Spring 5-10-2014

The Role of Lipocalin 2 In Early Stage Colon Cancer Metastasis To The Liver

Fides Elamparo

University of South Carolina - Columbia

Follow this and additional works at: http://scholarcommons.sc.edu/senior_theses

Part of the Biology Commons, and the Oncology Commons

Recommended Citation
THE ROLE OF LIPOCALIN 2 IN EARLY STAGE COLON CANCER

METASTASIS TO THE LIVER

SENIOR THESIS BY

FIDES ELAMPARO

Submitted in Partial Fulfillment
of the Requirements for
Graduation with Honors from the
South Carolina Honors College

May, 2014

Approved:

Dr. Maria Marjorette Peña
Director of Thesis

Dr. Hexin Chen
Second Reader

Steve Lynn, Dean
For South Carolina Honors College
Acknowledgements

I would like to express my utmost gratitude to the following people for their support throughout my entire time with the Peña lab, from the summer of my freshman year until the completion of my senior thesis. With their guidance, I have been able to mature not only as a person, student, and inquirer, but have also gained such an appreciation and understanding of the hard work that takes place in the remarkable field of scientific research.

Dr. Marj Peña or, Tita Marj, for so willingly accepting me into her lab, despite having no experience and little academic background, and for being an incredible mentor both in and out of the lab. I owe one of the most rewarding experiences of my undergraduate career to her kindness.

Tia Davis for providing an entire summer of learning various techniques, especially with PCR genotyping, counting tumors, etc, and for continuing to offer her help years later with electroporation. I thank her for her patience and for helping me to grow in many ways during my time with her.

Yu Zhang, Daniel Hughes, and Sapana Shah for graciously advising me throughout my research with lipocalin-2. They have been wonderful teachers who I have thoroughly enjoyed working with while going through this process.

Grishma Acharya

John Bonaparte

Nikeya Tisdale
# Table of Contents

**Thesis Summary** .................................................................................................................. 4

**Abstract** ............................................................................................................................... 6

**Introduction**

- Colorectal Cancer in Humans ......................................................................................... 8
- Previous Work ...................................................................................................................... 9
- Overview of Lipocalin 2 .................................................................................................. 12
- Objectives of the Study ..................................................................................................... 14

**Materials and Methods**

- Presence of Lcn2 in CT26 and CT26-FL3 Cell Lines by Western Blotting ............... 15
- Analysis of Lcn2 Secretion into the Media by ELISA .................................................. 17
- Injection of DNA into Mice by In Vivo Electroporation .............................................. 19
- Analysis of Serum Lcn2 Levels by ELISA ................................................................. 22

**Results**

- Intracellular Lcn2 in CT26 and CT26-FL3 Cell Lines by Western Blotting ........ 24
- Levels of Lcn2 Secretion from CT26 and CT26-FL3 Cell Lines .............................. 26
- Lcn2 mRNA Expression from CT26 and CT26-FL3 Cell Lines ............................... 27
- Kinetics of Lcn2 Expression During Time Course Experiment ............................. 28

**Discussion and Conclusions** ............................................................................................. 31

**References** ........................................................................................................................ 34
Thesis Summary

Worldwide for both men and women, colorectal cancer ranks third in prevalence and is responsible for around 694,000 deaths reported in 2012. In the United States, it is the second leading cause of cancer related fatalities. Though there are many known risk factors, such as family history and behavioral choices, colorectal cancer can develop seemingly and unsuspectingly over a period of 10 to 15 years. As people age, growths, known as adenomatous polyps, may form in the linings of the walls of the intestines without any outward symptoms. These are typically harmless, but due to a series of mutations within these tissues accumulating one after another, the regulation of growth becomes disrupted and can lead to the beginning stages of colorectal cancer. This disease is associated with a higher mortality rate because at the final stage of metastasis, or spreading of cancerous cells to other organs, there are very few effective curative options. The key to improving these outcomes is to further understand the mechanisms behind metastasis and identify possible biomarkers for earlier and more successful treatment of these patients.

