) CD62L negative activated T cells between normal and steatotic murine livers. Results showed a significant increase in the overall CD25 positive subpopulation of CD3ε positive cells in the steatotic livers when compared to normal livers. The percentage of CD4 positive helper T cells, CD8 positive cytotoxic T cells and $CD3\varepsilon^{+}CD62L^{-}$ activated T cells were not significantly different between steatotic and normal livers.

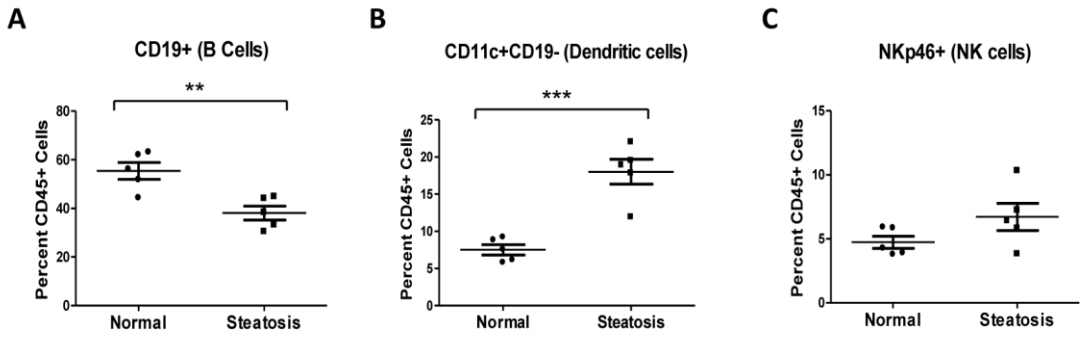


Figure 19: Changes in cell sub-populations of B cells, dendritic cells and natural killer cells in steatotic livers. Isolated CD45 positive cells from normal and steatotic livers were stained for markers for (A) B cells ($CD19^+$), (B) dendritic cells ($CD11c^+$, $CD19^-$) and (C) natural killers cells ($NKp46^+$) before being subjected to flow cytometry and gated as a fraction of $CD45^+$ cells to determine the percentage of different cell types present. Results demonstrate a significant decrease in B cells and a significant increase in dendritic cells in steatotic livers compared to normal livers. The percentage of natural killer cells were not significantly different between steatotic and normal livers.

difference in the natural killer cell subpopulation in the steatotic livers. To ensure inflammatory changes were specific to the steatotic liver and not generally associated with effects of obesity in other organs, we additionally profiled inflammatory subpopulations from the spleen. The resident inflammatory cell subpopulations in the spleen are shown in Table 1.

Inflammatory cell population changes in the setting of human NAFLD

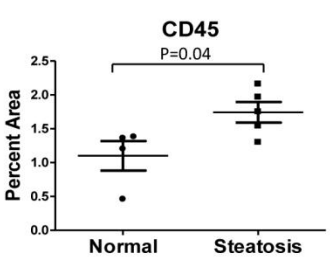
In order to test whether changes seen in inflammatory cell populations using the mouse model of high fat diet induced steatosis were corroborated in human samples with NAFLD, frozen liver sections were obtained from patients with and without steatosis and stained for inflammatory cell markers CD45, CD68, CD3ε, CD8 and CD56 as shown in Figure 20. We detected a significant increase in overall CD45 positive cells (P=0.04) as well as the total T lymphocytes as determined by the positive staining for CD3e marker (P=0.05) in steatotic livers. Staining for the CD8 subpopulation of T cells showed a slight increase in the steatotic livers, although this increase was not statistically significant. No significant differences were observed in the percent area stained for macrophages (CD68) or NK cells (CD56) by immunohistochemical analysis of normal and steatotic human liver samples, consistent with our findings in the steatotic murine livers.

Changes in cytokine profiles in a murine model of NAFLD

Changes in inflammatory cell populations are associated with activation and secretion of various cytokines such as TNFα, IL-6, MCP-1, IL-10, have been shown to be elevated in patient serum samples in the setting of NASH [43, 178–180]. These specific cytokines have additionally been proposed to play critical role in NAFLD

Table 1: Inflammatory cell profiles in the spleen of normal vs steatotic mice

Cell Type	**Normal %**	**Steatotic %**	**P Value**
$CD3\varepsilon^+$	27.58	36.275	**0.0036
$CD3\varepsilon^+$ $CD4^+$	51.02	50.975	0.9882
$CD3\varepsilon^+$ $CD8^+$	37.16	39.05	0.4916
$CD3\varepsilon^+$ $CD25^+$	10.592	14.45	*0.0234
$CD3\varepsilon^+$ $CD62L^-$	8.036	10.5975	0.2968
$CD11b^+$	9.72	8.825	0.6062
$CD11b^+$ $F480^+$	16.52	12.74	0.1948
$CD11b^+$ Gr1hi	23.84	14.9025	*0.0471
$CD11b^+$ $Ly6c^+$ $Gr1^+$	26.225	15.925	*0.0496
$CD11b^+$ $Ly6c^-$ $Gr1^+$	22.18	24.275	0.5718
$CD19^+$	66.2	64.625	0.6423
$CD11c^+$ $CD19^-$	1.54	1.8025	0.0868
$NKp46^+$	2.498	2.7525	0.7138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P=0.04
Percent Area
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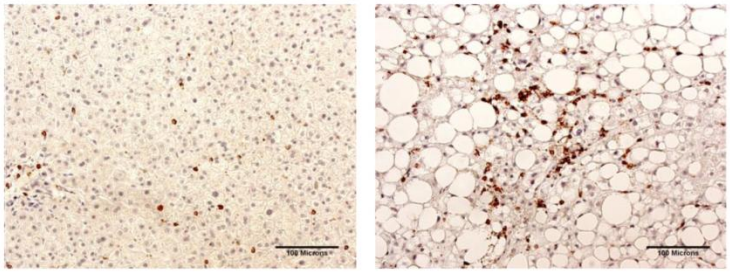
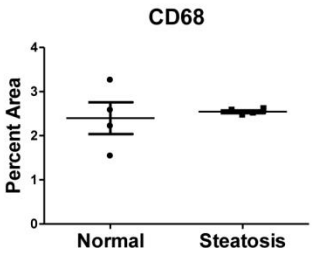
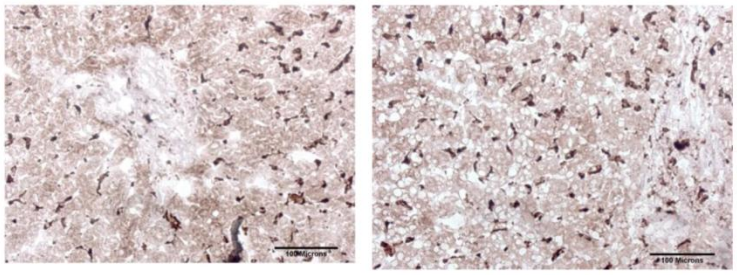
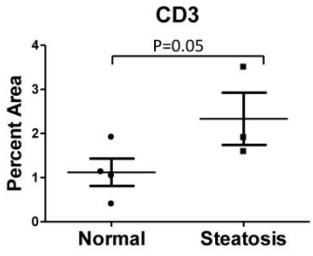
2.0
1.5
1.0
0.5
0.0
Normal
Steatosis




CD68
Percent Area
4
3
2
1
0
Normal
Steatosis




CD3
P=0.05
Percent Area
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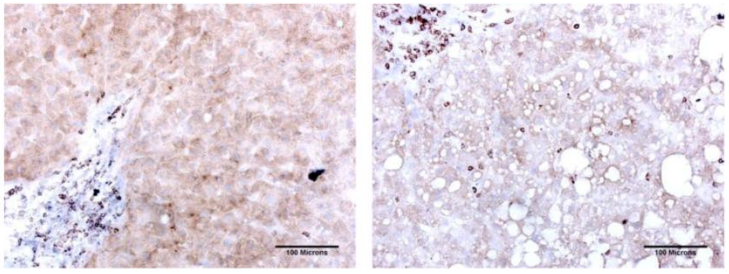
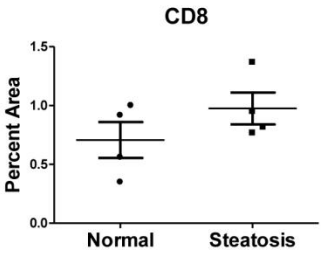
3
2
1
0
Normal
Steatosis




CD8
Percent Area
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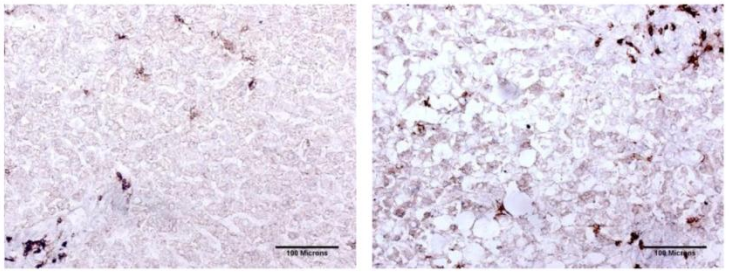
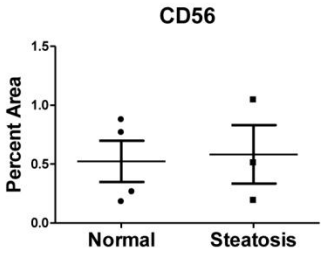
1.0
0.5
0.0
Normal
Steatosis

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Percent Area
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1.0
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0.0
Normal
Steatosis

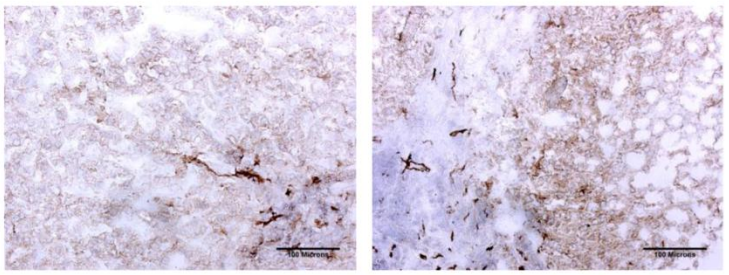

Figure 20: Quantification of inflammatory cell populations in human liver samples. Immunohistochemical staining was used to detect and quantify changes in inflammatory cell populations between normal and steatotic human liver samples. Positive immunoreactive staining (dark brown) was calculated as a percentage of total area for (A) CD45 positive inflammatory cells, (B) CD68 positive macrophages (C) CD3ε positive T lymphocytes (D) CD8 positive cytotoxic T cells and (E) CD56 positive NK cells in frozen sections of normal and steatotic human livers. Results demonstrate a significant increase in the number of CD45 and CD3 positive cells in the steatotic livers when compared to normal liver samples. CD68 macrophages, CD8 cytotoxic T cells, and CD56 NK cells were not significantly altered between sample sets. Images were acquired with a 20X objective, scalebar represents 100 microns.

pathogenesis [181–184]. To examine changes in additional cytokines we utilized a commercially available cytokine array to quantify the relative changes in various cytokine levels circulating in the plasma, in the liver tissue proper, and cytokines secreted from isolated CD11b positive myeloid cells of normal versus high fat diet induced steatotic mice. The cytokine array also contained some additional relevant adhesion molecules associated with the array. Array proteins that were significantly different are reported in Table 1 for each respective group. When comparing arrays from steatotic vs normal mice, several cytokines and relevant adhesion molecules were increased in both plasma and liver lysates, including Axl, CXCL16, Eotaxin, IL-13, IL-2, Leptin, CXCL4 (Platelet factor 4), P-selectin and VCAM-1. Further, CTACK (Cutaneous T cell-attracting chemokine), IL-6, IL-3 Rb and SCF (Stem cell factor) were elevated in the liver lysates and not plasma of steatotic mice. CTACK and IL-9 were significantly reduced in the plasma of steatotic mice when compared with plasma from normal mice.

Kupffer cells have been shown to undergo activation and play a role in progression of various liver diseases by secretion of cytokines that lead to activation of stellate cells and chemoattraction of inflammatory cell populations to the liver [173, 185]. Therefore, CD11b cells were isolated from dissociated normal and steatotic livers and used to generate conditioned media. Kupffer cells represent the majority (85% to 95%) of the CD11b positive population in the liver. Cytokine arrays were used to analyze differences in secreted cytokines from conditioned media of CD11b positive cells isolated from normal and steatotic livers. MIP-1r, MIP-2, RANTES, KC, sTNF RII and CXCL16 were elevated in the conditioned media from CD11b positive cells isolated from steatotic versus normal livers (Table 2).

Table 2. Fold change in cytokine levels of high fat diet fed mice.

Cytokine	Plasma	Liver Lysate	$CD11b^+$ Cell CM
Axl	2.13280389	1.831922538	2.443505541
CTACK	0.21229807	2.179987169	0.75720946
CXCL16	1.93180689	1.58065464	4.028336666
Eotaxin	1.65832784	2.007739833	0.130455649
IL-13	1.36055055	1.768719618	0.473546075
IL-2	2.34419611	2.618608513	0.859891851
IL-3	1.1356281	1.519534855	0.651248631
IL-3 Rb	1.09612642	2.513275758	0.858366742
IL-6	1.06119747	1.40169613	0.864977387
IL-9	0.46101177	1.508281212	0.874990301
KC	1.72431495	1.406850455	4.207922861
Leptin	1.86116246	1.840166242	0.840621661
L-selectin	1.36488563	1.520783331	0.722070716
MCP-1	1.01054898	1.340761806	0.694198945
MCP-5	1.02686785	1.319179552	0.657637641
MIP-1γ	1.68656519	1.178307719	11.43207136
MIP-2	1.47759187	1.656782016	4.711865379
PF-4	1.73472362	3.080963553	0.983425609
P-selectin	1.73297843	2.002406638	0.983425609
RANTES	1.02225724	1.36710967	6.589157964
SCF	0.9931903	2.093534246	2.293077231
sTNF R1	0.72735291	0.994273435	1.778782317
sTNF RII	2.5148749	1.022674689	4.160571141
TNF-α	1.20435513	1.290203739	0.793791961
VCAM-1	1.91774475	1.574854392	0.886619198

Table 2: Plasma, liver tissue lysates and conditioned media from $CD11b^+$ magnetically isolated cells Cytokine arrays were used to detect changes in cytokine levels in the plasma, perfused liver tissue lysate (Liver Lysate), and from conditioned media of isolated $CD45^+CD11b^+$ subpopulations ($CD11b^+$ Cell CM) between normal and steatotic samples. Cytokine values are presented as fold change with values greater than 1 representing increased levels and values less than 1 representing decreased levels in steatotic samples. (n=3). Axl (Tyrosine protein kinase 7), CTACK (Cutaneous T-cell attracting chemokine, CCL27), CXCL16 (CXC chemokine ligand 16), IL-2 (Interleukin-2), IL-3 (Interleukin-3), IL-6 (Interleukin-6), IL-9 (Interleukin-9), IL-13 (Interleukin-13), IL-3 Rb (Interleukin-3 receptor beta, CD131), KC (CXC chemokine ligand 1), MCP-1 (Monocyte chemotactic protein-1, CCL2), MCP-5 (Monocyte chemotactic protein-5, CCL12), MIP-1γ (Macrophage inflammatory protein-1 gamma), MIP-2 (macrophage inflammatory protein -2, CXCL2), PF-4 (Platelet factor 4 , CXCL4), RANTES (Regulated on activation normal T-cell expressed and secreted, CCL5), SCF (Stem cell factor), TNF (Tumor necrosis factor), VCAM-1 (Vascular cell adhesion molecule 1 , CD106).

