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The Effects of Elevated Circulating Levels of Lipocalin 2 on Liver Metastasis of Colorectal Cancer

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DEDICATION

This work is dedicated to:

My father, John David Bonaparte

the protector and provider that has shown me how to be a man and is responsible for the man I am today.

My mother, Tyronia Bonaparte

who is the matriarch of our family, you always know the right things to say and you are glue that holds our family together.

My sister, Nichelle Monique Bonaparte

My first friend and my best friend, and no matter what I can always depend on you to be there.

I love each and every one of you and thank you for pushing me to be the man I am today.

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ABSTRACT

In the United States, colorectal cancer (CRC) is the third most diagnosed cancer and the second leading cause of cancer deaths. In 2015, approximately 143,000 new cases will be diagnosed and 50,000 deaths are predicted. Liver metastasis is the major cause of death. To understand the molecular and cellular mechanisms of liver metastasis, we used an orthotopic mouse model of CRC liver metastasis that recapitulated all stages of tumor growth and metastasis to perform whole genome microarray analysis to identify genetic changes in the liver microenvironment in response to primary tumor growth in the cecum. We identified lipocalin 2 (*Lcn2*) as the most highly expressed gene in pre-metastatic liver. LCN2 is a 25 kD secreted glycoprotein that plays a key role in the innate immune response. LCN2 has been associated with various types of cancers including thyroid, cervical, breast, and renal. However, its role in the tumor microenvironment has not been fully elucidated and there is conflicting data on its role in tumor growth and liver metastasis of CRC. To determine the effect of elevated serum levels of LCN2 on liver metastasis of CRC, *Lcn2* was cloned into the pV1J plasmid and electroporated *in vivo* into syngeneic mice while control mice were electroporated with pV1J empty vector. MC38 CRC cells were then injected into the spleen of electroporated mice and the extent of liver metastases was determined after three weeks. The results showed that mice injected with *Lcn2* expressing plasmid had elevated serum levels of LCN2 and showed a significantly higher rate of liver metastasis as compared to mice electroporated with empty vector. The results suggest that elevated LCN2 expression in the

microenvironment is sufficient to enhance liver metastasis of CRC, underscoring its potential as a therapeutic target.

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List of Abbreviations

APC ..... Adenomatous Polyposis Coli

BAX ..... Bcl-2 Associated X Protein

CRC ..... Colorectal Cancer

CSF1R ..... Macrophage Colony-Stimulating Factor 1 Receptor

CXR4 ..... CXC Chemokine Receptor Type 4

CT ..... X-ray Computed Tomography

DMEM ..... Dulbecco's Modified Eagle Medium

ECM ..... Extracellular Matrix

EGFR ..... Epidermal Growth Factor Receptor

ELISA ..... Enzyme Linked Immunosorbent Assay

FAP ..... Familial Adenomatous Polyposis

FIT ..... Fecal Immunochemical Test

GI ..... Gastrointestinal

GSK-3β ..... Glycogen Synthase Kinase 3 Beta

gFOBT ..... Guaiac Based Fecal Occult Blood Test

IPTG ..... Isopropyl β-D-1-thiogalactopyranoside

LB ..... Lysogeny Broth

LCN2 ..... Lipocalin 2

LEF ..... LEF Transcription Factor

MMP9 ..... Matrix Metalloproteinase 9

MRI ..... Magnetic Resonance

MYC ..... V-Myc Avian Myelocytomatosis Viral Oncogene

P53 ..... Tumor Protein 53

PBS ..... Phosphate Buffered Saline

PTGS2 ..... Prostaglandin-Endoperoxide Synthase 2

qRT-PCR ..... Quantitative Reverse Transcription Polymerase Chain Reaction

TCF ..... T-Cell Factor

VEGFA ..... Vascular Endothelial Growth Factor A

VEGFR ..... Vascular Endothelial Growth Factor Receptor

Wnt ..... Wnt Proteins

X-Gal 5-Bromo-3-indoyl-β-D-galactopyranoside

CHAPTER 1

INTRODUCTION

1.1 COLORECTAL CANCER

Colorectal Cancer (CRC) is the third most common in both men and women, accounting for approximately 650,000 deaths per year worldwide. In the United States, it is the second leading cause of cancer-related fatalities (Society 2012). CRC is a term used for cancer that starts in the colon or the rectum. These cancers can also be referred to separately as colon cancer or rectal cancer, depending on where they start (Figure 1.1).

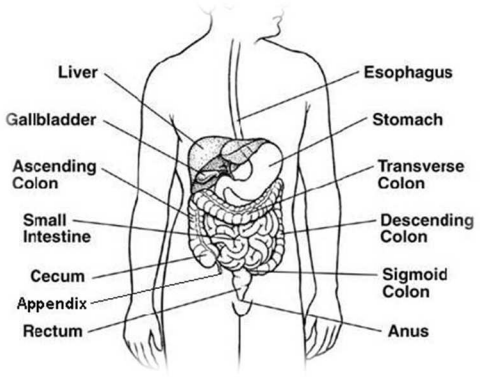

Figure 1.1 Diagram of the gastrointestinal system (GI). The colon and rectum are parts of the digestive system, which is also called the GI system. The large intestine is the portion of the digestive system. Food that cannot be digested is passed on to the small intestine through the ascending, transverse, descending, and sigmoid portions of the colon, and finally into the rectum for excretion (Society 2014)

Most colorectal cancers develop slowly over several years. Before a cancer develops, a growth of tissue or tumor usually begins as a non-cancerous *polyp* on the inner lining of the colon or rectum. A tumor is abnormal tissue and can be benign (not cancer) or malignant (cancer) (Society 2014). A polyp is a benign, non-cancerous tumor. Some polyps can progress into cancer but not all do. The chance of changing into a cancer depends on the kind of polyp. There are a number of different types of polyps that is encountered when dealing with colorectal cancer, but there are 2 main types that most common. One type is adenomatous polyps also referred to as adenomas. They are polyps that can change into cancer. Adenomas are considered as pre-cancerous condition. The second type of polyps are hyperplastic and inflammatory polyps, which are not pre-cancerous, but can become cancerous or might be an indication of a greater risk of developing adenomas and cancer, particularly when these polyps are growing in the ascending colon. Another kind of pre-cancerous condition is called dysplasia (Society 2012) wherein cells in an area in the lining of the colon or rectum appear abnormal when viewed under a microscope. These cells can develop into cancer over time. Dysplasia is usually observed in patients who have had diseases such as ulcerative colitis or Crohn's disease for many years (Society 2014).

Adenomatous polyps begin in the cells of glandular structures lining the colon, and are most likely to develop into cancer. Therefore, most colon cancers are adenocarcinomas. Once the cancer forms and grows from a polyp, colon cancer cells can eventually break through the intestinal wall of colon or rectum, spread into blood and lymphatic vessels, and travel to the lymph nodes and distant organs, such as the liver. Once these chain of events occur, this is referred to as *metastasis* (Society 2014).

In the United States, approximately 143,000 new cases of CRC were predicted to occur, and an estimated 51,000 fatalities are predicted in 2015 (Howlader N 2012). When patients are diagnosed at a localized stage, the five year survival rate after resection is approximately 90.8%; however, after metastasis has occurred, the five year survival rate decreases significantly to 11.7% (Howlader N 2012).