One method to studying cancer progression is to utilize established cancerous cell lines and observe which genes and proteins are more prevalent, suggesting they may have a more prominent role in the pathways involved. By previously developing a highly metastatic cell line, CT26-FL3, from a known lower metastatic cell line, CT26, and implanting these cells in mice, microarray analysis of the entire genome showed that lipocalin 2 (Lcn2) was one of the most highly expressed genes in comparison to mice that did not undergo the transplantation of CT26-FL3 cells. Protein detection by western blot analysis demonstrated an increase in Lcn2 as metastasis progressed.
Since Lcn2 is heavily involved in cell to cell and tissue communication, secretion into the media was investigated with both the lower and higher metastatic cell lines. This was examined by performing a western blot to discover the normalized intracellular expression of Lcn2 within the cells. As separate samples of these same cell lines grew to normalized confluency, the media was collected and levels of Lcn2 were quantified, revealing increased levels of overexpressed Lcn2 for CT26-FL3 in comparison to CT26.

In a conjunctural experiment with graduate student Daniel Hughes, expression of intracellular mRNA within CT26 and CT26-FL3 derivatives produced similar results. Within the stably transfected cell lines, it appears that Lcn2 has a positive association with metastatic conditions for colorectal cancer.

In vivo experiments began with creating a plasmid that could transfer Lcn2 into balb/c mice by in vivo electroporation. PBS by itself and 10µg of both the empty and Lcn2 inserted plasmid was injected in order to discover a potential treatment in the stable and continuous transfer of Lcn2. Mice were pre-bled days prior to electroporation. Once Lcn2 is taken in and released in the bloodstream, mice were bled on days 1, 4, and 6 with the subsequent data analyzed by ELISA, which is able to reveal the levels of protein expression in collected serum via antibodies against Lcn2. In vivo electroporation appears to be a promising treatment for investigating the effect of overexpressing Lcn2 on colon cancer metastasis to the liver. However, further varying treatments of dosage must be explored for maximum effectiveness.
**Abstract**

Metastasis, frequently from the colon to the liver, is the major cause of death with colorectal cancer, reducing the five-year survival to less than 6%. Metastasis occurs due to productive collaborations between tumor cells and host-derived cells in the tumor microenvironment, where a pre-metastatic niche is created to prime for cancer cell invasion into the target organ. In a highly metastatic colorectal cancer cell line implanted into the cecum of Balb/c mice, microarray analysis showed lipocalin 2 (Lcn2) is one of the most highly expressed proteins in the liver of tumor-bearing mice prior to metastasis.

When RT-PCR was performed, greater levels of Lcn2 mRNA were found in highly metastatic cells in contrast to less metastatic cells that had been stably transfected. Intracellular protein presence through western blot analysis and examination of media secretion by ELISA illustrated increased levels of Lcn2 secretion with the same cell lines. These results suggest that Lcn2 is highly associated with the promotion of colorectal cancer metastasis to the liver, most likely secreted from liver cells, with increased levels connected to the advancement of metastatic progression in vitro through the use of a highly metastatic mouse model.

After the construction, verification, and large-scale endotoxin-free purification of pSecTagA-Lcn2, this plasmid was utilized in in vivo electroporation of control groups PBS only, 10 µg of pSecTagA vector only, and 10 µg of experimental group pSecTagA-Lcn2 in order to explore the effectiveness of this method for the stable transfer and constant expression of Lcn2 within the blood stream. After 6 days, the experimental group expresses the greatest concentration of Lcn2 in comparison to both controls. In
vivo electroporation seems to be a viable method of continuously overexpressing Lcn2 in order to further examine colon cancer metastasis to the liver, though further studies must take place to identify the ideal dosage for maximized overexpression.
Introduction

Colorectal Cancer in Humans

Colorectal cancer is the third most common cancer in both men and women and has accounted for approximately 694,000 deaths worldwide in 2012 (WHO 2014). It is the second leading cause of cancer-related fatalities (CDC 2010) and the National Cancer Institute estimates 136,830 new cases will be diagnosed in 2014 within the United States. Coinciding with normal development over time, usually harmless tissue growths called adenomatous polyps may develop in the linings of the intestinal walls. However, some of these can become abnormally large and eventually become cancerous, though fewer than 10% progress to this stage (ACS 2011). This advancement is due to accumulated mutations in genes that control growth and repair of cells in the mucosa, or innermost layer, of the colorectal walls (NCI 2014).

