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LIPOCALIN 2 PROMOTES THE ESTABLISHMENT OF A PRO-TUMORIGENIC MICROENVIRONMENT IN PANCREATIC CANCER

by

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LIPOCALIN 2 PROMOTES THE ESTABLISHMENT OF A PRO-TUMORIGENIC MICROENVIRONMENT IN PANCREATIC CANCER

А

DISSERTATION

Presented to the Faculty of

The University of Texas

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and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Sobeyda B. Gomez-Chou, B.S.

Houston, Texas

May 2016

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Dedication

To my parents, your sacrifice has given me the strength to keep moving forwards in my education. I owe and dedicate this success to the both of you.

Para mis padres, su sacrificio me ha dado las fuerzas para seguir adelante en mis estudios. Este éxito se lo debo y lo dedico a Ustedes.

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LIPOCALIN 2 PROMOTES THE ESTABLISHMENT OF A PRO-TUMORIGENIC MICROENVIRONMENT IN PANCREATIC CANCER

Sobeyda B. Gomez-Chou, B.S.

Advisory Professor: Craig D. Logsdon

Pancreatic ductal adenocarcinoma (PDAC) is a disease characterized by a dismal prognosis with a 5-year survival rate of 7%. A unique hallmark of this disease is an abundant desmoplastic reaction that can account for up to 90% of the solid tumor volume. Key components of the PDAC stroma include the extracellular matrix (ECM) rich in collagen type I and III, activated pancreatic stellate cells (PSCs) and inflammatory cells such as neutrophils and macrophages. The main line of evidence has suggested a pro-tumorigenic role for the PDAC stroma as it has been shown to help enhance tumor growth, invasive potential and drug resistance. Recent reports however, have challenged these findings and shown that the stroma offered protection from tumor growth. Given the extensive presence of stroma and its presumed roles in PDAC, characterization of the function of molecules that can alter stromal composition is warranted. LCN2 is a molecule that has been confirmed to be differentially expressed in PDAC. Attempts to investigate its biological function in PDAC have unfortunately led to conflicting results. In this study, we examined the effect of LCN2 depletion on tumor growth in a syngeneic orthotopic model of PDAC. We report that LCN2 depletion delayed tumor growth, decreased changes in stromal composition, and

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diminished inflammatory cell infiltration leading to increased survival. In addition, we examined the role of LCN2 and its specific receptor SLC22A17 in human PSCs. We revealed for the first time that hPSCs don't produce LCN2, but do express LCN2 receptors. Moreover, ectopic LCN2 promoted hPSCs to produce inflammatory factors in a receptor-mediated manner. Finally, because LCN2 is involved in iron transport, we showed that LCN2's effects on hPSCs were also mediated by changes in iron levels. Overall, our results suggest a model for LCN2 function in the regulation of the tumor microenvironment and shown its ability to promote tumor growth in PDAC.

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Chapter 1: Introduction

Pancreatic Cancer

Human Pancreatic Ductal Adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in the United States (Fokas et al., 2015). Projected to become the second leading cause of cancer-related deaths by the year 2020, PDAC will only fall behind lung cancer (Rahib et al., 2014). Early stage PDAC is usually asymptomatic and the majority of cases are diagnosed when the disease is advanced and has metastasized to distant areas. According to the National Cancer Institute, nine percent of PDAC patients are diagnosed when the disease is localized to the pancreas while over fifty percent of patients are diagnosed when the disease has already spread to other organs. Because the five-year relative survival rate for patients with distant disease is just over two percent, the majority of patients diagnosed with PDAC have a very dismal possibility for surviving this malignancy.

Pancreatic pre-invasive lesions and PDAC development

Multiple observations have suggested that the development of non-invasive pancreatic intraepithelial neoplasia (PanIN) can be attributed to acinar cell to ductal cell metaplasia (Brune et al., 2006; Zhu et al., 2007). While other non-invasive pancreatic neoplasia have been found including mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN), PanINs are more common (Hruban et al., 2008). PanIN lesions appear to be present in non-cancerous pancreas and their prevalence increases with age (Schwartz and Henson, 2007). However, PanIN lesions alone do not have a high potential to develop to invasive cancer (Terhune et al., 1998). Further, three stages of PanIN lesions are found in the pancreas named PanIN-1, PanIN-2 and PanIN-3. PanIN-1 lesions contain

columnar epithelial cells with round nuclei. PanIN-2 lesions exhibit more extensive nuclear morphological changes, and PanIN-3 lesions form papillae and cribriform structures with more complex dysplasia (Hruban et al., 2008).

Genetic alterations have been reported as early as in PanIN-1 lesions. Mainly, an oncogenic point mutation in codon 12 of the Kras oncogene is found in over 95% of PDAC cases and has been detected in PanIN-1 lesions (Gharibi et al., 2016). The excessive activation of this oncogene via its mutation and stimulation by exogenous signals can lead to cell proliferation, differentiation and survival. Other alterations including the inactivation or deletion of the tumor suppressors CDKN2A (p16/Ink4A), TP53 and Smad4 are observed in the progression from PanIN-2/3 lesions to PDAC (Hruban et al., 2008). Despite the early accumulation of genetic alterations, a study has proposed that the progression from localized tumor to metastatic disease could occur over a decade (Yachida et al., 2010). This finding indicates a window of opportunity for the early detection of PDAC and for medical intervention before the disease metastasizes (**Figure 1**).

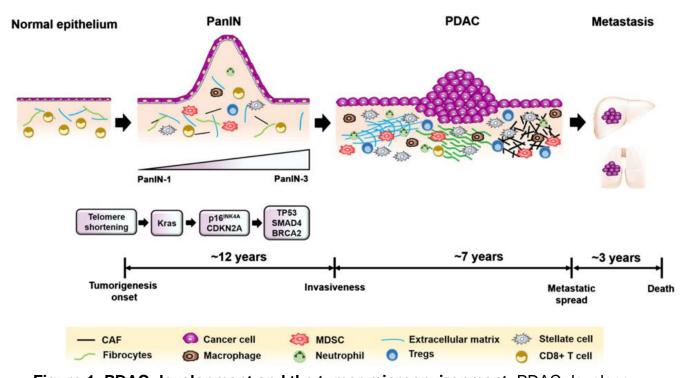


Figure 1. PDAC development and the tumor microenvironment. PDAC develops over a long period of time beginning with the formation of pancreatic pre-invasive neoplastic lesions called PanINs. Beginning in early PanIN-1, genetic mutations such as in the Kras gene are observed. Progression of PanIN-1 lesions to PanIN-2 and 3 occurs with additional genetic mutations, dysplasia and the accumulation of stromal components including the ECM, inflammatory cells, cancer associated fibroblasts and pancreatic stellate cells. An extensive stroma is a hallmark of PDAC. The time it takes to get from tumorigenesis onset to invasive PDAC suggests a potential window for medical intervention. (Figure 1 taken with permission from Fokas, E., O'Neill, E., Gordon-Weeks, A., Mukherjee, S., McKenna, W.G., and Muschel, R.J.Pancreatic ductal adenocarcinoma: From genetics to biology to radiobiology to oncoimmunology and all the way back to the clinic, Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, Volume 1855, Issue 1, January 2015, Pages 61-82. License ID: 3840020158964).

Pancreatic stroma

PDAC is a unique solid tumor in which an extensive desmoplastic reaction can constitute up to 90% of the tumor volume (Gharibi et al., 2016). This PDAC stroma is a heterogeneous and dynamic compartment implicated in the process of tumor formation, progression, invasion and metastasis (Chu et al., 2007). It contains various acellular and cellular components including the extracellular matrix (ECM), matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), cytokines and growth factors as well as stromal cells such as fibroblasts, pancreatic stellate cells (PSCs) and inflammatory cells (Feig et al., 2012). The ECM is comprised of fibrous proteins including collagens I and III, fibronectin and laminin as well as other proteins including glycoproteins, glycosaminoglycans and proteoglycans. Alterations in the composition of the ECM has been shown to regulate tumor invasion and metastasis (Hidalgo, 2010). MMPs are a family of proteolytic enzymes produced by tumor and stromal cells that degrade the ECM. Their expression can be induced by several growth factors, hormones and cytokines (Stamenkovic, 2000). Moreover, certain MMPs have the ability to activate other MMPs. For instance, MMP-3 has been shown to be able to activate the mature MMP-1, MMP-7 and MMP-9. MMPs can be further classified into six different types based on the substrates they bind such as collagens, gelatins, fibronectin and elastin among others. In pancreatic cancer, MMPs have been found to be upregulated and to correlate with malignancy (Bloomston et al., 2002). Specifically, the expression of MMP-1, MMP-3 and MMP-9, among other MMPs, has been correlated with degree of desmoplasia, tumor extent, nodal status and with survival in pancreatic cancer.

A milieu of cytokines and growth factors including TNF- α , TGF- α , TGF- β , Interleukins 1, 6, 8, 10, 17, 22 and VEGF among others have been identified in the PDAC stroma and are implicated in tumor initiation, progression and metastasis (Delitto et al., 2015). These factors can be produced by inflamed acinar cells, cancer cells, inflammatory cells and by PSCs. Human pancreatic stellate cells (hPSCs) have been extensively studied and are known to actively contribute to the establishment of the pancreatic tumor microenvironment. In fact, hPSCs are key producers of ECM components including collagens I/III and fibronectin, growth factors including TNF- α , TGF- β , VEGF and stromal derived factor 1 (SDF-1), MMPs, TIMPs, cytokines and chemokines (Duner et al., 2010; Mace et al., 2013; Omary et al., 2007).Quiescent hPSCs are present in the normal pancreas but they become activated upon acute and chronic inflammatory stimuli. Activated hPSCs are characterized by morphologic changes, expression of the α -smooth muscle actin (α -SMA), increased ECM deposition and increased proliferation (Omary et al., 2007). Moreover, hPSCs have been shown to be activated by platelet derived growth factor (PDGF), TNF- α , TGF- β , fibroblast growth factor (FGF) and Interleukins 1 β , 6, 8 that are released by cancer cells and leukocytes that have been recruited to areas of injury (Duner et al., 2010; Jaster, 2004; Omary et al., 2007). Thus, a crucial crosstalk between cancer cells, immune cells and hPSCs occurs in the PDAC stroma.

Consequently, many studies have examined the role of hPSCs and the stroma on PDAC development and growth. Several studies have shown that the co-culture of hPSCs or conditioned media from hPSCs with PDAC cells increases the proliferation, migration and invasion potential of cancer cells in vitro. Moreover, the

co-injection of hPSCs with PDAC cells has been shown to enhance tumor growth and metastasis (Erkan et al., 2012; Hwang et al., 2008; Vonlaufen et al., 2008). Numerous studies have also demonstrated the role of the stroma in promoting tumor growth and progression (Bachem et al., 2005; Duner et al., 2010; Korc, 2007; Mahadevan and Von Hoff, 2007). Interestingly a few recent reports have suggested a protective role for the stroma in tumor development and metastasis using genetic models that eliminate stellate cells during cancer initiation (Ozdemir et al., 2014; Rhim et al., 2014). These reports indicate the need for further studies to clarity the function of stromal components and their effect of PDAC.

Pancreatic cancer and inflammation

Inflammation of the pancreas, referred to as pancreatitis, has been suggested by many studies to be a risk factor for PDAC development (Lowenfels et al., 1993). Interestingly, the stromal components observed in pancreatitis are similar to those observed in PDAC (Delitto et al., 2015; Erkan et al., 2012; Lowenfels et al., 1993). Specifically, the activation of hPSCs, deposition of ECM components, expression of cytokines and growth factors and inflammatory cell infiltration is observed in pancreatitis (Guerra et al., 2007). Strong upregulation of the inflammatory mediators TNF- α , SDF-1, IL-1 β , IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1) and the complement component C5a has been implicated in the development of pancreatitis and progression to PDAC (Gukovsky et al., 2013; Matsuo et al., 2012). In addition to the upregulated presence of cytokines and chemokines, the presence of inflammatory cells also change in the progression from neoplastic lesions to PDAC. For instance, tumor associated

macrophages (TAMs) and myeloid derived suppressor cells (MDSCs) infiltrate the stroma early on during low grade lesions and persist through invasive PDAC development (Clark et al., 2007). These inflammatory cells have been shown to suppress the host immune system and may promote disease progression. Further, it has been suggested that the migration of these cells is mediated by proinflammatory cytokines and chemokines secreted by hPSCs including IL-6, IL-8 and MCP-1 among others.

Because PDAC is a very deadly disease and early detection is critical for medical intervention, many groups have attempted to dissect the differences in the inflammatory response seen in pancreatitis versus PDAC. Several analyses of human serum have shown that IL-6, IL-8 and TNF- α are significantly overexpressed in PDAC compared to benign patients and patients with pancreatitis (Blogowski et al., 2014; Ebrahimi et al., 2004). Similarly, a member of the lipocalin family, Lipocalin 2 (LCN2), has been found to be significantly upregulated in PDAC tissue and serum. Studies have shown weak to no LCN2 expression in normal tissue and pancreatitis samples compared to PDAC (Devireddy et al., 2001; Moniaux et al., 2008). Thus, the differential expression of LCN2 in PDAC suggests its use as a potential maker of this disease.

The Lipocalin family

The lipocalins are a large family of small proteins present in both prokaryotes and eukaryotes (Goetz et al., 2000). They are capable of binding, storing and transporting a large range of small hydrophobic molecules including vitamins and steroid hormones (Chakraborty et al., 2012; Du et al., 2015). Moreover, they have been implicated in a variety of different functions including transport of retinols and pheromones and synthesis of prostaglandins and immune cell recruitment among others (Flower, 1996; Flower et al., 2000). Many studies have performed structural analysis of several lipocalin family members to better understand their function. It has been found that this family of proteins share a varied amount of sequence conservation with some cases indicating sequence identity as low as twenty percent (Chakraborty et al., 2012; Flower, 1996). Nevertheless, lipocalins share a common secondary and tertiary structural feature named the lipocalin fold (Flower, 1994; Flower et al., 1991). This fold forms a cavity that aids ligand binding and the difference in amino acids that form this fold gives rise to the diverse amount of ligands bound by lipocalins (Du et al., 2015; Ganfornina et al., 2000; Schiefner and Skerra, 2015).

Identification and expression of Lipocalin 2

Lipocalin 2 (LCN2) is a molecule that has developed considerable research interest since its discovery decades ago. It was first identified as a small superinducible protein during the growth factor treatment of murine fibroblasts (Davis et al., 1991; Nilsen-Hamilton et al., 1982). Ever since, an extensive number of studies have examined the expression of this molecule in different species and

aimed to elucidate its function. Interestingly, results from such studies have led to a complicated and varied nomenclature for this specific lipocalin member. For example, Lipocalin 2 is also known as neutrophil glucosaminidase-associated lipocalin (NGAL), 24p3, migration stimulating factor inhibitor (MSFI), SIP24, Ch21, α1-microglobulin related protein, uterocalin and siderocalin (Chakraborty et al., 2012; Chan et al., 1988; Descalzi Cancedda et al., 1988; Hraba-Renevey et al., 1989). Thus, in order to get a thorough understanding on past and present LCN2 publications it is necessary to search under all of these names that have been given to this lipocalin member.

Human LCN2 was first found and isolated in a complex with the matrix metalloproteinase 9 (MMP-9) in neutrophils (Triebel et al., 1992). Following studies revealed that LCN2 was a secreted protein capable of being detected in human serum in quantities ranging from 40-109 ng/mL (Kjeldsen et al., 1993). Additional research revealed LCN2 expression in various human tissues including in breast, bone marrow, kidney, liver, trachea, lung, salivary glands, prostate, adipocytes and macrophages (Cowland and Borregaard, 1997; Cowland et al., 2003; Moreno-Navarrete et al., 2010). Further, LCN2 is expressed and upregulated in a variety of benign diseases. For instance, LCN2 is reported to be upregulated in inflammatory diseases including periodontitis, myocardidis, HIV and ulcerative colitis; in metabolic diseases including in diabetic and obese patients; in renal diseases including in kidney injury and chronic kidney disease (Landro et al., 2008; Sahinarslan et al., 2011; Van Dyke et al., 1985; Viau et al., 2010; Wang et al., 2007).

LCN2's role in iron transport via siderophores and specific receptors

Upon its discovery, several groups began to elucidate the three-dimensional structure of LCN2. Nuclear magnetic resonance (NMR) analysis revealed that the ligand binding cavity, or lipocalin fold, of LCN2 was significantly polar. Subsequently, one group used the results from LCN2 crystallography to identify a ligand for LCN2 (Goetz et al., 2002). A bacterial chromophore containing iron was found to bind tightly and specifically to LCN2. Further analysis revealed that this ligand was ferric enterobactin, a siderophore. Siderophores are proteins with high affinity to ferric iron (Fe³⁺) that make iron available to bacteria which is essential for their survival (Neilands, 1995). Moreover, it was found that LCN2 binds iron only in complex with a siderophore and not directly. Subsequent studies have also revealed the presence of mammalian siderophores called catechols that are able to bind LCN2 in the human body (Bao et al., 2010). These metabolites can be derived from plant sources and can also be synthesized in the body from dietary proteins. Specifically, the mammalian siderophore 2,5-dihydroxy benzoic acid (2,5-DHBA) was shown to bind LCN2 in the pro-B murine cell line FL5.12 (Devireddy et al., 2010).

