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The Effects of Lipocalin 2 on Lung Metastasis of Colorectal and Other Cancers

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THE EFFECTS OF LIPOCALIN 2 ON LUNG METASTASIS OF COLORECTAL AND OTHER CANCERS

SENIOR THESIS BY

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

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**Thesis Summary**

Colorectal cancer is the fourth most common cancer in the United States (US) and is estimated to be responsible for 49,190 deaths in this year alone. It is the second leading cause of cancer deaths in the US is ranked third in prevalence worldwide. Many factors can increase the risk for colorectal cancer including family history of colorectal cancer, gender, age, as well as behavioral and lifestyle choices. Most colorectal cancers are adenocarcinomas and often begin as a polyp, formed in the inner wall of the colon or rectum and carry no detectable symptoms. It is in this quiet beginning that colorectal cancer can develop unsuspectingly over a period of 10 to 15 years. When the polyp begins to invade deeper into to colon or rectum wall it moves closer and closer to the lymph nodes and the bloodstream that carry the now malignant cells throughout the body, causing the deadliest stage of cancer; metastasis. It is in prevention that colorectal cancer can be combated, using colonoscopies or sigmoidoscopies to explore the walls for polyps. It is also what drives research to improve outcomes, studies to attempt to understand what causes metastasis and to find therapeutic biomarkers to halt the disease in its tracks.

The first experiments were aimed at determining if a plasmid vector expressing Lcn2 can be used to increase its serum levels in mice via *in vivo* electroporation. Since Lcn2 secretion is vital for this process, the established cell line CT26-luc was transiently transfected and examined using a western blot. Intracellular and secreted levels of Lcn2 were determined and confirmed, validating both Lcn2’s positive association with metastatic conditions as well as pV1J’s role as a viable vector for Lcn2 expression and secretion into circulation during *in vivo* electroporation.
The empty vector pV1J and pV1J-Lcn2, expressing Lcn2 were isolated and purified under endotoxin free conditions at a concentration of 1 ug/uL. 50uL of either pV1J or pV1J-Lcn2 were used for electroporation into mice. In vivo electroporation was conducted in mice bearing tumors from MC38 mouse colon adenocarcinoma cell line in C57Bl/6 mice, 4T breast cancer cells in Balb/c mice, and B16-F10 melanoma cells in C57B1/6 mice. Mice were bled prior to electroporation (pre-bleed) and then bleed weekly after electroporation. Gross tumor analysis showed that elevated circulating levels of Lcn2 caused a significant increase in primary tumor growth and showed some increase in metastasis. However, further experiments using varying treatments of dosage must be explored to determine if the metastasis was significantly increased in these orthotopic mouse models of cancer.
Abstract

Lipocalin 2 (Lcn2) is a member of the lipocalin family of siderophore-binding molecules that can mediate iron trafficking. It acts during the innate immune response and has been implicated in conflicting roles in metastasis, leaving its role undefined. In breast cancer LCN2 was shown in previous studies to both inhibit or promote metastasis. Previous studies the Pena laboratory showed that increasing the serum levels of LCN2 caused a significantly increase in liver metastasis of the poorly metastatic MC-38 colon cancer cell line. In this study, we examined the ability of LCN2 to promote metastasis of colon and other cancers to the lung to determine its functionality as a multi-organ determinant of metastatic growth. The effect of elevated circulating levels of LCN2 on lung metastasis was tested on mouse models using MC-38 colon adenocarcinoma cells, 4T1 breast cancer cells, and B16-F10 melanoma cells using C57B1/6 (B6), Balb/c, and B6 mice, respectively. Isolated using endotoxin free methods, pV1J-LCN2 plasmid was utilized for in vivo electroporation of the mice, with the cancerous cells injected using tail-vein, mammary fat pad, and subcutaneous injections. The empty vector, pV1J was used as the control. Blood serum and in vivo bioluminescent images were obtained weekly, along with caliper measurements of the primary tumor. Mice were sacrificed after three weeks and the lungs, liver, and spleen were weighed and examined for metastasis post-mortem. Preliminary results indicate that LCN2 promoted a significant increase in tumor mass in all three cell lines. Though some metastasis was observed, further studies are required to determine the extent of the metastatic potential of LCN2.
Colorectal Cancer in Humans