Subsequently, these cancerous cells can affect nearby muscle layers and tissues, circulating through lymph nodes and the blood, and finally invading other organs, such as the liver or lungs (NCI 2011). This final step is known as metastasis. When observed early on, these malignant tumors can be removed to prevent any life-threatening damage, though the patient may experience recurrence later on. When patients are treated for colorectal cancer prior to metastasis, the survival rate is high at about 74%. After metastasis has occurred, the observed five year survival rate drops to six percent (ACS 2010). Unfortunately, those affected do not typically express outward symptoms of metastasis and it is often diagnosed when very little can be done for the patient. Most cases are treated with invasive surgery, including some chemotherapy, drug targeted therapy, radiation therapy, and other non-surgical methods (ACS 2013).
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 (Sethi and Kang 2011). Recent studies have shown that prior to the arrival of metastatic cancer cells to the target organ, molecular signals from the primary tumor to the bone marrow compartment can direct the recruitment of bone marrow derived cells to the target organ to establish a pre-metastatic niche, creating a fertile environment for cancer cell invasion (Kaplan et al. 2005). It is therefore 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.

**Previous Work**

Previous studies in Dr. Peña’s laboratory have shown that the gene encoding lipocalin 2, Lcn2, is one of the most highly expressed genes in the liver of tumor-bearing mice prior to metastasis.

**Use of a Highly Metastatic Mouse Model**

Mouse models have greatly aided in the advancement of understanding biological processes. Such models provide the proper environment to recognize the steps involved in the various pathways of both normal functioning and especially disease. The establishment of a highly metastatic colorectal cancer cell line, CT26-FL3, was
developed by utilizing a mouse model of colorectal cancer metastasis to the liver. In vivo education of flank cells was carried out by injecting a known low metastatic stable cell line, CT26, into the flank cells of Balb/c mice. After two weeks, cells were cultured and then implanted into the cecum of the same mice. Four to five weeks later, metastatic liver cells were retrieved from the mice to be cultured and re-injected into the cecum. This process was repeated using the metastatic liver cells for three rounds to achieve newly educated CT26-FL3 cells. It is significant to use a highly metastatic cell line for research to explore the conditions in which colorectal cancer metastasis to the liver occurs.

Figure 1: Process by which CT26 cells with low metastatic capabilities are educated to become highly metastatic CT26-FL3 cells.
Elevated Presence of Lipocalin 2

Gene expression analysis can determine which genes are expressed at transcriptional levels under specific circumstances. One technique that employs this type of profiling is microarray analysis, which allows for examination of the entire genome of an organism in specific circumstances (Nature 2014). The highly metastatic colorectal cancer cell line, CT26-FL3, 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 response to tumor growth in the cecum. As a control, liver samples from mice that have undergone sham surgery (no tumor cells injected) were also analyzed. These studies were done using the Agilent mouse whole genome microarray kit which allowed analysis of 44,000 genes. The results showed that
Lcn2 is expressed 34-fold higher in the liver of tumor bearing mice as compared to the liver in mice that have undergone sham surgery.

Rapid detection of the target gene at the translational level, or of the protein Lcn2, was investigated by western blot analysis. Using balb/c mice that underwent the same advancement to liver metastasis via CT26-FL3 cells, the circulating Lcn2 levels in blood sera increased with tumor progression, but not in mice that had undergone sham surgery.

<table>
<thead>
<tr>
<th>Week</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lcn2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3: Western blot analysis of sera from tumor bearing mice at various stages of metastasis.

**Overview of Lipocalin 2**

Lipocalin 2 (LCN2 in humans, Lcn2 in mice), also known as Neutrophil Gelatinase Associated Lipocalin (NGAL), is a protein expressed in human neutrophils and epithelial cells that is involved with a variety of processes within innate immunity and several kinds of pathologies in response to stress. The broader family of lipocalins contains a key structural feature, a β-barrel comprised of eight anti-parallel strands, which is highly conserved (Rodvold et al. 2012). With this structure, small, hydrophobic molecules are able to bind to lipocalins, including retinoids, hormones, and fatty acids, creating the potential for cellular and tissue interaction via the transport of these small molecules and defining the biological functionality of lipocalins (Rodvold).
For more than two decades since its initial discovery and classification, the protein LCN2 has become of increasing interest in biological research, including the areas of antimicrobial immunity, inflammation, infectious disease, renal cardiac disease or injury, and many other illnesses. Evidence has shown that LCN2 clearly plays a pathophysiologic role in each of these diverse states (Li and Chan, 2011). Additionally, it has also been abundantly present in a multitude of cancers, such as breast, pancreatic, ovarian, prostate, and colorectal carcinomas, though the function of LCN2 remains unclear in tumorigenesis (Rodvold).