Through its ability to bind iron-laden siderophores, LCN2 has been shown to regulate iron levels in mammalian cells. LCN2 was also first implicated to have a role in promoting cell death (Devireddy et al., 2001). One group identified a cell-surface receptor that allowed for LCN2-mediated iron uptake in FL5.12 cells (Devireddy et al., 2005). Two cell-surface proteins have been demonstrated to bind LCN2: solute carrier family 22 member 17 (SLC22A17) and low density lipoprotein receptor related protein (LRP2 or megalin) (Chakraborty et al., 2012). Both SLC22A17 and

LRP2 have been reported to bind and mediate the cellular uptake of LCN2 (Cabedo Martinez et al., 2016; Devireddy et al., 2005; Fang et al., 2007). While SLC22A17 has been shown to bind specifically to LCN2, LRP2 also binds numerous structurally unrelated molecules including aprotinin, vitamin D3 and lactoferrine (Hvidberg et al., 2005; Miyamoto et al., 2011a). While there is a limited amount of research published for SLC22A17, recent reports have indicated that its expression is correlated with malignancy. For instance, overexpression of both LCN2 and SLC22A17 has been associated with a worse prognosis in gliomas, hepatocellular and endometrial cancer (Liu et al., 2011; Miyamoto et al., 2011a; Zhang et al., 2012).

LCN2 and inflammation

Tight regulation of the immune system is essential for the body's defense against foreign pathogens and to prevent autoimmune malignancies. A myriad of molecules are involved in the complex activation and regulation of the inflammatory response. LCN2 has been implicated to function in a pro-inflammatory fashion and it is often times referred to as a cytokine. In a study using LCN2 deficient mice, it was found that macrophages, fibroblasts and endothelial cells produced and secreted high levels of LCN2 upon bacterial infection (Flo et al., 2004). In addition, a mouse model of allogenic cardiac transplant showed that LCN2 deficient mice exhibited significantly reduced levels of infiltrating neutrophils compared to control animals (Aigner et al., 2007). Other in vivo infection studies have also shown that LCN2 aids in the recruitment of neutrophils and is important in the activation and migration of these immune cells to sites of inflammation (Bachman et al., 2009; Schroll et al., 2012; Wu et al., 2010).

The fact that LCN2 expression is rapidly induced upon infection in different species including in humans, mice, and rats clearly implicates LCN2 in the immune response. The intricate relationship between LCN2, inflammatory molecules and its regulation has been examined by many (Chakraborty et al., 2012). In vitro and in vivo studies have shown that LCN2 expression can be induced by various proinflammatory cytokines and growth factors. Interleukins 1B, 1 α , 6, 17, 22, tissue necrosis factor α (TNF- α), nuclear factor κ B (NF- κ B), granulocyte monocyte colony stimulating factor (GMCSF), transforming growth factor a (TGF- α) and insulin like growth factor 1 (IGF-1) have been previously shown to induce LCN2 expression (Aujla et al., 2008; Cowland et al., 2006; Cowland et al., 2003; Fujino et al., 2006; Landro et al., 2008; Raffatellu et al., 2009; Sorensen et al., 2003). The LCN2 promoter contains a binding site for NF- κ B resulting in the positive regulation of LCN2 (lannetti et al., 2008; Shen et al., 2006). Of note, the ability of all these factors to upregulate LNC2 expression has been found to be cell or tissue dependent indicating the need to study their effect on LCN2 in tissues of interest.

LCN2 in cancer

In addition to being involved in inflammation, LCN2 is also upregulated in cancer. Studies have shown that LCN2 expression is abnormally high in thyroid, ovarian, breast, endometrial, colorectal, rectal, gastric, hepatocellular, oesophageal, lung, gliomas, chronic myeloid leukemia and in pancreas cancer (Bousserouel et al., 2010; Cho and Kim, 2009; El-Serag and Rudolph, 2007; Furutani et al., 1998; Santin et al., 2004; Villalva et al., 2008; Wang et al., 2010). A significant number of studies have also described the potential involvement of LCN2 in the biology of these

cancers. In breast cancer, excessive LCN2 expression has been shown to enhance tumor growth, migration, invasion and metastasis by inducing cell growth and promoting epithelial to mensenchymal transformation (Fernandez et al., 2005; Shi et al., 2008; Yang et al., 2009). LCN2 knockdown has shown to prevent thyroid cancer cell growth and tumor formation (Iannetti et al., 2008). LCN2 expressing endometrial tumor cells exhibited increased proliferation rates and invasive abilities (Miyamoto et al., 2011b). Lung cancer cells demonstrated upregulated LCN2 expression in response to apoptotic stimuli leading to increased survival (Tong et al., 2005). In colorectal cancer, LCN2 overexpression promoted cancer cell invasion potential and enhanced tumorigenesis in a xenograft model (Sun et al., 2011).

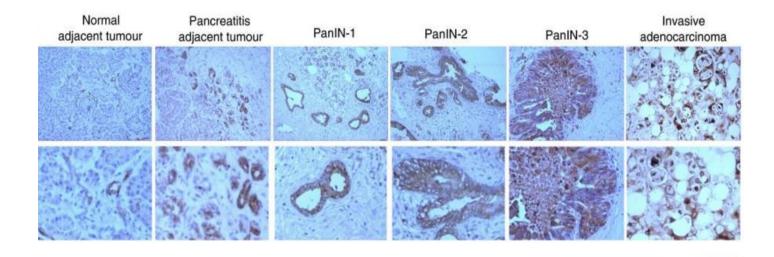
Some of these tumors, including in kidney and breast, have been shown to raise the levels of LCN2 in blood, urine and other biological fluids in addition to the primary tissue (Lippi et al., 2014). In addition, for many of these cancers, LCN2 expression has been shown to have a positive correlation with degree of differentiation, tumor aggressiveness and decreased survival (Lippi et al., 2014). In renal cell carcinoma, serum and urine LCN2 levels have been associated with mortality and shorter progression-free survival (Morrissey et al., 2011; Perrin et al., 2011). Higher plasma LCN2 levels have been found in patients with colorectal cancer and it has been associated with disease progression, stage, metastasis and mortality (Marti et al., 2013; Zhang et al., 2009). In gastric cancer, high serum LCN2 levels are associated with metastasis, invasion and mortality (Wang et al., 2010). LCN2 expression is associated with stage, invasion and tumor recurrence in hepatic cancer (Zhang et al., 2012). In breast cancer, LCN2 expression in tissue and serum

has been associated with tumor grade, estrogen and progesterone receptor status and with decreased disease-free survival (Bauer et al., 2008; Wenners et al., 2012). LCN2 expression is also associated with tumor aggressiveness, recurrence and mortality in endometrial cancer (Mannelqvist et al., 2012). The results from all these studies suggest the potential use of LCN2 as a diagnostic and prognostic marker in various cancerous malignancies.

LCN2 in PDAC

In a study designed to identify unique proteins produced by pancreatic ductal adenocarcinoma cells (PDAC), LCN2 was found to be differentially secreted by cancerous cells compared to normal tissue (Furutani et al., 1998). Other studies confirmed the upregulation of LCN2 in pancreatic cancer cells and in tumor tissue indicating its possible use as a PDAC marker (Han et al., 2002). It was further revealed that certain PDAC cell lines exhibited LCN2 upregulation approximately thirty-fold while other PDAC cells didn't express much higher levels than normal cells (Chakraborty et al., 2012). Another study examined LCN2 expression in the early stages of pancreatic cancer. LCN2 was observed to be differentially expressed beginning in early PanIN lesions which was positively correlated with the progression of the disease (Moniaux et al., 2008). More specifically, immunohistochemistry identified a very strong LCN2 staining pattern in PanIN-1, PanIN-2, PanIN-3 lesions and in invasive adenocarcinoma compared to pancreatitis and normal adjacent tissue (Figure 2). Prior reports have also demonstrated that LCN2 is highly upregulated in the serum of PDAC patients compared to chronic pancreatitis and normal healthy individuals (Kaur et al., 2013; Kaur et al.,

2014). These studies have thus indicated that LCN2 could be used as a potential marker for the detection of PDAC in conjunction with other specific PDAC biomarkers.



Pancreatic cancer progression

Figure 2. Immunohistochemical analysis of the progressive expression of

LCN2 in PDAC development. LCN2 expression is weak to non-existent in normal pancreas tissue. Upon inflammatory injury, LCN2 expression is moderately upregulated in pancreatitis. Its expression correlated with the progression of preinvasive lesions PanIN-1, 2, 3 and PDAC. (Figure taken with permission from Moniaux, N., Chakraborty, S., Yalniz, M., Gonzalez, J., Shostrom, V.K., Standop, J., Lele, S.M., Ouellette, M., Pour, P.M., Sasson, A.R., Brand, R.E., Hollingsworth, M.A., Jain, M., and Batra, S.K. Early diagnosis of pancreatic cancer: neutrophil gelatinase-associated lipocalin as a marker of pancreatic intraepithelial neoplasia. British Journal of Cancer. 2008;98(9):1540-1547. License ID: 3838480336765)

Further studies to identify a potential mechanism by which LCN2 may affect PDAC have so far been inconclusive. Two studies, one in 2008 and one in 2012, that made use of PDAC cell lines and xenograft mouse models to study LCN2, reported conflicting results. In the earlier study, LCN2 overexpression in two PDAC cell lines with low endogenous LCN2 levels significantly blocked the adhesion and invasion potential of these cells by decreasing focal adhesion kinase (FAK) phosphorylation and reduced the production of the vascular endothelial growth factor (VEGF) (Tong et al., 2008). In the same study, in vivo analysis demonstrated that the overexpression of LCN2 in a PDAC cell line implanted in an orthotopic nude mouse reduced tumor volume and metastasis. On the other hand, the later study reported that LCN2 depletion in two PDAC cell lines with high endogenous LCN2 levels significantly reduced adhesion, and increased invasion and tumor growth in subcutaneous SCID mice. Moreover, LCN2 was shown to promote VEGF expression which contributed to enhanced tumor vascularity (Leung et al., 2012). Thus, results from these studies have offered contradictory findings in the search for understanding LCN2's role in PDAC.

Hypothesis

Given the amounting evidence linking LCN2 to inflammation and PDAC and the lack of a clear biological role for LCN2 in this disease, we investigated if LCN2 depletion promoted tumor growth in a syngeneic orthotopic mouse model of PDAC. Previous studies for LCN2 in PDAC have made use of xenograft mouse models with compromised immune systems. Because of the important role inflammation plays in the development of PDAC and the fact that LCN2 may function as a cytokine, we

decided that studies in a syngeneic orthotopic model would more appropriately demonstrate tumor-host interactions. We hypothesized that LCN2 depletion would lead to decreased tumor growth along with a reduced immune response. To test this hypothesis, we implanted murine PDAC cells that naturally expressLCN2 in the pancreas of wild-type mice with genetic deletion of LCN2 expression. We then monitored tumor growth and assessed survival.

To obtain a better understanding of the role of LCN2 in the PDAC microenvironment, we examined the effect of LCN2 on hPSCs. While prior reports have already indicated the expression of LCN2 in PDAC cells, LCN2 expression and effects on hPSCs has not been reported. We hypothesized that LCN2, acting as a potential cytokine, could induce the synthesis and secretion of pro-inflammatory mediators by hPSCs. Many studies have previously shown the ability of cytokines to function in a paracrine and autocrine fashion in the crosstalk between PDAC cells and hPSCs. Thus, we tested our hypothesis by treating hPSCs with LCN2, knocking down its specific receptor SLC22A27 and assessing if iron was involved in LCN2mediated effects. Together, this work addressed a mechanism and biological function by which LCN2 could promote the establishment of a pro-tumorigenic microenvironment in PDAC.

Chapter 2: Materials and Methods

Cell Culture

All cell lines were cultured at 37 °C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and L-glutamine supplemented with 10% v/v fetal bovine serum (FBS) were routinely tested for mycoplasma, and found to be negative. BXPC3, HPAC, CAPAN2, MIAPACA2 and MPANC96 were obtained from American Type Culture Collection (ATCC). CAPAN1 cells were kindly provided by Drs. Collisson, Gray and McMahon (University of California and Lawrence Livermore Laboratory, San Francisco). Human pancreatic ductal epithelial (HPDEs) and Human pancreatic nestin-expressing cells (HPNEs) were obtained from Dr. Timothy Eberlein (St. Louis, MO) and Dr. Tsao (Ontario Cancer Institute, Toronto, ON, Canada) (Campbell et al., 2007; Lee et al., 2003; Qian et al., 2005). Human pancreatic stellate cells (HPSCs) were obtained from patient tissue by the outgrowth method and immortalized using lentiviral vectors with human telomerase (hTERT) or SV40 large T antigen (TAg) (Hwang et al., 2008). KPC cells were derived from a pancreatic tumor from a PDX-1-Cre, LSL-Kras^{G12D}, LSL-Trp53^{-/-} (KPC) genetically engineered mouse model (GEMM) and transfected with enhanced firefly luciferase (Ma et al., 2013; Olive et al., 2009).

Orthotopic model of pancreatic cancer

All animal experiments were reviewed and approved by the MD Anderson Institutional Animal Care and Use Committee. All animals used were C57BL/6 strain mice. LCN2 knockout (LCN2^{-/-}) animals were kindly provided by Dr. Ralph Arlinghaus (MD Anderson Cancer Center) (Flo et al., 2004). KPC-LUC cells in single suspension were prepared and mixed in HBSS and 20% Matrigel (BD Biosciences, Bedford, MA, USA) All surgeries were performed in a sterile environment under anesthesia. After 1-cm longitudinal skin incision made on the left upper axillary region of the abdomen of the mouse, the peritoneum was opened. The pancreatic capsule was cut and a suspension of 0.5x106 KPC-LUC cells in a total volume of 50 µL was carefully implanted via a 27 gauge needle into the pancreas. The pancreas was then put back into the abdominal cavity gently and the surgical opening was closed. Tumor growth was visualized by injecting mice with D-Luciferin (150 µg/mouse; Caliper Life Sciences) and measuring bioluminescence using the IVIS imaging system (Caliper Life Sciences) (Ma et al., 2013). All animals (LCN2^{+/+} n=20 and LCN2^{-/-} n=17) were imaged on a weekly basis until sacrifice was required.

Immunohistochemistry

Pancreatic tissue from each animal was fixed with 10% formaldehyde in phosphate-buffered saline overnight. After incubation, samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Further immunohistochemical staining was performed in pancreatic paraffin sections using the antibodies and conditions listed in **Table 1**. Briefly, after deparaffinization using the standard procedure of two washes with Xylene, 100% Ethanol, 95% Ethanol, and one wash of 80% Ethanol, antigen retrieval (1 x DAKO target retrieval solution, DAKO, Carpinetria, CA) was performed in a steamer for 20 minutes at 98°C. Then, endogenous blocking with H₂O₂ was done followed by washing and blocking, and primary antibodies were applied overnight (4°C). After washing with PBS and PBS containing 0.05% Tween20, sections were incubated with the appropriate secondary

antibodies. Positive labeling was detected by exposing sections to DAB+Substrate system (DAKO) and finally counterstaining with hematoxylin solution.

Collagen deposition was detected using a Picro-Sirius Red Stain Kit (Abcam, ab150681) in paraffin embedded pancreatic tissue. Briefly, the sections were deparaffinized and hydrated in distilled water. Adequate Picro-Sirius Red Solution was applied to tissue sections and incubated for 60 minutes. Next, the sections were rinsed quickly in 2 changes of Acetic Acid Solution followed by rinsing in absolute alcohol.

| Primary antibody | Source | Dilution | Secondary antibody, |
|-------------------|-------------------|----------|--------------------------------|
| | | | source |
| Rat anti F4/80 | e-Bioscience, 14- | 1:250 | Vectastatin Elite ABC Kit (rat |
| | 4801 | | IgG), Vector Laboratories, |
| | | | PK-6104 |
| Rabbit Anti-α-SMA | Abcam, ab5694 | 1:200 | Vectastatin Elite ABC Kit |
| | | | (rabbit IgG), Vector |
| | | | Laboratories, PK-6101 |
| Biotin rat-anti | BD Pharmingen, | 1:500 | NA |
| CD45 | 553078 | | |
| Rabbit anti Ki-67 | Sigma Aldrich, | 1:200 | Vectastatin Elite ABC Kit |
| | AB9260 | | (rabbit IgG), Vector |
| | | | Laboratories, PK-6101 |

Table 1: List of antibodies used for immunohistochemical staining of mouse pancreas

RNA isolation and quantitative RT-PCR

RNA was isolated from cells and mouse pancreas using TRIzol® Reagent (Life

Technologies). Briefly, cells were scrapped after adding TRIzol® Reagent while

snap-frozen pancreas tissue was homogenized for twenty seconds. Chloroform was added to each sample and centrifuged at 12,000 g for fifteen minutes. The upper aqueous phase containing RNA was collected and washed with 100% isopropanol and then with 75% ethanol. RNA was resuspended in RNase-free water and RNA concentration was measured using a NANODROP 1000 spectrophotometer (Thermo Scientific). cDNA was prepared by reverse transcription using the Verso cDNA Synthesis Kit (Thermo Scientific). Gene expression was determined by quantitative PCR using the primer sequences listed in **Table 2**.