Overall, the lifetime risk of developing colorectal cancer is about 1 in 20 (5%). This risk is slightly lower in women than in men. A number of other factors such as lifestyle related factors diet, weight, exercise, smoking, alcohol use, age, personal history with colorectal polyps or colorectal cancer, personal history with inflammatory bowel disease, and genetics can all affect a person's risk for developing colorectal cancer. The death rate (the number of deaths per 100,000 people per year) from colorectal cancer has been dropping in both men and women for more than 20 years (Society 2012; Society 2014). There are a number of likely reasons for this. One is that polyps are being found by screening and removed before they can develop into cancers. Screening is also allowing more colorectal cancers to be found earlier when the disease can still be curable. In addition, treatment for colorectal cancer has improved over the last several years. As a result, there are now more than 1 million survivors of colorectal cancer in the United States (Howlader N 2012).

Many cases of colon cancer have no obvious symptoms. Most symptoms indicating colon cancer are not specific, including abdominal pain and tenderness in the lower abdomen, blood in the stool, diarrhea, constipation, or other changes in bowel habits, narrow stools, and weight loss with no known reason (Society 2014). Even though there is not an exact cause for colon cancer, there are a number possible ways it can be

prevented. One of the most powerful ways to prevent CRC is through screening which allows early detection and more likely curative intervention. There are a number of screening tests that are used to detect polyps and CRC; some of these tests include the Guaiac-based fecal occult blood test (gFOBT) and fecal immunochemical test (FIT), where stool samples are checked for blood and cells that might indicate the presence of a polyp or cancer. Another test that may be used is the stool DNA test where a stool sample is checked for certain abnormal sections of DNA (genetic material) from cancer or polyp cells (Cunningham, Atkin et al. 2010).

Sigmoidoscopy is a method where a flexible, lighted tube is inserted into the rectum and lower colon to check for polyps and cancer. One of the most common tests performed today is a colonoscopy wherein a longer, flexible tube is used to examine the entire colon and rectum. Other tests that are less commonly used include the double contrast barium enema. This is an x-ray test of the colon and rectum and CT colonography (virtual colonoscopy) is a type of CT scan of the colon and rectum. The most common screening examinations to detect CRC and polyps are qFOBT, FIT, and stool DNA test (Smith 2012)

In addition to the methods above, CT or MRI scans of the abdomen, pelvic area, chest, or brain may be used to stage the cancer to discern if the cancer has spread .The staging is used to describe the extent of penetration of the cancer (Network 2013). Specifically in colon cancer, it is based on the depth by which the cancer has invaded into the intestine wall, whether or not it has reached nearby structures, and whether or not it has spread to the lymph nodes or distant organs. In the clinic, the stage of a cancer is viewed as the most important factor in determining the prognosis and treatments of

patients. Usually, there are two types of staging for colon cancer: clinical and pathologic stages, based on the physical exam, biopsy, and other tests. The pathologic stage is most often used, because it includes the results from surgery, and is more accurate (Cunningham, Atkin et al. 2010). The typical stages of CRC progression are described in Figure 1.2.

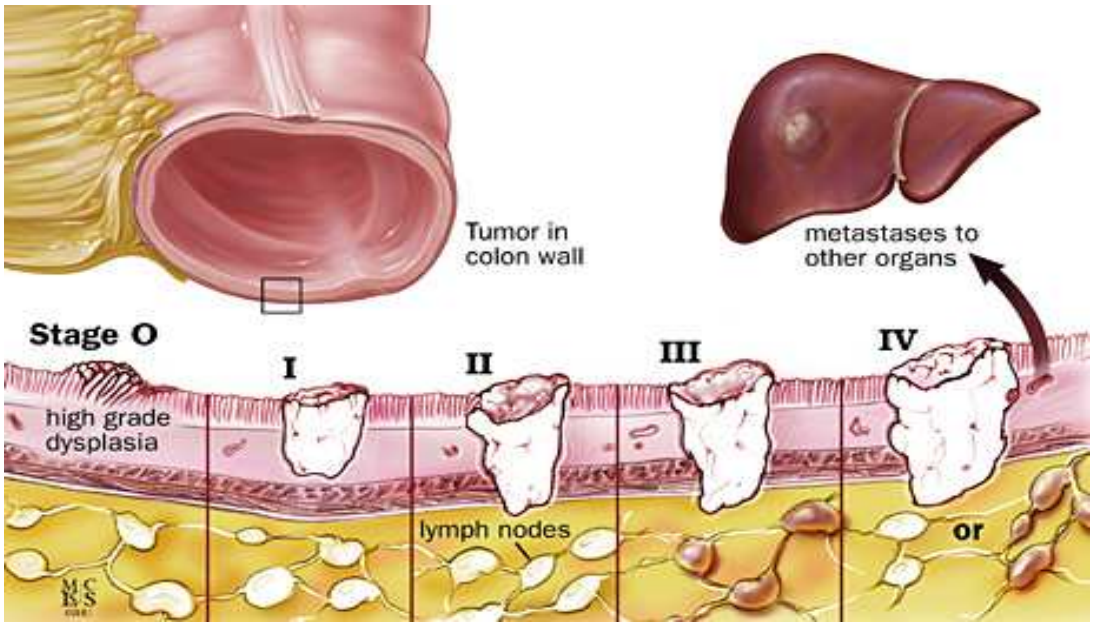

Figure 1.2. Progression of polyp development into cancer on the lining of the intestinal wall, Stage 0: Very early cancer on the innermost layer of the intestine; Stage I: Cancer is in the inner layers of the colon; Stage II: Cancer has spread through the muscle wall; Stage III: Cancer has spread to the lymph nodes; Stage IV: Cancer has spread to other organs outside the colon (John's Hopkins University)

Treatment of colon cancer depend on many factors, especially the stage of the cancer during patient diagnosis. Standard treatments include surgery to remove the primary tumor lesion that may be followed by chemotherapy to kill cancer cells, and radiation therapy to destroy cancerous tissue. For CRC at stages 0, I, II, and III, tumor resection and 6-8 months of adjuvant chemotherapy is a typical therapeutic plan,

sometimes combined with radiation therapy. For patients with stage IV disease with distant metastasis, treatments directed at metastatic lesion also need to be administered. However, the efficacy is typically very limited (Society 2012).

Among the complications of colon cancer, metastasis is undoubtedly the leading cause of death. Although metastasis occurs by the successful completion of multiple complicated steps by the metastatic cancer cells, these often occur without symptoms. Furthermore, early symptoms are not specific and when metastases occur, patients have already missed the opportunity to be treated successfully through surgery or irradiation (Chambers, Groom et al. 2002). The liver is one of the most common sites of metastatic spread of colon cancer (Hanahan and Weinberg 2011). At the time of diagnosis, approximately 20-25% of patients with colon cancer already present with liver metastasis. However, autopsy results revealed that up to 70% of colon cancer patients had liver metastases. Given these colorectal cancer statistics, it is effortless to conclude that liver metastasis is the major and direct cause of diminished survival in colon cancer patients (Schima 2005) and understanding its progression can lead to better diagnosis and treatment.

1.2 CANCER METASTASIS

A genetic model for describing the progression of CRC is the Vogelstein model (the Vogelgram), which was introduced 1990. In this model, the transformation from a normal to neoplastic cell begins with a mutation in the adenomatous polyposis coli (APC) tumor suppressor gene, which is a key protein in the Wnt signaling pathway (Nusse and Varmus 1992). This mutation is similar to that found in the hereditary variant of CRC

called familial adenomatous polyposis or FAP. The wild type APC protein forms a complex with axin and GSK-3β in the absence of Wnt signaling (Roelink, Wagenaar et al. 1992). The protein complex promotes the phosphorylation and subsequent proteasomal degradation of β-catenin, which is an intracellular signaling molecule. When APC is mutated, β-catenin escapes degradation and enters the nucleus and binds to TCF/LEF transcription factors, which activates transcription of genes such as MYC (c-Myc, a proto-oncogene) and possibly PTGS2 (COX-2) (Nusse and Varmus 1992; Gordon and Nusse 2006).