Cytokines increase hepatocyte proliferation in vitro

To determine whether alterations in cytokines could reflect changes in the observed increase in hepatocellular proliferation in the steatotic livers, we tested the effect of select cytokines (leptin, CXCL1, CXCL2 and CXCL16) that were increased in steatotic samples on hepatocyte proliferation. The effect of various cytokines on the growth of HepG2 human hepatocellular carcinoma cells and mouse SV40 transformed hepatocyte (HEPT) cells was determined using the MTT assay. Leptin, CXCL1, CXCL2 and CXCL16 significantly increased proliferation off both cell lines after 12 hours of treatment with different concentrations of each cytokine (Figure 21). Thus the increases in cytokine levels observed in the setting of steatosis could explain the increased hepatocellular proliferation determined by Ki67 staining.

Discussion

With the rising incidence of obesity, NAFLD is an increasing cause of chronic liver disease in the United States and the world, encompassing a spectrum of pathology marked by hepatic steatosis in the absence of significant alcohol consumption. Although simple steatosis follows a generally benign course, the more aggressive form, non-alcoholic steatohepatitis, can progress to cirrhosis and result in complications including hepatocellular carcinoma. A significant number of cases of hepatocellular carcinoma are occurring in the setting of NASH without underlying cirrhosis [40, 186]. A number of cellular and molecular mediators have been shown to be involved in the progression of NAFLD and some of these may be linked to tumor initiation and progression in the hepatic microenvironment of NAFLD. Accumulation of lipids in the liver cells can lead

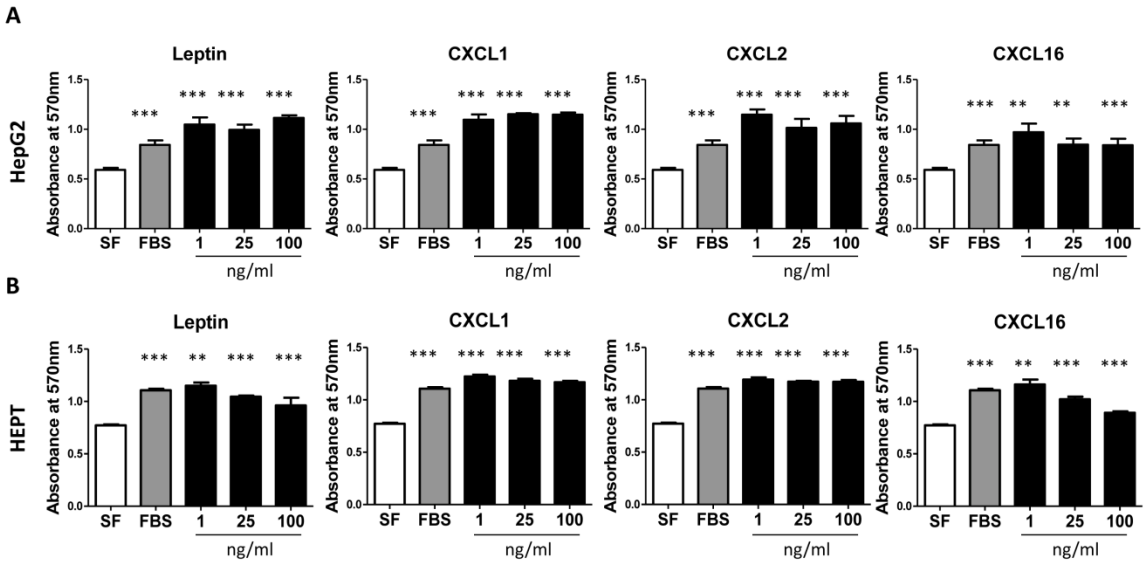

Figure 21: Cytokines effect hepatocyte proliferation in vitro. The effect of different concentrations of Leptin, CXCL1, CXCL2 and CXCL16 (1 ng/ml, 25 ng/ml and 100 ng/ml), added to serum free media, on proliferation of (A) HepG2 and (B) HEPT cells determined by MTT assay. 10% FBS was used as a positive control. ** represents P<0.001 and *** P<0.0001 when compared to Serum Free conditions.

to hepatocellular injury [79, 174, 175], one manifestation of which is apoptosis, which in turn can trigger a regenerative response. We have previously shown that mice fed a high fat diet for a prolonged period of time develop liver tumors [53]. To determine whether steatosis, progressive inflammation, and hepatocellular injury could impact hepatocyte proliferation, the total number of Ki67 positive cells as well as Ki67 positive hepatocytes were quantified and showed a statistically significant increase in hepatocellular proliferation in the steatotic livers as compared to normal livers. Repetitive cycles of apoptosis and regeneration/proliferation of these principal cells of the liver could lead to aberrant repair in some individuals culminating in tumor initiation.

Our current studies using a mouse model of diet-induced steatosis have shown that there are significant changes in specific inflammatory cell populations in the liver in the setting of steatosis. We observed significant increases in myeloid cell, T and B lymphocytes and dendritic cells populations. Accumulation of TGs in hepatocytes leads to the generation of lipid metabolites such as lysophosphatidylcholine (LPC), which has been associated with oxidative stress and hepatocellular death [172, 174, 178]. Cell death can lead to activation of inflammatory pathways such as the JNK and NF-κB pathways through release of damage-associated molecular patterns (DAMPs), which can then lead to the recruitment of inflammatory cells and contribute to the progression to NASH [99, 170, 173–175]. Kupffer cells are resident macrophages and act as the first responders to hepatic injury, they likely detect the expression of DAMPs on hepatocytes which have been injured by accumulation of triglycerides in the setting of steatosis [187, 188]. The subsequent production of TNFα and other chemoattractant cytokines by Kupffer cells

thus propagates the initial insult, leading to inflammation through the recruitment of inflammatory monocytes [189].

Recent studies by other groups have additionally shown that changes in Kupffer cell and dendritic cell populations can play an important role in the progression of NAFLD. Work by Henning et al. showed an increase in $CD11c^+$ dendritic cell populations and demonstrated a regulatory role for dendritic cells (DCs) in NASH by limiting sterile inflammation via their role in clearance of apoptotic cells and necrotic debris. They found that DCs increase regulatory T cell activation and production of the anti-inflammatory cytokine IL-10. Further, they showed that ablation of dendritic cells led to increased Toll-like receptor expression and cytokine production in innate immune effector cells in NASH, including Kupffer cells, neutrophils, and inflammatory monocytes [177]. In concordance with their data, we detected a significant increase in the percentage of $CD11c^+$ dendritic cells in the high fat diet induced steatotic livers compared to normal livers. Though we were not able to detect any changes in the percentage of $CD11b^+F480^+$ cells (Kupffer cells) as a percentage of the $CD11b^+$ myeloid cells between normal and steatotic livers, we did see a significant increase in the percentage of $CD11b^+$ cells in the steatotic livers compared to normal livers. Additionally, we were able to detect changes in cytokine production from conditioned medium (CM) obtained in vitro from isolated $CD11b^+$ myeloid cells. Since the Kupffer cells make up the majority of this population (85%-95%), cytokine alterations reflect changes in the resident Kupffer cells of the liver and their activation status.

Multiple chemokines and cytokines have been implicated in the development of steatosis and the progression to NASH; including IL-6, TNFα, MCP-1 and IL-10 [182,

183]. The development of NASH in human patients and in murine models of NASH have each exhibited elevated serum levels of TNF-α and increased expression of TNFα transcripts in liver as well as adipose tissue [43, 190, 191]. Our results comparing plasma and liver tissue samples from normal and steatotic livers, showed a slight increase in IL-6 and TNFα with steatosis (Table 2). Park et al recently reported that proinflammatory cytokines IL-6 and TNFα are important for the progression from hepatic steatosis to steatohepatitis in obese mice and that absence of either IL-6 or TNFR1 reduced lipid accumulation in the liver and also reduced influx of macrophages and neutrophils in livers of mice fed a high fat diet [43]. However, other studies have shown that IL-6 deficiency or blockade reduced liver inflammation without affecting the development of steatosis suggesting a role for IL-6 only in promoting liver inflammation [192, 193].

MIP-1γ, MIP-2, RANTES, CXCL1, sTNFRII and CXCL16 represented the most significantly elevated cytokines specifically from the CD11b conditioned media. MIP-1γ has been shown to attract dendritic cells and immature myeloid cells that possess the CCR1 chemokine receptor [194] and its expression in tumor cells aids colon cancer metastasis to the liver and accumulation of immature myeloid cells [195]. Increased expression of MIP-2 has been shown to contribute to neutrophil and lymphocyte recruitment [196] which could help propagate the inflammatory response observed in NAFLD. CXCL1 is expressed by macrophages, neutrophils and epithelial cells, and has neutrophil chemoattractant activity. It is involved in inflammation and gene expression levels are elevated in patients with NASH [182, 197]. Recent studies have shown that elevated RANTES and sTNFRII levels correlate with the progression of NAFLD [198, 199] though further functional studies will need to be carried out for these and other

altered cytokines in the setting of NAFLD. A recent study identified CXCL16 in preoperative serum as a marker for poor prognosis and high level of recurrence of liver metastasis in patients with HCC [200–202].

Specifically decreased in the conditioned media of CD11b cells from steatotic livers were IL-13, MCP-5 and MCP-1. IL-13 is a Th2 cytokine that plays a central role in various inflammatory diseases [203]. IL-13 induces tissue fibrosis by stimulating and activating TGF-β1 and was shown to play a role in progression from NASH to fibrosis in a rat model fed a choline deficient diet [204]. However, no studies have evaluated levels at early stages of NAFLD. MCP-5 specifically attracts eosinophils, monocytes and lymphocytes and is therefore found predominately in lymph nodes and thymus under normal conditions, yet its expression can be induced in macrophages [205]. It has been shown to play a role in exacerbation of pulmonary fibrosis by recruitment of bone marrow derived fibrocytes to the lung [206, 207], but its role in progression of NAFLD is yet to be assessed. Several studies have reported an important pathological role of MCP-1 in the progression of NAFLD. However we detected no significant differences in cytokine levels from plasma or tissue samples. In NASH, the role of MCP-1 is controversial; MCP-1 deficiency in mice fed a methionine choline deficient diet didn't affect the development of steatohepatitis, but actually decreased fibrosis [180], and didn't impact liver disease progression [208]. Recently, pharmacological inhibition of MCP-1 or the lack of CCR2 expression (MCP-1 receptor) in a murine model of NASH was shown to decrease liver inflammation and steatosis without affecting hepatic fibrogenesis [209, 210] The current study provides evidence that various cytokines are differentially expressed during the early stages of NAFLD in a mouse model of diet-induced steatosis

and further studies are required to determine the precise role of each of cytokines at different stages of NAFLD.

While effects of microenvironmental as well as systemic alterations of chemokine levels have been demonstrated for the recruitment of inflammatory cells, less is known about the effects of these chemokines on hepatocytes. We were able to show that high fat diet-induced steatosis results in a significant increase in hepatocyte proliferation compared to normal livers. Interestingly, Sydor et al. were able to also demonstrate a significant increase in proliferation in steatotic livers after partial hepatectomy, which correlated with increased levels of leptin [211]. Leptin has been shown to directly increase proliferation of chick hepatocytes [212], although conversely administration of leptin was unable to restore replicative competence after partial hepatectomy [213], leaving conflicting evidence for the ability of leptin to promote hepatocellular proliferation. CXCL16 and Axl have both been shown to increase epithelial proliferation, yet we detected an increase in growth when hepatocytes were exposed to CXCL16. MCPs can induce proliferation, yet hepatocytes are known to not express CCR2 and therefore hepatocytes should not directly respond to these chemokines. Hepatocytes do constitutively express CXCR2 and can respond to MIP-2 [214, 215] and possibly CXCL1. We found both CXCL1 and CXCL2 to be upregulated in conditioned media from CD11b isolated myeloid cells in steatotic livers and both of these cytokines were able to increase the growth of murine conditionally immortalized hepatocyte cells (HepT) and human HepG2 cells hepatocytes *in vitro*. Further, ELR-CXC chemokines have been shown to induce hepatocyte proliferation in culture [215]. We were further able to show that leptin was also capable of inducing growth of hepatocytes *in vitro*. Indirectly,

increased activating chemokines in the steatotic liver may also influence resident stromal cells to impact the growth of hepatocytes.

In summary, this study demonstrates that there are significant changes in hepatocellular proliferation, influx of inflammatory cell populations and cytokine levels in the steatotic liver. The recognition of their roles in progression of NAFLD to end stage liver disease and a potential tumor-initiating role in the steatotic liver microenvironment may open the door for modulation of these cell populations and cytokines as part of novel therapies, especially for difficult-to-treat cancers such as HCC. Additional investigations are needed to understand the mechanisms by which these changes in inflammatory cell populations, cytokines, and the proliferation of hepatocytes have on the progression of NAFLD.

CHAPTER IV

MMP13 PROMOTES TUMOR CELL EXTRAVASATION AND ESTABLISHMENT OF METASTASIS IN THE STEATOTIC LIVER MICROENVIRONMENT

The contents of this chapter have been accepted for publication the journal *Molecular Cancer,* December 22, 2014

Introduction

The obesity epidemic has been closely linked with an increased incidence of non-alcoholic fatty liver disease (NAFLD) [216]. NAFLD results from accumulation of fat in the liver which alters the local liver microenvironment leading to changes in lipid profiles, recruitment of inflammatory cells and changes in cytokine expression [169, 217]. Changes in the tissue microenvironment can affect cell-cell interactions and influence the development of both primary and metastatic tumors. Epidemiological studies show that NAFLD has been linked to an increase in the risk for development of primary liver cancer [218, 219] but very little is known about the effect of steatosis on tumor metastasis to the liver. The liver is a frequent site of metastasis for several cancers such as colorectal cancer, breast and pancreatic cancer. In addition, obesity is an independent risk factor for the development of these tumor types among others [34]. Previous studies from our laboratory have shown that mice with high fat diet induced steatosis have an increase in metastatic burden compared to mice with normal livers [53]. Metastasis represents the end-stage of cancer progression and is responsible for

most cancer related deaths [220]. Improved understanding of the metastatic tumor microenvironment is important in devising therapies to impact this stage of the disease.

Matrix Metalloproteinases (MMPs) are a family of zinc dependent proteases that are capable of cleaving various components of the extracellular matrix. Apart from matrix molecules, they can also cleave adhesion proteins, activate growth factors such as TGF-β and VEGF as well as release and activate cytokines [114, 221]. MMPs are produced by tumor cells, stromal cells and infiltrating inflammatory cells and facilitate host-tumor interactions. Members of the MMP family have been associated with progression through multiple stages of cancer, from initiation to acquisition of metastatic properties [104, 105]. However, MMPs have also been shown to have anti-tumorigenic functions and are important for normal developmental and wound healing responses [222]. Early clinical trials, broadly targeting the MMP family as a whole, led to unintended clinical side effects. Improved understanding of specific roles for individual MMPs and development of pharmaceutical agents that selectively target individual MMPs may be part of effective therapeutic strategies in the future [131].