| Gene | Forward Sequence | Reverse Sequence |
|-----------|------------------------------|------------------------------|
| hLCN2 | GAAGTGTGACTACTGGATCAGGA | ACCACTCGGACGAGGTAACT |
| hSLC22A17 | GCTGCCTGCCGGTGCTCTTCGTG | GCTGGCGGCGCTGGCTGCTA |
| hLRP2 | TCATGGATGGCAGCAACCGTAAAG | AAACCGAGAGTCAACCCAGTAAAC |
| mLCN2 | TGGCCCTGAGTGTCATGTG | CTCTTGTAGCTCATAGATGGTGC |
| mSLC22A17 | TTTGGCCGTCGTGGGATTG | GGCGCATCAGGTAGACACC |
| mLRP2 | AAAATGGAAACGGGGTGACTT | GGCTGCATACATTGGGTTTTCA |
| 18S | GAGCGGTCGGCGTCCCCCAACTT C | GCGCGTGCAGCCCCGGACATCTA A |
| hIL6 | ACTCACCTCTTCAGAACGAATTG | CCATCTTTGGAAGGTTCAGGTTG |
| mIL6 | TAGTCCTTCCTACCCCAATTTCC | TTGGTCCTTAGCCACTCCTTC |
| hMCP1 | GAGGAACCGAGAGGCTGAGACTA A | GGGGAATGAAGGTGGCTGCTAT |
| mMCP1 | TTAAAAACCTGGATCGGAACCAA | GCATTAGCTTCAGATTTACGGGT |

| hIL8 | CTAGGACAAGAGCCAGGAAGAA | GGGTGGAAAGGTTTGGAGTATG |
|--------------|--------------------------|--------------------------|
| hIL1B | ATGATGGCTTATTACAGTGGCAA | GTCGGAGATTCGTAGCTGGA |
| mIL1B | GCAACTGTTCCTGAACTCAACT | ATCTTTTGGGGTCCGTCAACT |
| hMMP1 | GGCCCACAAACCCCAAAAG | ATCTCTGTCGGCAAATTCGTAAGC |
| hMMP3 | GACCCAAATGCAAAGAAAGTGACA | AAAAATGACCGGCAAGATACAGAT |
| hMMP9 | TGTACCGCTATGGTTACACTCG | GGCAGGGACAGTTGCTTCT |
| mMMP9 | GCAGAGGCATACTTGTACCG | TGATGTTATGATGGTCCCACTTG |
| ha-SMA | TTCAATGTCCCAGCCATGTA | GGAAACGTTCATTTCCGATG |
| hC5 | ACAGTCATAGAGTCTACAGGTGG | CCAACTGGTCAAGCGAATCTT |
| hFTH1 | TCCTACGTTTACCTGTCCATGT | GTTTGTGCAGTTCCAGTAGTGA |
| mFTH1 | CAAGTGCGCCAGAACTACCA | GCCACATCATCTCGGTCAAAA |
| hTFR1 | GTGTGCAGTGTCGGAGCAT | CATCGGATGGAATGACGCTTT |
| mTFR1 | ATGCCGACAATAACATGAAGGC | ACACGCTTACAATAGCCCAGG |
| hFerroportin | TGGATGGGTTCTCACTTCCTG | GTCAATCCTTCGTATTGTGGCAT |

Table 2: List of human (h) and mouse (m) primer sequences used in studyImmunoblotting

Lysates from cultured cells and mouse pancreatic tissue were prepared using RIPA buffer (50 mM Tris-HCI [pH 7.4], 150mM NaCl, 1% Triton X-100, 1mM EDTA, 0.5% deoxycholate), 1mM sodium orthovanadate, 1mM sodium fluoride, 1 mM

phenylmethylsulfonyl fluoride and a cocktail of protease inhibitors (Roche, Indianapolis, IN). Protein concentrations were determined using the Protein Assay Dye Reagent (Bio-Rad, Hercules, CA). Conditioned media was prepared by collecting media from treated or untreated cells in 10mm plates and concentrating the media ten-fold by using Ultra-15 centrifugal filter units (EMD Millipore, Billerica, MA). Samples were prepared to have equal amounts of protein per volume and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane, blocked with 5% milk in PBST for one hour and incubated with primary antibodies (**Table 3**) overnight at 4 °C. Membranes were then incubated with corresponding secondary antibodies labeled with IRDye® near-infrared fluorescent dyes for one hour. Protein signal was detected by visualizing near-infrared fluorescent signals with the Odyssey® imaging system (Li-Cor, Lincoln, NE).

| Primary antibody | Source | Dilution | Secondary antibody, |
|-------------------|-------------------|----------|----------------------------|
| | | | source |
| Monoclonal Rat | R&D Systems | 1:1000 | IRDye® 800CW Goat anti- |
| anti LCN2 | (Minneapolis, MN) | | Rat, 1:10,000, (Li-Cor, |
| | | | Lincoln, NE) |
| Rabbit polyclonal | Thermo Scientific | 1:1000 | IRDye® 650 Goat anti- |
| anti-SLC22A17 | | | Rabbit, 1:10,000, (Li-Cor, |
| | | | Lincoln, NE) |
| Rabbit polyclonal | Abcam (Cambridge, | 1:1000 | IRDye® 650 Goat anti- |
| anti-C5 | MA) | | Rabbit, 1:10,000, (Li-Cor, |
| | | | Lincoln, NE) |

Table 3: List of primary used in immunoblotting

Lentivirus-mediated SLC22A17 knockdown

Mission shRNA bacterial glycerol stock plasmids for SLC22A17 were obtained from Sigma Aldrich (St. Louis, MO) with the following sequence:

CCGGGCGATTCCTACAGCGAATGATCTCGAGATCATTCGCTGTAGGAATCGCT TTTTG. Non-targeting controls were used along with both shRNA plasmids. 2.3 μ g of the each respective plasmid was con-transfected with the envelope PMD2.G (2 μ g) and packaging PsPAX2 (4.7 μ g) plasmids into 293FT cells using Lipofectamine 2000 (Life Technologies). After an overnight incubation, fresh media containing 10% FBS was added to the cells. 24 and 48 hours later, virus-containing media was collected and filtered through 0.45 μ m filters. HPSCs were cultured in 6-well plates and incubated with 500 μ L of virus supernatant along with 4 μ g of polybrene for 24 hours. Stable knockdown cells were selected using 1 μ g/mL puromycin. SLC22A17 was also transiently knockdown using plasmids and siRNAs. siRNA transfection was performed using 12 μ L of HiPerfect transfection reagent (QIAGEN, Valencia, CA) and 50nM of the respective siRNA for 24 hours.

Recombinant human LCN2 and Deferoxamine treatments

HPSCs with endogenous or knockdown of SLC22A17 were plated at 2 x 10⁵ cells per well in 6-well plates or at 1 x 10⁶ in 10mm dishes. The next day, cells were washed twice with PBS and serum-starved overnight. Before treatment, cells were again washed twice with PBS. Recombinant human LCN2 (rhLCN2) (R&D Systems, Minneapolis, MN) was resuspended in 25mM MES and 150mM NaCl at pH 6.5. Cells were treated with different concentrations of rhLCN2 at different time points in order to determine the optimal concentration and treatment time. Most experiments

were conducted using 100 or 500 ng/mL rhLCN2 for 24 to 48 hours. Deferoxamine (DFO) (Sigma Aldrich, St. Louis, MO) was diluted in water and was added to cells at a concentration of 20µM for 24 to 48 hours.

Iron colorimetric assay

The BioVision colorimetric iron assay kit (BioVision, Milpitas, CA) was used to measure ferrous iron (Fe²⁺). Briefly, cells were lysed in Iron Assay Buffer and centrifuged at 12,000 g for ten minutes. 30µg of cell lysate with a total volume of 50µL was added to a 96-well plate along with 55µL of Iron Assay Buffer. A standard curve of 0, 2, 4, 6, 8, and 10 nMol was generated using the Iron Standard provided. Samples and iron standards were incubated at room temperature for thirty minutes. 100µL of Iron Probe was then added to each well and incubated at room temperature at 593nm in a microplate reader.

Cytokine protein array

Concentrated conditioned media (200 uL) was used to detect the expression of cytokines using the Proteome Profiler human cytokine array panel A (R&D Systems, Minneapolis, MN). Conditioned media was diluted in array buffers 4 and 5 and then incubated with 15uL of the biotinylated cytokine antibody cocktail for one hour. Each sample was added to a nitrocellulose membrane spotted in duplicate with selected capture antibodies and incubated overnight at 4 °C. The following day, each membrane was washed and incubated with Streptavidin-HRP for thirty minutes at room temperature. After a second round of washing, membranes were incubated with chemiluminescent reagents and exposed to X-ray film for ten minutes. Positive

signals were analyzed with the ImageJ software protein array analyzer plug-in (created by Gilles Carpentier, Faculté des Sciences et Technologies, Université Paris) to obtain the pixel density for each spot on the array.

LCN2 ELISA

Human and mouse LCN2 Quantikine ELISA kits (R&D Systems, Minneapolis, MN) were used to measure LCN2 levels in human and mouse serum samples. Briefly, 50µL of serum diluted in Assay Diluent RD1-34, as well as LCN2 standards, were added to a 96-well microplate pre-coated with LCN2 monoclonal antibody and incubated for two hours at room temperature. After a series of four washes, an enzyme-linked monoclonal antibody against LCN2 was added to each well and incubated for two hours at room temperature. After washing, 100µL of substrate solution was added to each well and incubated for thirty minutes while protected from light. 100µL of Stop solution was then added and the optical density was determined using a microplate reader se to 450nm.

Statistics

Statistical analysis was performed using the Prism 5 software program (GraphPad Software San Diego, CA). Results are expressed as the mean ± standard error of the mean. A *t*-test or one-way analysis of variance was performed on sex-matched littermates. The percentage of survival was analyzed using the Mantel-Cox method. The median survival in days was presented. P levels less the 0.05 were considered significant.

Chapter 3:

Lipocalin 2 depletion attenuates ECM remodeling, immune cell infiltration and delays pancreas cancer growth with increased survival

Within the last two decades, many animal experimental models have been developed to reproduce and understand pancreatic cancer (Guerra and Barbacid, 2013; Herreros-Villanueva et al., 2012). Many of these models have focused on the genetic activation of mutant KRas oncogenes. In addition to knocked-in mutant kras, additional mutations and deletions of genes known to be altered in PDAC development such as *trp53*, *ink4a* and *smad4* have been explored. In particular, the PDX-1-Cre; LSL-*Kras^{G12D}*; LSL-*Trp53^{R172H/-}* (KPC) model of pancreas cancer has been well studied and shown to be clinically representative of human disease. These animals express both mutant KRas and Trp53 genes in tissue progenitor cells of the developing mouse pancreas which is driven by the pancreatic-specific promoter PDX-1. The pancreas of such animals are then able to develop the full range of pancreatic intraepithelial neoplasia observed in humans (Hingorani et al., 2005). Specifically, PanIN-1 lesions have been observed early in four week old mice. With increasing age, these animals develop higher-grade PanINs, PDAC within two to three months and display a mean survival of five months. Similarly, animals with complete deletion of Trp53 generate the same lesions with a shortened median survival of three months (Guerra and Barbacid, 2013).

In addition to genetically engineered mouse models, tumor xenograft mouse models of PDAC have been commonly used in the last decade (Herreros-Villanueva et al., 2012). Some of these models rely on the injection of culture-grown human PDAC cells into mice or even the transplantation of a human tumor mass (Herreros-Villanueva et al., 2012). Appropriately, severely compromised immunodeficient mice (SCID) (with affected B and T lymphocytes) or athymic nude animals (unable to

generate mature T lymphocytes) are used in such studies. In addition to having the option to use variable human tumor sources and animals, the location of the implant is also variable. The subcutaneous xenograft model allows for the implantation of tumor cells or mass in between the dermis and the underlying muscle of a flank, the back or footpad of an animal. An advantage of this model is the ability to visually assess tumor growth or response to therapy over time (Herreros-Villanueva et al., 2012). This model is widely used as a preclinical model to test response to certain therapies which can indicate new potential therapeutic options. However, one of the biggest disadvantage to using this model is the irrelevant subcutaneous tumor microenvironment. This has been suggested as a reason for which drug treatments that have been shown to be successful in this model are not effective for treating human disease.

Orthotopic xenograft models on the other hand, allow for the study of appropriate organ specific biology. In this model, tumor cells are directly injected into the pancreas of a mouse and therefore allows for gene expression studies in the presence of relevant tumor-host interactions. Disadvantages in using this model includes a technically challenging procedure, longer recovery times and lower throughput imaging methods. Despite these challenges, the orthotopic model is preferred due to its clinical relevance.

Although many prefer the use of GEMMs, the use of syngeneic (allograft) orthotopic models is becoming widespread. The syngeneic model consists of tumor tissue derived from and implanted into animals with the same strain background. The most significant advantage to using this model is that the host immune system

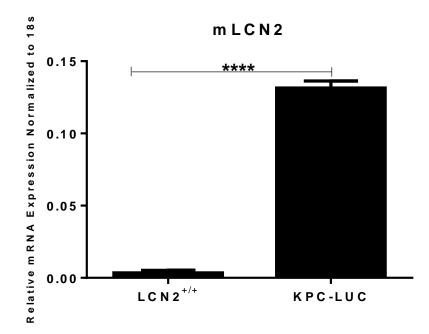
is normal and it may much closely represent relevant tumor-host interactions compared to any other xenograft models. Further, because of their intact immune system, syngeneic models are becoming the "go to" reliable model for immunotherapy studies.

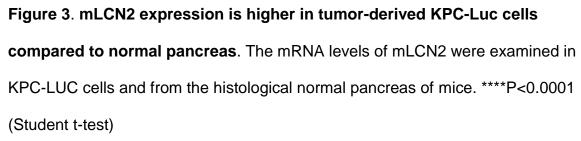
Previous in vivo studies have reported contradicting roles of LCN2 in PDAC. The first study made use of an orthotopic xenograft model in nude mice in which PDAC cells modified to overexpress LCN2 were injected directly into the mouse pancreas (Tong et al., 2008). Tumors obtained from LCN2 overexpressing cells were found to be significantly smaller than in the control animals. In the most recent study, PDAC cells overexpressing LCN2 or with LCN2 knockdown were injected subcutaneously into SCID mice. In this model, tumors from LCN2 overexpressing cells were markedly larger than tumors developed from cells with silenced LCN2 expression. These contradicting findings highlight the need of further studies in order to understand the role of LCN2 in PDAC.

LCN2 depletion delays tumor growth in a syngeneic orthotopic PDAC model

Given the important crosstalk between tumor cells and the tumor microenvironment in PDAC, in vivo studies were performed in a syngeneic orthotopic model. LCN2 has been previously shown to be present in significant higher levels in both the serum and the pancreas of KPC mice with high-grade neoplastic lesions and cancer compared to normal mice (Slater et al., 2013). Tumor cells derived from a PDX-1-Cre; LSL-Kras^{G12D}; LSL-Trp53^{-/-} (KPC) mouse with PDAC were first analyzed for LCN2 expression. KPC cells derived from tumor

demonstrated significantly higher mRNA levels of LCN2 compared to normal pancreas. (Figure 3)





Half a million KPC-LUC cells were directly injected into the pancreas of C57BL/6 mice with either wild type LCN2 (LCN2^{+/+}) or whole-body LCN2 silenced (LCN2^{-/-}) expression. Tumor growth was visualized by injecting mice with D-Luciferin (150 µg/mouse; Caliper Life Sciences) and measuring bioluminescence using the IVIS imaging system (Caliper Life Sciences) (Ma et al., 2013). All animals (LCN2^{+/+} n=20 and LCN2^{-/-} n=17) were imaged on a weekly basis until sacrifice was required. We first assessed the presence of KPC-LUC cells by imaging all animals the day

after tumor cell implantation. The pancreas of each mice revealed a bioluminescent signal indicative of the presence of KPC-LUC cells (**Figure 4**). In fact, there seemed to be no difference in the bioluminescent signal given off by LCN2^{+/+} mice compared to LCN2^{-/-} animals at one week. This indicated that similar amounts of KPC-LUC cells were injected and present in both groups of animals.

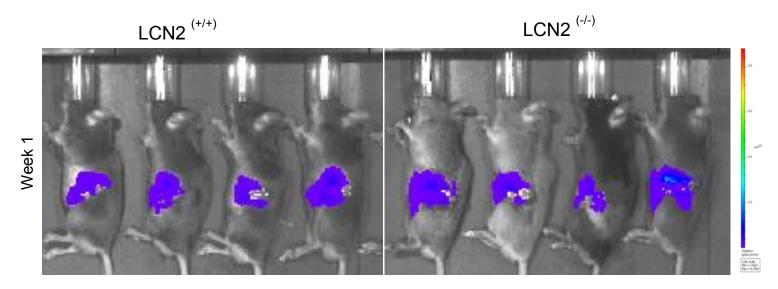
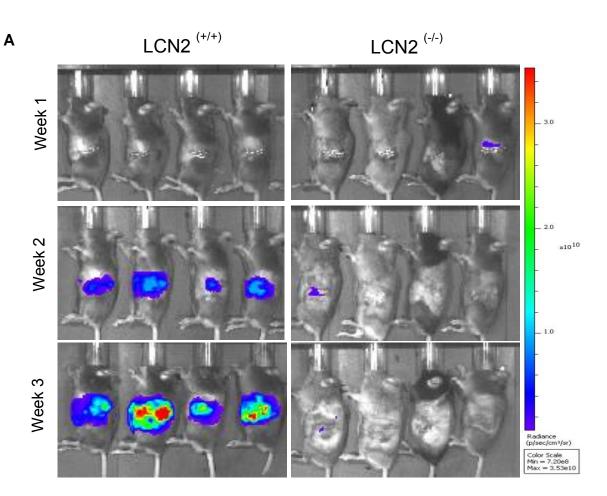


Figure 4. **KPC-Luc cells are present in mouse pancreas after injection**. The bioluminescent signal detected via the IVIS imaging system indicates the presence of tumor cells in the pancreas of mice after surgery.