In the United States, colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer death in men and women.\textsuperscript{4} It was estimated that in 2016, approximately 136,830 new incidences of CRC will be diagnosed and 50,310 deaths would result from colorectal cancer.\textsuperscript{16} Commonly diagnosed in older patients, colorectal cancer begins when healthy cells in the epithelial lining of the colon or rectum begin to proliferate, forming a mass called a tumor. The tumor most often begins as a polyp, a noncancerous growth that develops on the inner wall of the colon or rectum that form as a part of aging.

There are five stages used to diagnose colon cancer. Stage 0, also termed “carcinoma in situ”, is diagnosed when abnormal cells are found in the innermost layer (mucosa) of the colon wall.\textsuperscript{5} The stage is termed “in situ” since the cancer is in the “original place”, as the Latin term suggests. The next four stages progress as the cancer spreads deeper into colon, to the surrounding organs, to the lymph nodes, and then finally to metastasis. Metastasis is involved in cancers that commonly have the poorest prognosis, and occurs when cancer cells break away from the primary tumor and travel through the blood or lymph system to form new tumors in other areas of the body.\textsuperscript{11}

Metastasis occurs in multiple steps and often has no symptoms, making it particularly difficult to diagnose and to treat. Early detection can improve diagnosis; the five-year survival rate of those with localized stage colorectal cancer is approximately 90%. However, when diagnosed at a later stage after metastasis has occurred, the five-
year survival rate drops to less than 12%. At the time of diagnosis, approximately 32% of patients already have metastasis, and autopsy results often reveal that as many as 70% of those with colorectal cancers die from metastasis. Common sites of metastasis for colorectal cancers include the liver, lung, and peritoneum. For patients with metastasis, nearly 50% have metastasis in the liver, with resection as the only curative treatment option. One of the known factors to influence metastasis is the epithelial- mesenchymal transition, or EMT. However, a re-differentiation resembling the mesenchymal-epithelial transition is detectable in the metastases themselves, suggesting that malignancy is based on dynamic processes including genetic alterations that are then up regulated by the tumor microenvironment. Understanding the genetic and molecular mechanisms of liver and lung metastasis is critical to the development of better methods of detection and treatment that can block metastatic recurrence or progression.

The Pre-Metastatic Niche

Prior to the arrival of metastasizing cells to the target organ, the primary tumor sends molecular signals to recruit bone marrow derived cells (BMDCs) to the liver where they create a pre-metastatic niche (PMN). Specifically, the up-regulation of fibronectin and clustering of bone marrow derived cellular infiltrates coexpressing matrix metalloproteinases in distant tissues prior to tumor cell arrival appear to be invaluable for the initial stages of metastasis. Thus, these bone marrow-derived hematopoietic progenitors, especially those that express endothelial growth factor receptor 1, mobilizes in response to an unknown unique combinations of growth factors produced by the primary tumor. This is crucial for the growth of secondary tumors because the PMN
provides sites where cancer cells can attach as well as a fertile environment where metastasizing cells can proliferate. Identifying these molecules by parsing out the early cellular and molecular events in cancer dissemination could provide biomarkers for diagnosis or therapeutic targets to block metastatic progression.

The Tumor Microenvironment

The landmark paper, *The Hallmarks of Cancer*\(^8\), established six underlying commonalities in cancer that govern the transformation of benign to malignant cells; evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, and sustained angiogenesis. The follow up to that seminal work, *The Hallmarks of Cancer: The Next Generation*\(^7\) found two “emerging hallmarks”, reprogramming of energy metabolism and evading immune destruction. The paper also found that in addition to the cells, the tumors themselves provided another layer of complexity to the acquisition of the hallmarks. As more research is done with these hallmarks, the importance of the tumor microenvironment is becoming increasingly explored. The tumor microenvironment includes critical stromal support cells, as well as the surrounding blood vessels, various bone marrow-derived cells such as mesenchymal stem cells, macrophages and other immune cells, non-neoplastic cells (infiltrating immune cells, fibroblasts, and endothelial cells), and the extracellular matrix (ECM).\(^7\) The TME can secrete growth factors, chemokines, cytokines, and reactive oxygen species. Though previously thought to be recruited by molecular signals from cancer cells, the cells of the tumor microenvironment are influenced and work with cancer cells to support tumor progression by enhancing the
growth of the primary tumor as well as directing its metastatic spread to distant organs. A large issue in understanding metastasis is in identifying the sets of genes (“metastatic signatures”) that correspond and appear to orchestrate the establishment of macroscopic metastases in specific tissues. However, it is almost certainly dependent upon the establishment of a permissive tumor microenvironment. Thus, it is understanding the establishment of the tumor microenvironment, its features and capabilities, that we can begin to understand cancer metastasis.