Beyond the defects in the Wnt signaling pathway, other mutations must occur for the cell to become cancerous. The p53 protein, a product of the *TP53* gene, normally monitors cell division and targets cells for apoptosis cells if the Wnt pathway is defective. Eventually, mutated cells acquire a mutation in the *TP53* gene and transforms the tissue from a benign epithelial tumor into an invasive epithelial cell cancer. Sometimes the gene encoding p53 is not mutated, but another protective protein named BAX is mutated instead (Isobe, Emanuel et al. 1986; Cho, Gorina et al. 1994).

Metastasis is a multi-step process that is silent and undetectable in the early stages. Tumor invasion is the initial event in the metastatic cascade and is primarily mediated by regulated interaction of tumor cells with the surrounding extracellular matrix (ECM). This step of metastasis requires tumor cells to attach, proteolyze and migrate through the basement membrane and enter into the circulation (Fidler 2003). Once tumor cells detach from the primary tumor mass, they enter into the circulatory or lymphatic system. This process is called intravasation. Once in circulation, they no longer get the survival signals they normally receive from cell-cell and cell-ECM interactions (Kaplan, Rafii et al.

2006). Tumor cells with metastatic potential acquire several mechanisms to evade this process and acquire resistance to immune cells that are trying to kill them. This resistance not only provides them with increased survival but also facilitates reattachment and colonization at secondary sites in a process called extravasation (Gupta, Minn et al. 2005; Kaplan, Rafii et al. 2006).

The last and final stage of the metastatic cascade is the colonization of the secondary site. Metastasis is not a random process. After performing a large number of autopsies, Stephen Paget proposed the 'seed and soil' hypothesis wherein he proposed that certain disseminated tumor cells (seed) preferentially colonize to specific organs whose microenvironment (soil) is compatible for their growth and proliferation (Paget 1989). For example, breast cancers frequently metastasize to lungs, liver, bone, and brain whereas prostate cancers metastasize to the bone. This hypothesis was refuted by James Ewing who proposed that distant metastasis at specific sites is primarily determined by the vasculature at the primary site (Ewing 1928). Recent advances and emerging data strongly support Paget's hypothesis and stresses the concept of a viable tumor microenvironment or 'pre-metastatic niche' where metastasizing tumors cells can attach and engraft at distant sites (Kaplan, Rafii et al. 2006).

1.3 TUMOR MICROENVIRONMENT AND ROLE OF LCN2 IN CANCER

The progression of CRC not only depends on genetic events. Changes in tissue microenvironment and expression of growth factors and other signaling factors are also of major importance (Woodhouse, Chuaqui et al. 1997; Perl, Wilgenbus et al. 1998). A tumor is not only made up of dividing, mutated epithelial cells. It also consists of vascular, inflammatory, and other activated stromal cell. Interactions between tumor cells

and cells within the stroma through soluble molecular factors have a profound impact on cell proliferation, movement, and differentiation (Egeblad and Werb 2002).

The tumor microenvironment, which is also the tumor-associated stroma is thus the cellular milieu in which the tumor exists, and includes the surrounding blood vessels, non-neoplastic cells such as infiltrating immune cells, fibroblasts, and endothelial cells, adipocytes, various bone marrow-derived cells such as mesenchymal stem cells, as well as macrophages and other immune cells, and the extracellular matrix (ECM) (Hanahan and Coussens 2012). It is considered as an important hallmark of cancer, which contributes to the cancer development and progression. They secrete growth factors, chemokines, cytokines, and reactive oxygen species. It was previously postulated that cellular components were only recruited by molecular signals from cancer cells. Currently, it has been shown that these cells are influenced by cancer cells and work closely with them to support tumor progression by not only enhancing the growth of the primary tumor but also directing its metastatic dissemination to distant organs (Matrisian, Cunha et al. 2001). The immune cells in the tumor microenvironment represents the major inflammatory component of the stroma of many tumors (breast, prostate, glioma, lymphoma, bladder, lung, cervical and melanoma) and are able to affect different aspects of the neoplastic tissue (Matrisian, Cunha et al. 2001). For example, tumor-infiltrating immune cells can promote invasion, metastatic dissemination and seeding of cancer cells via their presence at the invading margins of the tumor; tumor associated fibroblasts can also modulate tumor cell invasion and metastasis; while endothelial cells have been shown to regulate angiogenesis (Ahmed and Bicknell 2009).

These findings provide examples of the bidirectional interactions that occur between tumor cells and nearby stroma and contribute important factors to the biology of the tumor. Previous works focused on the tumor as a single cancer cell type but current findings suggest that the tumor should be treated as an organ in which there is a heterogeneous collection of cancer cells that collaborate with an equally heterogeneous collection of tumor stromal cells that contributes to such function as: initiation, proliferation and invasion (Mantovani 2010). Carcinoma cells stimulate the formation of an inflamed stroma, and the latter reciprocates by supplying various growth factors and cytokines thereby enhancing the malignant traits of the carcinoma cells. This depends on back-and-forth reciprocal heterotypic signaling between cancer cells and supporting stromal cells during the stepwise progressing of the tumor which establishes a potentially self-amplifying positive feedback loop (Xu, Rajagopal et al. 2010).

According to Weinberg et al, the transition from normal to benign to metastatic is not just driven by events inside the tumor cell itself but also by events around it. Throughout this intricate process, the tumor stroma is viewed as an integral part in the progression of multistep tumorigenesis. During the transformation of normal tissue into malignant, both neoplastic cells and stromal cells around then change and this histopathological progression must reflect underlying changes in heterotypic signaling between tumor parenchyma and stroma (Hanahan and Weinberg 2011).

Therefore, this reciprocal interaction between cancer and host environment appear to be a dynamic duo that assist each other by heterotypic signaling during tumor progression in which leads to metastasis. When analyzing the communications between the tumor and host microenvironment it is imperative to fully understand the mechanisms

and infrastructure because this will help us conquer cancer metastasis by unlocking the underlying mystery of the cancer cells and the multiple networks and support them in hopes to destroy them (Cheng, Chytil et al. 2008).

In recent years, there have been a number of studies with the goal and intent to reverse the tumor-enhancing effects of the microenvironment and recreate a repressive host tumor interaction while metastasis is in progress. While, there are a number of therapeutic agents that target certain stromal cells to block the host-tumor interactions like VEGFA and VEGFR inhibitors, EGFR and CSF1R antagonists, chemokine inhibitors, including CXR4 antagonist, TNF-α inhibitors, and protease inhibitors. Their effects have shown some positive signs but over time the emergence of resistance puts a halt to all progress. In order to figure out the complex problem of metastasis further understanding of host-tumor interaction is vital (Hiratsuka, Nakamura et al. 2002; Shojaei, Wu et al. 2007).

1.4 PREVIOUS STUDY REVEALS *LCN2* WAS MOST HIGHLY EXPRESSED GENE IN LIVER MICROENVIRONMENT.

In previous studies by Zhang et al., the CT26 mouse colon adenocarcinoma cell line was used to develop a mouse model for spontaneous metastasis to the liver in syngeneic Balb/c mice. This metastatic model required the injection of CT26 cells into the flank of Balb/c mice; tumors were removed and a single cell suspension was obtained. These CT26-F1 cells were then injected into the cecum of the Balb/c mice, which increased the incidence of metastasis to the liver from 8 to 50%. However, with three sequential passages of these cells through the liver, the highly metastatic CT26-FL3 cell line was obtained. The CT26-FL3 cell line was ten times more metastatic than the

CT26 cells, 90% versus 8% frequency of metastasis, respectively (Zhang, Davis et al. 2013).