By week three after surgery, most LCN2^{+/+} animals had developed large tumors. The size of the tumors was visualized by a strong bioluminescent signal as seen in **Figure 5a**. At this time point, it was quite obvious that LCN2 depletion delayed tumor growth as observed by the small amount of signal detected in the LCN2^{-/-} mice. Of note, the signal previously seen in week one appears to be almost absent due to the normalization of all images at the same scale. Further, the radiance given off by each signal was measured using the Living Image® software. Quantification of the bioluminescent signal indicated that LCN2^{+/+} animals developed a significantly higher fold change in average radiance compared to LCN2^{-/-} mice starting at three weeks post-surgery as seen in **Figure 5b**.



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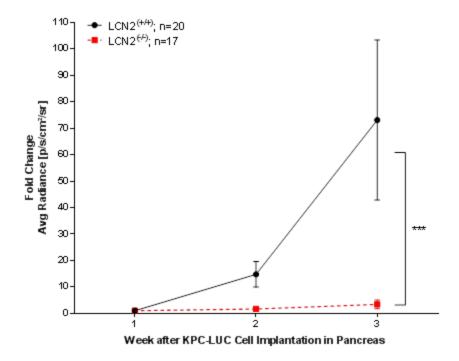
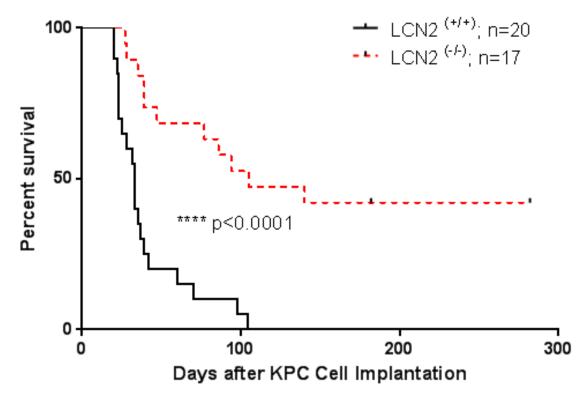


Figure 5. LCN2 depleted animals display delayed tumor growth. After injecting KPC-LUC cells to either LCN2^{+/+} or LCN2^{-/-} animals, luciferase signal representing tumor growth was measured every week. (A) By the third week, LCN2^{+/+} animals demonstrated very high signal while LCN2^{-/-} animals presented significantly lower signal. (B) Living Image® software was used to quantify the average radiance given off by each animal. The graph demonstrates that significance in signal was achieved at week 3. ***P<0.001 (student t test)

LCN2 depletion increases survival

Continued monitoring of these animals allowed us to notice that LCN2^{+/+} mice developed bigger tumors at a faster rate than the LCN2^{-/-} animals. In fact, the survival analysis of these experiments revealed that the median survival of LCN2^{-/-} animals was over three-fold higher than LCN2^{+/+} mice, 105 days versus 33 days respectively (Figure 6). Further, all of the LCN2^{+/+} mice had to be sacrificed due to morbidity by 104 days after KPC-LUC cell implantation while about fifty percent of LCN2^{-/-} animals were still alive at that time. In fact, several of the LCN2^{-/-} animals were sacrificed without signs of morbidity at 282 days after KPC-LUC cell implantation in order to analyze the pancreas of this group of animals. The pancreas of these animals showed no sign of bioluminescence (Figure 7c) revealing that KPC-LUC cells were not present in the pancreas after 282 days. In contrast, the small number of LCN2^{-/-} mice that did develop tumor showed signs of KPC-LUC cell presence in some areas of their pancreas. (Figure 7b). Moreover, the pancreas from most LCN2^{+/+} animals showed very high amounts of bioluminescence in the whole pancreas tumor (Figure 7a).





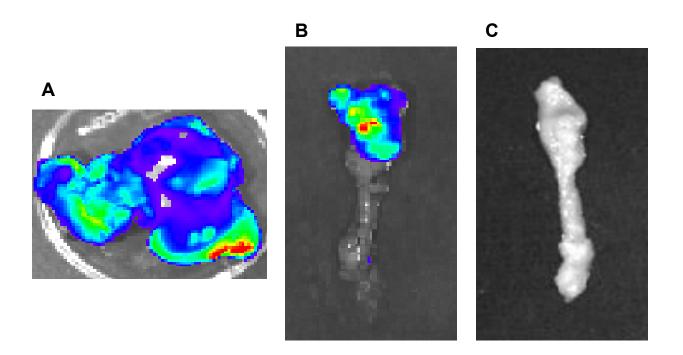
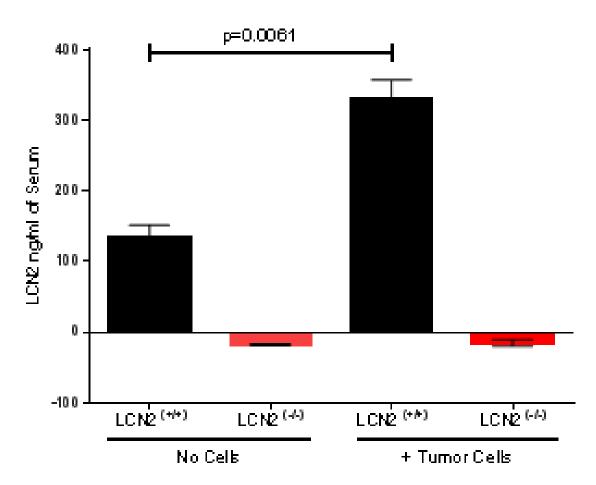
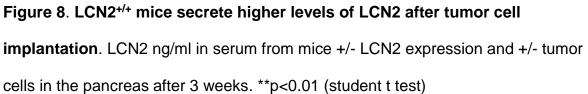


Figure 7. KPC-LUC cells do not survive in all LCN2^{-/-} **mice.** (A) Pancreas resected from a LCN2^{+/+} animal 37 days after tumor cell implantation. (B) Pancreas from a LCN2^{-/-} animal showing some tumor growth 86 days after tumor cell implantation. (C) The pancreas from a LCN2^{-/-} animal showing no sign of KPC-LUC cell presence 282 days after implantation.

As LCN2 is a secreted molecule and is detected in serum, we examined if serum LCN2 levels changed in mice after tumor cell implantation. We observed that serum LCN2 levels significantly increased in LCN2^{+/+} mice after KPC tumor cell injection compared to control animals without any tumor cells. On the other hand, LCN2 serum level changes were undetectable in LCN2^{-/-} animals (**Figure 8**). The fact that LCN2 serum levels did not significantly increase in LCN2^{-/-} mice also point out the lack of KPC-LUC cell presence in most of these animals at three weeks after implantation.





LCN2 depletion attenuates ECM remodeling

Upon closer examination of the pancreas of all animals, we observed that most LCN2^{-/-} mice had little to no ECM remodeling compared to LCN2^{+/+}animals. (**Figure 9**) The Hematoxylin and eosin (H&E) stain of the LCN2^{+/+}animals, shown in the first panel in **Figure 9a**, showed the extensive presence of tumor with just a small amount of adjacent seemingly normal pancreas. In contrast, we observed a mostly normal pancreas with unperturbed pancreatic acinar cells in LCN2^{-/-} mice as shown in the first panel in **Figure 9b**. Picrosirius red staining of the pancreas of these animals (second panel in **Figure 9**) revealed an extensive remodeling of the ECM in LCN2^{+/+}mice demonstrated by an increase in the presence of collagen networks. Specifically, this staining showed expression of Collagens I and III which was not visible in the pancreas of LCN2^{-/-} animals. Additionally, staining for α -SMA was positive for LCN2^{+/+}animals compared to LCN2^{-/-}mice (third panel in **Figure 10**). This observation indicated the presence and activation of mouse pancreatic stellate cells which are key regulators of the pancreas stroma.

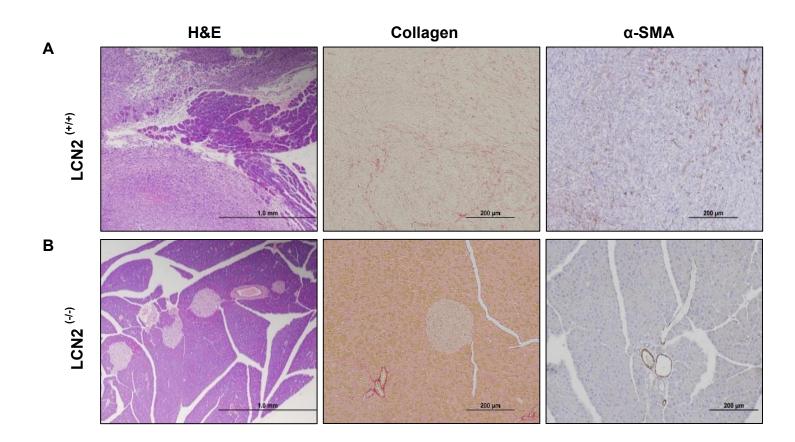
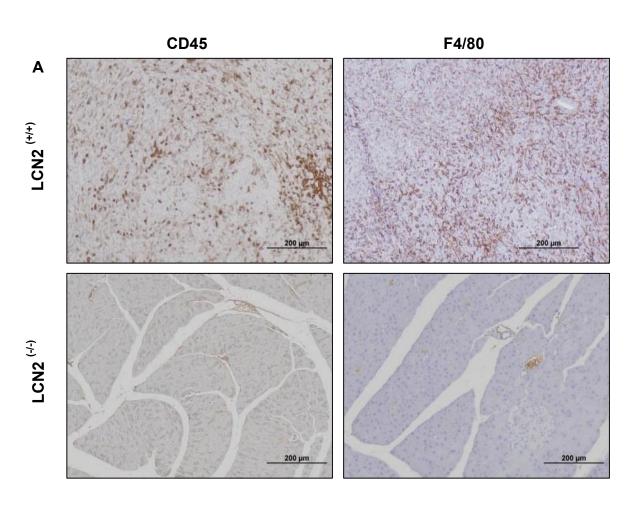


Figure 9. **LCN2 depletion attenuates ECM remodeling**. H&E staining, Picrosirius red staining (indicating presence of Collagens I and III) and staining for α-SMA (indicating presence of mouse pancreatic stellate cells) for (A) LCN2 wild type and (B) LCN2 null animals injected with KPC-LUC cells.

LCN2 depletion attenuates the inflammatory response in mouse pancreas

LCN2 has been previously shown to play a role in innate immunity due to its upregulation after bacterial infection and secretion by neutrophils and macrophages (Meheus et al., 1993; Nielsen et al., 1996). In addition, inflammation is also responsible for driving the expression of LCN2 (Rodvold et al., 2012). Proinflammatory cytokines such as IL-1B, TNF-a and IL-17 are capable of inducing LCN2 expression (Li and Chan, 2011). A study examining the effect of LCN2 depletion in an obesity mouse model revealed that LCN2 deficiency attenuated inflammation in adipose tissue (Law et al., 2010). Given the amounting evidence linking LCN2 to inflammation, we decided to assess the effect of LCN2 depletion in our syngeneic orthotopic PDAC model. We performed IHC staining for the leukocyte marker CD45 and the macrophage maker F4/80 (Figure 10a). Results from the staining demonstrated a significant infiltration of inflammatory cells in the pancreas of LCN2^{+/+}animals. However, in LCN2^{-/-}mice, we observed little to no presence of these inflammatory cells. Further, LCN2^{+/+}animals were found to secrete significantly higher levels of the macrophage-colony stimulating factor (M-CSF) (Figure 10b). M-CSF is a cytokine that influences the differentiation of hematopoietic cells into macrophages. In addition to secreting M-CSF, analysis of bulk pancreas tissue revealed that LCN2+/+animals synthesized significantly higher levels of the proinflammatory cytokines IL-6, IL-1B and MCP1 Figure 11. Thus, these results suggest a role for LCN2 in promoting an inflammatory response in the tumor microenvironment.



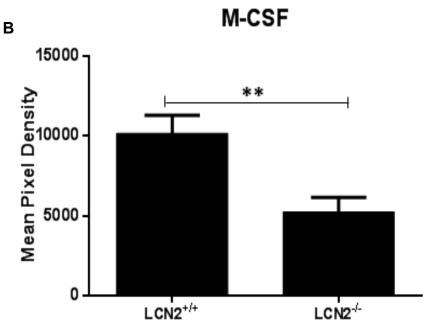


Figure 10. LCN2 depletion attenuates immune cell infiltration. (A) IHC staining for the leukocyte marker CD45 and the macrophage maker F4/80 revealed an immune cell infiltration in LCN2^{+/+}mice. (B) The cytokine M-CSF responsible for hematopoietic cell differentiation to macrophages is significantly upregulated in LCN2^{+/+}mice. **p=<0.01 (student t-test)

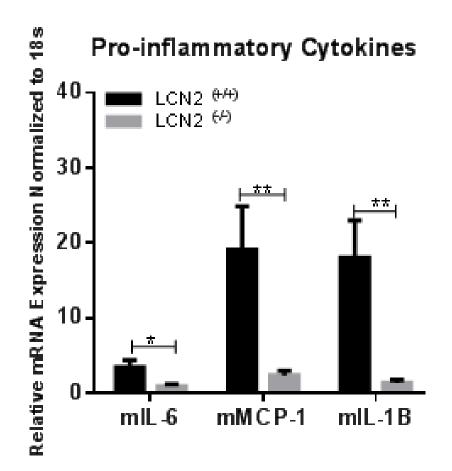
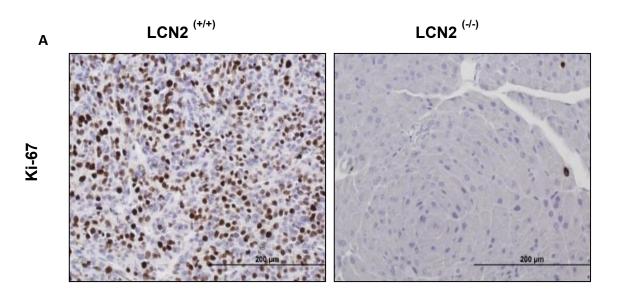


Figure 11. LCN2^{+/+} mice synthesize high amounts of pro-inflammatory

cytokines. IL-6, MCP-1 and IL-1B mRNA levels were highly upregulated in LCN2^{+/+} animals compared to in LCN2^{-/-}mice. *p=<0.05, **p=<0.01 (student t-test)

Ki-67 is an important biological marker indicative of cellular proliferation (Schluter et al., 1993). This molecular marker is commonly used in cancer studies as an index to characterize proliferating tumor cells (Ammendola et al., 2015; Kalogeraki et al., 1997). In fact, Ki-67 has been previously used as an index of PDAC growth in both human and mouse tissue samples (Ammendola et al., 2015; Gardovskis et al., 2012; Yamamoto et al., 2004). We examined cellular proliferation by staining LCN2^{+/+} and LCN2^{-/-} mouse tissue for Ki-67 (**Figure 12a**). The resulting staining patterns clearly indicated a vast amount of proliferating cells in LCN2^{+/+} animals. In contrast, little to no actively growing cells were visible in LCN2^{-/-} mice.

In addition to examining Ki-67 as an index of tumor growth, we further analyzed the serum of these animals for other putative PDAC biomarkers including sICAM-1, IGFBP-1 and TIMP-1 (Brand et al., 2011; Slater et al., 2013; Wolpin et al., 2007). (**Figure 12b**) shows that LCN2^{+/+} animals secreted significantly higher quantities of all three molecules compared to LCN2^{-/-} mice. These data suggest the potential of LCN2 to be used as a putative prognostic marker for PDAC in conjunction with these other molecules.



PDAC markers

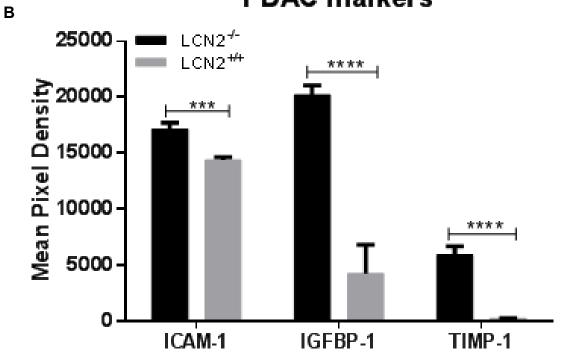


Figure 12. LCN2 depletion decreases the expression of PDAC tumor markers.

(A) Ki-67, a commonly used marker for tumor cell proliferation, is highly expressed in the pancreas of LCN2^{+/+} mice. (B) The putative PDAC biomarkers sICAM-1, IGFBP-1 and TIMP-1 are secreted in significant higher levels in LCN2^{+/+} mice. ***p=<0.001, ****p=<0.0001 (student t-test)

Tumors from LCN2 depleted animals demonstrate attenuated stromal remodeling

While 7 out of 17 LCN2 null animals did not develop any tumors, the remaining 10 animals did develop tumors. When compared to LCN2^(+/+) animals, the LCN2 null tumors exhibited attenuated collagen, α -SMA, CD45, F4/80 and Ki67 staining (**Figure 13**). This again demonstrated that even though tumors grew, the presence of proliferative cells was diminished in animals lacking LCN2. Thus, these results suggest a role for LCN2 in delaying tumor growth and stromal remodeling in a mouse model of PDAC.