There is conflicting evidence on their role in cancerous growth; some evidence suggests that lipocalins can inhibit the proliferation of cancer cells (Lee et al. 2006), while others suggest that they promote its progression (Leng et al. 2009). However, more studies that have been conducted in recent years seem to suggest the latter, especially in colorectal cancer. In 2009, Barresi et al. found that LCN2 can be characterized as a negative prognostic marker related to a higher progression risk from stage I colorectal cancer. Similarly, preoperative serum NGAL levels were elevated, especially with higher neoplastic tissue volume, in patients with colorectal cancer than in comparison to a normal population (Marti et al. 2013). In regards to more aggressive carcinomic stages, LCN2 appears to be associated with higher rates of metastasis and poorer outcomes of survival for colon cancer development and metastasis within affected patients (Maier et al. 2014). Though these positive associations with advancement of colorectal cancer are consistent, it is clear that further research must be undertaken to determine the role of LCN2 in establishing cancerous conditions and promoting metastasis.
Objectives of the Study

The overall goal of my project is to investigate the role of Lcn2 in promoting the early stages of colon cancer metastasis to the liver. Using the mouse model described above, I will assess Lcn2 protein levels in isogenic colon cancer cell lines with different capabilities to metastasize. I will also begin to determine whether increasing levels of Lcn2 coincides with the progression to early colorectal metastasis to the liver in vivo by overexpressing Lcn2 levels in tumor cells and blood sera on tumor cell invasiveness and ability to metastasize.
Materials and Methods

Presence of Lcn2 in CT26 and CT26-FL3 Cell Lines by Western Blotting

Western blot analysis is a widely used technique that is able to detect the presence of particular proteins depending on which antibodies are incorporated during the procedure. To start, CT26, CT26-FL3, CT26 mCherry, CT26-FL3 mCherry, and stably transfected cell lines of CT26 mCherry and CT26-FL3 mCherry with the pCMV6 empty vector and pCMV6-Lcn2 in each were taken out of storage within the liquid nitrogen tank and kept in a 37°C water bath for five minutes. 10 mL of DMEM with 10% fetal bovine serum culture media was added to labeled petri dishes. 1 mL of thawed cells were then added and distributed evenly. With a 20 mL pipette, media and cells were distributed throughout the dish. All dishes were then placed in a 37°C incubator to grow. After two days, cells were broken up by single cell suspension. They were first washed with 5mL of HBSS and then the liquid was aspirated. 1mL was added and allowed to react with cells for 30 seconds. Single cell suspension was observed under the microscope at 40X. 5 mL of DMEM culture media was then added to stop the reaction and was mixed with culture well. All culture was transferred to sterile 15 mL test tubes and centrifuged at 2000 rpm for 4 minutes. The medium was aspirated and the pellet was agitated manually. 5 mL of PBS was added in each test tube, vortexed, and then centrifuged at 2000 rpm for 4 minutes. These last two steps were repeated. Afterward, 1 mL of PBS was used to separate the cells in the pellets of each test tube and mixed to then be transferred to 1mL Eppendorf tubes. These were centrifuged at 2000 rpm for 4 minutes and the PBS
supernatant was aspirated as closely as possible. All samples were then stored at -80°C for future usage.

In order to test the concentration of each cell in order to normalize in preparation for western blotting, cells were broken with respective protein concentrations measured in a spectrophotometer (Beckman DU-600). 200 µL of Mammalian Protein Extraction Reagent and 1 mL of 500X Protease Inhibitor were both added to each sample after they were thawed. After being extensively mixed by pipetting and vortexing, they were placed on ice for 10 minutes and then in the low temperature centrifuge at 11,000 rpm for 1 hour at 4°C. 200 µL of Bio-Rad Protein Assay Dye Reagent Concentration, 797 µL of deionized water, and 3 µL of each protein sample were analyzed and corresponding concentration readings recorded in µg/mL.