Figure 1. The cells of the tumor microenvironment. Not shown are the premalignant stages in tumorigenesis, which also have distinctive microenvironments created by the abundance and characteristics of the assembled cells.
Previous Work

Dr. Pena’s lab has conducted a number of experiments exploring liver metastasis and others with LCN2. In previous studies, members of Dr. Pena’s lab examined changes in gene expression in the liver microenvironment in tumor bearing mice prior to and after the arrival of metastasizing cells to the liver using microarray analyses. Studies were conducted with the goal to identify genes that were activated in the liver by signals from the primary tumor, whose products were essential for establishing a PMN that will enhance tumor homing and engraftment, and that were required for supporting tumor growth upon arrival into the liver. LCN2 was found to be the most abundant mRNA that was expressed 10-fold higher in pre-metastatic liver and 50-fold higher in metastatic liver as compared to liver from non-tumor bearing mice. Furthermore, overexpression of serum levels of LCN2 by intramuscular in vivo electroporation of a plasmid expressing LCN2 prior to splenic injection of tumor cells (an experimental model of liver metastasis) significantly increased liver metastasis of a poorly metastatic MC-38 colon cancer cell line.

![Figure 2. Comparison of LCN2 serum levels in LCN2 knockout mice, wild type mice, and mice that were in vivo electroporated with pV1J-LCN2 or pV1J plasmid. Proteins were normalized against albumin protein²](image-url)
Figure 3. Spleen and liver weights, analyzed four weeks after splenic injections of
2 × 10^5 MC38 cells in C57BL/6 mice. Mice were injected with 50ug of pV1J-Lcn2 or
pV1J plasmids and electroporated. Increased liver weight in the Lcn2 group indicates an
increase in metastasis^2

Lipocalin 2: Its Significance and Role

Lipocalin 2 (Lcn2) is a member of the lipocalin family of siderophore-binding
molecules that can mediate iron trafficking.⁹ Also known as Neutrophil Gelatinase
Associated Lipocalin (NGAL), Lcn2 is a protein expressed in epithelial cells and
neutrophils. Lipocalins as a family have a key structural component, a β-barrel with eight
anti parallel strands, creating a structure that easily binds to small hydrophobic molecules
(including hormones, retinoids, and fatty acids).¹⁵ It can act during the innate immune
response, sequestering iron that would otherwise be taken up by invasive bacterium. Lcn2
has been implicated in conflicting roles in metastasis, thus its role has yet to be firmly
established. In breast cancer, \textit{Lcn2} was shown to inhibit or promote metastasis, while in CRC, its expression was inversely correlated with metastatic potential, although its role might be influenced by its specific location in the tissue.\textsuperscript{1,14} \textit{Lcn2} is over-expressed in many types of non-microbially-associated cancers including breast, pancreatic, and ovarian carcinomas.\textsuperscript{15} \textit{Lcn2} also has been shown to have a role in promoting tumorigenesis, done through enhancing tumor cell survival and proliferation.\textsuperscript{15} It was discovered that LCN2 can induce the epithelial to mesenchymal transition (EMT), which is instrumental to metastasis. In colon cancer specifically, LCN2 was found to promote EMT through RAC1. Further, it was found that in the up-regulation of LCN2 in a poorly metastatic colon cancer cell line the authors generated more invasive cells.\textsuperscript{13} However, an over-expression of \textit{Lcn2} in the aggressive metastatic colon cancer cell line KM12SM markedly reduced its invasive behavior in vitro and in vivo.\textsuperscript{13} In an additional study, it was found that LCN2 expression correlates with aggressive tumor formation in mouse breast tumor cell lines, with the most aggressive from the 4T1 and 4T07 cells.\textsuperscript{14} Although LCN2 is a secreted protein, there have been no in vivo studies examining its impact on the tumor microenvironment particularly in the target organ of metastases.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{lcn2_expression.png}
\caption{LCN2 expression in various cancer types.}
\end{figure}
Objectives of Study