MMP13 is an interstitial collagenase that is capable of cleaving multiple collagens, preferentially collagen II, as well as other matrix substrates such as gelatin, fibronectin, and aggrecan relevant to tumor metastasis [154]. This protease has been previously linked to the progression of fibrotic liver disease [162]. In addition, increased expression of MMP13 has been associated with poor prognosis in patients with colorectal cancer metastasis to the liver [161]. MMP13 was identified as a part of the breast cancer metastasis signature and was associated with decreased overall survival and metastasis in breast cancer and renal cell carcinoma [223–225]. Furthermore, stroma-derived MMP13

was found to be involved in the growth of liver, lung, brain and heart metastases of melanoma cells [155]. In this study we show that both stromal and tumor derived MMP13 play important roles in modulating the liver microenvironment and facilitating the establishment of liver metastasis.

Results

MMP13 expression is elevated in the steatotic liver

Previous studies in our lab have found a significant increase in the metastatic tumor burden to the liver in the setting of steatosis as compared to normal mouse livers [53]. Additionally, our group has identified MMP9 as an important mediator of tumor metastasis to the liver [226] and other groups have shown several additional MMPs to be important in metastasis [227]. Therefore, we wanted to determine which MMPs were altered in the steatotic microenvironment and whether these MMPs contributed to the increased metastasis. We assessed the relative gene expression levels of a panel of MMPs, associated with tumor progression, in the liver tissue of mice with steatosis compared to normal mice. We found that *Mmp12* and *Mmp13* were significantly upregulated in the liver of mice with steatosis compared to normal livers (Figure 22A). MMP13 has previously been associated with fibrotic liver disease [162] and its expression in tumors has been associated with poor prognosis in patients with colorectal cancer metastasis to the liver [161]. MMP13 protein levels were evaluated in the mouse liver and were found to be increased in the steatotic livers compared to normal livers (Figure 22B). To determine whether changes in MMP13 levels were relevant to the human progression of NAFLD, we evaluated the MMP13 protein levels by western blot

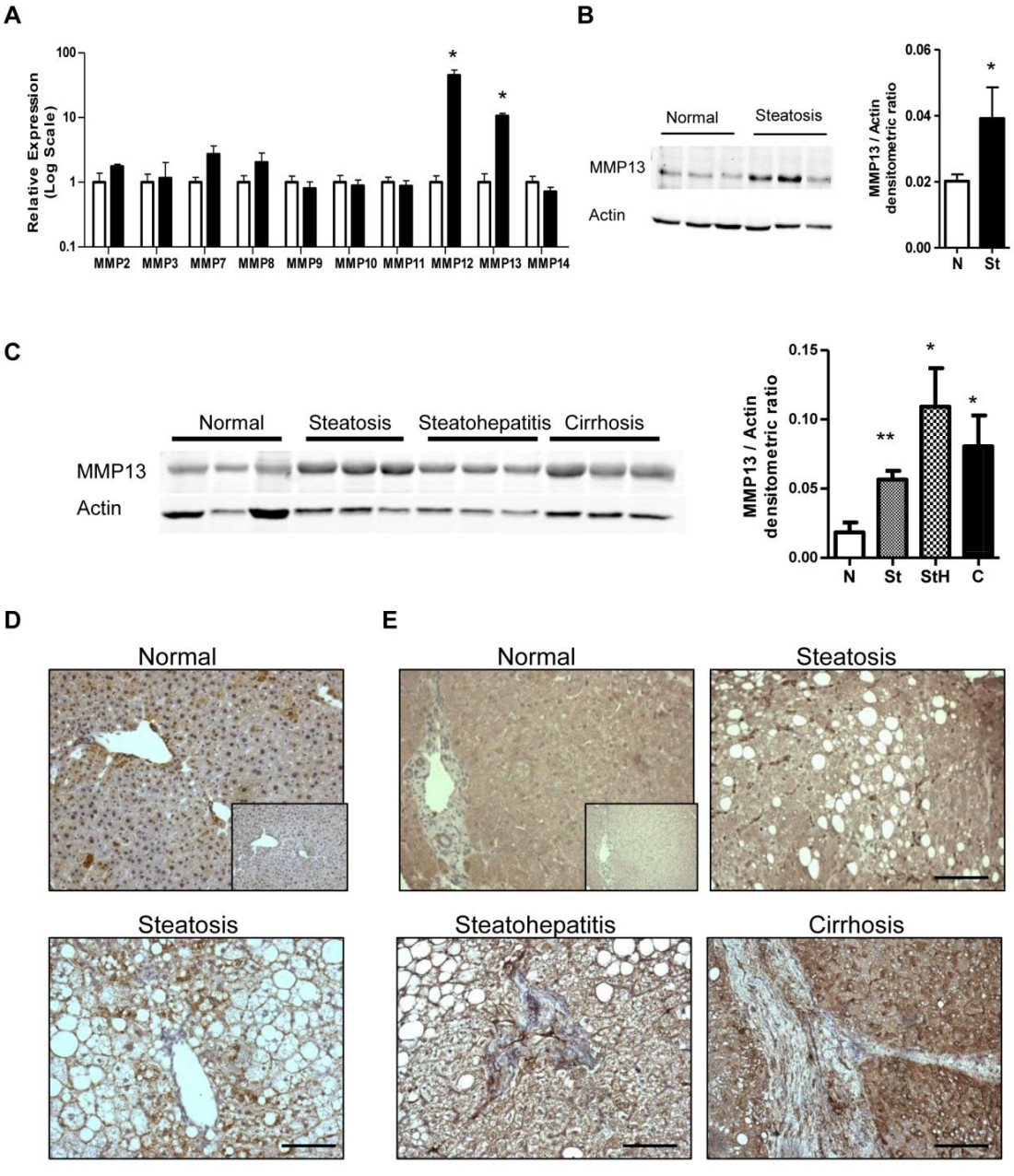
A
Relative Expression (Log Scale)
100
10
1
0.1
MMP2
MMP3
MMP7
MMP8
MMP9
MMP10
MMP11
MMP12
MMP13
MMP14
B
Normal
Steatosis
MMP13
Actin
MMP13 / Actin densitometric ratio
0.06
0.04
0.02
0.00
N
St
C
Normal
Steatosis
Steatohepatitis
Cirrhosis
MMP13
Actin
MMP13 / Actin densitometric ratio
0.15
0.10
0.05
0.00
N
St
StH
C
D
Normal
Steatosis
E
Normal
Steatosis
Steatohepatitis
Cirrhosis

Figure 22: MMP13 is elevated in the steatotic liver. (A) Relative gene expression of matrix metalloproteinases (MMPs) in liver tissue of mice with steatosis (black bars) compared to normal (white bars) mice. Transcript levels were normalized to GAPDH and expressed as fold change relative to normal controls. Values represent the mean (n = 3 per group). Statistical analysis was carried out using 2-way ANOVA followed by Bonferroni post-test between normal and steatotic samples for each MMP tested (*, $P < 0.05$). (B) Western Blot and densitometric quantification of MMP13 protein levels relative to actin levels in livers of wildtype C57bl/6 mice with and without steatosis. (C) Western Blot and densitometric quantification of MMP13 protein levels relative to actin levels in human liver samples through multiple stages of non-alcoholic fatty liver disease (NAFLD), (n=9 per group). Statistical analysis was carried out using a 2-tailed t-test with respect to normal livers. No significant differences were observed between the different stages of progression of NAFLD. (D) Representative immunohistochemical staining (IHC) of MMP13 in murine normal and steatotic livers. Inset represents IgG control. (E) IHC of human MMP13 at different stages of NAFLD. MMP13 staining is detected at low levels in the normal liver hepatocytes and is increased in the stromal cells of livers with steatosis and steatohepatitis. Additionally, MMP13 is present in select hepatocytes in cirrhotic livers. Images were taken at 20X, Scalebar represents 100 microns. N, Normal; St, steatosis; StH, steatohepatitis; C, Cirrhosis.

from liver lysates of normal livers and compared them to livers with steatosis, steatohepatitis or NAFLD related cirrhosis. Figure 22C shows that MMP13 protein expression is increased with the progression of NAFLD. Immunohistochemical analysis of MMP13 expression shows increased staining of MMP13 with steatosis in the murine liver (Figure 22D) and varied distribution of the protein in both stromal cells as well as hepatocytes which is increased with disease progression of NAFLD in human patient samples (Figure 22E). Thus, MMP13 is elevated both in human NAFLD progression as well as in our mouse model of diet-induced steatosis.

Loss of host derived MMP13 leads to decreased tumor metastasis to the liver

Elevation of MMP13 in the steatotic liver suggested that it could play an important role in priming the steatotic liver microenvironment for tumor establishment. To elucidate the role of host MMP13 on tumor metastasis to the liver, *Mmp13* null (*Mmp13*-/-) mice were fed a high fat diet to induce steatosis. *Mmp13*-/- mice developed steatosis comparable to the wildtype counterparts (Figure 23). Subsequently, wildtype and *Mmp13*-/- mice with and without steatosis were inoculated with 5×10^5 syngeneic MC38 colon cancer cells through the intrasplenic/portal route to generate experimental liver metastases. After 2 weeks, mice were sacrificed and livers were harvested. The liver weight and percent liver weight to total body weight ratios were measured to determine metastatic burden (Figure 24B,C), and livers were fixed, dissected and stained by H&E (Figure 24A) to record the incidence (Figure 24D) and area (Figure 24E) of metastasis. We found that the tumor burden and incidence of metastases increased in the steatotic compared with normal mice in both the wildtype and *Mmp13*-/- mice. However, the tumor burden, incidence and area of liver metastases was markedly reduced in the *Mmp13*-/- mice compared to the

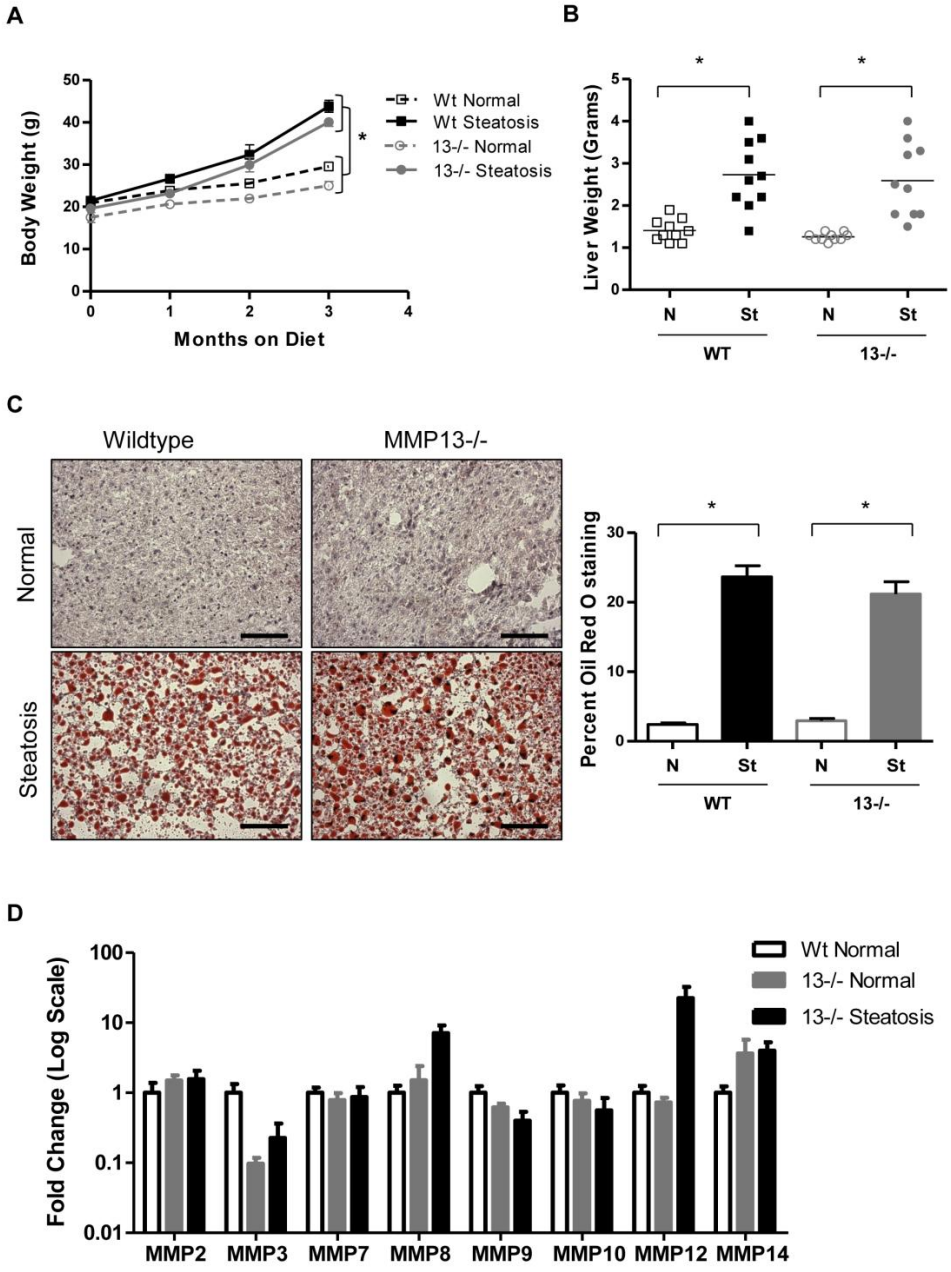

Figure 23: Characterization of *Mmp13* -/- mice. Wildtype and *Mmp13* deficient mice were fed regular (Normal) or high fat diet (Steatosis) for 3 months to determine overall body weight and liver weight. (A) Graph illustrating total body weight over time with respect to diet and genetic status show that *Mmp13*-/- mice gain weight similar to wildtype mice (n=5). (B) Graph illustrating total liver weight after 3 months on diet, which trends the same as body weight (n=10). (C) Lipid accumulation visualized as red staining via Oil Red O lipid dye and graphic quantification (n=10). (D) Relative gene expression of matrix metalloproteinases (MMPs) in liver tissue of *Mmp13*-/- mice with or without steatosis compared to normal wildtype mice. Relative transcript levels were normalized to GAPDH and expressed as fold change relative to normal controls. Values represent the mean (n = 3). N, normal; St, steatosis; WT, wildtype; 13-/-, *Mmp13*-/-.