To prepare these samples for western blot analysis, previous protein concentrations are normalized in order to have even distribution of overall protein present in loading samples. All are boiled at 99°C for 10 minutes. 10 µL of Bio Rad Plus Precision Dual Standards ladder and 18 µL of samples were added to wells of pre-made gels and immersed in 1X SDS Page Buffer. This gel ran at 200V for 30 minutes. Afterward, the gel was removed and added to sandwich of filter paper and transfer membrane, somewhat immersed in transfer buffer, into the Semi-Dry Transfer Trans-Blot. This ran at 15V for 1 hour. During this time, the first cocktail composed of 0.5g of Bio-Rad Blotting-Grade Blocker Nonfat dry milk was combined with 9.5 mL PBST to make 5% milk in PBST. After successful membrane transfer, 5% milk in PBST was added to the membrane, shaking at room temperature for 30 minutes. A second cocktail composed of 0.5g BSA, 9.5mL PBST and 20 µL of the first antibody, R&D Mouse
Lipocalin-2/NGAL Affinity Purified Polyclonal Goat IgG Antibody (diluted 1:500) was added to membrane after first washing with 10 to 20 mL PBST 3 times. The membrane was left shaking at room temperature for 1 hour. The first antibody was then washed off with 10 to 20 mL PBST four times, lasting 10 minutes each. A third cocktail with 0.5 g BSA, 9.5 mL PBST and 2 µL of the second antibody, Bio-Rad EIA Grade Affinity Purified Rabbit Anti-Goat IgG (H+C)-HRP Conjugate (diluted 1:5000), was added to the membrane shaking for 30 minutes at room temperature. The membrane was then washed against with 10 to 20 mL PBST for twice for 5 minutes and 10 minutes. 500 µL of each Immobilin Western Chemiluminescent HRP Substrate was added to the membrane and exposed for 10 seconds. In order to test for β-actin levels, a protein conserved in all cells, to ensure for even loading, the membrane was first washed with PBST twice for 5 minutes. A first cocktail of 0.2 g BSA, 9.8 mL PBST, and 2 µL of the first antibody, mouse (diluted 1:5000) was added for 30 minutes, shaking at room temperature. This was followed by two PBST washes at 5 minutes each. A second cocktail of 0.2 g BSA, 9.8 mL PBST, and 2 µL of second antibody, anti-mouse (diluted 1:5000) was added for 20 minutes, shaking at room temperature, followed by two PBST washes at 5 minutes each. 1 mL of each ECL Western Blot Detection reagent was mixed and added to the membrane, later exposed for 30 seconds. Images were quantified with Image J provided by the National Institutes of Health.

Analysis of Lcn2 Secretion into the Media by ELISA

Followed procedure for waking up cells with same cell lines as previously mentioned, up until termination of single cell suspension, adding split cells to labeled 4,
6-well plates. Counted with hemacytometer in order to plate around 200,000 cells per well on each plate. On subsequent days, media was collected from samples when they each reached around 70% to 80% confluency, as consistently as possible. Media was collected with 5 mL syringe to inhibit passage of cells. Samples were stored in 15 mL sterile test tubes at -80°C. The procedure from R&D Systems Quantikine ELISA Mouse Lipocalin-2/NGAL kit was then followed. This procedure follows a sandwich ELISA model where the sample is first recognized by a detecting antibody, with added first and second antibodies on top, followed by the enzyme-substrate reaction with the enzyme attached to the second enzyme and added substrate. All reagents and samples were brought to room temperature. Standards were created as well as reagents Mouse Lipocalin-2 Control reconstituted, Wash buffer made, and standards from Mouse Lipocalin-2 Standard combined with Calibrator Diluent RD5-24 (1X). 50 µL of both Assay Diluent RD1-34 and standards, controls, and samples were added to each well. They were then covered and incubated for 2 hours at room temperature on the bench top. Each well was then aspirated and washed with 400 µL Wash Buffer for a total of four washes. Next, 100 µL of Mouse Lipocalin-2 Conjugate was added to each well with all wells covered and incubated for another 2 hours at room temperature on the bench top. The aspiration and washing step was repeated. 100 µL of Substrate Solution was added to each well and incubated for 30 minutes at room temperature, protecting from light. A color change was subsequently observed. 100 µL of Stop Solution was then added to each well, tapping the plate for thorough mixing. The optical density was immediately determined with a microplate reader at 450 nm, with wavelength correction at 540 nm twice. All data analysis was done on Microsoft Excel and ReaderFit.
Injection of DNA into Mice by In Vivo Electroporation