The CT26-FL3 cell line was implanted into the cecum of Balb/c mice. Liver samples from tumor bearing mice were analyzed prior to metastasis by microarray analyses to identify genetic changes in the liver in the response to tumor growth in the cecum. As a control, liver samples from mice injected with PBS were also analyzed. For this study an Agilent mouse whole genome microarray kit was used to identify genes that were differentially expressed in the liver microenvironment in response to primary tumor growth in the cecum. The most highly expressed gene, lipocalin 2 (LCN2) (Iannetti, Pacifico et al. 2008) was found to be upregulated in the pre-metastatic liver prior to the presence of metastasis. Immunoblot analysis further showed that tumor-bearing mice displayed increasing levels of LCN2 in the serum during tumor progression to metastasis. Elevated levels of lipocalin protein was not seen in mice that had undergone sham surgery and injected with PBS (Zhang, Davis et al. 2013).

1.5 THE SIGNIFICANCE AND ROLE OF LCN2 IN CANCER

Lipocalin 2 (LCN2) is a 25 kD secreted glycoprotein that plays a key role in the body's innate immune response (Leng, Ding et al. 2009). LCN2 binds to small lipophilic molecules such as retinols, prostaglandins, pheromones, catecholates, and matrix metalloproteinase 9 (MMP9). Lcn2 is known as neutrophil gelatinase associated lipocalin and as mouse oncogene 24p3. There are about 20 types of lipocalin lipoproteins, and all share an eight strand anti-parallel β-barrel around a central binding pocket (Berger, Cheung et al. 2010). Lipocalin 2 is an antimicrobial product similar to β-defensin and

binds to microbial siderophores that are chatecholate-like and sent from microbial cells to scavenge iron from the environment.

LCN2 interacts with the 24p3 receptor, also known as megalin, with or without a bound moiety and transports the moiety into the cell. In an infected state, neutrophils are the first leukocytes to an infection where they can secret Lcn2 to scavenge iron in order to reduce the metabolically essential minerals for infectious bacteria (Srinivasan, Aitken et al. 2012). LCN2 was first discovered in neutrophils and is highly expressed in tissues that have high exposure to bacteria such as esophagus, bone, lung, bone marrow, stomach, and salivary glands (Flower 1996). LCN2 not only binds bacteria siderophores, but it can also bind mammalian chatechols and transport iron throughout the body, as seen mostly in the kidney.

LCN2 has been shown to have altered expression in multiple cancers, including colorectal, breast, renal, cervical, and thyroid during their progression (Bao, Clifton et al. 2010). However, there are conflicting data over the effects of LCN2 both on the primary tumor and in metastasis. According to Iannetti et al, LCN2 expression was found to have an inverse correlation with metastatic potential in a human colon cancer cell line (Iannetti, Pacifico et al. 2008). In breast cancer one report showed that inhibition of LCN2 did not block metastasis, while others showed that inhibition of LCN2 blocked metastasis (Iannetti, Pacifico et al. 2008) (Rius, Guma et al. 2008). A previous study conducted by Iannetti et al, on thyroid tumors gave some understanding as to how LCN2 could possibly have multiple roles in tumorigenesis, carcinogenesis, and metastasis (Seandel, Butler et al. 2008).

While lipocalin-2 has been implicated in various types of cancers including thyroid, cervical, breast, and renal (Flower 1996; Lee, Lee et al. 2006; Iannetti, Pacifico et al. 2008; Seandel, Butler et al. 2008; Bao, Clifton et al. 2010), its has not been explored in the microenvironment when levels of LCN2 have been elevated. Little is also known about its role in tumor growth and liver metastasis. Therefore, the data gained from this project could identify novel roles of LCN2 in the microenvironment that might promote metastasis. These findings might also clarify the contrasting roles of Lcn2 in colorectal cancer progression.

CHAPTER 2

THE EFFECT OF MICROENVIRONMENT SECRETION OF LCN2 ON LIVER METASTASIS OF COLORECTAL CANCER CELLS

2.1 GOAL AND OBJECTIVE OF CURRENT STUDY

CRC metastasis is a multistep process that is fatal and is not well understood because it can develop undetected until it is too late. More research must be done on the biological and molecular hallmarks that direct the early stages of metastasis, a stage that if diagnosed can provide the best opportunity for therapy to block its progression. Metastasis takes place through multiple steps mediated by complex interactions between the primary tumor and the target organ to promote malignant growth. It is important to identify the genetic and molecular changes taking place in the target organ in response to the growth of cancer cells in the primary tumor. Understanding the mechanisms they direct will ultimately aid in significantly lessening the impact of colorectal cancer on the patient.

The overall goal is to determine the role of Lcn2 in promoting the early stages of colon cancer metastasis to the liver. The research question being examined is: Does increased circulating levels of LCN2 in mice promote the metastasis of CRC to the liver? We hypothesize that elevated the serum levels of LCN2 is sufficient to enhance tumor growth and liver metastasis of MC38 colon adenocarcinoma cells. Because LCN2 can be

secreted by hematopoietic cells in the tumor microenvironment that are involved in CRC metastasis, the results from this study will complement previous research that was conducted by Zhang et al. The mouse model developed by Zhang et al. will be used in combination with intramuscular *in vivo* electroporation to increase serum levels of LCN2 to examine the effect on liver metastasis of CRC. In order to complete this task, three aims were proposed:

Aim One: To construct a plasmid that will overexpress *Lcn2* that will be injected into C57Bl/6 mice.

Aim Two: To inject C57Bl/6 mice intramuscularly with 50 μg of purified endotoxin free LCN2 overexpressing plasmid, followed by *in vivo* electroporation to increase serum levels in the mice. We will determine the kinetics of *Lcn2* expression after electroporation by analysis of serum levels by ELISA.

Aim Three: To examine the rate of metastasis to the liver under conditions of physiological or elevated LCN2 levels by splenic implantation of MC38 mouse colon tumor cells after *in vivo electroporation* of *Lcn2* over-expressing plasmids. We will measure the weights of the spleens and livers after implantation (four weeks) to assess the effect of LCN2 on primary tumor growth and liver metastasis of CRC cells.

2.2 MATERIALS AND METHODS

Construction of the pV1J-LCN2 plasmid

The pV1J-LCN2 plasmid was constructed from pCMV6-LCN2 plasmid and pV1J plasmid. The *Lcn2* gene in the pCMV6-LCN2 plasmid was PCR amplified with forward primer 5' TCGACTGGATCCGGTACCGAGGAGATCTGC3' and reverse primer 5'GCCGAATTCGGCCGTTTAAACCTTATCGTC 3' that contained the restriction sites for KpnI and EcoRI at the 5' and 3', respectively. The PCR product was electrophoresed on a 1% agarose gel. The 752 bp fragment containing *Lcn2* was excised from the gel and was purified by PCR Clean-up System from Promega. The purified LCN2 fragment was cloned into the pGEMT-Easy vector. The ligated fragments were transformed DH5α competent cells purchased from Life Technologies (Carlsbad, CA). The pGEMT-Easy vector and LCN2 fragment were incubated on ice for 20 minutes, followed by heat shock at 42° C for 2 minutes, and then the mixture was placed on ice for 2 additional minutes. Then 950 μL of LB media (no antibiotics) was added the mixture and it was allowed to incubate on a shaker for one hour. 200 μL of the transformation mix was placed on LB plates containing Ampicillin (100 ug/mL), X-Gal, and IPTG. The plates were incubated overnight at 37°C. The following day the screening was conducted by picking white colonies from the plate. The colonies were then grown in liquid LB media with Ampicillin (100 ug/mL). Plasmids were purified using Qiagen plasmid mini-prep kit following manufacturer's instructions. A restriction enzyme digestion was then performed to determine the ligation of LCN2 into the pGEMT-Easy vector. A sequential digestion was performed with EcoR1 and Kpn1, to release the 752bp fragment of LCN2.