The goal of this study is to test the hypothesis that elevated circulating levels of LCN2 can promote metastasis of multiple cancers to the liver and lung by recruitment of bone marrow derived cells that promote tumor homing, attachment, and growth in the target organ. We will use in vivo electroporation to increase circulating levels of LCN2 and test its ability to promote lung and liver metastasis of CRC, breast cancer, and melanoma cells using orthotopic and experimental models of lung and liver metastases.
Materials and Methods

Presence of Lcn2 in CT26-luc Cell Lines by Western Blotting

Western blot analysis is a widely used technique to detect the presence of proteins, a process dependent on the antibodies incorporated in the procedure of the blot. The procedure began by waking up CT26-luc cells, removing from liquid nitrogen and kept in the 37°C water bath for approximately five minutes. Taking care to keep the vial sterile, cells were resuspended 10 mL of DMEM with 10% fetal bovine serum (FBS) after being spun down in the centrifuge. Cells were incubated until they reached 80-90% confluency and then were transiently transfected with pV1J or pV1J-Lcn2 plasmids for 72 hours. Cells were washed with 5mL of HBSS and then 1 mL of Trypsin was added to the cells and then placed in the 37°C incubator for 5 minutes. After 5 minutes DMEM was added and the cells were placed in a 15mL Eppendorf tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and the pellet re-suspended in 5mL of PBS to wash it again, repeating the wash and centrifugation. The final time, the pellet was re-suspended in 1mL of PBS and then centrifuged at 2000 rpm and 5 minutes. The final cells were then re-suspended in 200 uL of Mammalian Protein Extraction Reagent adding 1uL of 500X Proteinase Inhibitor. The sample was vortexed and put on ice for 10 minutes, centrifuged at 11,000 rpm for an hour at 4°C. Protein samples were quantified by the Bradford assay using an Epoch plate reader (Biotek Instruments, Inc Winooski, VT).

The previous protein concentrations were normalized, and 18uL of that sample was placed into a PCR tube and the BIOL program was run (putting the sample at 99°C
for 10 minutes). 10μL of Bio Rad Plus Precision Dual Standards Ladder and 18 μL of samples are added to wells of a precast Mini-PROTEAN Bio-Rad gel and immersed in 1X SDS Page Buffer. The gel is run at 80V until the sample is through the loading gel, and then raised to 100V for approximately 60 minutes. Follow the gel electrophoresis the gel is removed and added to a “sandwich” of filter paper and transfer membrane. This was then placed into the Semi-Dry Transfer Apparatus, and run at 15V for 1 hours, while in a cocktail of 0.5g of Bio-Rad Blotting Grade Blocker Nonfat dry milk and 9.5 mL of PBST. After transfer, 5% milk in PBST was added to the membrane and placed in a shaker at room temperature for 30 minutes. The antibody against the Lcn2 was used as a probe, and incubated with the primary antibody overnight at 4°C, washed 4 times with PBST for 10 minutes each, and the secondary antibody (Bio-Rad EIA Grade Affinity Purified Rabbit Anti-Goat IgG (H+C)-HRP Conjugate, diluted 1:5000) was added to 2% BSA solution and incubated at least one hour at room temperature. The membrane was subsequently washed twice for 5 minutes and twice for 10 minutes in PBST. 1 mL of each ECL Western Blot Detection reagent was mixed and added to the membrane, later exposed in the dark room for approximately 30 seconds.