To increase the serum levels of LCN2 in balb/c mice, intramuscular in vivo electroporation was performed using the LCN2 plasmid pSecTagA purified under endotoxin free conditions.

Figure 4: Restriction map of pSecTagA-Lcn2 vector.
Purification of pSecTagA and pSecTagA-Lcn2 DNA was performed with Promega Pure Yield Plasmid Miniprep System. This procedure allows for the cloning of pSecTagA vector with Lcn2 DNA in *E. coli* cultures after pSecTagA has been ligated with Lcn2. The bacteria are able to incorporate the vector and target DNA after quickly heat shocking and placing back on ice. Clones are selectively grown by the addition of antibiotic Hygromycin B, which the pSecTagA vector is resistant to. Well-formed colonies are chosen for further growth with incubation at 37°C to create concentrated inoculum. Subsequently, Qiagen Plasmid Plus Giga Kit was used for large scale endotoxin-free purification of pSecTagA and pSecTagA-Lcn2 to prepare for this experiment. The concentration of Lcn2 DNA was measured by a spectrophotometer.

![Figure 5: Verification of Lcn2 DNA within pSecTagA vector transformants. This is a picture taken from agarose gel after restriction enzyme digestion. On the left is the “DNA” ladder that serves as a control to compare molecular weights of DNA fragments.](image)
Prior to surgery, the mice were anesthetized from inhalation of 5% isoflurane in oxygen delivered at 1 L/minute. As the mice were each put into the induction chamber, the concentration was lowered to 2.5% in oxygen. When successfully anesthetized, the mice were placed on the sterile operating table with a nose cone delivering the same concentration of isoflurane in oxygen. When laid down on its back, the thigh hair of the mice is shaved and a small incision is 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 must be cut to expose the quadriceps muscle. 10 µg of pSecTagA or pSecTagA-Lcn2 were injected in a final volume of 15 or 30 µL PBS into the quadriceps, while holding the syringe vertically and perpendicular to the muscle and quickly pulling out afterwards. The BTX ECM 830 ElectroSquare Porator applied 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.

To determine kinetics of Lcn2 expression after electroporation, 9 balb/c mice, all born in January, were electroporated: 3 with PBS, 3 with pSecTagA vector at 10µg, and 3 with pSecTagA-Lcn2 at 10µg. Blood samples were taken from these mice days prior to electroporation for a pre-bleed sample, and then on days 1, 3, and 6 following surgery. Blood samples were obtained from the retro-orbital sinus using capillary tubes which were then centrifuged for 5 minutes to separate red blood cells from blood sera containing Lcn2. Sera samples were stored at -80°C until further analysis.