Plasmids containing the *Lcn2* fragment were sequenced by GeneWiz Inc. (Research Triangle Park, NC).

Upon verification of the LCN2 sequence, the pGEMT-Easy-LCN2 plasmid was digested with Kpn1 and EcoR1. The same restriction enzymes were used to excise the pV1J expression plasmid. The pV1J and LCN2 fragments were purified, ligated and transformed into DH5α competent cells and plated in LB plated containing Kanamycin (50 ug/mL), X-Gal and IPTG. Plasmids were purified from transformed cells and verified by digestion with KpnI and EcoRI as previously described.

Purification of Endotoxin Free pV1J-*Lcn2* and pV1J plasmid

Bacteria transformed with pV1J and pV1J-*Lcn2* DNA were grown overnight in 2.5 liters LB containing Kanamycin. Endotoxin free plasmid preparations for in vivo electroporation were purified using Qiagen Plasmid Plus Giga Kit following manufacturer's instructions. The concentration of pV1J and pV1J-*Lcn2* DNA were measured by a spectrophotometer.

Cell Culture

The C57BL/6-derived mouse colon carcinoma cell line MC38 was purchased from American Type Culture Collection (ATCC), and cultivated in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% Penicillin/Streptomycin (Pen/Strep, Mediatech, Manassas, VA) at 37°C and 5% CO2 in a humidified atmosphere. pV1J or pV1J-*Lcn2* was transiently transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY)

following manufacturer's instructions.

Western blotting

Expression of LCN2 in total cellular protein extract. Cells that were 80-90% confluent and had been transiently transfected with pV1J or pV1J-*Lcn2* plasmids for 72 hours were washed with 5 mL of HBSS and was aspirated. 2 mL of Trypsin was added and the cells were placed in the 37° C incubator for 5 minutes. After 5 minutes DMEM was added to stop the reaction of the Trypsin. Next the cells were placed in a 15 mL Eppendorf tube and centrifuged at 2000 rpm for 5 minutes. The supernatant above the pellet was aspirated and the cells were re-suspended in 5 mL of PBS and washed. The wash and centrifugation was repeated. After the last wash, the supernatant was aspirated and the pellet was re-suspended in 1 mL of PBS and centrifuged at 2000 rpm and 5 minutes. The cells in the final pellet were re-suspended in 200 μL of Mammalian Protein Extraction Reagent and 1 μL of 500X Protease Inhibitor was added to each sample. The samples were vortexed and placed on ice for 10 minutes and then centrifuged at 11,000 rpm for 1 hour at 4°C. Protein samples were quantitated by the Bradford assay using an Epoch plate reader (Biotek Instruments, Inc Winooski, VT).

Equal protein amounts were subjected to electrophoresis on a 10% SDS-PAGE gel, followed by electrophoretic transfer to nitrocellulose filters. After successful membrane transfer, 5% milk in PBST was added to the membrane, and incubated at room temperature for 1hour. The blot was then washed 3 times for 10 minutes with PBST. A goat monoclonal antibody against the Lcn2 protein was used as a probe: (Abcam, Cambridge, MA). The blot was incubated with primary antibody (1:1000) overnight at

4°C, washed three times with PBS/0.01% Triton X-100, followed by HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA)(1:5000) for 1 hour at room temperature. The blots were visualized using an ECL enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). As internal controls for equal protein loading, blots were stripped and probed with antibodies against albumin (Santa Cruz Biotechnology, Santa Cruz, CA).

Expression of LCN2 in protein in media. Media from cell culture plates containing cells that were 80-90% confluent and had been transiently transfected with pV1J or pV1J-Lcn2 plasmids for 72 hours, was collected in a 15 mL Eppendorf tube. The cell media was then concentrated using Centriprep Centrifugal Filters from Millipore (Merck KGaA of Darmstadt, Germany). After the media was concentrated it was diluted 1:10 with molecular grade water. Then 5 µL of diluted sample was mixed with 10 µL of molecular grade water and 3 µL of 6X for a total of 18 µL. Samples were boiled at 100 °C for 10 minutes. SDS-PAGE electrophoresis and transfer to nitrocellulose was performed as described above. Then antibody against the LCN2 protein was used as a probe: (from Abcam, Cambridge, MA). The blot was incubated with primary antibody (1:1000) overnight at 4°C, washed three times with PBS/0.01% Triton X-100, followed by HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA)(1:5000) for 1 hour at room temperature. The blots were visualized using an ECL enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). As internal controls for equal protein loading, blots were probed with antibodies against albumin (Santa Cruz Biotechnology, Santa Cruz, CA).

Splenic injection of MC38 mouse colon cancer cells

Sub-confluent MC38 cells were harvested and washed in phosphate buffered saline (PBS) just prior to implantation. Eight-week-old male C57BL/6 mice were anesthetized by inhalation of 2% isoflurane in oxygen and placed in supine position. A midline incision was made to exteriorize the spleen. Using a 33-gauge micro-injector (Hamilton Company, Reno, NV), 2×10^5 cells in 10-15 uL were injected into the spleen. The injection site was sealed with a tissue adhesive (3M, St. Paul, MN) and sterilized with 70% alcohol to kill cancer cells that may have leaked out. The spleen was placed back into the cavity and the abdominal wall and skin closed with 6-0 polyglycolic acid absorbable sutures (CP Medical, Portland, OR). This process was conducted with mice twenty-four hours after *in vivo* electroporated of pV1J and pV1J-*Lcn2* plasmids.

Measurement of serum levels of pV1J-*Lcn2* after in vivo electroporation

Sera from mice electroporated with pV1J and pV1J-*Lcn2* plasmids were collected at the indicated time points after electroporation and analyzed by immunoblotting. Using antibodies against the LCN2 protein was used as a probe: (from Abcam, Cambridge, MA) as described above. As internal controls for equal protein loading, blots were stripped and probed with antibodies against albumin (Santa Cruz Biotechnology, Santa Cruz, CA).

Alternatively, LCN2 levels in sera were analyzed by ELISA using Lipocalin-2/NGAL Mouse ELISA kit from Abcam following manufacturer's instructions. Sera from mice was allowed to clot for 2 hours at room temperature then centrifuged at 2000 rpm for 20 minutes. Standard solutions were prepared in a concentration range of 156 to 5000 pg/mL. Standards, their duplicates, and properly diluted sera samples were

aliquoted at 100 uL per well in a 96-well plate. The plate was sealed and incubated at 37°C for 90 minutes. The cover was removed and the plate contents discarded and blotted onto paper towels. 100L of biotinylated anti-mouse LCN2 antibody working solution was then added into each well and incubated at 37°C for 60 minutes. The plate contents were discarded and the plate was washed 3 times with create 0.01 M PBS washing buffer, each time allowing the buffer to stay in wells for 1 minute. Buffer was discarded and the plate blotted. 100 uL of ABC working solution was added into each well and incubated at 37°C for 30 minutes. The plate content was discarded and the plate was washed 5 times with washing buffer, each time allowing the buffer to stay in wells for 1 to 2 minutes. Buffer was discarded and plate blotted. 90 uL of TMB color developing agent was added into each well and incubated at 37°C for 20 to 25 minutes. Next, 100 uL of TMB stop solution was added into each well which resulted in a color change to yellow immediately. OD absorbance was read at 450 nm using BioTek's Epoch Micro-Volume Spectrophotometer System and concentration of standard solution in pg/mL was graphed against optical density readings of standards.