Isolation of pV1J and pV1J-Lcn2

In order to determine the effects of Lcn2 on metastasis and the tumor microenvironment, plasmid needed to be isolated. pV1J was used as the vector due to its previous success for optimal gene expression intramuscularly by in vivo electroporation. Additionally, it had previously been show to be an efficient plasmid to increase circulating levels of the gene of interest.
Bacteria transformed with pV1J and pV1J-Lcn2 DNA were grown overnight in a starter culture containing 5mL containing Kanamycin. The day following those 5mL starter cultures were grown overnight with incubation at 37°C, in 1.5L LB containing Kanamycin. This process enables the cloning of the pV1J vector with Lcn2 DNA, and the Clones were selectively grown by the addition of Kanamycin, which the vector is resistant to. Endotoxin free plasmid isolations for in vivo electroporation were purified using Qiagen Plasmid Plus Mega Kit following manufacturer’s instructions. Plasmid concentrations were measured by a spectrophotometer, and concentrated or diluted to a concentration of 1ug/μL.
Injection of DNA into Mice by In Vivo Electroporation

Prior to surgery, mice were anesthetized from inhalation of 5% isoflurane in oxygen, delivered at 1 L/minute, and after approximately 1 minute in the induction chamber, the concentration was lowered to 2.5% isoflurane in oxygen. After the mice in the induction chamber were successfully anesthetized, mice were removed individually shaved in the anterior portion of the thigh, clearing the field for the quadriceps. The mouse was then placed on the sterile operating table with a nose cone to deliver the 2.5% isoflurane in oxygen to the mouse in question. Laid on its back, a small incision was made in the skin just above the knee, and the slit was expanded to expose the quadriceps muscle. 10 ug of pV1J or pV1J-Lcn2 were injected (by a volume of 10 uL), achieved while holding the syringe vertically and perpendicular to the muscle and pulling out afterwards in a twisting motion, preventing loss of plasmid to an area other than the quadriceps muscle. The BTX Electroporator delivered 5 pulses of 100 volts of electricity at 50 milliseconds each to the site, allowing the muscle to take in the plasmid and express Lcn2 to be secreted into the bloodstream. The surgical site was closed by staples and the mice were monitored to assure surgery did not produce undue stress.
Various Cell and Mouse Types for In Vivo Electroporation

In order to determine the kinetics of Lcn2 expression after electroporation, several combinations of cell line, cell injection site, and mouse type were used. The MC38 colon adenocarcinoma cell line was used in a previous experiment to determine kinetics of Lcn2 within colon cancer. $2 \times 10^5$ cells were implanted into the spleen of eight week old C57BL/6 mice the day following in vivo electroporation of pV1J-Lcn2 plasmid or the empty pV1J vector. Sera were harvested weekly after electroporation and three weeks after spleen injection, the mice were humanely sacrificed. The 4T1-rfp-luc breast cancer cell line was used in Balb/c mice to determine kinetics of Lcn2 within breast cancer. An initial experiment was conducted with injections of 100,000 4T1 cells into the inguinal mammary fat pads, in the 4th fat pad on either the left or right side of the mouse. Sera were harvested weekly after electroporation and four weeks after fate pad injection, the mice were humanely sacrificed.
To determine the kinetics further, the 4T1/Balb C experiment was repeated using 5,000 4T1-rfp-luc cells, injected in the same manner in the same area of the mouse. Electroporated Bi-weekly, sera were collected on the weeks not containing electroporation. Mice were humanely sacrificed after 8 weeks of this treatment.

The final exploration of kinetics involved the highly metastatic melanoma cell line in C57B1/6 mice. Following electroporation, $2 \times 10^5$ cells were injected subcutaneously. Mice were electroporated on days 0 and 14 and sera was collected on days 7, 21, and 28, as well as prior to experiment. Mice were humanely sacrificed after 4 weeks. In all mouse treatments, blood samples were obtained from the retro-orbital sinus using capillary tubes, were centrifuged for 5 minutes to separate red blood cells from blood sera containing Lcn2, and stored at -80°C for further analysis.
**Gross Analysis of Tumors**

Weekly, mice of all treatments were weighed and their primary tumors measured by caliper. IVIS (in vivo imaging system involving luciferase) was utilized on a bi-weekly basis to determine if metastasis had occurred. After the mice were sacrificed, tumor bearing lungs, liver, and spleens of mice as well as the primary tumors were fixed in 4% paraformaldehyde. The primary tumor and the spleen were weighed as well.
Results

Intracellular/Extracellular Lcn2 in CT26-luc Cell Line by Western Blotting

The presence of Lcn2 was confirmed in the stably transfected CT-26-luc cell line by western blot analysis. The data indicates with the presence of Lcn2 as compared to the β-actin control.