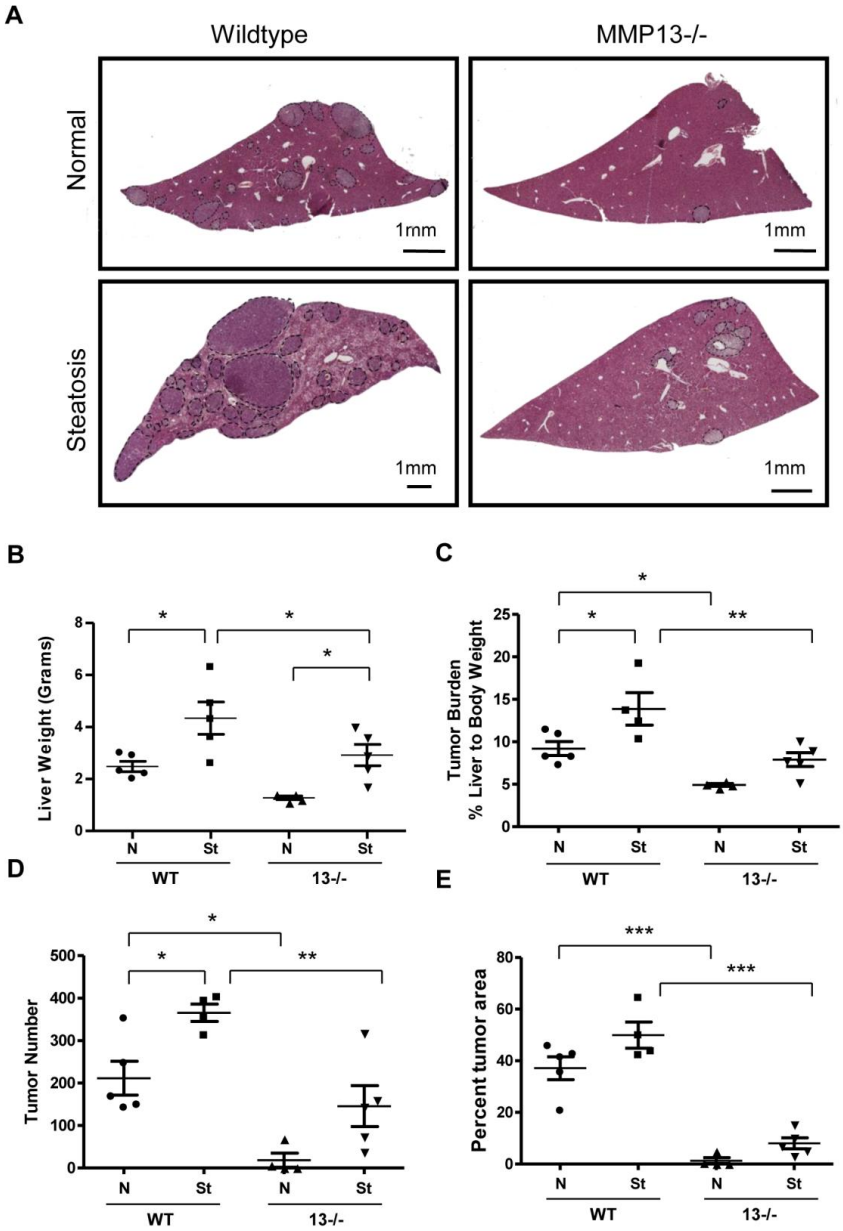

Figure 24: Loss of host derived MMP13 leads to decreased tumor metastasis to the liver. (A) Representative liver cross sections stained with Haematoxylin & Eosin of wildtype and *Mmp13*-/- mice with normal or steatotic livers show an increased tumor burden in wildtype mice compared to *Mmp13*-/- mice. Tumors are denoted by black dashed line. Quantification of metastatic tumor burden by (B) liver weight, (C) tumor burden measured as percent liver weight to total body weight, metastatic seeding by quantification of (D) tumor number and (E) tumor area per liver section in normal and steatotic livers. Statistical analysis was performed using GraphPad Prism software. Data was analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. P values are represented by stars where: * ≤.05, ** ≤ .01, and *** ≤ .001. N, normal; St, steatosis; WT, wildtype; 13-/-, *Mmp13*-/-.

wildtype mice after tumor cell injection, thus supporting the role of MMP13 in metastatic tumor growth to the liver both in normal and steatotic mice.

Additionally, liver metastases were stained for Ki67 (proliferation marker) and cleaved caspase-3 (apoptosis marker) to further examine the role of MMP13 in promoting the survival and outgrowth of metastatic tumors (Figure 25A, B). The percentage of tumor cells proliferating or undergoing apoptosis was similar between the wildtype and *Mmp13*-/- tumors of comparable size, suggesting no difference in the rate of tumor growth between the WT and *Mmp13*-/- mice. Additionally, there was no difference in tumor vascularity as determined by vWF staining (Figure 25C). To determine whether there were changes in tumor outgrowth, we plotted a histogram of tumor size distribution (Figure 26). *Mmp13*-/- mice had fewer tumors and the distribution curve shifted to the left compared to WT mice, however the tumors were still capable of becoming large; suggesting that loss of host MMP13 could affect some of the earlier steps in the metastatic cascade such as tumor cell survival and seeding to the liver or ability of tumor cells to adhere to the vasculature and extravasate into the liver tissue.

Loss of host MMP13 affects the ability of tumor cells to extravasate from the vasculature

Since we observed a decrease in tumor burden with the loss of MMP13 in both normal and steatotic mice, we focused on the role of MMP13 on tumor metastasis to the liver irrespective of the diet. To determine whether the decrease in tumor burden in the *Mmp13*-/- mice compared to wildtype mice resulted from a difference in the ability of the tumor cells to survive in circulation and seed the liver, we injected normal wildtype or *Mmp13*-/- mice with 1×10^6 MC38 tumor cells, labeled with cell tracker red, and sacrificed the mice 24 and 48 hours post injection. Livers were harvested, processed,

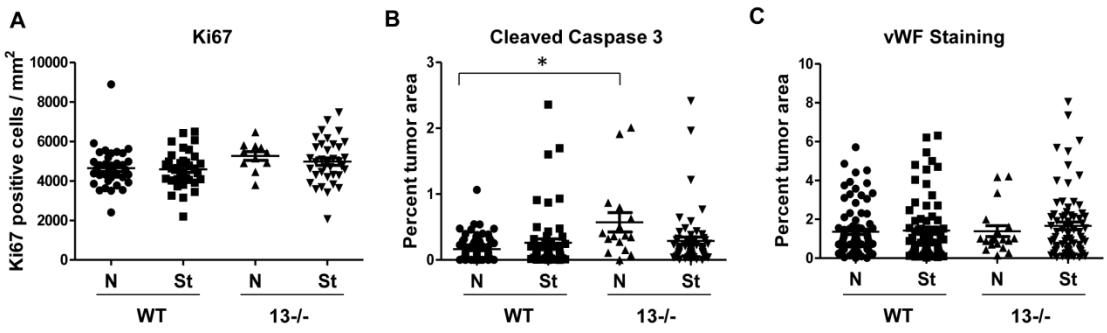

Figure 25: Effect of loss of host MMP13 on tumor proliferation, apoptosis and vascularity. (A) Quantification of tumor proliferation by Ki67 staining determined by plotting number of Ki67 positive cells per unit tumor area. (B) Change in apoptosis within tumors in wildtype and *Mmp13*-/- mice determined by percentage of cleaved caspase 3 staining per unit tumor area. (C) Quantification of tumor vascularity as determined by percentage area of von Willebrand factor staining per unit tumor area. Quantification was done using Metamorph software. Statistical analysis was performed using GraphPad Prism software. Data was analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test (* ,$P \leq 0.05$). N, normal; St, steatosis; WT, wildtype; 13-/-, *Mmp13-/-*.

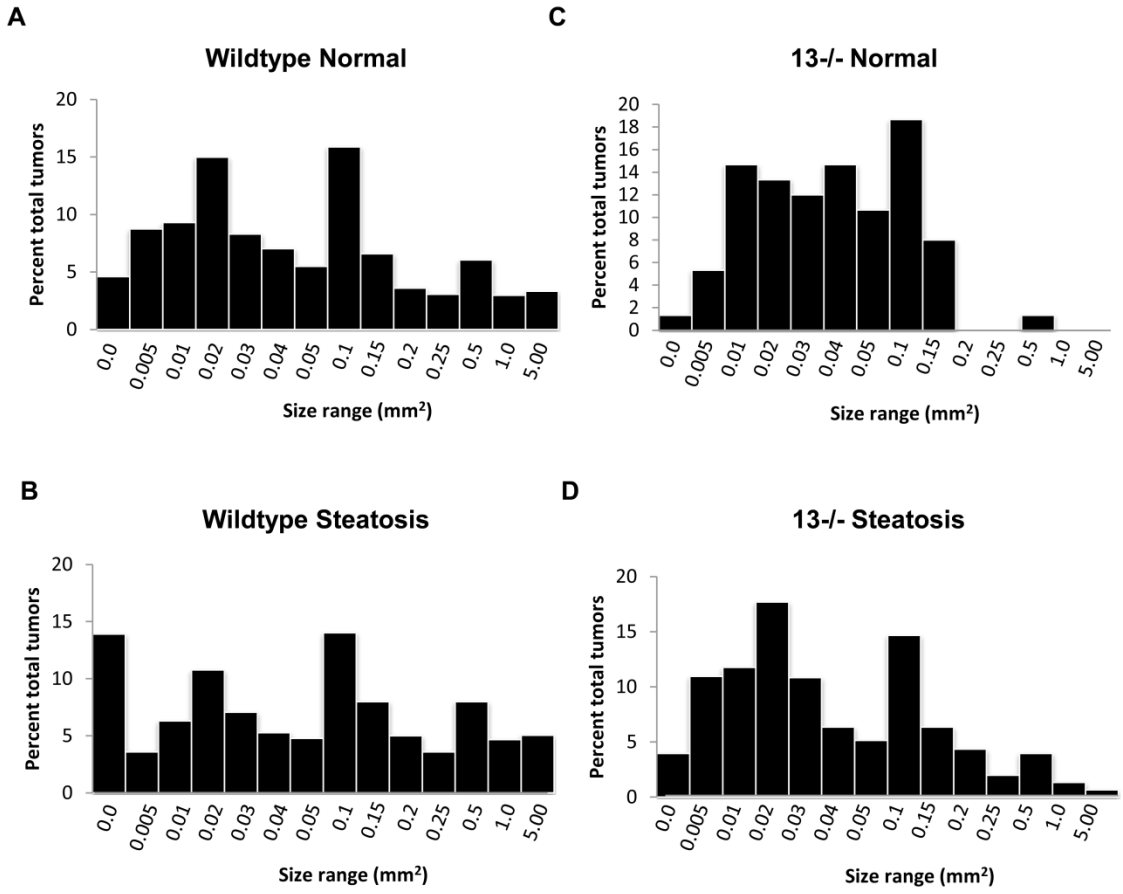

Figure 26: Tumor size distribution in Wildtype and *Mmp13*-/- mice. Histogram of MC38 tumor size distribution plotted as a percentage of total number of tumors in (A) wildtype normal, (B) wildtype steatotic, (C) *Mmp13*-/- normal and (D) *Mmp13*-/- steatotic mice.

sectioned and the number of tumor cells per section were quantified (Figure 27A). These results demonstrate no significant differences in the number of tumor cells present in the livers of these mice of wildtype and *Mmp13*-/- mice at 24 and 48 hours post injection and therefore no difference in survival or seeding of the cells to the liver.

To investigate the role of host MMP13 on the ability of MC38 colon cancer cells to extravasate from the vasculature, we adapted the methodology developed by Martin et al. [166]. MC38 cells were labeled with cell tracker red and injected into normal wildtype or *Mmp13*-/- mice. Mice were sacrificed at 24 and 48 hours post-injection, their livers perfused with saline and their vasculature labeled with tomato lectin. The liver explants were then imaged using a confocal microscope to visualize individual tumor cells relative to the vasculature and to compare the percentage of tumor cells that had extravasated in wildtype versus *Mmp13*-/- mice at each time point. We observed a significant decrease in the percentage of tumor cells that had extravasated in the *Mmp13*-/- mice compared to that of the wildtype mice at 48 hours post tumor cell injection (Figure 27B).

The hepatic vascular volume was evaluated from the 3-D vascular reconstructions (Figure 27C, D) of wildtype and *Mmp13*-/- mice liver. No difference in quantity of vascular staining was observed between the wildtype and *Mmp13*-/- mice indicating that changes in extravasation do not result from changes to the vascular capacity (P=0.7 using 2-tailed t-test).

Loss of tumor derived MMP13 leads to decrease in migratory and invasive properties of cells *in vitro*

MMP13 is a part of the breast cancer metastasis signature and tumor cell expression of MMP13 is linked with increased invasiveness and ability to metastasize in

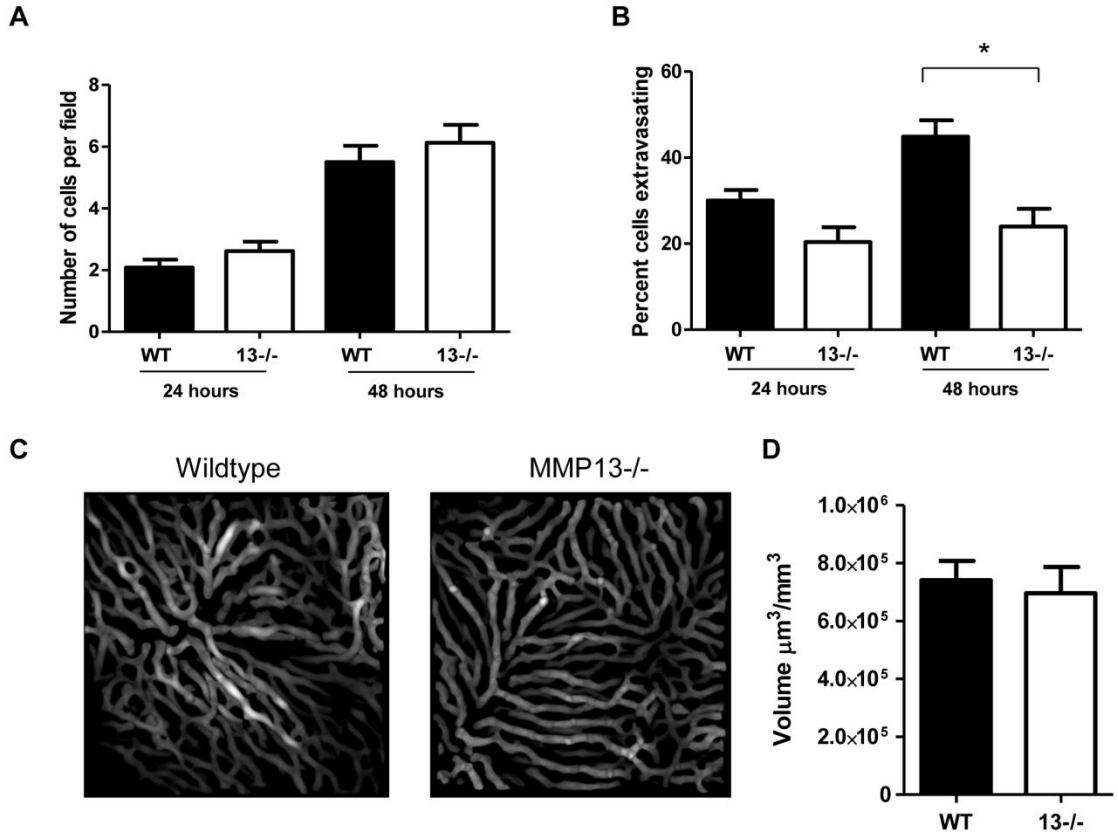

Figure 27: Lack of stromal MMP13 leads to decreased tumor cell extravasation from the microvasculature. (A) Number of tumor cells in the liver at 24 and 48 hours post intrasplenic injection of MC38 tumor cells quantified as average number of cell tracker red labeled cells per 10X field from 5 random images per mouse (n=3). (B) Quantification of the percentage of tumor cells extravasating in the liver at 24 and 48 hours post injection of wildtype and *Mmp13*-/- mice (n=3). Statistical analysis was performed using the 2-tailed t-test. (C) Representative image of 3D reconstruction of wildtype and *Mmp13*-/- hepatic vasculature and (D) quantification of hepatic vascular volume in wildtype and MMP13-/- mice (n=5). Statistical analysis was performed using the 2-tailed t-test. WT, wildtype; 13-/-, *Mmp13-/-*.