In vivo Electroporation

Prior to surgery, the mice were anesthetized by inhalation of 5% isoflurane in oxygen delivered at 1 L/minute. Anesthesia was maintained in the induction chamber at 2.5% in oxygen. The mice were placed on the sterile operating table with a nose cone delivering the same concentration of isoflurane in oxygen. The thigh hair was shaved and a small incision was made in the skin at the bottom of the thigh just above the knee. This slit is expanded by further careful handling of scissors to uncover fatty tissue which was removed to expose the quadricep muscle. 50 μg of pV1J or pV1J-*Lcn2* plasmid in PBS

were injected in a final volume of 15 or 30 μL into the quadriceps, while holding the syringe vertically and perpendicular to the muscle and quickly pulling out afterwards. The BTX ECM 830 ElectroSquare Porator was used to apply three pulses of electricity at 100V for 50 milliseconds to allow the muscle to take in the plasmid and consequently express LCN2 protein to be secreted into the bloodstream. Animal procedures have been approved by the USC Institutional Animal Care and Use Committee.

Assessment of tumor burden and metastasis

Tumor burden and metastasis was analyzed after mice were humanely sacrificed. Spleens and livers were weighed to determine the growth of primary tumor and the rate of metastasis to the liver.

2.3 RESULTS AND CONCLUSION

In order to assess the effects of elevated circulating levels of LCN2 on liver metastasis of colorectal cancer, a plasmid needed to be constructed to manipulate LCN2 levels in vivo.

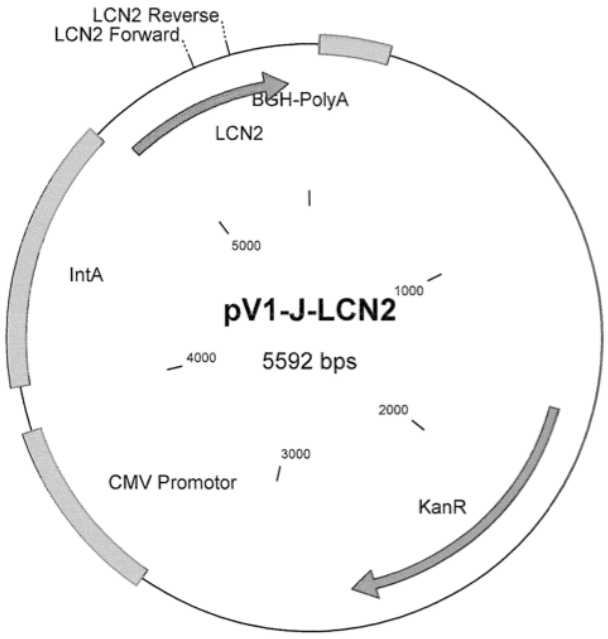

Figure 2.1. Map of plasmid pV1J-*Lcn2*. *Lcn2* is constitutively expressed by the CMV Promoter and the plasmid confers Kanamycin resistance to transfected cells.

The pV1J backbone (Figure 2.1) was chosen because it has been optimized to express encoded genes when delivered intramuscularly by *in vivo* electroporation and it has been shown to be an efficient plasmid to increase circulating levels of the gene of interest. The plasmid map of pV1J- *Lcn2* is shown in Figure 2.1.

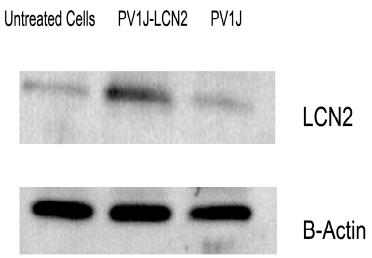

Figure 2.2. Examination of Intracellular protein from MC38 cells that were either untransfected, transfected with pV1J-*Lcn2*, or pV1J vector only. The cells were transiently transfected for 72 hours, harvested, and total protein extracts were obtained. The proteins were separated on a 0.8% SDS-PAGE and analyzed by immunoblotting.

After transiently transfecting MC38 cells for 72 hours with pV1J- *Lcn2* plasmid, intracellular protein was analyzed to determine the uptake of the plasmid into the cells and expression of LCN2. If the cells successfully uptake the plasmid and express it, the muscle cells would also be able to uptake the plasmid and spread it systemically throughout the mouse for expression. Immunoblot analysis of transiently transfected cells showed that the plasmid was up-taken and expressed LCN2 (Figure 2.2). The figure shows that the LCN2 levels in cells transfected with pV1J- *Lcn2* is expressed higher than levels in cells transfected with the pV1J empty vector or untransfected cells.

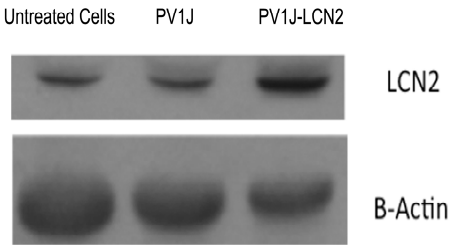

Figure 2.3 Immunoblot analysis of extracellular LCN2 in media from MC38 cells. Cells were transiently transfected for 72 hours and harvested. Cells were either untreated, transiently transfected with pV1J vector only or pV1J-*Lcn2* plasmids. The protein extracts were normalized against B-actin protein.

Since LCN2 is typically secreted, the extracellular protein levels in the cell cuture media were also analyzed. Immunoblot analysis of the cell culture media from transiently transfected cells showed a higher level of LCN2 protein was present in the media from cells transfected with pV1J- *Lcn2* as compared cells transfected with the empty vector or untransfected cells (Figure 2.3). These data indicate that LCN2 can be expressed from the pV1J vector and is secreted and therefore, the plasmid can potentially be used to increase its circulating levels in the blood.

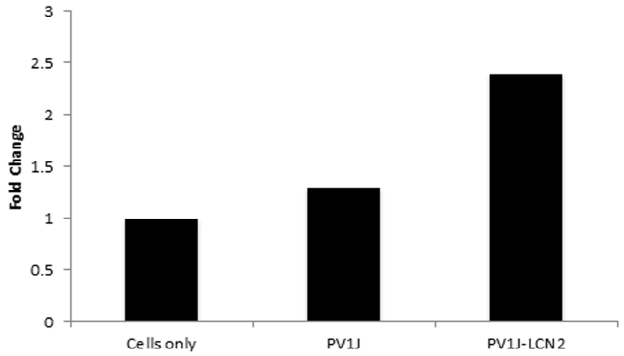

Figure 2.4: Fold change of extracellular protein levels between non-transfected MC38 cells and cells transfected with pV1J vector or pV1J-*Lcn2* plasmid. Cells were transiently transfected for 72 hours and harvested. Protein was quantitated by ELISA that probed with an antibody against LCN2.

The media collected from the cells was further analyzed by ELISA, which is a common laboratory technique which is used to measure the concentration of an analyte (usually antibodies or antigens) in solution. The results showed that cells transfected with pV1J- *Lcn2* secreted 2.5-fold higher LCN2 than cells transfected with the pV1J empty vector and untransfected cells (Figure 2.4). This is expected since LCN2 is being overexpressed in the cells.