Figure 8: CT26-luc stably expressing high levels of Lcn2
Figure 9. CT26-luc extracellular Lcn2 levels, using DMEM+10% FBS as an additional control

Since the Lcn2 is also secreted, the extracellular proteins levels in cell culture media were also analyzed. Western blot analysis of CT-26 transient transfected cells showed an increased level of LCN2 protein in pV1J-Lcn2 cells as compared to those with the empty vector. These results validate pV1J as a vector to secrete Lcn2 and enable its use to increase its circulating levels in the blood, specifically for in-vivo electroporation.
Gross Analysis of Tumors

The results for the initial experiment involving 4T1 cells injected into Balb/c mice show large increases in tumor growth. Tumor volume was measured via caliper weekly and calculated using the formula $V=(W(2) \times L)/2$. By Day 28, 41% of pV1J mice ($n=12$) and 72% ($n=11$) of pV1J-Lcn2 had the primary tumor spread from the 4th mammary fat pad to the 5th. Additionally, preliminary IVIS imaging on Day 28 indicated that 2 of the pV1J-Lcn2 mice had metastasis near the pleural cavity, possibly to the lungs. Figure 10
clearly displays that though both mice treated with pV1J and pV1J-Lcn2 show an increase of tumor volume over time, the pV1J-Lcn2 mice showed significantly more growth.

Figure 11. Tumor Mass, Mass Percentage, and Volume from injection of 5,000 4T1-luc cells in the Mammary fat pad. Data collected approximately 8 weeks after injection.
The 4T1 mammary fat pad experiment was repeated with a reduction in the initial tumor injection and had a reduction in the number of electroporations conducted, broadening the kinetics of the tumor progression. Tumor Volume was determined using the equation $V = (L \times W^2)/2$. Tumor mass was measured after mice were humanely sacrificed, and spleen weight was taken as well. Tumor mass percentage was done by comparing the mass of the tumor to the mass of the mouse. Of the 12 mice, 6 pV1J and 6 pV1J-Lcn2, 5 pV1J and 5 pV1J-Lcn2 had metastases of some form at the time of the sacrifice. Figure 11 clearly displays the same trend found in the first 4T1 experiment, that pV1J-Lcn2 mice have larger tumors in terms of volume, mass, and mass percent.

**Figure 11.** Tumor Mass and Mass Percentage of B16-F10 Melanoma in B6 mice, approximately 4 weeks after subcutaneous injection.
The C57Bl/6 mice injected with B16 F-10 cells similar results. Of the mice (n=15) at the end of the experiment, only one had lung metastasis, and that mouse was in the pV1J-Lcn2 treatment. Spleen masses were also measured, and 42.8% (n=7) of pV1J mice had spleens weighing over 0.2 g, as well as 62.5% (n=8) of pV1J-Lcn2 mice had spleens above that benchmark. Overall, Figure 12 clearly displays a significant difference in tumor growth between the pV1J and pV1J-Lcn2 mice.
Discussion/Conclusions

In order to understand cancer’s progression, one must study metastasis. Within that scope, it is also necessary to determine what factors enable or increase that metastasis, be it the development of a pre-metastatic niche or the role of the tumor microenvironment. Regardless, it is of vital importance to attempt to find metastatic biomarkers to the progression of colon and other cancers to metastatic potential. The confirmation and detection of such a target protein or molecule would prove to be invaluable to early screening and treatment. In previous studies exploring the development of CT26-FL3 cells, a highly metastatic cell line, the gene encoding the protein Lcn2 was found to be highly expressed in the metastatic liver both against other genes and the transcriptional genome of the sham control group of mice not injected with CT26-FL3 cells. Previous studies within Dr. Pena’s lab also found LCN2 to be the most abundant mRNA that was expressed 10-fold higher in pre-metastatic liver and 50-fold higher in metastatic liver as compared to liver from non-tumor bearing mice.