melanoma and breast cancer [155, 223]. Immunohistochemical analysis of human colorectal cancer metastases to the liver and murine experimental MC38 colon carcinoma tumors in the liver show MMP13 staining within these tumors (Figure 29A, B). We evaluated *Mmp13* gene expression in murine and human colorectal cancer cell lines and found that *Mmp13* is expressed by the MC38 (murine) and HCT116 (human) colorectal cancer cell lines. To assess the role of tumor-derived MMP13 on the ability of tumor cells to metastasize to the liver, we developed *Mmp13* stable knockdown cell lines (Figure 28) using RNA interference. We found that knock down of *Mmp13* does not have a significant effect on cell proliferation as determined by the MTT assay in the MC38 nor the HCT116 cell line (Figure 29C, F). To study the effect of MMP13 on cell migration, control and *Mmp13* knockdown cells were seeded in a modified Boyden chamber. Both MC38 and HCT116 cell lines showed a decrease in transwell migration compared with the respective control cells (Figure 29D, G). Next, the knockdown cell lines were evaluated for their ability to invade through matrigel with the modified Boyden chamber and demonstrated a decreased invasive ability with the knockdown of *Mmp13* in both cell lines (Figure 29E, H). For the HCT116 cell line, there was an average 9 fold decrease in transwell invasive ability between the knockdown cell lines and controls, and a 2.5 fold decrease in transwell migration, suggesting loss of MMP13 effects cell invasion in addition to its effect on cell migration. These results suggest that tumor derived MMP13 is essential for migration as well as invasion.

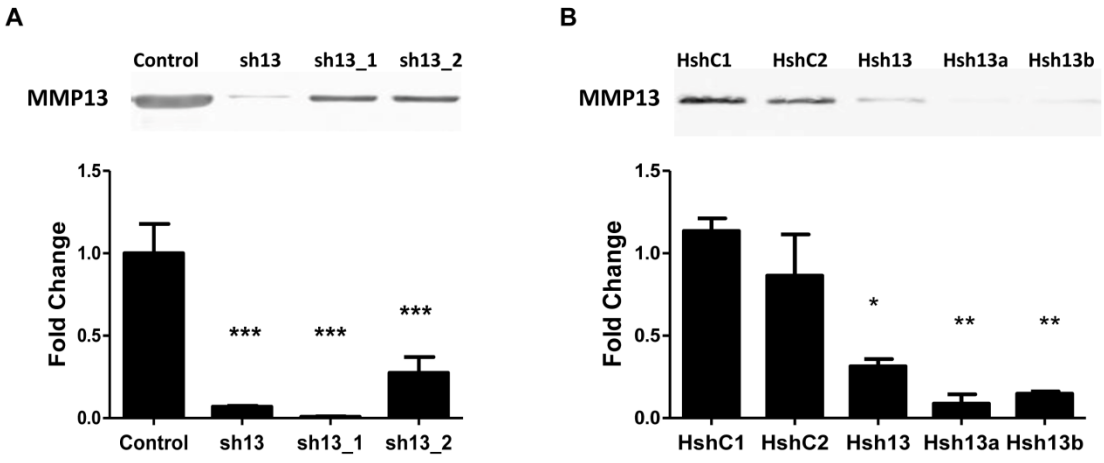

Figure 28: Establishment of stable MMP13 knockdown cell lines. Stable knockdown of MMP13 cell lines were developed using RNA interference. (A) Western blot of MMP13 protein levels secreted into the media and quantification of mRNA expression of *Mmp13* in MC38 control and knockdown cell lines. sh13 is a pooled MMP13 knockdown population. sh13_1 and sh13_2 are expanded cell lines from individual clones. (B) Western blot of MMP13 protein levels secreted into the media and quantification of mRNA expression of *MMP13* in HCT116 control and knockdown cell lines. HshC1 and HshC2 are expanded clonal cell populations of control shRNA treated HCT116 cells. Hsh13 is a pooled population of MMP13 shRNA treated cells. Hsh13a and Hsh13b are derived from single clones.

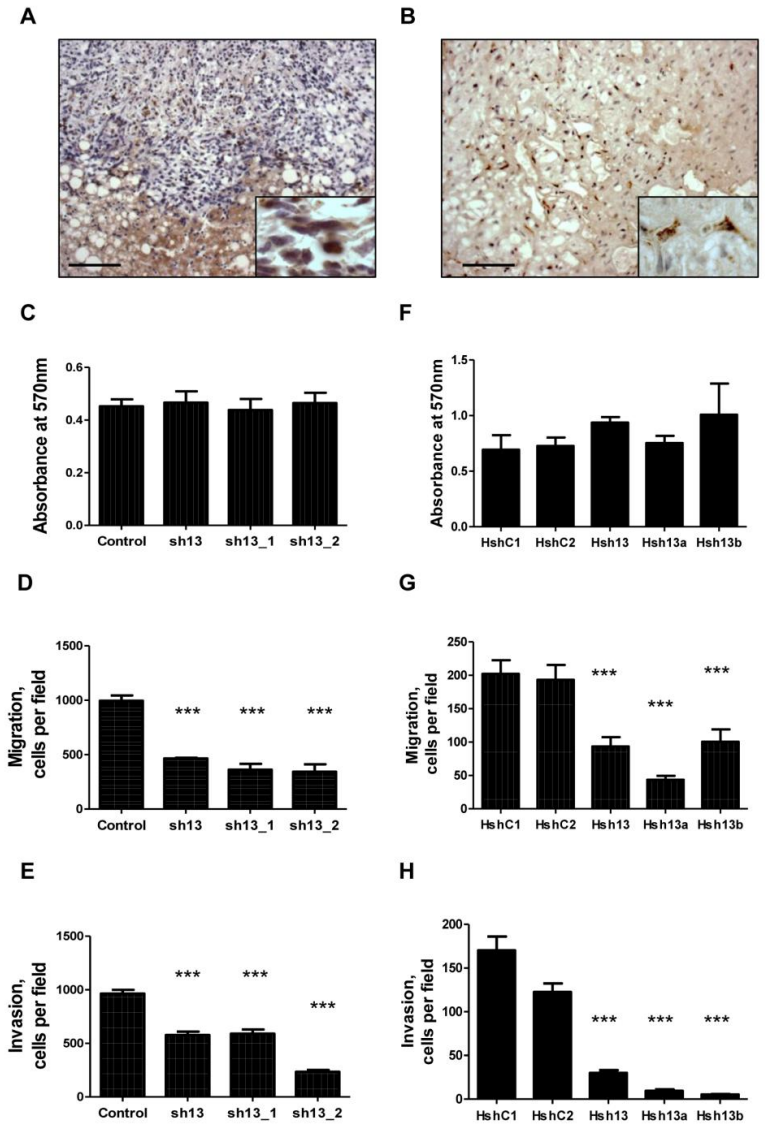

Figure 29: Effect of knockdown of *MMP13* in tumor cells on various hallmarks of cancer *in vitro*. Immunohistochemical staining for MMP13 in representative sections of (A) murine MC38 tumor in the mouse liver and (B) human colorectal cancer metastasis to the liver taken with a 20X objective. Scalebar represents 100 microns. Inset shows individual tumor cells at 63X magnification. MTT assay shows no change in proliferation after loss of MMP13 *in vitro* in (C) MC38 or (F) HCT116 control and MMP13 knockdown cell lines. Knockdown of MMP13 leads to decreased ability of tumor cells to migrate *in vitro* as determined by a transwell migration assay in (D) MC38 and (G) HCT116 cell lines. Knockdown of MMP13 leads to decreased ability of tumor cells to invade *in vitro* as determined by the transwell invasion assay in (E) MC38 and (H) HCT116 control and knockdown cell lines. Statistical analysis was performed using GraphPad Prism software. Data was analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. P values are represented by stars where: *** $P \leq 0.0001$ when compared to the respective non-silencing control treated cell lines.

Loss of tumor-derived MMP13 reduces the ability of tumor cells to metastasize *in vivo*

To test the importance of tumor cell derived MMP13 on establishment of metastasis to the liver, we utilized the splenic injection model to deliver 2.5 x 10^5 *Mmp13* knockdown or control MC38 cell lines into wildtype C57bl/6 mice. After 21 days, mice were sacrificed and livers harvested. The liver weight and percent liver weight to total body weight ratio was measured to determine metastatic burden (Figure 30B, C). Liver sections were stained by H&E (Figure 30A) to record the incidence (Figure 30C) and area (Figure 30D) of metastasis. We found that the tumor burden and incidence of metastases decreased in the mice injected with *Mmp13* knockdown cells compared to control cells. Liver metastases were stained for Ki67 (proliferation marker) and cleaved caspase-3 (apoptosis marker) to examine the role of MMP13 in promoting the survival and outgrowth of metastatic tumors (Figure 31A, B). The percentage of tumor cells proliferating or undergoing apoptosis were similar between the wildtype and *Mmp13*-/- tumors of comparable size, suggesting no difference in the rate of tumor growth between the WT and *Mmp13*-/- mice. Additionally, there was no difference in tumor vascularity as determined by vWF staining (Figure 31C). These results indicate that tumor-derived MMP13 facilitates the establishment of metastases in the liver without affecting metastatic outgrowth.

Combined loss of both stromal and tumor derived MMP13 leads to further decrease in the ability of tumor cells to metastasize *in vivo*

To test the importance of combined loss of both stromal MMP13 and tumor cell derived MMP13 on establishment of metastasis to the liver, we utilized the splenic injection model as described previously to deliver 2.5 x 10^5 *Mmp13* knockdown or

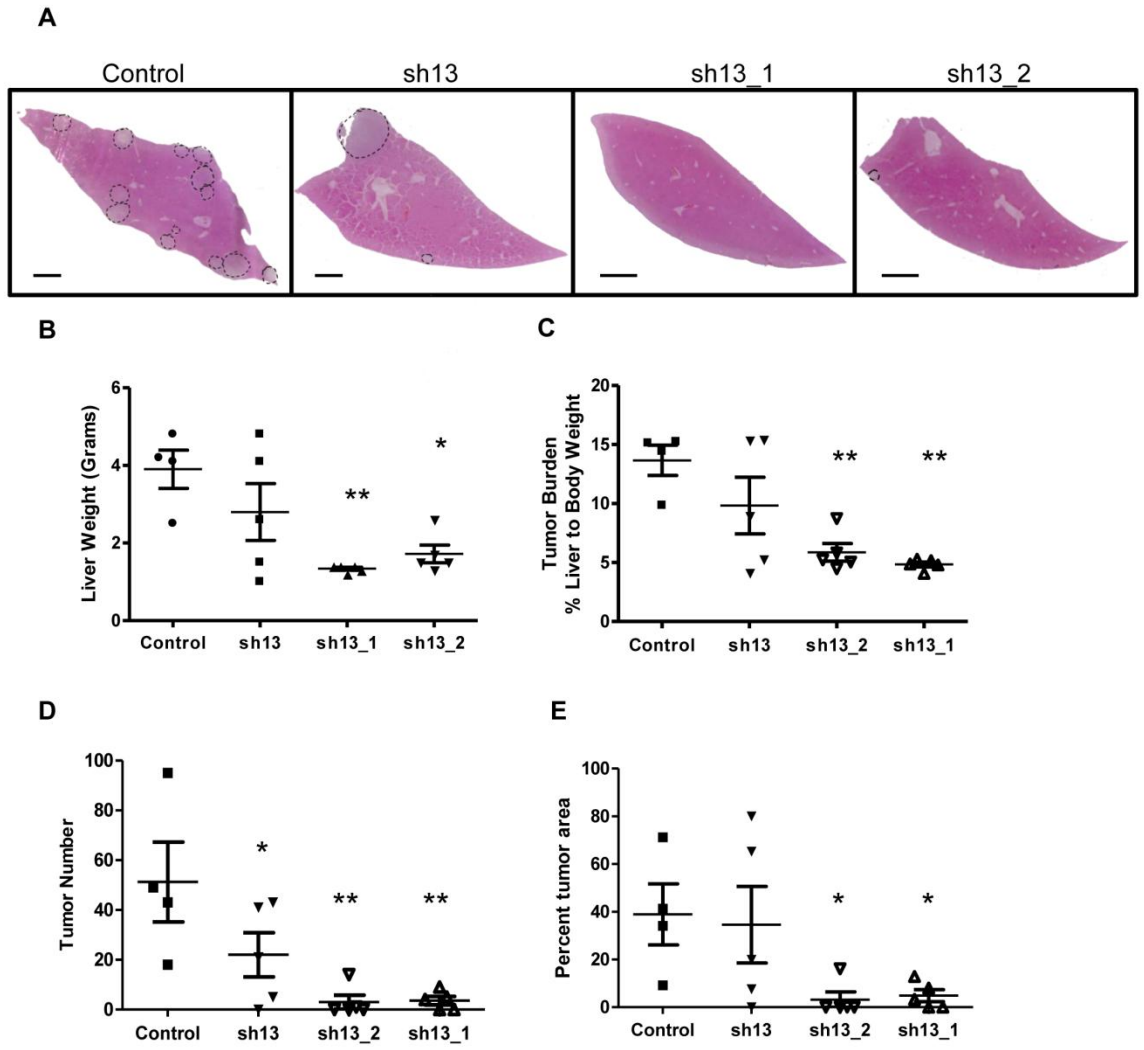

Figure 30: Knockdown of tumor derived MMP13 leads to decreased tumor metastasis to the liver. (A) Representative liver cross sections stained with Haematoxylin & Eosin of wildtype mice injected with control or MMP13 knockdown MC38 cell lines (MSh13 pooled, MSh13-1, and MSh13-2). Dashed black lines denote tumors. Quantification of metastatic tumor burden measured by (B) liver weight, (C) tumor burden as a percent liver weight to total body weight, (D) metastatic seeding by quantification of tumor number and (E) percentage of tumor area to total liver area in normal and steatotic livers (n=5 per group). Statistical analysis was performed using GraphPad Prism software. Data was analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. P values are represented by stars where: *P ≤.05, **P ≤ .01, and ***P ≤ .001 when compared to mice injected with non-silencing control cell lines.