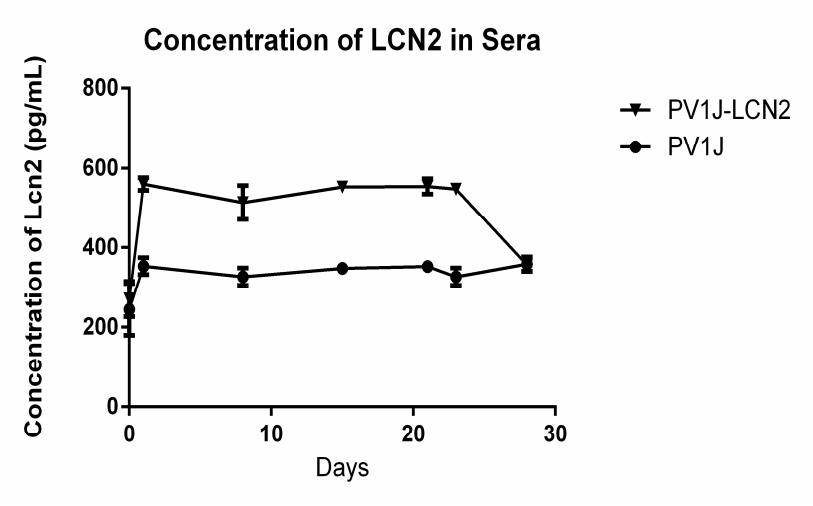

Figure 2.5 Measurement of concentration of LCN2 in sera at various time points after *in vivo* electroporation with pV1J-*Lcn2* or pV1J. Sera were quantitated by ELISA that probed with an antibody against LCN2.

To determine if the plasmids were capable of increasing circulating levels in sera, mice were electroporated with pV1J or pV1J- *Lcn2* plasmids. Blood was withdrawn prior (Pre-bleed) to electroporation and then every week for 4 weeks to determine how long the LCN2 levels stay elevated after electroporation. The concentration of LCN2 in blood sera was analyzed by ELISA. Figure 2.5 shows that electroporation of pV1J- *Lcn2* resulted in a three-fold increase in serum levels of LCN2. Elevated levels were maintained for 4 weeks after which they dropped to the same levels as mice injected with pV1J empty vector. Electroporation of pV1J empty vector caused a slight increase in LCN2 over the pre-bleed levels of approximately 0.1-fold. Therefore, plasmid pV1J-*Lcn2* is capable of increasing circulating levels of LCN2 after intramuscular in vivo electroporation into recipient mice.

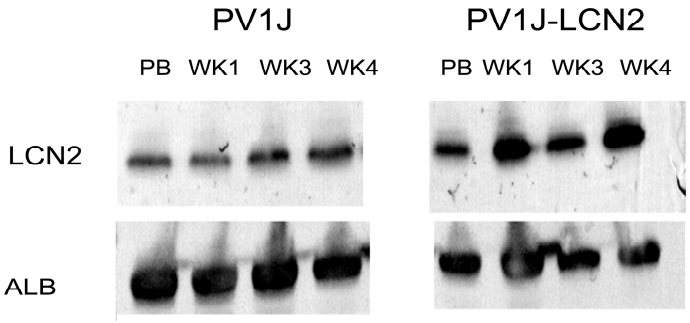

Figure 2.6 Comparison of LCN2 serum levels before and after electroporation with pV1J or pV1J-*Lcn2*. Samples were examined at various time points. The proteins were separated on a 0.8% SDS-PAGE and was normalized against albumin protein.

We further determined LCN2 levels in sera by western blotting. Sera from pre-bleed, and weeks 1, 3, and 4 after electroporation were analyzed. Consistent with the results from ELISA, LCN2 levels in sera from mice electroporated with pV1J empty vector was fairly constant throughout the experiment while sera from mice electroporated with the pV1J- *Lcn2* plasmid showed an increased LCN2 levels in the gene throughout the progression of the experiment (Figure 2.6). The western blot was normalized by albumin.

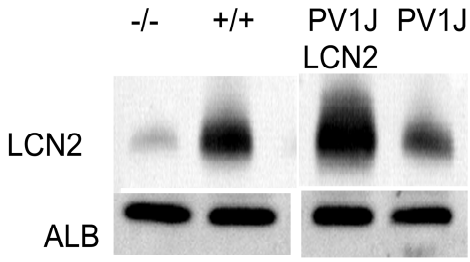

Figure 2.7: Comparison of LCN2 serum levels in LCN2 knockout mouse, wildtype mice, and mice that were *in vivo electroporated* with pV1J-*Lcn2* or pV1J plasmid. The proteins were normalized against albumin protein.

The expression of LCN2 was analyzed in *Lcn2*$^{-/-}$ knockout mice, wild tyoe mice, and mice electroporated with pV1J-*Lcn2* mouse and pV1J empty vector. The knockout mouse is shows a faint band of similar size to LCN2 , in which it should not, but from some previous studies the immunoglobulin G (IgG) heavy chains may be being cross-reacting (Figure 2.7). PCR analysis confirms that the mouse is an *Lcn2* knockout. The wild type mouse and the mouse electroporated with the pV1J plasmid mouse expression express comparable levels of LCN2, while the mouse electroporated with pV1J-*Lcn2* overexpression expression is expressing approximately three-fold higher LCN2 levels than the previous two groups (Figure 2.7). These samples were normalized for equal loading using albumin.

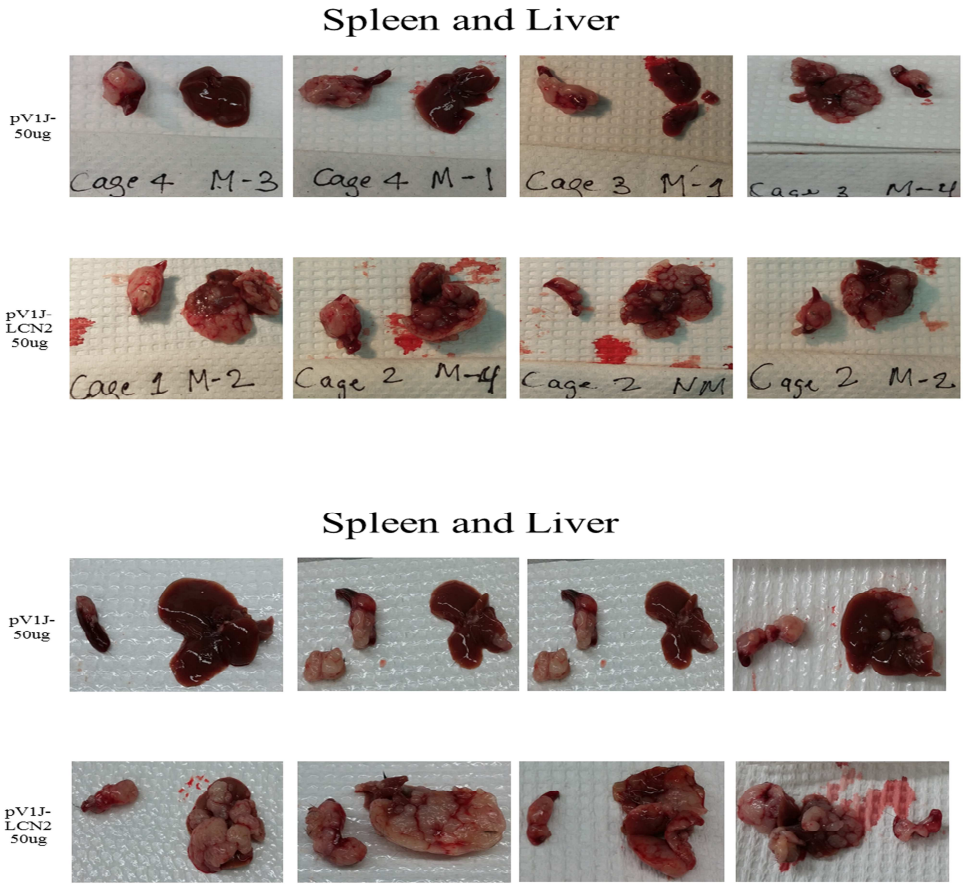

Figure 2.8: Assessment of rate of liver metastasis in mice injected with 50 µg of pV1J-*Lcn2* or pV1J plasmids. The weights of the spleen and liver were recorded to determine the rate of metastasis.