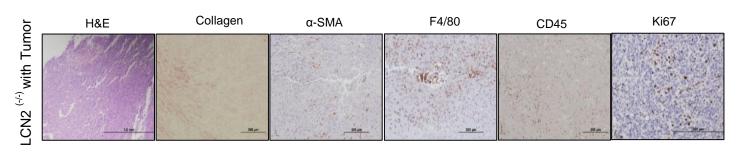


Figure 13. LCN2 depletion attenuates ECM remodeling, immune infiltration and tumor cell proliferation in LCN2 null mice with tumors. H&E staining, IHC for collagen, α -SMA, F4/80, CD45 and Ki67.LCN2 null animals injected with KPC-LUC cells that did develop tumors still maintained lower collagen, a-SMA expression, inflammatory cell infiltration and tumor cell growth compared to tumors from LCN2^(+/+) animals.

LCN2 null animals express lower levels of the LCN2-specific receptor SLC22A17 and iron responsive genes

SLC22A17 and LRP2 are the two known receptors that are able to bind and endocytose LCN2 (Chakraborty et al., 2012). While LRP2 is also able to bind a series of other ligands including the iron binding molecules transferrin and lactoferrin, SLC22A17 is LCN2 specific (Chakraborty et al., 2012). In our syngeneic study, LCN2^{-/-} animals showed a significant reduction in SLC22A17 mRNA expression in the pancreas (**Figure 14 A**). These results seem to suggest a previously unidentified role for SLC22A17 in PDAC malignancy. Moreover, this suggestion is further aided by the fact that high LCN2 and SLC22A17 expression has been correlated with malignancy in hepatocellular and endometrial cancer.

In addition, the pancreas of LCN2 depleted animals expressed lower levels of the iron responsive molecules ferritin (FTH1) and the transferrin receptor (TFR1) (**Figure 14 B**). Because FTH1 mRNA levels increase in response to higher iron levels, these results suggest a higher iron content in pancreas tissue after tumor cell implantation in animals with endogenous LCN2 expression. While TFR1 expression typically increases when iron levels decrease, in our study, TFR1 levels were significantly higher in animals with wild type LCN2 expression. TFR1 expression has been linked to a malignant cell presence in pancreas cancer (Ryschich et al., 2004). Thus, it is possible that the higher levels of TFR1 in LCN2^{+/+} mice is due to increased presence of malignant cells after tumor cell implantation.

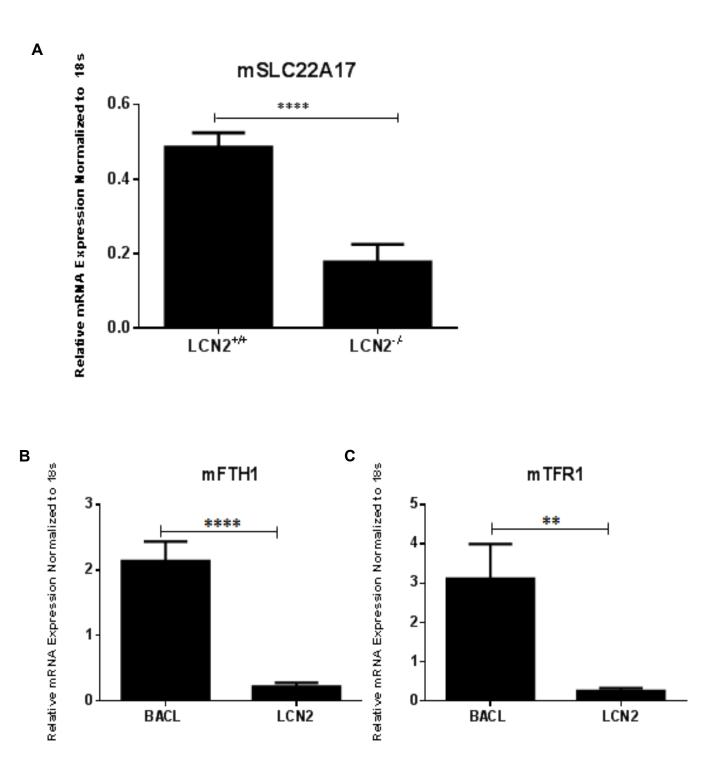


Figure 14. **LCN2 depleted animals express lower levels of the LCN2 receptor SLC22A17 and iron responsive molecules**. (A) mSLC22A17 mRNA levels are significantly higher in the bulk pancreas of LCN2^{+/+} mice. (B) A decrease in FTH1

expression indicates reduced amounts of iron levels. (C) Decreased TFR1 would usually indicate higher iron levels however, TFR1 has been shown to be a marker of malignancy in PDA. This data suggests increased malignancy in $LCN2^{(+/+)}$ animals. **p<0.01, ***p<0.001 (student t-test) Chapter 4:

Lipocalin 2 Stimulates a Receptor and Iron-Mediated Pro-Inflammatory Response in Human Pancreatic Stellate Cells

LCN2 was first isolated over two decades ago as a complex with MMP9 (Hraba-Renevey et al., 1989). Six years later, LCN2 was first detected in the plasma of healthy individuals (Axelsson et al., 1995). In the following years, it was found that LCN2 was synthesized and secreted by adult neutrophils, macrophages, adipocytes, breast, lung and liver among other normal adult tissue (Chakraborty et al., 2012; Cowland and Borregaard, 1997; Kjeldsen et al., 2000; Wang et al., 2007). The fact that LCN2 was expressed by immune cells and in tissues that are often exposed to microorganisms suggested a role for LCN2 in regulating the inflammatory response. This was confirmed as LCN2 was found to be able to combat bacterial infections by binding bacterial siderophores that have a high affinity to iron (Clifton et al., 2009; Yang et al., 2002). LCN2 thus deprived bacteria from essential iron sources leading to diminished bacterial growth and survival (Chakraborty et al., 2012). Moreover, it was revealed that LCN2 altered iron stores in mammalian cells by transporting iron through its receptors SLC22A17 and LRP2 (Devireddy et al., 2005). Given that iron has important roles in both immunity and cancer, these studies suggest potential mechanisms as to how LCN2 may modulate the inflammatory response in benign and malignant diseases.

Aberrant LCN2 expression has been reported in thyroid, ovarian, breast, endometrial, colorectal, gastric, hepatocellular, oesophageal, lung and pancreatic cancers (Bauer et al., 2008; Candido et al., 2014; Li and Chan, 2011; Lippi et al., 2014; Moniaux et al., 2008; Nielsen et al., 1996). Many studies have shown that LCN2 overexpression correlates with tumor size, aggressiveness and invasiveness implicating LCN2 as a marker of malignancy (Chakraborty et al., 2012; Lippi et al.,

2014). In pancreatic cancer, LCN2 was first found as a secreted molecule in eight human pancreatic cancer cell lines with weak expression in normal pancreatic tissue (Furutani et al., 1998). This was later confirmed in additional PDAC cell lines with varied levels of expression ranging from very weak to up to a thirty-fold increase compared to normal pancreatic tissue (Han et al., 2002). An immunohistochemical assessment revealed that all human PDAC tissue samples were positive for LCN2 expression while normal tissue stained very weakly for LCN2 (Moniaux et al., 2008). More importantly, this study demonstrated that LCN2 expression was detectable in pre-neoplastic lesions as early as PanIN-1. The majority of the staining patterns were localized to pancreatic duct or cancer cells with a small amount of staining in the tumor stroma. When we examined LCN2 expression using a microarray to measure RNA levels in 15 PDAC, 16 pancreatitis and 10 normal tissue samples, we found that LCN2 is significantly overexpressed in PDAC samples compared to both normal and pancreatitis samples (Figure 15a). Moreover, LCN2 was differentially regulated in PDAC compared to other lipocalin family members (Figure 15b). These results suggest the potential use of LCN2 as a potential PDAC biomarker. Others have continued to investigate LCN2 in PDAC with the main focus placed on the effect of overexpressing or silencing LCN2 expression in PDAC cell lines. While it is well known now that LCN2 expression in PDAC cell lines is variable, there have been no studies that indicate whether LCN2 is expressed in human pancreatic stellate cells (hPSCs).

hPSCs are active members of the pancreatic tumor stroma. Extensive studies have shown that hPSCs have roles in pancreatic inflammation, modulation of the

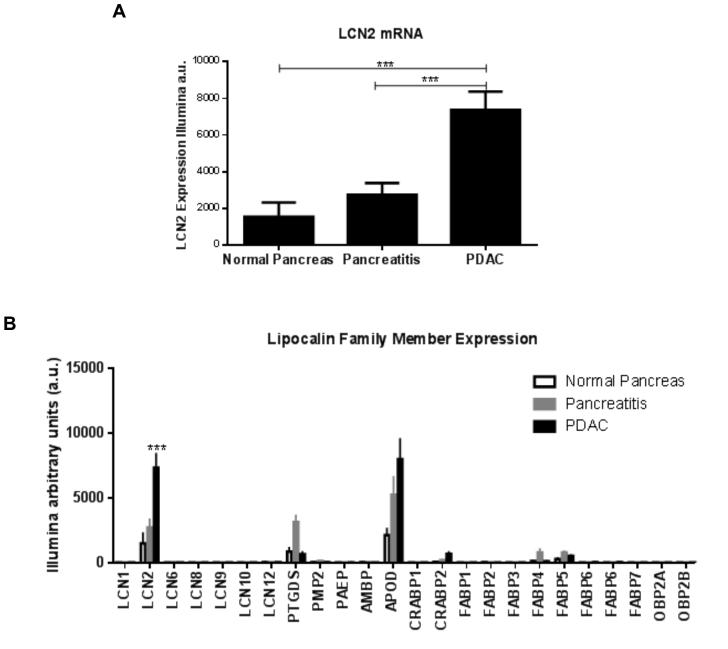


Figure 15. LCN2 is differentially upregulated in PDAC. PDAC (n=15), pancreatitis (n=16) and normal (n=10) tissue samples were analyzed using an Illumina microarray for LCN2 expression. (A-B) LCN2 is significantly and differentially upregulated in PDAC compared to other lipocalin members in pancreatitis and normal samples. ***p<0.001 (student t-test)

ECM and in PDAC (Duner et al., 2010; Liu and Du, 2015; Omary et al., 2007; Pandol and Edderkaoui, 2015). Specifically, hPSCs have the ability to synthesize and secrete cytokines, growth factors and ECM molecules that allow for the establishment of the tumor microenvironment. Examples of the molecules secreted by hPSCs include Collagen I, Laminin, Fibronectin, Matrix Metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), stromal-derived factor 1 (SDF-1), FGF, IGF-1, PDGF, Interleukins 1, 6, 8 and M-CP1 in addition to other molecules (Andoh et al., 2000b; Duner et al., 2010; Fokas et al., 2015; Omary et al., 2007). These factors are then able to exert a paracrine effect on neighboring cells which can alter the proliferation, migration and invasion potential of tumor cells. Additionally, cytokines and growth factors can also have an autocrine effect on hPSCs resulting in the chronic activation and secretion of these factors. Moreover, hPSCs have been shown to have the potential to regulate macrophages and Myeloid-derived suppressor cells (MDSCs) via secretion of IL-6 (Mace et al., 2013; Shi et al., 2014). Given the important role of hPSCs in the host-tumor crosstalk as well as its important role in balancing the pancreatic stromal architecture, we investigated the role of LCN2 in these cells and whether any resulting effects were mediated by the LCN2 receptors and iron.

Human Pancreatic Stellate Cells express LCN2 receptors but lack LCN2

In order to determine how LCN2 expression could impact hPSCs, we first assessed if LCN2 was synthesized and secreted by hPSCs. We isolated RNA and cDNA from a series of two immortalized "normal" pancreatic epithelial cells, six PDAC cells and two different immortalized hPSCs according to the methods

indicated in chapter two. Human pancreatic ductal epithelial cells (HPDEs) and human pancreatic nestin-expressing cells (HPNEs) were included in this analysis as a negative control for LCN2 expression as many groups have reported weak to no LCN2 presence in the normal pancreas. The six different PDAC cells included BXPC3, HPAC, CAPAN1, CAPAN2, MIAPACA2 and MPANC96 cells which have been previously examined for LCN2 expression (Leung et al., 2012; Tong et al., 2008). Similar to these previous studies, we confirmed that out of our group of cells, CAPAN1 and CAPAN2 had the highest levels of LCN2 expression followed by BXPC3, HPAC and MPANC96 and that Miapaca2 cells did not express LCN2 (Figure 16a). The corresponding levels of LCN2 secretion or lack thereof have been previously shown (Leung et al., 2012; Tong et al., 2008). More importantly, we reported for the first time that neither hPSC line expressed any LCN2 mRNA levels. Moreover, upon examining cell supernatants and using recombinant human LCN2 (rhLCN2) as a positive control, we further confirmed that hPSCs do not secrete LCN2 (Figure 16b).

SLC22A17 was first identified as an LCN2 receptor after being isolated from a murine cell derived cDNA library (Devireddy et al., 2005). The clone identified actually matched a protein called brain type organic cation transporter (BOCT) suggesting a physiological role in the brain. Although the OCT/SLC22 family members have been shown to be able to transport various organic cations, anions and zwitterions, a rat BOCT/SLC22A17 was unable to transport any of the typical SLC22 substrates (Bennett et al., 2011). Nevertheless, SLC22A17 has been reported to bind and mediate the cellular uptake of LCN2 (Cabedo Martinez et al.,

2016; Devireddy et al., 2005; Fang et al., 2007). Megalin, LRP2, has also been shown to be a receptor for LCN2 although it also binds numerous structurally unrelated molecules including aprotinin, vitamin D3 and lactoferrine (Hvidberg et al., 2005; Miyamoto et al., 2011a). Upon examining the expression of these two LCN2 receptors, we observed that both hPSC lines expressed both SLC22A17 and LRP2 (**Figure 15c-d**). Moreover, both of these cell lines expressed significantly higher levels of both receptors compared to PDAC cells and normal epithelial pancreatic cells. To the best of our knowledge, we are the first group to investigate and report the presence of these cell membrane receptors on hPSCs and on pancreatic normal and cancer cells.

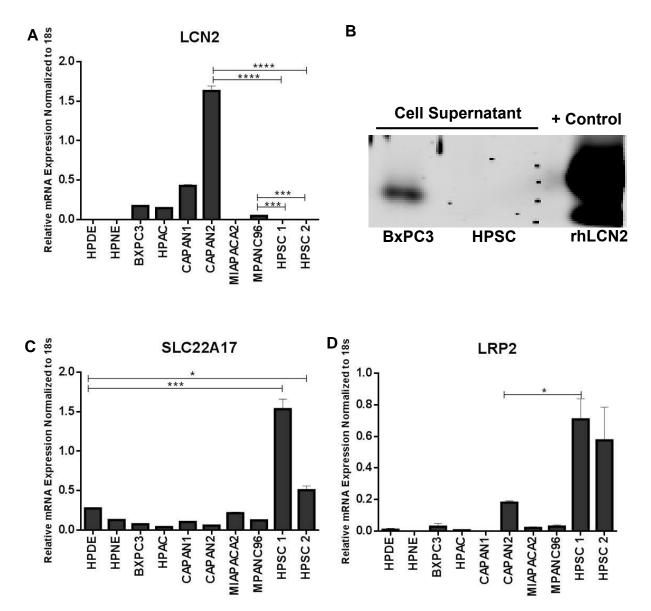


Figure 16. Human Pancreatic Stellate Cells (HPSCs) express LCN2 receptors SLC22A17 while lacking LCN2 expression. (A) LCN2 mRNA levels were examined for human pancreatic immortalized normal epithelial cells (HPDE, HPNE), PDAC cell lines (BXPC3, HPAC, CAPAN 1&2, MIAPCA2, MPANC69) and two immortalized human pancreatic stellate cells (HPSC1 &2) (B) Western blot of cell supernatants from BXPC3 confirm LCN2 secretion while HPSC do not secrete LCN2 (C) HPSCs express the LCN2-specific receptor SLC22A17 (D) HPSCs express the

second known LCN2 receptor LRP2. *p<0.05,***p<0.001, ****p<0.0001 (student t-test)

Ectopic rhLCN2 induces the expression of a pro-inflammatory cytokine and an iron responsive molecules

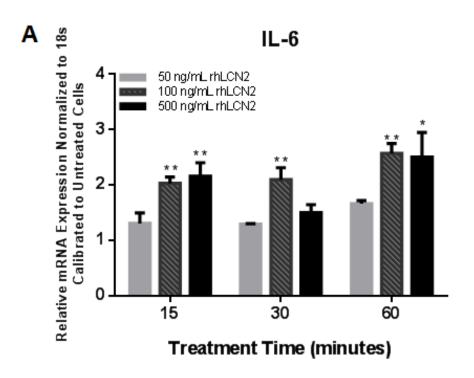
The fact that PDAC cells and inflammatory cells secrete LCN2 and hPSCs express LCN2 receptors led us to ask whether LCN2 had any effect on the expression of pro-inflammatory molecules. Interleukin-6 (IL-6) is a well-studied proinflammatory cytokine that has been reported to be upregulated during pancreatitis in humans and in mice (Talar-Wojnarowska et al., 2009).Many groups have identified IL-6 as one of the cytokines secreted by activated hPSCs (Fokas et al., 2015; Omary et al., 2007). Consequently, IL-6 can act in both a paracrine and autocrine manner to stimulate neighboring tumor cells and further activate hPSCs (Lunardi et al., 2014). Moreover, IL-6 has been shown to mediate PanIN formation and PDAC development via downstream signaling involving STAT3 (Lesina et al., 2011). A more recent study indicated that while IL-6 expression was not necessary for PanIN formation, it was a key player in the maintenance and progression of PanIN lesions in a Kras driven model (Zhang et al., 2013).