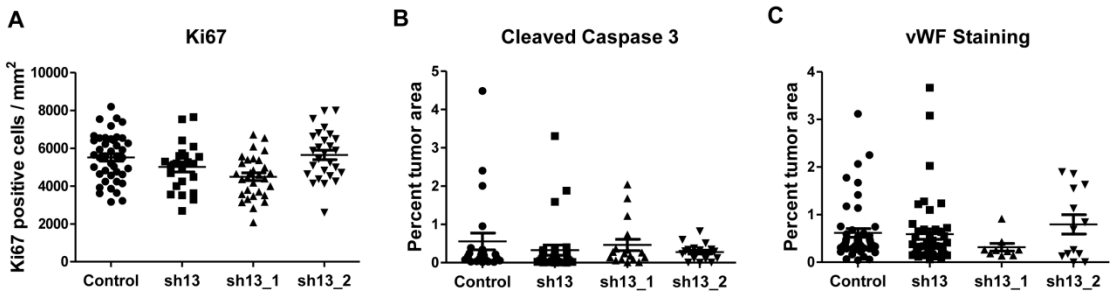

Figure 31: Effect of loss of tumor derived MMP13 on tumor proliferation, apoptosis and vascularity. (A) Quantification of tumor proliferation by Ki67 staining determined by plotting number of Ki67 positive cells per unit area. (B) Change in apoptosis within tumors in determined by percentage of Cleaved caspase 3 staining per unit tumor area. (C) Quantification of tumor vascularity as determined by percentage area of von Willebrand factor (vWF) staining per unit tumor area. Quantification was done using Metamorph software. Statistical analysis was performed using GraphPad Prism software. Data was analyzed by using one-way ANOVA and showed no significant differences between different test groups.

control MC38 cells into *Mmp13*-/- C57bl/6 mice. At the endpoint of 21 days post injection, mice were euthanized and their livers harvested. The liver weight and percent liver weight to total body weight ratio was measured to determine metastatic burden (Figure 32B,C). Liver sections were stained by H&E (Figure 32A) to record the incidence (Figure 32C) and area (Figure 32D) of metastasis. We found that the tumor burden and incidence of metastases decreased in the mice injected with *Mmp13* knockdown cells compared to control cells. These results suggest that combined loss of both stromal and tumor derived MMP13 lead to a further decrease in tumor burden and targeting both stromal and tumor derived MMP13 would be beneficial to prevent establishment of metastases in the liver.

Discussion

The liver is a common site of metastasis of several types of cancer [228] and identification of molecular effectors that can prevent metastasis to the liver is of important clinical relevance. NAFLD is increasingly becoming recognized as the most common cause of liver disease and it is associated with increased risk of development of primary liver cancers, even prior to establishment of cirrhosis [216]. Progression of NAFLD results in changes in the liver microenvironment and alterations in the extracellular matrix, which effects cancer progression and outcome [78, 100]. The MMPs are an important class of proteases that can alter the extracellular matrix and influence the microenvironmental integrity. There is considerable evidence supporting the role they play at different steps of malignant tumor metastasis including tumor cell intravasation and extravasation [229]. We initially evaluated the steatotic microenvironment for

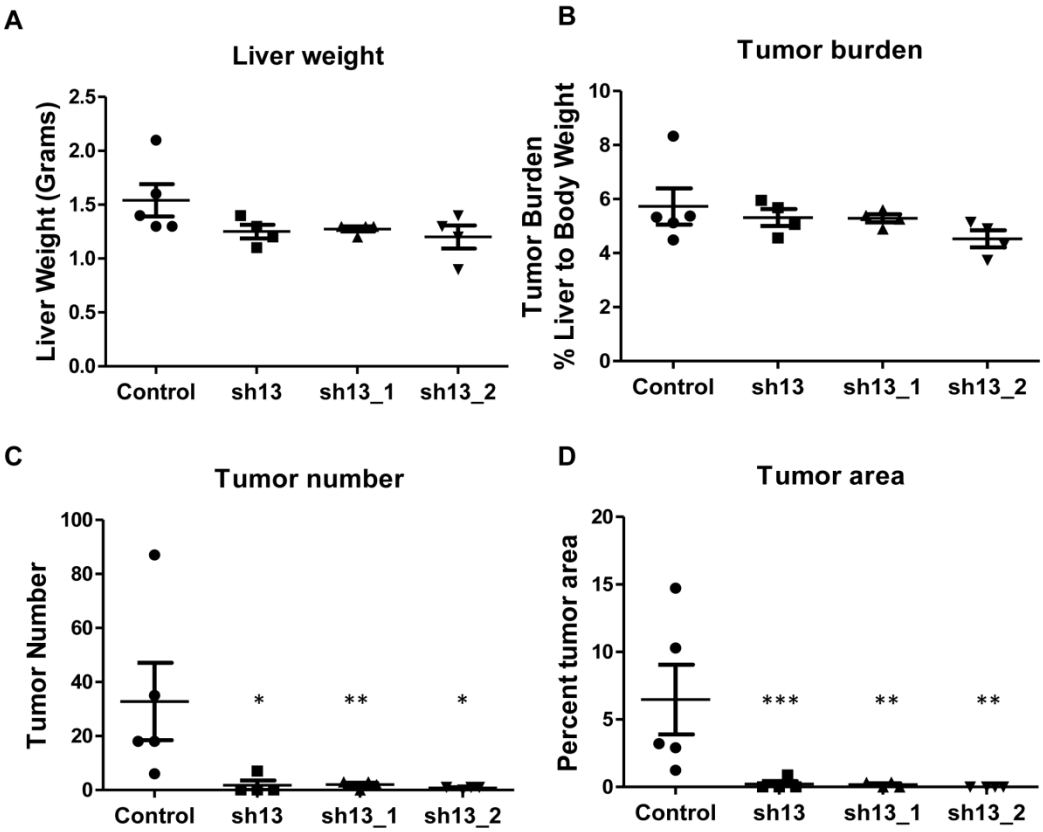

Figure 32: Combined loss of both tumor derived and host MMP13 lead to additional decrease in metastatic tumor burden to the liver. Normal *Mmp13*-/- mice were injected with either Control or *Mmp13* knockdown MC38 colon cancer cell lines (sh13, sh13_1,sh13_2). Quantification of metastatic tumor burden measured by (A) liver weight, and (B) tumor burden as a percent liver weight to total body weight. Metastatic seeding was determined by quantification of (C) tumor number and (D) percentage of tumor area to total liver area in normal and steatotic livers. Statistical analysis was performed using GraphPad Prism software. Data was analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. P values are represented by stars where: *P $\leq$.05, **P $\leq$ .01, and ***P $\leq$ .001 when compared to mice injected with non-silencing control cell lines.

alterations in the gene expression levels for a panel of MMPs that have been previously associated with cancer progression. Our results demonstrate that MMP13, an interstitial collagenase, is elevated in the steatotic liver in our murine model as well as with progression of NAFLD in human patient samples. Others had previously shown that MMP13 was elevated with fibrotic liver disease [162]. Several studies also link elevated levels of MMP13 in either the stroma or tumor cells with cancer progression [155, 161, 223, 225], however there are reports that alternately suggest a protective role for MMP13 [230, 231]. There still remains a limited understanding of the role of MMP13 in the liver microenvironment and its influence on the establishment of hepatic metastases. Here, we evaluated the role of both stromal and tumor cell derived MMP13 on the establishment of metastases in the liver.

Using mice genetically deficient in *Mmp13*, we have shown that loss of stromal MMP13 leads to a significant decrease in the tumor burden in the liver. This effect was seen in both normal and steatotic livers, suggesting that elevation of MMP13 in the liver plays a role in tumor metastasis. In addition, elevated MMP13 in the setting of other liver diseases such as fibrosis and hepatitis may cultivate a pro-metastatic liver microenvironment. Although we saw a significant decrease in tumor burden in the *Mmp13* deficient mice, differences in proliferation or apoptosis were not observed within the tumors, nor were any differences in the vascularity of the tumors. This suggested that the difference in metastastic burden was due to early events in the metastatic cascade. Similar levels of metastatic cell dissemination and early cell survival were observed in wildtype compared to MMP13 deficient livers, yet the loss of host MMP13 diminished the ability of tumor cells to extravasate. Several factors can influence the ability of tumor

cells to adhere to the vascular walls and extravasate into the surrounding tissues. Studies have shown that changes in expression level and structure of collagen surrounding the vasculature are important in the ability of tumor cells to extravasate [231–233]. Since MMP13 is a collagenase, we evaluated the levels of collagen I, II and IV in the liver but found that mice lacking MMP13 had no significant differences in collagen immunofluorescence staining pattern compared to the wildtype mice (Figure 33). Although we did not observe changes in collagen in the liver, MMP13 may mediate release or activation of critical factors involved in tumor cell extravasation. MMP13 can cleave, release and activate cytokines thereby altering recruitment and or activation of inflammatory cells such as neutrophils and macrophages that have been shown to facilitate tumor cell extravasation [61, 234–236]. We evaluated changes in the lymphoid and myeloid lineage inflammatory cell sub populations within the liver of wildtype and *Mmp13*-/- mice, in the absence of tumor cells (Figure 34). I found an increase in the CD3ε positive lymphoid population in the *Mmp13*-/- mice on regular diet but did not observe significant differences within the myeloid cell subpopulations. Changes in the lymphoid cell subpopulations as well as the activation state and function of the immune cells could explain the differences in tumor burden observed with the loss of MMP13. In the tumor studies, the spleens were removed following splenic injection of tumor cells, to prevent the interference of "primary" tumors developing in the spleen that could provide a continuous supply of circulating tumor cells to the liver and impact the assessment of metastatic tumor burden. Additionally, splenectomy prevents development of large "primary" tumors in the spleen. The spleen plays an important role in the immune response, and a splenectomy could influence the immune cells infiltrating the liver and

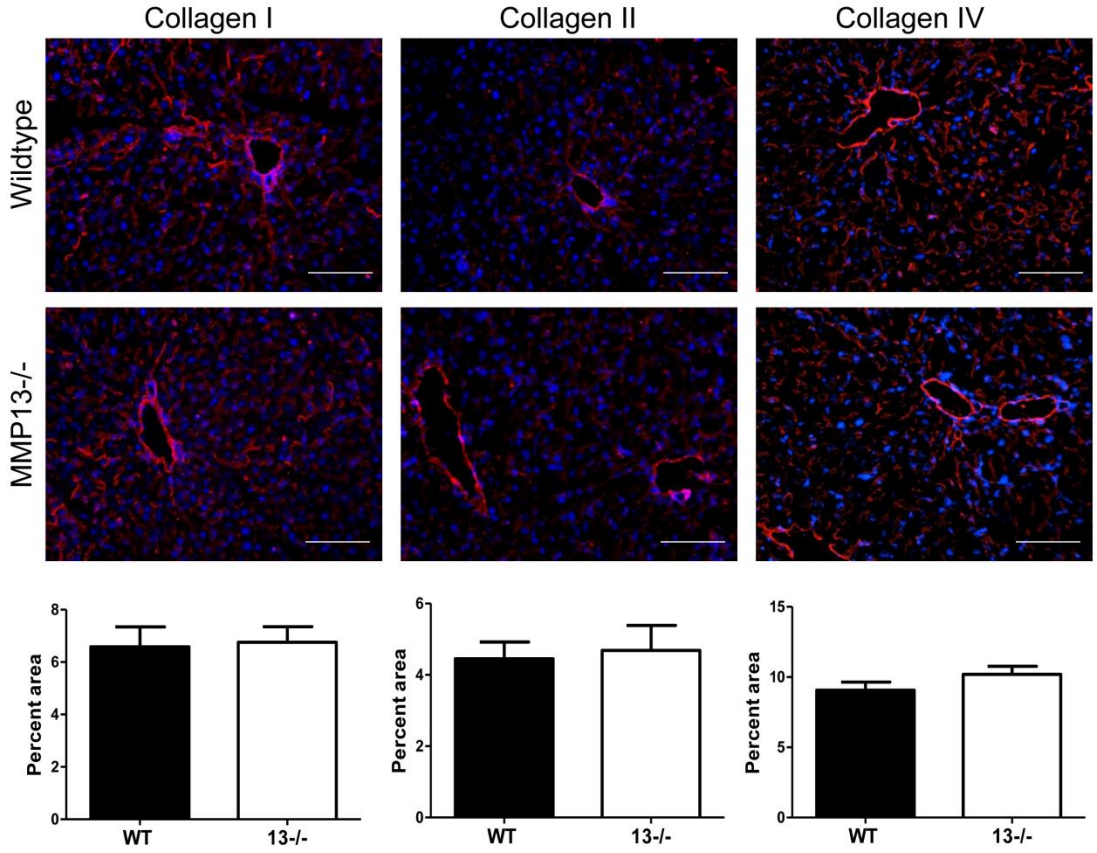

Figure 33: Immufluorescence of collagen in the livers of wildtype and *Mmp13*-/- mice. (A) Representative images of immunoflourescent staining of Collagen I, Collagen II and Collagen IV in the livers of wildtype and *Mmp13*-/- mice. (B) Quantification of the percent area stained over set threshold show no significant differences in the amount of collagen stained within the livers of wildtype and *Mmp13*-/- mice (n=6). WT, wildtype; 13-/-, *Mmp13-/-*.

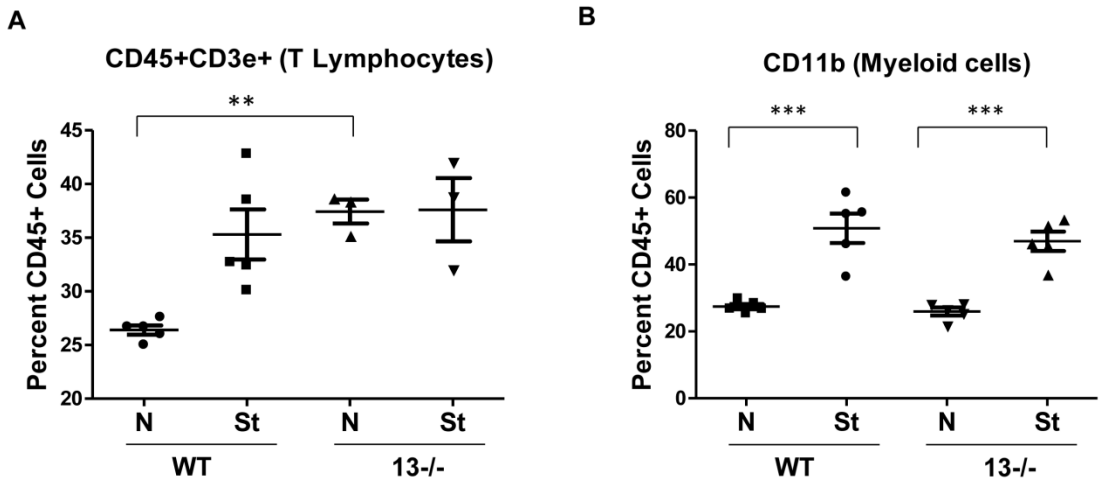

Figure 34: Effect of loss of MMP13 on inflammatory cell populations in the liver. CD45 positive cells were isolated from wildtype and *Mmp13*-/- digested liver samples with and without steatosis using immune-magnetic beads and then stained for the immune markers CD3ε and CD11b before being subjected to flow cytometric analysis. CD45 positive cells were then gated for the percentage of (A) CD3 positive T lymphocytes or (B) CD11b positive myeloid cells. Data was analyzed by using one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. P values are represented by stars where:
*P ≤.05, **P ≤ .01, and ***P ≤ .001. N, normal; St, steatosis; WT, wildtype; 13-/-, *Mmp13-/-*.

impact tumor growth. To ensure consistency, spleens were removed in all study groups. Even without the spleen, the study takes into consideration the changes in the immune complement within the liver microenviromnet and recruitment of circulating inflammatory cells and from the bone marrow.