Analysis of Serum Lcn2 Levels by ELISA

Enzyme-linked immunosorbent assay (ELISA) is a relatively rapid method of qualitative detection and quantitative measurement of protein levels. The blood sera was first allowed to clot for 2 hours at room temperature. It was then spun at 2000 rpm for 20 minutes. The kit used was ABCAM Lipocalin-2 / NGAL Mouse Elisa Kit, which first instructed to create reagents and proper conditions including 0.01M PBS as washing buffer, dilution of all samples, reconstituting mouse Lcn2 standard of 10000 pg/mL to create new standards from 156 pg/mL to 5000 pg/mL, biotinylated anti-mouse Lcn2 antibody and working solution both diluted, avidin-biotin-peroxidase complex (ABC) and working solution diluted, noting that ABC and TMB color developing agent had to be pre-warmed In 37°C for 30 minutes before usage. Standards, their duplicates, and properly diluted sera samples were aliquoted 100µL per well. The plate was then sealed
and incubated at 37°C for 90 mins. Afterward, the cover was removed, with the plate content discarded and blotted onto paper towels. 100 µL of biotinylated anti-mouse LCN2 antibody working solution was then added into each well and incubated at 37°C for 60 mins. The plate content was discarded and the plate was washed 3 times with created washing buffer, each time allowed to stay in wells for 1 minute. Buffer was discarded and plate blotted. 100 µL of ABC working solution was added into each well and incubated at 37°C for 30 mins. The plate content was discarded and the plate was washed 5 times with washing buffer, each time allowing to stay in wells for 1 to 2 mins. Buffer was discarded and plate blotted. 90 µL of TMB color developing agent was added into each well and incubated at 37°C for 20 to 25 mins. Next, 100 µL of TMB stop solution was added into each well. Observe 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. All data was analyzed on Microsoft Excel.
**Results**

Intracellular Lcn2 in CT26 and CT26-FL3 Cell Lines by Western Blotting

Figure 7: CT26 and CT26-FL3 cells stably expressing high levels of Lcn2.

The presence of Lcn2 is confirmed in stably transfected CT26 and CT26-FL3 associated cell lines with the pCMV6 and pCMV6 Lcn2 vectors by western blot analysis. Figure 4 illustrates CT26, CT26 mCherry, CT26 mCherry pCMV6 empty vector, CT 26 mCherry pCMV6 Lcn2, CT26-FL3, CT26-FL3 mCherry, CT26-FL3 mCherry pCMV6 empty vector, and CT 26-FL3 mCherry pCMV6 Lcn2 with their associated Lcn2 and loading control β-actin occurrence. This data indicates relatively the same amount of Lcn2 expressed intracellularly for each cell line.
Figure 8: Lcn2 protein levels from western blot normalized against β-actin loading control.

Using the program Image J provided by the National Institutes of Health, it is possible to quantify the results from western blot analysis. Though there are variations within the Lcn2 protein expression of each CT26 derived cell line, these results must be placed against the expression of the loading control indicative of the amount of sample added. Once the expression of the β-actin, a protein which is normally expressed and highly conserved, is also determined and compared with the coinciding expression of Lcn2 for each cell line, it is clear that these differences are caused by the volume of sample added. Therefore, within each of these cell lines, the intracellular levels of Lcn2 expressed are about the same, though this is not indicative of a difference for the Lcn2 protein that may be transcribing and ultimately secreted extracellularly in order to communicate with other cells or tissues.
Levels of Lcn2 Secretion from CT26 and CT26-FL3 Cell Lines

Figure 9: Concentration of Lcn2 (pg/mL) secreted in harvested media by stably transfected CT26 and CT26-FL3 cell lines.

Results from examining secretion of Lcn2 into the media of cultured cells reveal several points of interest. As expected of the lower metastatic cells, with a success rate of around 5%, the levels of Lcn2 secretion are almost nonexistent, whereas with the higher metastatic cell line, with a success rate of around 90%, Lcn2 concentration are more prevalent even without overexpression. Within both groups of cell lines, there is a clear distinction in overexpression of CT26 and CT26-FL3 mCherry pCMV6-Lcn2 cells and
their control counterparts, indicative of successful overexpression during the process of transfection. What is most notable is the delineation between these two groups; the level of overexpression for CT26-FL3 mCherry pCMV6-Lcn2 is significantly higher than that of CT26 mCherry pCMV6-Lcn2, suggesting a positive correlation of Lcn2 protein secretion for conditions prone to metastasis.

Lcn2 mRNA Expression from CT26 and CT26-FL3 Cell Lines

Figure 10: mRNA transcription levels of Lcn2 within CT26 derived cell lines.

In a conjunctional experiment with graduate student Daniel Hughes, real-time polymerase chain reaction (RT-PCR) was used to measure intracellular levels of Lcn2 mRNA transcription. This study shows increased Lcn2 transcription for the
overexpressing plasmids of each respective cell lines, as well as greater mRNA expression between the lower metastatic CT26 and the higher metastatic CT26-FL3 cells.