To determine the effect of elevated circulating levels on tumor growth or liver metastasis, $2x10^5$ MC38 cells were implanted into the spleen of eight-week-old C57BL/6 mice one day after in vivo electroporation of pV1J- *Lcn2* plasmid or pV1J empty vector Sera were harvested weekly after electroporation. Three weeks after spleen injection, the mice were humanely sacrificed and spleen and livers were harvested and weighed. Two

sets of experiments were conducted with N=20 for both experiments. 50 µg pV1J- *Lcn2* or pV1J was injected into each mouse. Figure 2.8 shows the size of the primary tumor in the spleen and metastatic lesions in the liver for each mouse. Examination of these photos suggest that primary tumor size in the spleen is not affected by elevated LCN2 circulating levels; however the size and number of liver lesions is much higher in mice electroprated with pV1J- *Lcn2*. The spleens (primary tumor) and livers (metastasis) were weighed. The results shown in Figure 2.9 show that in both sets of experiments, there was no significant difference in the weights of the primary tumor in the spleen. On the other hand, the mice that were injected with pV1J- *Lcn2* had larger livers and more metastases as compared to mice injected with the empty vector. p-values for these differences in liver weight in both experiments were 0.029 and 0.0066, respectively. Collectively, these data suggest that elevated LCN2 levels in the sera can promote liver metastasis of MC38 colon cancer cells whose LCN2 expression is not altered.

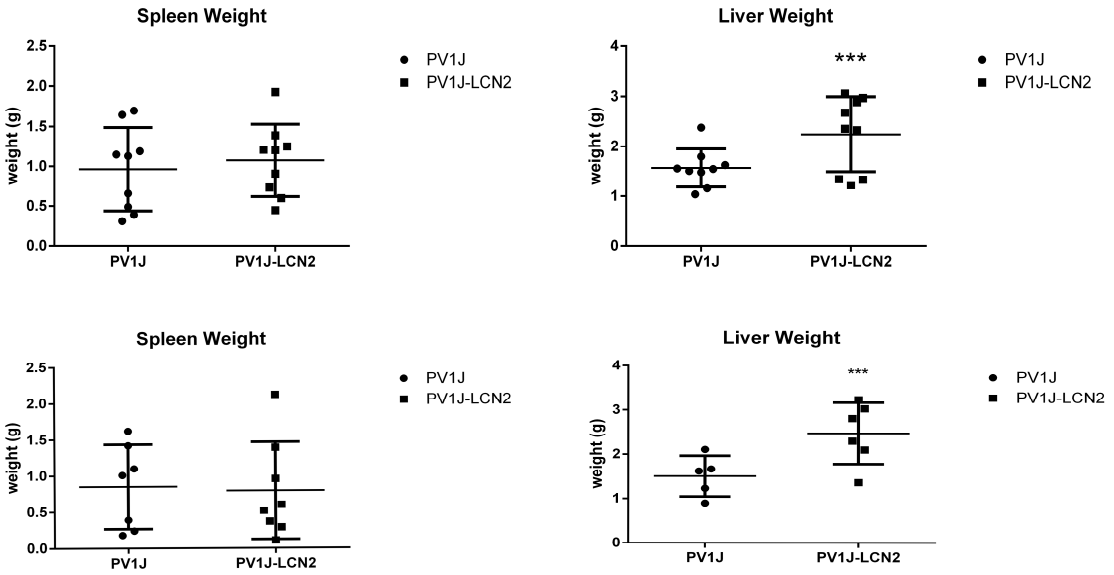

Figure 2.9: Spleen and liver weights were analyzed four weeks after splenic injections of MC38 cells and *in vivo electroporation* of pV1J-*Lcn2* or pV1J plasmids.

2.4 DISCUSSION

It is important to study potential biomarkers and their roles when examining the key transition of colon cancer metastasis to the liver. If it is confirmed that the upregulated presence of a target protein or other molecule coincides with the progression to the exceedingly lethal stage of cancerous cells invading other organs, then its rapid detection would be very useful in the clinical setting for early screening and treatment. From previous studies involving the development of CT26-FL3 cells, a highly metastatic cell line, the gene encoding the protein Lcn2 was found to be greatly expressed in the metastatic liver both against other genes and the transcriptional genome of the sham control group of mice that were not injected with CT26-FL3 cells.

To begin studying the effect of overexpressing LCN2 on colon cancer metastasis within the living mouse model, the plasmid pV1J-*Lcn2* was constructed, purified, and purified under large-scale endotoxin-free conditions to prepare for *in vivo* electroporation. This method was chosen to be able to continuously express Lcn2 in the bloodstream over extended periods of time via entry by a stable plasmid. Mice were chosen to be in the following treatment groups: 50 μg of pV1J empty vector, and 50 μg of pV1J-*Lcn2*. These dosages were injected into the quadriceps muscle of the mice and incorporated into the blood stream by *in vivo* electroporation at 100V for 50 milliseconds. Blood was taken from the retro-orbital eye sinus 1 day before and once a week after electroporation for further analysis of LCN2 levels in the blood sera. Averaged samples for each group measured from ELISA determined increasing circulation of Lcn2 in the blood stream for the experimental group injected with pV1J-*Lcn2*, while for the other controls group pV1J empty vector expression decreased after 1 week.

The findings from *in vivo* electroporation seems to be a viable method for the stable and continuous expression of LCN2. Mice injected with the pV1J-*Lcn2* plasmid show an increase in the *Lcn2* gene in blood sera over the 4 week study. The initial pre-bleed concentrations (before plasmid injection and cell implantation) were similar. After the injection of the gene there is nearly a 2 fold change in the concentration in the sera after weekly electroporation. The pV1J-*Lcn2* and pV1J empty vector mice concentrations returned to similar levels when mice were not electroporated with stable plasmids (pV1J-*Lcn2* and pV1J empty vector), suggesting that LCN2 may have a role in colorectal metastasis. A western blot was also conducted to analyze the blood sera in mice with pV1J empty vector and pV1J- *Lcn2* and the findings show an increase in the *Lcn2* gene

over the course of the study.

The increased levels of LCN2 showed a positive correlation in metastasis. There was significance between the livers of pV1J- *Lcn2* and pV1J empty vector mice. The rate of metastasis was much higher and level of metastasis was higher on the appearance of the actual liver suggesting that something is happening in the tumor microenvironment with the gene *Lcn2*. What exactly is happening is a question that needs to examine more thoroughly and will take more experiments to answer this questions. Conversely, the spleen (primary tumor) of pV1J- *Lcn2* and pV1J empty vector, there was no significance difference between the two groups.

Future experiments can continue directly from these conclusions, the identification of Bone Marrow Derived Cells found in the hepatic microenvironment throughout tumor progression in our orthotopic mouse model. Also, determine if increased LCN2 is positively correlated with metastasis and carcinogenesis in our mouse model through invasion assays. Analogous studies can be performed with Lcn2 inhibition whether it is by *Lcn2* gene knock out, silencing *Lcn2* by siRNAs, or other similar methods to study the influence of absence of LCN2 in colon cancer metastasis to the liver.

If these results follow in line with current findings, rates of metastasis should decrease without stimulus from *Lcn2*. Since LCN2 limits bacterial growth by sequestering iron carrying siderophores, it may alter cancerous cell proliferation by affecting iron in some way, though this is entirely speculation at this point. Overall, these are merely the beginning investigations on the role of LCN2, though they show some

promise in its possible crucial involvement for the progression of colon cancer metastasis to the liver. Additional studies must be undertaken to further elucidate LCN2 exact molecular interactions within the tumor microenvironment and beyond.

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