Cytokine array analysis with liver protein lysates showed no significant changes between lean wildtype or lean *Mmp13*-/- mice. Steatotic mice did have observable increases in multiple cytokines compared to lean mice for both wildtype and *Mmp13*-/- mice. We noted a decrease in the level of IGF binding proteins in livers of *Mmp13*-/- with steatosis compared to wildtype mice with steatosis. IGF signaling has been previously shown to be important in regulating the liver microenvironment in the setting of obesity and can facilitate liver metastasis [237]. Changes in IGF signaling with the loss of MMP13 could impact the liver microenvironment with steatosis and warrant further study.

MMP13 is produced by resident stellate cells and Kupffer cells that are activated by the accumulation of fat in the liver MMP13 is also produced by infiltrating inflammatory cells into the liver [163, 238]. Identification of the cellular source responsible for the increase in MMP13 would be important to target these cells for treatment and limit enhanced MMP13 production. To understand whether resident or bone marrow derived cells were responsible for MMP13 production and important for the establishment of metastases, I carried out bone marrow transplant experiments where wildtype bone marrow was replaced with bone marrow from *Mmp13*-/- mice and *vice versa*. Post tumor cell injection, the tumor burden observed exceeded previous studies in all study groups. This could be a result of the irradiation required for the bone marrow

transplants which can affect the liver microenvironment. These results could not clearly identify the bone marrow derived cells as being responsible for changes in the metastatic burden observed in the *Mmp13*-/- mice (Figure 35).

MMP13 expression has been observed in invasive malignant tumors such as breast carcinomas, squamous cell carcinomas (SCCs) of the head and neck and vulva, primary and metastatic melanomas, and transitional cell carcinoma of the urinary bladder [159]. Our results indicate that both the MC38 murine colon cancer line and the HCT116 colon cancer cells express MMP13. Knockdown of MMP13 utilizing lentiviral shRNA resulted in a decreased ability of tumor cells to migrate and invade *in vitro* and correspondingly led to the development of fewer metastasis *in vivo*. Coupled together, both tumor cell and host derived MMP13 promote the establishment of metastases.

The use of selective MMP13 inhibitors may be an important step to control tumor growth and metastasis to the liver, however, the clinical use of MMP inhibitors has been hampered by their lack of specificity [239]. Fortunately, progress is being made toward developing specific inhibitors and a few MMP13 selective inhibitors are currently being studied [224, 240]. It will therefore be important to determine whether MMP13 selective inhibitors can pharmacologically block metastasis to the liver.

In conclusion, we have shown that MMP13 is elevated in the setting of hepatic steatosis and that both tumor and stromal derived MMP13 are involved in attenuating metastatic tumor burden in the liver. Collectively, these data suggest that MMP13 could represent a new therapeutic target in the management of metastasis to the liver.

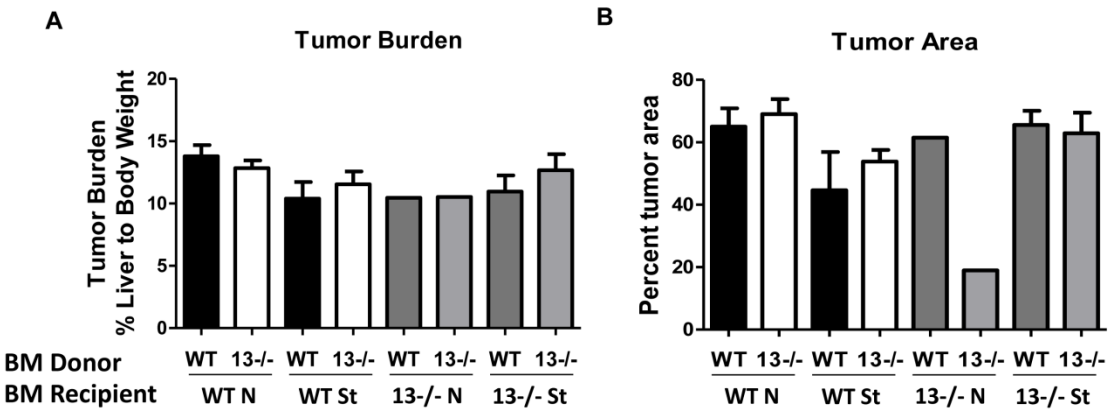

Figure 35: Effect of loss of bone marrow derived MMP13 on tumor development in the liver. Quantification of metastatic tumor burden measured by (A) tumor burden as a percent liver weight to total body weight and (B) percent tumor area in normal and steatotic livers of wildtype and *Mmp13* deficient mice with either wildtype or *Mmp13* deficient bone marrow show no significant differences in tumor burden. N, normal; St, steatosis; WT, wildtype; 13-/-, *Mmp13-/-*.

CHAPTER V

CONCLUDING REMARKS

Summary

The overall goal of this thesis was to characterize alterations in the liver microenvironment of NAFLD that influence the establishment of tumor metastasis to the liver. The increasing recognition of NAFLD as an important disease has spawned a relatively new field of study. There is still a limited understanding of the pathogenesis of the disease and the complications associated with its incidence. NAFLD is associated with an increased risk of development of primary HCC as discussed earlier. However, there is very limited understanding of the impact of NAFLD on tumor metastasis to the liver. We previously demonstrated a significant increase in the tumor burden and number in steatotic livers compared to normal livers [53]. Additionally, Wu et al. showed that elevated IGF-1 in the setting of obesity alters macrophage number and function in the liver and promotes cancer metastasis to the liver [237]. These studies indicate that obesity, and associated NAFLD, are important in the establishment of metastases in the liver. I thus hypothesized that molecular and cellular changes in the steatotic liver, relative to the normal liver contributed to a more permissive microenvironment for tumor growth and establishment of metastases in the liver. To test this hypothesis I evaluated the changes in inflammatory cell populations and cytokines in steatotic compared to normal livers. To further determine changes in the steatotic liver that could be responsible for this difference in metastatic tumor burden in the liver, I adopted a candidate approach

and performed a microarray between mRNA from normal and steatotic livers, and found that *Mmp12* and *Mmp13* were elevated. Figure 36A summarizes some of the current knowledge in the field of liver metastasis in the setting of NAFLD. The results and contributions that my dissertation work has made to this field are represented in Figure 36B and summarized below.

Characterization of the steatotic livers showed a significant increase in the number of CD45 positive inflammatory cells that infiltrated into the liver upon development of steatosis. Inflammatory infiltrates contained CD11b positive myeloid and CD3ε positive lymphocytic populations. Additionally, infiltrates contained a decrease in the B cell sub-population and an increase in the dendritic cell populations. Using cytokine array analysis, the secretion of several cytokines in the plasma, liver tissue and conditioned media from isolated myeloid cells were found to be altered in the setting of steatosis and are listed in Table 2. Leptin, CXCL1, CXCL2 and CXCL16 were all found to be increased in plasma and in the conditioned media of cultured myeloid cells. These cytokines were further shown to directly increase the proliferation of hepatocellular cell lines following treatment in vitro. The proliferative capability of these cytokines is one explanation for the overall increase in hepatocyte proliferation observed with Ki67 staining in both human and mouse liver tissue sections. Therefore, I believe that the early inflammatory stages of hepatic steatosis provide a proliferative microenvironment that could potentiate the growth of metastatic foci as well as potentiate the development of primary HCC associated with NAFLD.

Dysregulation of MMPs has been associated with liver disease and have also been shown to be important in all stages of tumor progression, playing key roles in metastatic

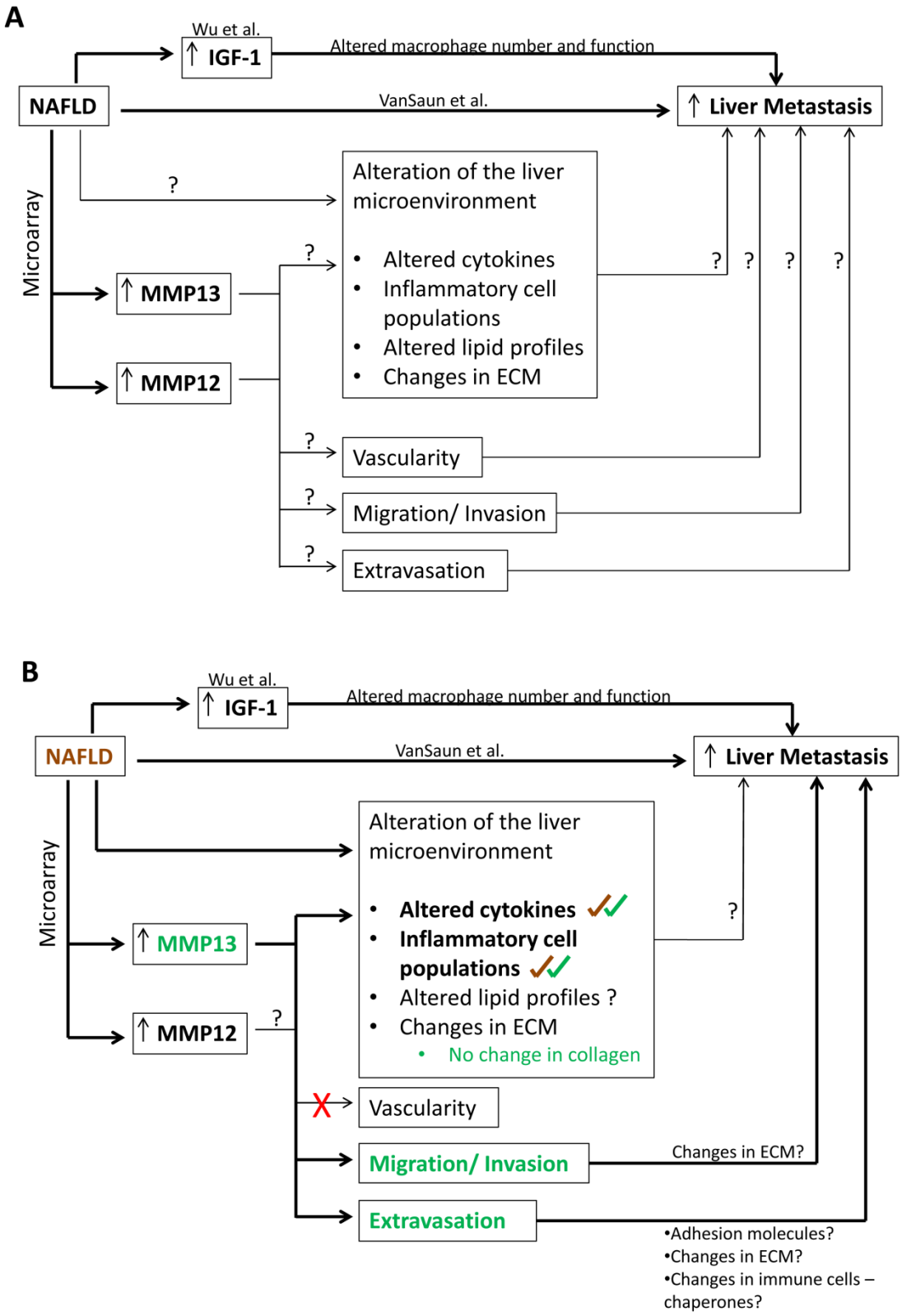
A
Wu et al.
↑ IGF-1
Altered macrophage number and function
NAFLD
VanSaun et al.
↑ Liver Metastasis
Microarray
?
Alteration of the liver microenvironment
• Altered cytokines
• Inflammatory cell populations
• Altered lipid profiles
• Changes in ECM
↑ MMP13
↑ MMP12
Vascularity
Migration/ Invasion
Extravasation
B
Wu et al.
↑ IGF-1
Altered macrophage number and function
NAFLD
VanSaun et al.
↑ Liver Metastasis
Microarray
Alteration of the liver microenvironment
• Altered cytokines
• Inflammatory cell populations
• Altered lipid profiles ?
• Changes in ECM
• No change in collagen
↑ MMP13
↑ MMP12
Vascularity
Migration/ Invasion
Changes in ECM?
Extravasation
•Adhesion molecules?
•Changes in ECM?
•Changes in immune cells – chaperones?

Figure 36: Proposed mechanisms of liver metastasis in the setting of non-alcoholic fatty liver disease (NAFLD). (A) Summary of knowledge in the field of NAFLD and the potential mechanisms of cancer metastasis to the liver prior to this dissertation. VanSaun et al. observed that NAFLD leads to increased risk of colorectal cancer metastasis to the liver [53]. Wu et al. showed that IGF-1 alters the liver micoenvironment in the setting of obesity, leading to increased liver metastasis [237]. Potential mechanisms for this increase in liver metastasis include alterations in the liver microenvironment. Changes in Matrix Metalloproteinases (MMPs), particularly MMP13 could further alter the liver microenvironment, impact tumor vascularity, tumor cell migration, invasion and extravasation, thus influencing establishment of metastasis in the liver. (B) Modifications to the field based on data from this dissertation. These results show that NAFLD leads to alterations in inflammatory cell populations and cytokines in the steatotic liver. MMP13 impacts the ability of tumor cells to migrate and invade *in vitro* and extravasate from the vasculature *in vivo*. Bold text indicates what is known. Un-bolded text indicates proposed mechanisms that have not been fully elucidated. Changes as a result of alterations in MMP13 are shown in green and those observed in NAFLD in brown.

potential of tumor cells and alteration of the tissue microenvironment at secondary sites of metastasis [229]. To gain further insight into alterations in MMPs, I evaluated the change in mRNA expression for a panel of MMPs between normal and steatotic livers. I found that *Mmp12* and *Mmp13* were significantly elevated in the steatotic murine liver compared to normal livers. The role of MMP13 on tumorigenesis in the setting of steatosis became the principle focus of my thesis. In addition to MMP13 from the stromal cells in the steatotic liver, I was able to demonstrate that the tumor cells themselves were a source of MMP13. The aims of my studies were thus to assess the contribution of stromal and tumor derived MMP13 on the establishment of colorectal cancer metastasis to the liver. Loss of stromal MMP13 led to a decrease in the ability of tumor cells to metastasize to the liver in both a normal and steatotic setting compared to wildtype mice. This decrease in metastatic tumor burden can be explained in part by the significant decrease in the ability of the tumor cells to extravasate from the vasculature in the absence of MMP13 48 hours post injection determined using confocal microscopy (Figure 27). Additionally, stable MMP13 knockdown cell lines were used to demonstrate that reduction of tumor derived MMP13 decreased migratory and invasive properties in vitro and decreased metastatic burden in vivo. Taken together, the loss of both stromal and tumor derived MMP13 have an additive effect on preventing the development of metastasis. These results suggest that MMP13, both stromal and tumor derived, contribute to the establishment of metastases in the liver. Furthermore, this suggests that MMP13 could be a potential therapeutic target for treatment of patients with primary CRC, that are at risk of developing liver metastases, by blocking the motility of tumor cells and their ability to exit from the vasculature into the liver.