**Kinetics of Lcn2 Expression During Time Course Experiment**

![Average Lcn2 During In Vivo Electroporation](image)

Figure 11: Concentrations of Lcn2 for each control and experimental group averaged during in vivo electroporation experiment.

Results from ELISA analysis of in vivo electroporation of control and experimental groups show differences in Lcn2 expression after collecting blood sera at various time points. It should be noted that initial levels of Lcn2 expressed in blood sera differ in general as Lcn2 is naturally expressed in blood neutrophils. The highest
expression of Lcn2 actually occurs one day after experimentation with the PBS sham control group. This is most likely due to a response in inflammation following surgery. On average, the PBS group also has the lowest concentration of Lcn2 found 6 days after in vivo electroporation. Differences between these groups can be further elucidated when examining the same data in the following figure.

Figure 12: Averaged concentrations of Lcn2 detected in blood sera for treatment groups over time.

Differences over time are more clearly perceived when the ELISA data for different time points are graphed linearly. Again, the PBS control group experiences the most dramatic decrease in measured protein during the course of this experiment. The
pSecTagA vector only group, on average, also declines in Lcn2 expression in a similar fashion even below measured basal levels, while the experimental group of pSecTagA with Lcn2 increases at least 6 days following in vivo electroporation. The injection of 10 µg of pSecTagA-Lcn2 plasmid seems to be feasible for the initial overexpression of Lcn2 in the blood sera, though it is questionable whether the same or greater levels will be stably expressed continuously.
**Discussion and Conclusions**

It is significant 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.

In order to further study the role of Lcn2 in colon cancer metastasis to the liver, both in vitro and in vivo experiments were performed. The first experiment involved western blot analysis for intracellular protein detection of Lcn2 within CT26, CT26 mCherry, CT26 mCherry pCMV6 empty vector, CT26 mCherry pCMV6-Lcn2, CT26-FL3, CT26-FL3 mCherry, CT26-FL3 mCherry pCMV6 empty vector, and CT26-FL3 mCherry pCMV6-Lcn2 transfected cell lines. Quantification of protein levels against loading control β-actin for all samples by Image J indicated that Lcn2 expression remains normalized between all cell types. However, RT-PCR for these CT26 derived cells showed that the greatest levels of intracellular Lcn2 mRNA are being transcribed by the higher metastatic CT26-FL3 cells with the pCMV6-Lcn2 plasmid vector overexpressing Lcn2. When the same numbers of CT26 and CT26-FL3 cells are cultured and their media collected at the same rates of confluency, the secretion of Lcn2 protein is also varied.
Compared to the lower metastatic cell line, all CT26-FL3 derived cells secrete more Lcn2, with the cells stably transfected with the Lcn2 promoting plasmid releasing the highest concentration of Lcn2 into the media. These in vitro experiments indicate that Lcn2 is positively associated with the greater tendency to metastasize by secreting the protein (most likely) from the liver extracellularly in order to interact with other cells and tissues.

To begin studying the effect of overexpressing Lcn2 on colon cancer metastasis within the living mouse model, the plasmid pSecTagA-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: PBS only, 10 µg of pSecTagA empty vector, and 10 µg of pSecTagA-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 ms. Blood was taken from the retro-orbital eye sinus 2 days before and 1, 4 and 6 days after surgery 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 pSecTagA-Lcn2, while for the other controls Lcn2 expression decreased at the 6 day mark. Due to these findings, in vivo electroporation seems to be a viable method for the stable and continuous expression of Lcn2.

Future experiments can continue directly from these conclusions. Increasing dosages of treatments must be explored to find the maximal amount of plasmid that can
be injected for the greatest, continuous expression of Lcn2 within the blood stream. The results from these experiments can be analyzed with ELISA similar to my experiments and compared for the most desired outcomes. Once this dosage has been discovered, CT26 and CT26-FL3 tumor cells can be injected into the spleen when the Lcn2 serum levels are most highly expressed. Following metastasis to the liver, the spleen and liver can be harvested and weighed to examine rate of tumor growth with the diameters of the primary tumors measured as well. It is predicted that the increased amounts of metastasis will occur with the higher metastatic cell line at a greater frequency, though metastasis may also be amplified compared to the usual 5% rate of metastasis for CT26 cells.

Conversely, 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.
References