To explore Lcn2 further, in vivo experiments were conducted using colon, breast, and skin cancer to determine metastatic potential not only in colon cancer but as a more general biomarker. A western blot analysis was performed using CT26-luc cells transiently transfected to determine intracellular and extracellular Lcn2 expression. The analysis revealed both intracellular Lcn2 as well as extracellular Lcn2, which provides validation that pV1J vector could be utilized to secrete Lcn2 and enable its use to increase its circulating levels in the blood, specifically for in-vivo electroporation.

To begin viewing Lcn2 and its effects on metastasis within the living mouse models, the plasmid pV1J-Lcn2 was constructed, isolated, and purified under large-scale
endotoxin free conditions. In vivo electroporation was chosen due to its ability to continuously express Lcn2 in the bloodstream over extended time period via entry by stable plasmid. There were 3 types of mouse and three cell lines explored, the previously validated MC38 colon adenocarcinoma cell line injected into the C57Bl/6 mice, the 4T1 breast cancer cell line injected into Balb/c mice, and the B16-F10 melanoma cell line injected into C57Bl/6 mice. Mice were electroporated with 10ug of either 10uL of pV1J plasmid or 10uL of pV1J-Lcn2. The plasmid was injected into the quadriceps muscle and then released into the bloodstream via electroporation at 100V for 50ms. Blood was collected from the retro-orbital sinus prior to surgery as well as weekly after electroporation.

Gross analysis of tumor progression show that Lcn2 significantly increases primary tumor growth in both 4T1 and B16-F10 treated mice. Previous experiments displayed Lcn2’s ability to significantly increase liver weight in MC38 treated mice as well. Additionally, Lcn2 was shown in the initial 4T1 treatment with 100,000 cells to possibly increase lung metastasis, with 2 mice showing luciferase activity near the pleural cavity, both in the Lcn2 treatment group. Repetition with a much smaller initial injection of 4T1 cells (5,000) allowed the metastatic potential to be explored further, and though it showed similar increases in tumor volume as the initial experiment, both pV1J and pV1J-Lcn2 mice had metastasis in equal measure at the final measure of the experiment. The B16-F10 melanoma cells progressed rapidly, producing often flat tumors difficult to measure with the caliper, but at the close of the experiment it was determined that Lcn2 treated mice significantly increased tumor mass and mass percent. Of the mice treated, only one had a lung metastasis, a pV1J-Lcn2 treated mouse.
The results confirmed some previous experiments while providing some new insights as well. Lcn2 clearly has a significant effect on the growth of the primary tumor, regardless of cell line or mouse type. Additionally, it seems to have some slight correlation with metastasis to the lung, but no significant data was found. The findings also confirmed in vivo electroporation as viably method to explore Lcn2 as a metastatic biomarker.

Future experiments could be conducted to further explore Lcn2 and its metastatic potential. Sera collected could be analyzed with ELISA to determine the expression of Lcn2 within the bloodstream in the three treatments of the in vivo experiments above. Additionally, electroporations of the B16-F10 mice could be explored with a reduction in initial subcutaneous injection to determine that if the tumor kinetics ran longer if more metastasis would have occurred. Additionally, the 5,000 cell 4T1 mice could be sacrificed earlier, or more IVIS images could have been taken to determine whether Lcn2 increases metastasis or if metastasis simply occurred because the treatment ran longer. Analogous studies also could be preformed with Lcn2 inhibition rather than up-regulation, be it Lcn2 gene knockout, silencing Lcn2 by siRNAs, or other methods to study the absence of Lcn2 in colon, skin, or breast cancer metastasis. To confirm the results found in this study, Lcn2 inhibition should show a vast decrease in tumor mass and volume as well as a decrease in metastasis. The identification of Bone Marrow derived cells in any of the areas of metastasis in the orthotopic mouse model would provide insight to the tumor microenvironment and its interaction with Lcn2. Future studies could very well be conducted to determine the role of Lcn2 in the tumor microenvironment and its molecular interactions within that environment and beyond.
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