These studies define some of the changes in the steatotic liver microenvironment. We have identified differences in inflammatory cell infiltration and cytokine levels in the steatotic liver. We further demonstrate that MMP13 is an important mediator of metastasis in this setting and confirmed that both stromal and tumor derived MMP13 play a role in establishment of metastasis in this setting. Though much progress has been made in understanding the changes in the liver microenvironment with NAFLD, there are still questions to be answered to fully understand the complexities of this microenvironment and its effect on tumor development and progression, which are discussed below.

Future Directions

As the incidence of obesity continues to increase, NAFLD has become a major health concern. The risks of disease associated co-morbidities increases with progression of NAFLD. Initial stages of NAFLD, prior to the development of fibrosis, can be revert back to normal liver with proper diet and exercise regimens in some cases [241]. As such, development of safe noninvasive methods to stage NAFLD would have important clinical implications in the treatment of patients with NAFLD. NAFLD is increasingly being recognized as the most common risk factor for the development of HCC and further understanding of the cellular and molecular factors involved with this disease progression are necessary to prevent cancer associated co-morbidities. My studies have identified changes in inflammatory cell populations and cytokine levels in the steatotic livers but their effects on activation of signaling pathways and development of primary and metastatic tumors needs to be further assessed. I found additional molecular factors including MMP12 and MMP13 to be markedly upregulated in the steatotic liver microenvironment. My results show that MMP13 is important in the motility of tumor

cells and their ability to extravasate form the vasculature and thus plays an important role in establishment of metastases in the liver. However, I did not evaluate the role of MMP13 on the development of primary tumors in the liver.

In addition to the importance of MMP13 in this disease, increased levels of MMP12 in the steatotic setting could have additional effects on modulating the steatotic liver microenvironment and affect tumor development and progression in the setting of NAFLD. Further understanding of the molecular and cellular factors altered in NAFLD, and the signaling pathways they trigger, could provide potential drug targets and therapeutic options that would have great clinical implications. Importantly, targeted therapeutics could prevent progressive disease and/or the development of primary or metastatic liver cancers. Important future directions in the area of hepatic steatosis and NAFLD are discussed below.

Identification of biomarkers for the progression of NAFLD

Currently there are no reliable non-invasive techniques to distinguish patients with steatosis from NASH, which is clinically important as prognosis and treatment of each stage of the disease is different. Reliable methods to make this assessment require a liver biopsy which is an invasive procedure. Therefore, the development of non-invasive biomarkers for the different stages of NAFLD has become of major interest. Several studies have used a combination of serum markers and advanced imaging techniques but still lacked the capability to reliably distinguish simple steatosis from NASH [242].

One of the prominent features of the development of NAFLD is the accumulation of lipids in the liver. Determination of changes in lipid composition and function are only now being examined [81]. Several lipid mediators typically associated with lipotoxicity

such as diacylglycerols (DAGs), oxysterols, ceramides and free fatty acids (FFA) are suggested as potential key links in the mechanism of disease progression towards NASH. Previous studies were able to link changes in lipid composition in plasma with that of the liver in the setting of NASH [243, 244]. We hypothesized that analyzing specific changes in lipid composition in the liver and accessible body fluids such as plasma/urine could provide a unique and reliable set of non-invasive biomarkers in the blood/urine that represent the changes taking place in the liver and could distinguish the different NAFLD stages.

To identify unique lipid species, in collaboration with the laboratory of Alex Brown, lipids were profiled from liver biopsies, plasma, and urine samples in a double-blinded study from 88 individuals diagnosed as either normal (n=31), steatotic (n=17), NASH (n=20), or cirrhotic (n=20). Additionally, metabolites of the citric acid cycle, glycolytic pathway, nucleotides, and CoA-derivatives were profiled. Finally, gene expression from liver samples was analyzed by RNA-Seq and the results were correlated with both lipid and intermediary metabolite levels on a sample-by-sample basis.

Our lipidomic and metabolomic based analysis significantly discriminated between the different stages of NAFLD. Sphingolipids and glycerophospholipids were the most reliable predictive indicators of a given disease stage. Cirrhosis which represents end stage liver disease, with alterations in liver function is clearly distinguishable from the normal and intermediate stages of NAFLD with a small panel of analytes and transcripts. Distinguishing between normal, steatosis and NASH poses more of a challenge due to the high variability in lipid expression and overlap between the different stages of NAFLD. We were able to identify a diverse panel of just 20 plasma lipid and

aqueous metabolites that efficiently separated all disease states by linear discriminant analysis. This study provides a signature that could be used to non-invasively stratify patients based on the stage of NAFLD. Further validation on a larger sample set is required before implementation in the clinic, but our analysis provides a viable alternative to a liver biopsy. (Manuscript in revision, Journal of Lipid Research).

The role of MMP13 in primary tumors of the liver

My studies showed a 10-fold increase in mRNA expression of *Mmp13* in the steatotic liver compared to normal liver. Our lab has further demonstrated that the steatotic liver microenvironment promotes the establishment of tumor metastases in the liver [53]. In addition to metastasis, NAFLD has been associated with an increased incidence of primary HCC [186]. It would thus be important to determine whether increased MMP13 regulates primary tumor growth and progression in addition to the role it plays on tumor metastasis to the liver in the setting of NAFLD.

Our previous studies demonstrated that prolonged consumption of high fat diet results in the development of primary liver tumors in mice [53]. Using mice deficient in *Mmp13*, we did not see tumor growth at 12 months of age, suggesting that loss of *Mmp13* in the mice may be protective against primary tumor development in the setting of steatosis. We have shown that *Mmp13* deficient mice indeed develop steatosis similar to wildtype mice. Although the difference in incidence of primary tumors is intriguing, the low numbers of mice in these studies did not provide statistical power for definitive results. For future studies, we propose that both wildtype and *Mmp13*-/- maintained on a high fat diet be exposed to a "second hit" with DEN (Diethylnitrosamine) which could decrease the lag time for tumor development and increase the aggressiveness of the

tumors [245]. Inclusion of longitudinal screening with CT would further provide evidence for the onset of primary HCC and rate of tumor progression in either wildtype or *Mmp13*-/- mice. Endpoint histological analysis and grade of tumors would be determined by a pathologist.

Alternative methods to generate primary tumors in the setting of steatosis would be to utilize syngeneic primary HCC cell lines, which are injected into the liver via spleen of either wildtype or *Mmp13*-/- mice. The splenic injection model allows seeding of primary tumor cells into the orthotopic liver site via the portal vein circumventing any added injury that would be caused by direct injection to liver. MMP13 expression of the primary HCC cell lines before and after tumor establishment can be assessed as well to determine whether tumor cells express MMP13 that could play a role in tumor progression and metastasis. Additionally, we could control the expression of MMP13 from the primary tumor cells as we demonstrated in our colorectal metastatic model.

Studies such as these would be able to determine whether loss of MMP13 was protective against development of primary HCC. This would provide viable information as to whether MMP13 or its downstream effectors could be targeted to prevent tumor onset and progression in NAFLD patients at high risk of developing HCC.

The use of MMP13 inhibitors in treatment of liver metastasis

MMPs have been associated with incidence of cancer for a long time and there was extensive preclinical data that led to clinical testing of synthetic MMP inhibitors (MMPIs) for the treatment of cancer [131]. The results of these Phase III clinical trials were disappointing and did not increase patient survival [131]. Several reasons for their failure have been now identified, particularly the fact that these trials used broad

spectrum MMP inhibitors on patients with advanced disease. It is now known that even though MMP inhibition can slow tumor progression, several MMPs are important for normal function and some MMPs exert a protective role. Therefore, use of broad-spectrum inhibitors of MMPs could in some cases worsen the prognosis by blocking protective actions of MMPs [246]. Selective MMPIs may yet have therapeutic potential in the appropriate clinical setting, but a detailed understanding of each MMP in specific stages of disease progression is required before these reagents can be effectively translated to clinical benefit.

Evidence presented in this dissertation showed elevated MMP13 in the setting of steatosis, which facilitated metastatic tumor cell extravasation in the liver. Additionally, tumor derived MMP13 was also shown to enhance the migratory and invasive potential of tumor cells. Selective MMP13 inhibitors could thus be a viable drug target to block establishment of metastases in the liver. Although the clinical use of MMP inhibitors has been hampered by their lack of specificity [239], progress is being made toward developing specific inhibitors and a few MMP13 selective inhibitors are currently being studied [224, 240]. Shah et al. show that the use of an MMP13 selective inhibitor Cmpd1 is successful in limiting breast cancer metastasis to the bone with little toxicity to the animal at the presented dosages, suggesting that developing drugs targeting specific MMPs with limited toxicities is a possible line of future treatment [224]. It will therefore be important to determine whether MMP13 selective inhibitors can pharmacologically block metastasis to the liver.

Role of proteolytic function of MMP13 on tumor progression

Historically, the function of MMPs has been attributed to their ability to proteolytically cleave substrates in order to remodel the ECM, activate/deactivate growth factors and generically degrade other substrates [247]. Recently, studies have identified several non-proteolytic functions of MMPs that are just beginning to be understood [110, 248, 249]. Dufour et al. showed that the proteolytic activity of MMP9 is not required for MMP9-induced cell migration in COS-1 cells through the use of structural domain swapping and truncated MMP9 proteins lacking catalytic activity. They demonstrated that it was the hemopexin domain of proMMP-9 that was responsible for increased cell migration and not the catalytic domain [110]. Thus, even though the collagenase function of MMP13 is thought to breakdown the basement membrane and facilitate tumor cell escape, this would require further testing. Using cells expressing mutated or collagenase dead (catalytically inactive) MMP13, it would be possible to determine whether the proteolytic function of MMP13 is necessary for tumor cell motility and extravasation from the vasculature. Additional truncated versions of the MMP13 protein lacking the hemopexin domain and other regions of the protein will determine whether MMP13 has non-proteolytic functions that are important in tumor progression as well. Understanding the role of each domain of MMP13 would be important to develop drugs that would specifically target each individual domain and its related functions.

Role of MMP12 in tumor metastasis to the liver

MMP expression analysis in steatotic compared to normal livers demonstrated that MMP12 (macrophage metalloelastase) was significantly upregulated in the steatotic liver (Figure 22). *Mmp13*-/- mice maintained on the high fat diet also had elevated

Mmp12 mRNA expression (Figure 23). Though *Mmp13*-/- mice had a significant decrease in metastatic tumor burden in both normal and steatotic livers compared to respective wildtype livers, we still observed an increase in the tumor burden in the MMP13-/- steatotic livers compared to *Mmp13*-/- normal livers. This suggests that MMP12 and MMP13 together could have an additive effect on tumor burden in the liver, and elevated MMP12 could explain the increased tumor burden in the *Mmp13*-/- mice with steatotic relative to normal livers. MMP12 could regulate inflammatory infiltrates and damage during steatohepatitis and therefore increases the susceptibility to the development of primary or metastatic tumors to the liver.

The role of MMP12 in tumorigenesis is controversial and studies have shown both pro [250–252] and anti [253, 254] tumorigenic effects. Overexpression of MMP12 in the tumors was found to be an indicator of poor prognosis in patients with HCC [255]. Paradoxically, MMP12 expression levels were higher in primary CRC without liver metastasis compared to those with liver metastasis [256] MMP12 expression was found to suppress orthotopic tumor growth of colon cancer cells as a result of decreased VEGF (vascular endothelial growth factor) expression and increased angiostatin levels [257]. These contradictory findings regarding the role of MMP12 in tumor progression suggest that additional microenvironmental cues in the setting of steatosis might govern whether MMP12 functions in either a pro or anti-tumorigenic fashion, thus warranting further study.

MMP12 deficient mice were placed on diet similar to the MMP13 studies discussed. Preliminary studies using *Mmp12*-/- mice show that loss of *Mmp12*-/- leads to an increase in the number of macrophage crown like structures in the steatotic liver.

Additionally, an increased CK19 positivity in MMP12 deficient steatotic livers suggested development of ductal reaction in the setting of steatosis compared to wildtype mice. Further studies to characterize inflammatory cell populations and cytokine levels in the liver are underway. We would like to examine how the loss of MMP12 leads to increased macrophage crown like structures (CLS) in the liver. Increased prevalence of CLS have been observed in the peripheral fat tissues surrounding injured and dying adipocytes [258]. The CLS in the liver might be playing a similar function in the *Mmp12*-/- mice and the elevated numbers could be a result of increased hepatocyte injury in *Mmp12*-/- mice.

Significance

Steatosis and steatohepatitis result in unique microenvironments in the liver. Although the setting of NAFLD has been previously recognized to be conducive to the development of progressive fibrosis and primary hepatocellular carcinoma [171], there is still a need to understand the molecular factors altered in the setting of NAFLD that could potentiate this tumor promoting microenvironment. The work presented in this dissertation has identified unique changes in the inflammatory cell populations and cytokine profiles as a result of steatosis, both in our murine model as well as in human patients with NAFLD. Alteration of several cytokines in the setting of NAFLD increased the proliferation of liver cell lines in vitro and hepatocytes in vivo. These results implied that steatosis induces a proliferative liver response that could potentially facilitate the development of HCC. Further understanding of these changes could help determine the risk of development of HCC and help to devise strategies for the prevention of this devastating cancer.

Additionally, I identified MMP13, a member of the matrix metalloproteinase family, to be elevated in the setting of steatosis. MMP13 had been previously shown to be associated with fibrotic liver disease and the wound healing response, suggesting a role in modulating the stromal response in the steatotic liver [162]. The findings that both stromal and tumor derived MMP13 are important in the establishment of metastasis in the liver is highly clinically relevant. The loss of stromal and tumor derived MMP13 led to a decrease in tumor burden in both instances, suggesting that inhibition of MMP13 could be an ideal therapeutic target. MMP13 is unique in that it is expressed during early development and bone remodeling and typically not in adult human tissue, except during the wound healing processes and in pathologic settings such as cancer. Elevated MMP13 in steatosis might be the result of a healing response to counteract the fibrogenic process initiated by the activation of HSCs upon liver injury. Thus use of inhibitors selectively targeted for MMP13 could circumvent several of the side effects seen with broad spectrum MMPs drugs and could potentially be used to treat primary or metastatic cancers of the liver in patients with NAFLD.

As the incidence of NAFLD continues to increase worldwide, it is very important to identify and understand the pathophysiology that characterizes this disease. We demonstrate that NAFLD results in molecular and cellular alterations in the liver microenvironment that lead to an increased risk in the development of both primary and metastatic liver cancer. The results presented in this dissertation show alterations in cytokine levels in the liver that have a proliferative effect on the resident cells in the liver, and may play a role in tumorigenesis. Further we show that MMP13 plays a key role in establishment of metastasis in the liver by facilitating tumor cell motility and

extravasation from the vasculature. These results suggest that counteracting liver inflammation and controlling the levels of MMP13 in the liver could prevent development of primary and metastatic tumors in the liver in the setting of NAFLD.

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