As IL-6 is implicated in both inflammation and in PDAC, we examined if LCN2 could induce IL-6 expression in hPSCs. 2.0 x 105 cells were plated in six-well plates, they were serum-starved overnight and then treated with either 50, 100 or 500 ng/mL of recombinant human LCN2 (rhLCN2) for either 15, 30 or 60 minutes. RNA and cDNA were isolated after this treatment and RT-qPCR was performed to assess IL-6 mRNA levels. We observed that while all doses resulted in higher IL-6 levels

compared to untreated cells, the 50 ng/mL dose had minimal effects at all three time points that did not reach significance (**Figure 17a**). The 100 ng/mL dose however, allowed for the significant upregulation of IL-6 expression at all time points with the highest effect occurring at 60 minutes of treatment. Similarly, the 500 ng/mL dose induced significant IL-6 expression at comparable levels to that of the 100 ng/mL dose. Because the serum LCN2 levels in pancreatic patients have been found to be around 111 ng/mL, we decided to continue further experiments using either 100 or 500 ng/mL rhLCN2. This experiment served as the first evidence that LCN2 had an effect on hPSCs.

Due to the fact that LCN2 is involved in iron transport, we asked whether LCN2 could also mediate iron regulation in hPSCs. Iron is an essential mediator of cellular growth, proliferation and metabolism. It is involved in oxygen transport, DNA synthesis and in the activity of many enzymes serving as an essential cofactor in many biological processes (Beard, 2001; Cherayil, 2011; Yang et al., 2002). Due to this, iron transport, storage and excretion are tightly regulated as either diminished or excess iron could result in malignancies. Ferritin (FTH1) is an iron-storage molecule that can store up to 4,500 iron atoms (De Domenico et al., 2008). It is a multimer comprised of heavy and light subunits with the heavy subunit containing ferroxidase activity. Ferritin is thus able to oxidize ferrous (Fe²⁺) to ferric (Fe³⁺) iron within its shell and store ferric iron. Along with other iron storage and transport molecules, ferritin is post-transcriptionally regulated by iron-regulatory proteins (IRPs) (De Domenico et al., 2008). During iron deficiency, IRPs bind iron responsive elements (IREs) that are present in ferritin mRNA and prevent the translation of the

mRNA (Rouault, 2006). In iron-replete conditions, IRPs don't bind IREs resulting in the unhindered mRNA translation of ferritin. Thus, ferritin mRNA expression levels have been and are continued to be used as indicators of changes in iron levels in cells. Therefore, we examined if treating hPSCs with different doses of rhLCN2 at different time points led to changes in ferritin expression. Treatment with 50, 100 and 500 ng/mL rhLCN2 induced the expression of ferritin mRNA at 15, 30 and 60 minutes (**Figure 17b**). Moreover, the highest induction was observed after 60 minutes of treatment with 100 ng/mL rhLCN2 when ferritin levels were upregulated three-fold. These results indicated LCN2's ability to affect an iron responsive molecule and potentially mediate the regulation of iron levels inside hPSCs.







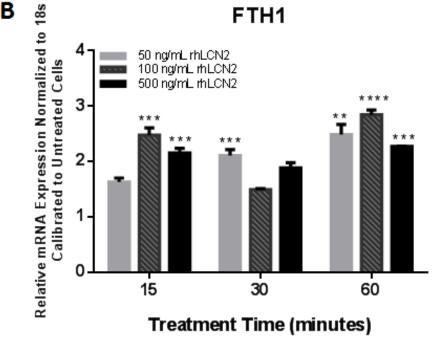


Figure 17. **rhLCN2** induces the expression of IL-6 and the iron-responsive **molecule ferritin (FTH1) in hPSCs**. (A) 50, 100 or 500 ng/mL of rhLCN2 was ectopically added to hPSCs for either 15, 30 or 60 minutes. Treatment with rhLCN2 led to the increased expression of IL-6 (B) rhLCN2 treatment similarly led to the increased mRNA expression of FTH1 indicating LCN2's potential to regulate iron levels in hPSCs. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (student t-test)

Knockdown of the LCN2-specific receptor SLC22A17

After determining that LCN2 induces inflammatory and iron-responsive gene expression changes in hPSCs, we then studied if these observations could be potentially mediated by the LCN2 receptor SLC22A17. In order to do this, we performed cellular shRNA-mediated knockdown of SLC22A17. We purchased several shSLC22A17 bacterial glycerol stocks from a commercial source, purified shSLC22A17 plasmids and packaged them into lentiviral particles as detailed in the methods chapter 2. HPSCs were then transfected with these viruses and selected with puromycin to develop stable cell lines. Subsequently, we assessed the SLC22A17 mRNA levels in these hPSCs cells (Figure 18a). Results indicated that our vector control, HPSC PGZIP, expressed similar SLC22A17 mRNA levels as our untreated hPSCs. Further, one of our constructs, shSLC 4, expressed significantly reduced SLC22A17 mRNA levels. Over seventy-three percent SLC22A17 mRNA knockdown was achieved with this plasmid. Protein analysis of our vector control and shSLC22A17 4 cell lines confirmed knockdown of the SLC22A17 protein (Figure 18b).

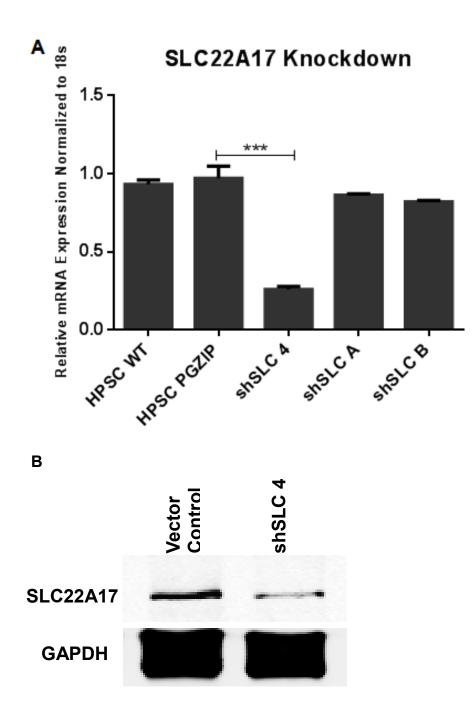


Figure 18. **SLC22A17 knockdown in hPSCs**. (A) Different shSLC22A17 plasmids were assessed for their ability to knockdown SLC22A17 mRNA expression. shSLC 4 showed over seventy-three percent knockdown of SLC22A17 mRNA (B) SLC22A17 knockdown was confirmed at the protein level in hPSCs. ***p<0.001 (student t-test)

LCN2 induces a receptor-mediated expression of pro-inflammatory molecules

After confirming the knockdown of SLC22A17, we examined the effects of rhLCN2 in hPSCs with or without the SLC22A17 receptor. Given our results examining rhLCN2 treatment at different doses, we decided to continue further experiments using either 100 or 500 ng/mL rhLCN2. Moreover, we extended the treatment times to examine effects several hours to two days after rhLCN2 treatment. In addition to looking at IL-6, we assessed the expression of other cytokines including the monocyte chemotactic protein 1 (MCP1), Interleukin-8 (IL-8) and Interleukin 1B (IL-1B) as they have also been shown to be secreted by hPSCs.

As mentioned earlier, we first decided to examine IL-6 expression as it is a proinflammatory cytokine that is implicated in the progression of PDAC. Similar to our previous results, we observed that IL-6 mRNA expression was significantly upregulated after one hour of treatment with 100 ng/mL rhLCN2 (**Figure 19a**). Chiefly, this experiment indicated that IL-6 expression was further upregulated after 24 and 48 hours of rhLCN2 treatment. Upon knocking down the expression of SLC22A17, LCN2's positive effect on IL-6 expression was abrogated. This significant reduction in LCN2's effects was true at all time points indicating that SLC22A17 may mediate IL-6 upregulation by LCN2.

MCP-1 is a chemokine that is synthesized and secreted by many cell types including fibroblast, monocytes and macrophages among others (Schall, 1991). It is a potent recruiter of macrophages, memory T-cells and dendritic cells to sites of inflammation (Takaya et al., 2000). Further, MCP-1 expression has been implicated in the recruitment of these inflammatory cells in breast, ovarian, prostate and

pancreatic cancers (Lazar et al., 2010). MCP-1 is expressed by PDAC cell lines as well as by hPSCs. One study has previously indicated that high serum levels of MCP-1 are more likely to occur in patients with PDAC (Sullivan et al., 2011). Given the implications of MCP-1 involvement in inflammation and PDAC, we examined the effects of rhLCN2 treatment on MCP-1 expression by hPSCs. Significant upregulation of MCP-1 mRNA expression was observed after 24 and 48 hours of rhLCN2 treatment (**Figure 19b**). This upregulation was diminished in hPSCs with SLC22A17 knockdown. These results indicate that both LCN2 and SLC22A17 may play a role in MCP-1 expression leading to the infiltration of immune cells at sites of pancreatic injury.

IL-8 is a pro-inflammatory activator and chemoattractant for neutrophils (Andoh et al., 2000a; Takaya et al., 2000). It has been found to be secreted by several PDAC cell lines and further upregulated by other pro-inflammatory molecules (Andoh et al., 2000a; Andoh et al., 2000b). In addition, IL-8 has been shown to be a potential activator of hPSCs which in turn leads to sustained secretion of IL-8 and other cytokines (Omary et al., 2007). In PDAC, IL-8 has been shown to be upregulated compared to in benign patients (Frick et al., 2008; Hussain et al., 2010). Moreover, it has been proposed that IL-8 can serve as a serum diagnostic and prognostic marker for PDAC (Blogowski et al., 2014; Chen et al., 2012; Shaw et al., 2014). For these reasons, we decided to analyze the changes in IL-8 expression upon treating hPSCs with rhLCN2. Our results showed that rhLCN2 does not affect IL-8 expression up until after 24 or 48 hours of treatment (**Figure 19c**). IL-8 mRNA was upregulated upwards to three-fold at these two time points compared to

untreated cells. Again, SLC22A17 knockdown was able to alleviate the expression of this chemokine upon LCN2 treatment.

The last inflammatory cytokine we studied was Interleukin 1B (IL-1B). As with IL-6 and IL-8, IL-1B has been shown capable of inducing the activation of hPSCs and to be secreted by the same cells (Omary et al., 2007). Moreover, IL-1B can induce the expression of other cytokines including IL-6, IL-8 and MCP-1 making IL-1B a potent pro-inflammatory molecule (Takaya et al., 2000). In PDAC, IL-1B is implicated in tumor progression and has been associated with a poor histopathologic response to neoadjuvant therapy (Delitto et al., 2015). Likewise, IL-1B has been studied and proposed as a potential serum biomarker for discriminating between PDAC patients and benign individuals (Shaw et al., 2014). Therefore, it was of no surprise to find that rhLCN2 also significantly induced the expression of IL-1B after 24 and 48 hours of treatment (**Figure 19d**). Accordingly, this induction was shown to be mediated by SLC22A17 expression. The fact that LCN2 induces the expression of potent proinflammatory cytokines and chemokines in activated hPSCs indicates that LCN2 promotes an inflammatory response in the pancreas microenvironment.

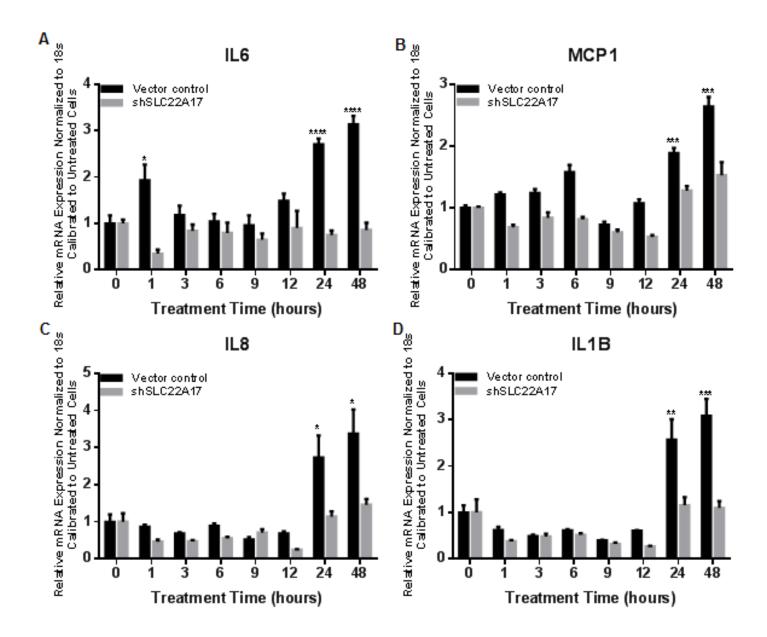


Figure 19. **rhLCN2 induces a SLC22A17-mediated pro-inflammatory cytokine response in hPSCs**. 100 ng/mL of rhLCN2 was ectopically added to hPSCs with or without SLC22A17 knockdown at several different time points. The resulting effect on the mRNA expression of (A) IL-6 (B) MCP-1 (C) IL-8 and (D) IL-1B was examined. rhLC2 induces a significant upregulation of all molecules at the later time

points of 24 and 48 hours after treatment. This effect is diminished upon knocking down SLC22A17. *p<0.05, **p<0.01, ****p<0.001, ****p<0.0001 (student t-test)

LCN2 induces a receptor-mediated expression of Matrix Metalloproteinases

In addition to producing pro-inflammatory cytokines and chemokines, activated hPSCs also secrete molecules that can alter the physical structure of the ECM. Matrix metalloproteinases (MMPs) are enzymes that play an important role in tissue remodeling by degrading the ECM and basement membrane components (Sorsa et al., 2004). This family of over twenty enzymes can be divided into six groups based on their substrate specificity, sequence similarities and organization of their domains (Visse and Nagase, 2003). They are secreted by various cell types including fibroblasts, endothelial cells, macrophages, neutrophils and lymphocytes (Verma and Hansch, 2007). Consequently, these molecules are involved in a milieu of physiological and pathological processes including immune response, inflammation, wound healing and in cancer (Verma and Hansch, 2007). Several MMPs are upregulated and have been correlated with the invasive and metastatic potential of several cancers (Foda and Zucker, 2001; Rundhaug, 2003). MMP-1, MMP-3 and MMP-9 have all been implicated in tumor progression (Foda and Zucker, 2001). Moreover, the expression of these three MMPs has been shown to correlate with the degree of desmoplasia, metastases and survival in pancreatic cancer (Bloomston et al., 2002).

MMP-1 falls under the collagenase group of MMPs as it is able to degrade collagens I, II and III among other proteins (Visse and Nagase, 2003). It has been specifically associated with aiding cell migration and proliferation. In pancreatic

cancer, MMP-1 is expressed in tumor cells as well as in stromal fibroblasts. Moreover, its expression in primary tumors has been correlated with poor prognosis (Ito et al., 1999). Thus, due to its role in regulating the structure of the tumor microenvironment and the fact that it is secreted by hPSCs, we examined if whether LCN2 regulated the expression of MMP-1 in hPSCs. We treated hPSCs with 100 ng/mL rhLCN2 for either 24 or 48 hours (**Figure 20a**). After 24 hours of treatment, LCN2 upregulated MMP-1 expression over three-fold compared to untreated cells. A significant upregulation was also seen at 48 hours but knockdown of the LCN2specific receptor abrogated these changes at both time points.

MMP-3 is a stromelysin capable of degrading multiple molecules including collages II, III, IV, IX, X, fibronectin, laminin and elastin among others (Visse and Nagase, 2003). Of note, MMP-3 plays a role in the generation of other fully active MMPs including MMP-1, MMP-7 and MMP-9 (Bini et al., 1996). Accordingly, MMP-3 is associated with tumor invasion (Moilanen et al., 2003). In PDAC, tumor cell expression of MMP-3 has been shown to be a prognostic factor for poor survival and promote PDAC (Mehner et al., 2014; Mehner et al., 2015). In fact, hPSCs secreting higher levels of MMP-3 were found to promote tumor growth and invasion (Ikenaga et al., 2010). We also investigated if LCN2 could promote the expression of MMP-3 in hPSCs. Our results indicated that rhLCN2 treatment can in fact lead to the elevated expression of MMP-3 (**Figure 20b**). This was observed at both at 24 and 48 hours after treatment with the more significant induction occurring at 24 hours after treatment. SLC22A17 also seemed to mediate MMP-3 upregulation as its knockdown reverted MMP-3 expression back to those of untreated cells.

MMP-9 is a gelatinase that can degrade gelatins and collagens IV and V among other molecules (Visse and Nagase, 2003). It has been shown to be expressed by tumors cell and by stromal immune cells (Thomas et al., 1999). Moreover, MMP9 can be found in a complex with LCN2 in the extracellular space where macrophages and neutrophils are thought to be the sources of this complex (Chakraborty et al., 2012). This then leads to the stabilization of MMP-9 by preventing its degradation and potentially a more invasive tumor (Candido et al., 2016). In PDAC, MMP-9 is secreted by both tumor cells and hPSCs (Lunardi et al., 2014). Additionally, it has been found to correlate with the degree of desmoplasia and nodal status (Bloomston et al., 2002). Thus, the interaction between LCN2 and MMP9 appears to contribute to a more pro-tumorigenic stroma. Correspondingly, we assessed if LCN2 affected MMP-9 expression in hPSCs. Upon 24 and 48 hours of treatment, rhLCN2 induced a significant upregulation of MMP-9 (**Figure 20c**). This also was mediated by the presence of SLC22A17.

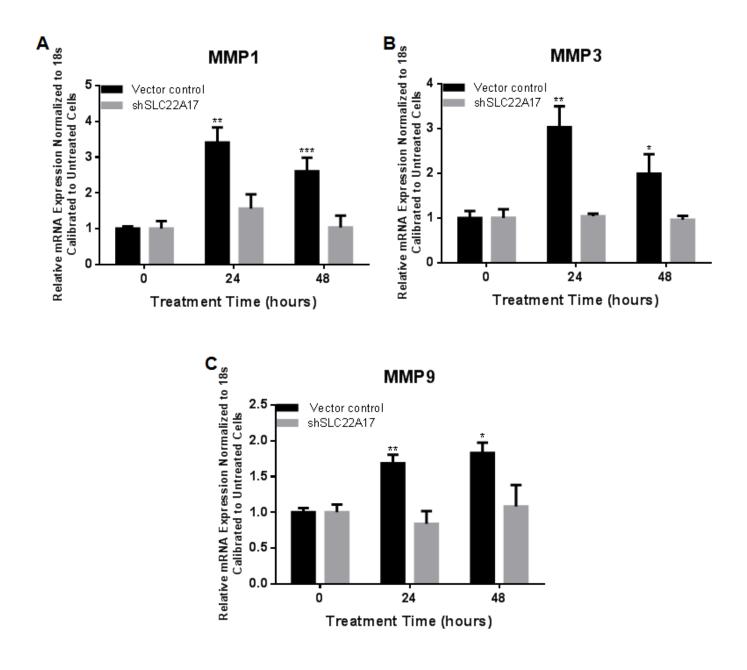


Figure 20. rhLCN2 induces the SLC22A17-mediated expression of matrix metalloproteinases in hPSCs. 100 ng/mL of rhLCN2 was ectopically added to hPSCs with or without SLC22A17 knockdown at 24 and 48 hours. The resulting effect on the mRNA expression of (A) MMP1 (B) MMP3 and (C) MMP9 was examined. rhLC2 induced a significant upregulation of all MMPs which diminished upon knocking down SLC22A17. *p<0.05, **p<0.01, ***p<0.001 (student t-test)

LCN2 induces further activation of hPSCs

Pancreatic stellate cells are quiescent components of the pancreas until pancreatic injury results in their activation. Growth factors, cytokines, and transcription factors have been found to activate hPSCs (Omary et al., 2007). This activation results in morphological changes and expression of certain molecules that distinguish them from their quiescent state. Upon activation, hPSCs gain the expression of alpha smooth muscle actin (α -SMA), a molecule involved in cell motility and structure. It is these activated cells that secret the myriad of molecules that allow them to become a source of fibrosis in PDAC. Therefore, since we observed in this study that LCN2 induces the expression of pro-inflammatory and ECM components known to be secreted by hPSCs, we examined if LCN2 could also affect the expression of α -SMA. For these studies we utilized hPSCs that were immortalized to allow continuous in vitro growth. Stellate cells are somewhat activated in culture. Nonetheless, ectopic rhLCN2 led to a significant upregulation of α-SMA at 24 and 48 hours after treatment (Figure 21). α-SMA expression reverted to normal levels in cells with SLC22A17 knockdown. Thus, LCN2 could be added to the list of molecules that can lead to hPSC activation.

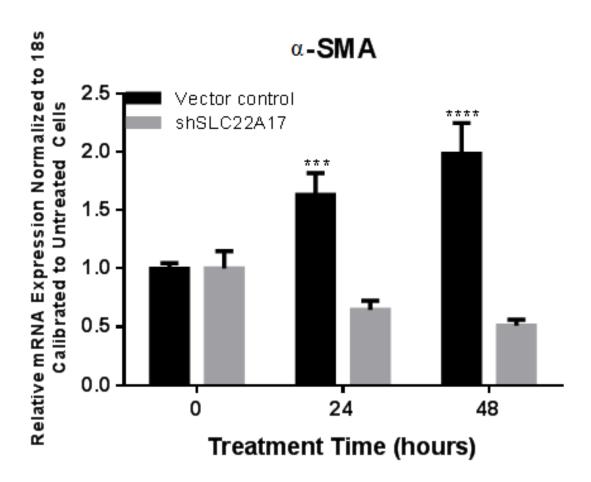


Figure 21. rhLCN2 induces the SLC22A17-mediated expression of α -SMA, the marker of active hPSC. 100 ng/mL of rhLCN2 was ectopically added to hPSCs with or without SLC22A17 knockdown at 24 and 48 hours. α -SMA was found to be upregulated upon treatment in a receptor-mediated fashion indicating LCN2's ability to further incite the activation of these cells. ***p<0.001 (student t-test)

LCN2 mediates the secretion of potent pro-inflammatory cytokines in hPSCs

Our results have indicated that LCN2 has the potential to promote hPSCs to synthesize pro-inflammatory cytokines, chemokines and MMPs. All these molecules have been implicated in inflammation and in cancer. More specifically, they have been found to be able to promote an inflammatory response and the remodeling of the stroma to create a more favorable tumor microenvironment for tumor growth and metastasis. Therefore, we decided to assess if any of these molecules were secreted by hPSCs upon treatment with rhLCN2. We employed the use of a human cytokine array panel to detect cytokine differences between LCN2 treated and untreated cells.

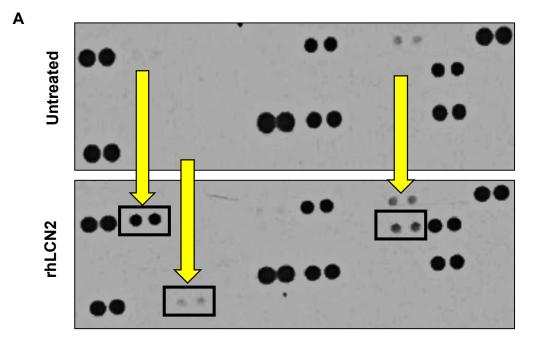
After treating hPSCs with 500 ng/mL rhLCN2 for 48 hours, we collected cell supernatants, concentrated the samples ten-fold using centrifugal filter units and assessed cytokine levels using a cytokine array kit. Briefly, cell supernatants were incubated with a cocktail of biotinylated detection antibodies and a cytokine array panel membrane, then incubated with Strepavidin-HRP and finally subjected to X-ray film for chemiluminescence detection. The resulting positive signals on the developed film were analyzed using a protein array analyzer tool set for the ImageJ software. Here, the average pixel density was analyzed for each signal, background signal was subtracted and resulting pixel densities were graphed to determine relative changes in cytokine expression. Upon developing the nitrocellulose membranes containing different capture cytokine antibodies, we noticed obvious differences between LCN2 treated and untreated cells. In the membrane incubated with treated cell supernatants we observed the notable appearance of three different

sets of dots as highlighted in **Figure 22a**. According to the kit array coordinates, we quickly identified these sets of dots to belong to the complement component 5/5a, IL-6 and the stromal cell-derived factor 1 (SDF-1).

The complement system is a key part of the innate immunity. C5 is a component protein member of the complement system that can be produced through both the classical and alternative pathways. C5a is a pro-inflammatory fragment that is cleaved from C5 via the C5 convertase (Morgan and Harris, 2015). It is a potent chemoattractant for neutrophils and macrophages (Darling et al., 2015; Ward, 2004). Thus, C5a has been implicated in many diseases including cancer due to its powerful inflammatory effect. Moreover, high C5a levels have shown to stimulate tumor progression, invasion and migration (Gunn et al., 2012; Kim et al., 2005; Nitta et al., 2013). In addition, C5a has been shown to be able to activate PSCs and high C5a levels have been found to be significantly elevated in the serum of PDAC patients compared to healthy individuals (Sendler et al., 2015; Tonack et al., 2013). Quantification of the mean pixel density clearly indicated a significant upregulation in C5a secretion by hPSCs upon LCN2 treatment (Figure 22b). This further corroborates LCN2's potential role as an important pro-inflammatory molecule in the PDAC tumor microenvironment.

In addition to C5a, the mean pixel density analysis confirmed the significant expression of IL-6, SDF-1, IL-8 and ICAM-1 after LCN2 treatment. This confirms our previous results in which we saw induction of IL-6 mRNA in hPSCs due to LCN2. SDF-1 is an efficient chemoattractant of lymphocytes and monocytes (Bleul et al., 1996). It has been shown to be secreted by fibroblasts resulting in MMP expression

and increased tumor invasion potential (Orimo et al., 2005; Tjomsland et al., 2011). Moreover, hPSCs have been shown to produce SDF-1 which may mediate paracrine functions on PDAC cells (Duner et al., 2010; Mace et al., 2013). Thus the secretion of these three factors suggest that LCN2 promotes hPSCs to produce potent inflammatory cell chemoattractants leading to a significant remodeling of the tumor stroma.



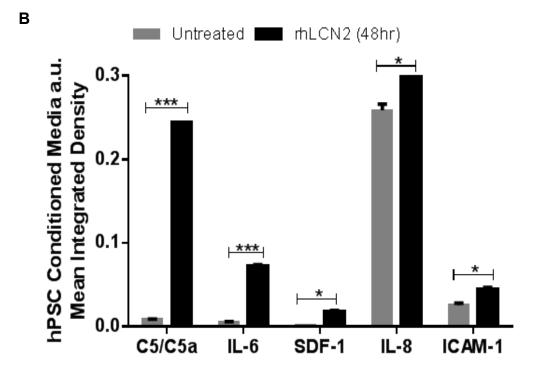


Figure 22. rhLCN2 induces the secretion of pro-inflammatory molecules in hPSC. A human cytokine array panel was used to detect differences in cytokines in

cell supernatants after treating hPSCs with 500 ng/mL of rhLCN2 for 48 hours. (A) The cytokine array membrane panel clearly indicates a difference in the secretion of cytokines after rhLCN2 treatment belonging to Complement component C5a, IL-6 and stromal cell-derived factor 1 (SDF-1), IL-8 and ICAM-1 (B) The mean pixel density quantification indicated a significant upregulation in the secretion of these molecules. *p<0.01, ***p<0.001 (student t-test)

LCN2 induces a receptor-mediated expression of Complement component C5a and its receptor C5aR1 in hPSCs

Because C5a is such a potent mediator of inflammation and immune cell infiltration to areas of injury, we investigated if its expression in hPSCs could also be mediated by SLC22A17 in addition to by LCN2. After treatment with 100 ng/mL rhLCN2 for 24 and 48 hours, we observed a significant upregulation of C5/C5a mRNA confirming our human cytokine array results (**Figure 23a**). However, we saw that SLC22A17 knockdown mitigated this induction indicating its role in LCN2 mediated C5a expression. Additionally, we assessed the effect of LCN2 on the expression of the C5a receptor C5aR1. This receptor is critical in mediating several downstream signaling cascades that are impacted by secreted C5a including the MAPK pathway in neutrophils (Ward, 2004). Moreover, C5aR1 expression was found on the cancerous epithelium of the pancreas compared to normal tissue (Nitta et al., 2013). We found that C5aR1 mRNA expression in hPSCs was induced by LCN2 and that this was mediated by SLC22A17 expression (**Figure 23b**).

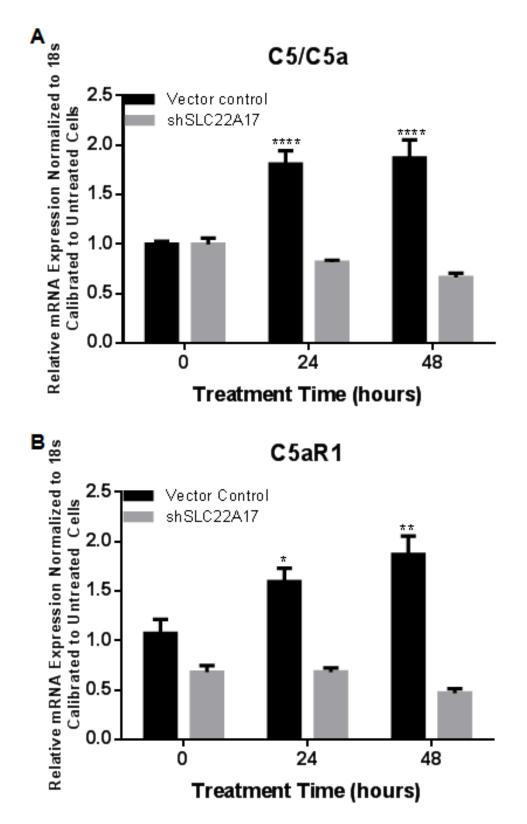


Figure 23. rhLCN2 induces a SLC22A17-mediated expression of Complement

component C5a and its receptor C5aR1 in hPSCs. hPSCs with or without SLC22A17 were treated with 100 ng/mL of rhLCN2 for 24 or 48 hours. (A-B) The mRNA expression of C5a and C5aR1 were upregulated by rhLCN2 but diminished upon SLC22A17 knockdown. *p<0.01, **p<0.01, ****p<0.0001 (student t-test)

LCN2 induces a receptor-mediated increase in iron and iron-responsive genes in hPSCs

LCN2's main biological function involves iron trafficking into and out of cells. SLC22A17 was found to mediate iron-uptake and iron-depletion by LCN2 in HeLa cells (Devireddy et al., 2005). The ability of LCN2 to regulate iron levels in cells has become an active area of research as the tight regulation of iron is crucial to many cell biological functions. The labile iron (Fe2+) pool in cells can modulate energy generation, DNA synthesis and the expression of the many iron responsive genes. Malignant cells have been previously shown to contain higher amounts of labile iron (Torti and Torti, 2011, 2013).

Given LCN2's role in iron regulation, we examined if LCN2 could induce iron level changes in hPSCs. An iron colorimetric assay was used to analyze iron level changes after LCN2 treatment. Upon treatment with 100 ng/mL rhLCN2 for 24 and 48 hours, we observed an upregulation of ferrous iron (Fe²⁺) in hPSCs (**Figure 24a**). This was shown to be regulated by SLC22A17 expression. Further, we examined the expression of iron responsive molecules as a read out of iron level changes. Ferritin (FTH1) is an iron storage molecule that is upregulated in the presence of increased iron. Indeed, we confirmed that iron levels were higher in hPSCs after LCN2 treatment as FTH1 mRNA levels increased accordingly (**Figure 24b**).

Likewise, the mRNA levels of the iron exporter ferroportin (SLC40A1) are upregulated in response to increased iron. We confirmed this to be true upon LCN2 treatment (**Figure 24c**). The transferrin receptor (TFR1) aids in the endocytosis of the iron-binding molecule transferrin. TFR1 expression has been shown to decrease upon increased iron levels in cells in order to preserve iron homeostasis. We however, observed that TFR1 mRNA levels were upregulated upon LCN2 treatment (**Figure 24d**). TFR1 levels have been actually shown to be differentially upregulated in PDAC (Ryschich et al., 2004). Thus, hPSCs may be a source the TFR1 upregulation observed in PDAC. These results show that LCN2 can regulate iron levels in hPSCs via its receptor.

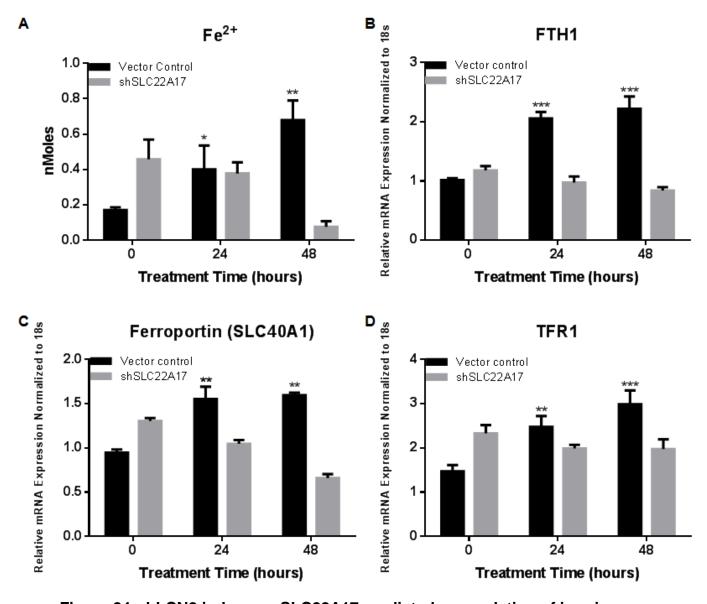


Figure 24. **rhLCN2 induces a SLC22A17-mediated upregulation of iron in hPSCs**. hPSCs with or without SLC22A17 were treated with 100 ng/mL of rhLCN2 for 24 or 48 hours. (A) An iron colorimetric assay was used to assess changes in ferrous iron levels in hPSCs. LCN2 induced a receptor-mediated increase in iron in hPSCs. (B-D) Iron level regulation was further confirmed by examining the mRNA expression of iron-responsive molecules in hPSCs. *p<0.01, **p<0.01, ****p<0.0001 (student t-test)

The iron chelator Deferoxamine diminishes the effects of LCN2 on hPSCs

Deferoxamine (DFO) is a small molecule that binds iron with high affinity. It has been previously used to treat patients suffering from iron overload as well as cancer patients (Torti and Torti, 2013). Since LCN2 regulates iron levels in hPSCs, we asked whether iron could be responsible for inducing the pro-inflammatory changes we have shown. In order to study this, we treated hPSCs with 500 ng/mL rhLCN2 with or without 20 µM DFO. This simulated a competition assay as LCN2 would compete with DFO to acquire any iron available from the cell culture media. Our results indicated that the addition of both LCN2 and DFO to hPSCs diminished the stimulation MCP-1, IL-1B and MMP-1 compared to just LCN2 alone (**Figure 25 A-C**). Moreover, we confirmed that the addition of DFO reduced the expression of the iron responsive molecules FTH1 and ferroportin indicative of a decrease in iron levels inside hPSCs (**Figure 25 D-E**). Thus, our results suggest that iron is at least partially responsible for promoting an LCN2-mediated pro-inflammatory phenotype in hPSCs.

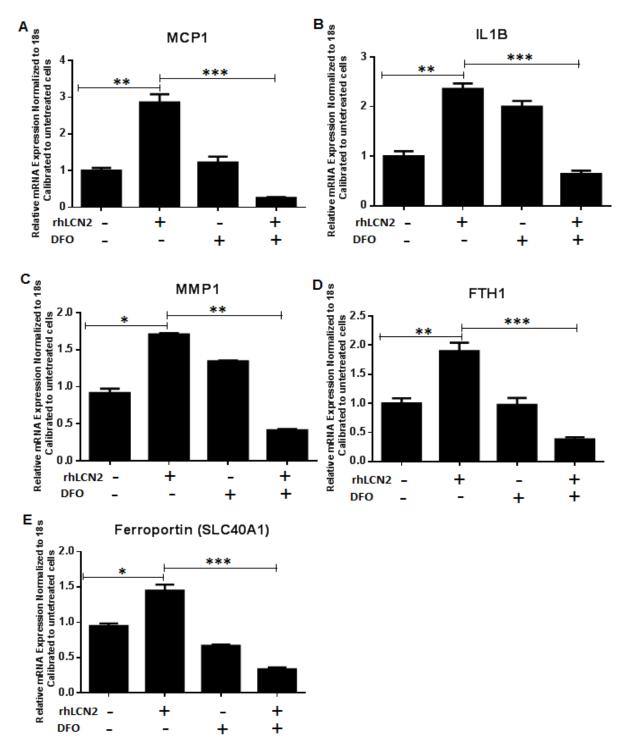


Figure 25. The iron chelator DFO diminishes the effects of LCN2 on hPSCs.

hPSCs were treated with 500 ng/mL rhLCN2 with and without 20 µM DFO (A-C) DFO competition indicated that iron mediated the upregulation of MCP1, IL-1B and MMP1 by LCN2 (D-E) DFO decreased iron levels inside hPSCs as determined by a

decrease in FTH1 and ferroportin mRNA expression. *p<0.01, **p<0.01,

****p<0.0001 (student t-test)

LCN2 induces SLC22A17-mediated pro-inflammatory response in another hPSC line

In order to confirm that LCN2 indeed affects hPSCs in a receptor mediated fashion, we transiently knockdown SLC22A17 in another hPSC line using siRNA. Using a non-targeting scrambled siControl RNA, we confirmed the significant mRNA decrease in SLC22A17 expression (**Figure 26 A**). After confirming this knockdown, we then treated these cells with 500 ng/mL rhLCN2 24 hours after transfection for a period of 24 hours. We confirmed that the expression of IL-6, C5a, MMP-1 and FTH1 increased after LCN2 treatment in cells transfected with siControl (**Figure 26 B**). As with our stable knockdown cell line, we observed that transient SLC22A17 knockdown significantly diminished the effects of LCN2. Thus, we confirmed that LCN2 induces a receptor-mediated production of pro-inflammatory mediators and an iron responsive molecule in another hPSC line.

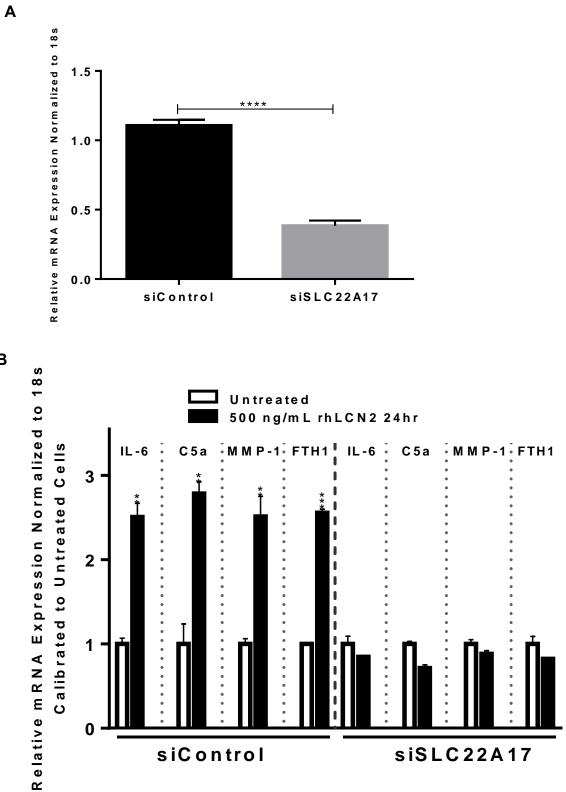




Figure 26. rhLCN2 induces a SLC22A17 mediated pro-inflammatory response in another hPSC line. (A) SLC22A17 was transiently knockdown in another hPSC line using siRNA. (B) hPSCs were treated with 500 ng/mL rhLCN2 with and without SLC22A17. LCN2 treatment induced the upregulation of IL-6, C5a, MMP1 and FTH1 in receptor-mediated fashion. **p<0.01, ***p<0.001, ****p<0.0001 (student t-test) Chapter 5: Discussion

Pancreatic ductal adenocarcinoma is a deadly disease projected to become the second leading cause of cancer related deaths in the next fifteen years (Rahib et al., 2014). The dismal prognosis for PDAC has been attributed to the fact that the disease has spread at the time of diagnosis. Furthermore, over fifty-three percent of patients have advanced PDAC when diagnosed. Due to this, only a small percentage of patients are eligible for surgical tumor resection leaving chemotherapy as the only treatment option for the majority of patients. Low chemotherapy drug efficiency has been suggested to be caused by the high amount of tumor stroma content and low vasculature present in this solid tumor (Fokas et al., 2015). In many cases, the strong desmoplastic reaction observed in PDAC accounts for the majority, 50-90%, of the tumor volume. Thus, modifying the stroma to allow for increased drug penetrability has been an active area of research.

Important components of the PDAC stroma include ECM proteins, cytokines, growth factors, MMPs, hPSCs and inflammatory cells. These components have been shown to appear early on during pre-invasive lesions, pancreatitis and remain present through the development of invasive PDAC. It has been previously shown that an important crosstalk between tumor cells and stromal components can mediate tumor growth and progression. Chiefly, factors secreted by tumor cells can activate hPSCs resulting in the deposition of ECM proteins and the secretion of pro-inflammatory mediators that recruit immune cells to areas of injury. LCN2 is a molecule that is differentially upregulated in PDAC compared to in pancreatitis or a normal pancreas (Moniaux et al., 2008). Prior studies have revealed its correlation with malignant disease, its regulation by pro-inflammatory mediators and its ability to

induce inflammatory cell recruitment (Chakraborty et al., 2012). Attempts to investigate its biological function in PDAC have unfortunately led to conflicting results. Given that LCN2's potential use as a PDAC marker has been suggested by many, it is essential to clarify its role in PDAC and the microenvironment. In this study, we examined the effect of LCN2 depletion on in vivo tumor growth and investigated a potential mechanism by which LCN2 may promote the establishment of a pro-tumorigenic microenvironment in PDAC.

Role of LCN2 in tumor growth, ECM remodeling and survival

We made use of a syngeneic orthotopic mouse model of PDAC to preserve the important tumor-host interactions. Aggressive tumor cells derived from a KPC mouse model of PDAC were injected directly into the pancreas of mice with endogenous or whole-body depleted LCN2 expression. Within this experimental model, we examined if re-introduction of LCN2 expression via KPC cells specifically in the pancreas of LCN2 null mice would affect tumor growth. Murine KPC cells have been used by many and shown to be able to result in tumor growth in syngeneic orthotopic studies (Ma et al., 2013; Olive et al., 2009). Starting from the second week after surgery, we visually observed a decrease in tumor growth in LCN2 null animals. At three weeks, it became evident that whole-body LCN2 depletion significantly delayed tumor growth. Previous studies have shown LCN2's potential to promote cell and tumor growth (Fernandez et al., 2005; Mishra et al., 2004). However, modification of LCN2 expression in PDAC cells has shown to have no effect on cell growth rate in vitro (Leung et al., 2012; Tong et al., 2008). Upon histological examination, we observed that pancreatic tissue from LCN2 null animals

exhibited minimal staining of the cell proliferation maker Ki67. In fact, even in LCN2 animals that did develop tumors at a later time (86 days), Ki67 staining was diminished in comparison to tumors from LCN2^(+/+) animals (37 days). Given that LCN2 expression was not modified in KPC cells themselves, our results suggest that other factors, including changes in the tumor microenvironment due the systemic depletion of LCN2, may have led to decreased tumor cell growth.

The PDAC stroma, a hallmark of this solid tumor, is rich in collagen type I and III content (Hezel et al., 2006). Increased collagen expression in human tissue has been previously associated with a poor prognosis and increased metastasis (Ramaswamy et al., 2003). Collagenous matrices have been shown to alter the morphology, growth, migration and adhesion of normal cells (Kleinman et al., 1981). Moreover, alterations in collagens I and III content has been shown to increase tumor cell proliferation, migration and survival (Li et al., 2014). The tumors formed in LCN2^(+/+) animals demonstrated the development of extensive collagen networks compared to LCN2 null animals with or without tumor. PicroSirius red staining indicated specifically the accumulation of collagens I and III in LCN2^(+/+) animals. Studies in PDAC cells have shown that collagen I and III enhance tumor cell proliferation, motility and reduce drug penetration (Miyamoto et al., 2004; Shields et al., 2011). Thus, we propose that the development of extensive collagen networks in LCN2^(+/+) mice could be responsible increased tumor cell growth observed in these mice as shown by Ki67 staining.

We also observed the activation of PSCs in $LCN2^{(+/+)}$ animals as shown by increased α -SMA staining. It has been established that PSCs are key regulators of

ECM deposition and cytokine/chemokine secretion upon inflammatory stimulus. Further analysis of the pancreas of these animals revealed a significant upregulation in the mRNA expression of IL-6, IL-1 β , MCP-1 and MMP-9. In PDAC, IL-6 has been shown to be required for the maintenance and progression of pancreatic preinvasive lesions (Zhang et al., 2013). This group also reported that IL-6 null animals were no longer able to maintain active fibroblasts and IL-1 β expression was also diminished. IL-6 and IL-1 β are activators of PSCs and are themselves produced by PSCs. Thus, the significantly lower levels of these two cytokines observed in LCN2 null animals may be due to the diminished amount of activated PSCs.

In addition to crosstalk between tumor and PSCs, stromal mediators are also able to regulate each other (Feig et al., 2012). For instance, IL-1 β is a major activator of IL-6 expression and it has been proposed to also regulate the expression of MMP-9 (Wu et al., 2009). Moreover, increased IL-1 β expression can upregulate the expression of MCP-1 and IL-6 can upregulate ICAM-1 and M-CSF (Chomarat et al., 2000). Interestingly, we observed a significant upregulation of ICAM-1 and M-CSF in the serum of LCN2^(+/+) animals. Further, the expression of all these factors may explain why we observed an increased infiltration of leukocytes and macrophages in the pancreas of LCN2^(+/+) mice. IL-6, IL-1 β , MCP-1, ICAM-1 and M-CSF all serve as chemo-attractants for macrophages. In fact, previous studies have shown that LCN2 depletion in murine marrow cells prevented the development of chronic myelogenous leukemia (CML) and solid tumor, implicating LCN2 expression in tumor formation (Leng et al., 2008). Tumor associated macrophages are known to infiltrate the pancreas during the formation of pre-invasive lesion and persist

throughout the development of invasive carcinoma. Increased macrophage infiltration has been previously correlated with PDAC cell and tumor growth, drug resistance and survival (Pandol and Edderkaoui, 2015). In addition to affecting these biological functions, macrophages produce and secrete cytokines, growth factors and even LCN2.

This study's results may help explain the significant increase in survival observed in LCN2 null mice. Moreover, we observed a significant decrease in the expression of the LCN2 specific receptor SLC22A17. High LCN2 and SLC22A17 have been previously correlated with malignancy and poor prognosis in glioma, endometrial and hepatocellular cancers. Thus, depletion of LCN2, as suggested in this study, may contribute to decreased tumor growth and increased survival in PDAC.

LCN2 activates hPSCs in a receptor and iron dependent manner

In an effort to understand how LCN2 depletion could lead to decreased malignancy, we examined the effect of LCN2 on hPSCs. Upon examination of LCN2 expression, we discovered that hPSCs did not synthesize or secrete LCN2 like most PDAC cells did. Instead, we found that hPSCs expressed the receptors SLC22A17 and LRP2 which have been shown to mediate the cellular endocytosis of LCN2. Moreover, hPSCs expressed the SLC22A17 receptor in significant higher quantities than PDAC cells. Given that LCN2 is implicated in promoting the immune response, we asked if LCN2's effects could be mediated in a receptor and iron mediated fashion.

We first achieved the knockdown of SLC22A17 in hPSCs and treated hPSCs with or without SLC22A17 expression with LCN2. As hypothesized, we observed that LCN2 induced a receptor mediated expression of the pro-inflammatory cytokines IL-6, IL-8, IL-1 β and MCP-1. These results recapitulated the in vivo observations given the increased IL-6, IL-1ß and MCP-1 mRNA expression in the pancreas of LCN2^(+/+) mice. Moreover, the expression of LCN2 and increased levels of SLC22A17 in LCN2^(+/+) animals would facilitate an increased activation of hPSCs. Indeed, we observed that LCN2 induced the synthesis of α -SMA in a receptor mediated manner indicating further activation of hPSCs. This too may help explain the increased α -SMA staining seen in the pancreas of LCN2^(+/+) mice. Further, endogenous LCN2 upregulated the expression of MMP-1, MMP-3 and MMP-9. These molecules are involved in ECM degradation and have been previously shown to correlate with poor prognosis in PDAC. Additionally, because LCN2 can form a complex with MMP9, it is possible that LCN2 in pancreas cancer also promotes the stability of this MMP. Because the induction of these MMPs was diminished upon SLC22A17 knockdown, these results implicate this receptor in LCN2's ability to promote the remodeling of the ECM.

LCN2 also promoted the secretion of C5a, IL-6, SDF-1, IL-8 and ICAM-1 (**Figure 26**). Others have previously reported the ability of IL-6, IL-8 and ICAM-1 to recruit macrophages to areas of pancreatic injury. SDF-1 has been implicated in the development of pancreatitis, progression to PDAC and in the recruitment of macrophages. Just recently, a group has also reported that SDF-1 protein levels are significantly upregulated in PDAC compared to normal samples (Fan et al., 2016).

C5a expression is known to be a result of the activation of the complement system. It is a potent chemoattractant for neutrophils and macrophages and is expressed during pre-invasive lesions, pancreatitis and invasive PDAC. Recently, it was also reported that C5a has a role in mediating the development of fibrosis and can lead to PSC activation (Sendler et al., 2015)

Finally, we assessed if LCN2's ability to promote the induction of all these proinflammatory factors could be mediated by iron. First, we observed that endogenous LCN2 treatment resulted in increased ferrous iron levels in hPSCs as well as the modulation of the iron responsive molecules ferritin, transferrin receptor 1 and ferroportin. Increased labile iron pools in cells have been associated with a malignant phenotype. Iron can affect many cellular processes such as metabolism, DNA synthesis, cell growth and cell death (Evstatiev and Gasche, 2012). Thus, tight regulation of iron levels is essential for cell survival. Ferritin is an iron storage molecule whose expression is induced upon increased cellular iron levels. Ferroportin, and iron exporter, also responds to iron in a similar fashion. The expression of the transferrin receptor however, is decreased if cellular iron levels increase in order to maintain homeostasis. In our experiments, increased iron levels and increased ferritin and ferroportin were indicative of LCN2's ability to mediate iron regulation in hPSCs. Moreover, this iron modulation was SLC22A17 dependent. On the other hand, expression of the transferrin receptor was induced in this setting. This is not a normal response to increased iron levels but upregulated levels of this receptor have been shown to be associated with a malignant phenotype in PDAC. Likewise, in our in vivo study we observed that the pancreas of LCN2^(+/+) mice

expressed significantly higher mRNA levels of both ferritin and the transferrin receptor. These results suggest that LCN2 may mediate iron homeostasis in the PDAC microenvironment. To obtain a second confirmation, we treated hPSCs with LCN2 along with the iron chelator DFO. The results from this experiment would further indicate if LCN2 was responsible for increased iron levels in hPSCs. We observed that upon competition with DFO, ectopic LCN2 was no longer able to induce the expression of MCP-1, IL-1B or MMP-1. This indicated that the ability of LCN2 to induce these factors was not only receptor dependent but also iron dependent. Further, DFO competition reduced the expression of both ferritin and the transferrin receptor. Thus, here we report a mechanism by which LCN2 activates hPSCs and mediates their ability to produce pro-inflammatory factors that can alter the composition of the PDAC tumor microenvironment.

Conclusion and future perspectives

Our results indicate the participation of LCN2 in the feed-forward loop created by the crosstalk between tumor cells, hPSCs and inflammatory cells (**Figure 27**). We propose a model in which malignant pancreatic cells secrete cytokines, growth factors and LCN2 during the formation of pre-invasive lesions which leads to the activation hPSCs and recruitment of macrophages to the site of injury. Activated hPSCs are then able to produce ECM components and inflammatory mediators, partly regulated by LCN2 and its receptor SLC22A17, that act in both a paracrine and autocrine fashion to allow for tumor cell growth, increased macrophage recruitment and to sustain their own activation. Macrophages are then also able to

secrete inflammatory mediators in addition to LCN2 to allow the persistence of pancreatic malignancy.

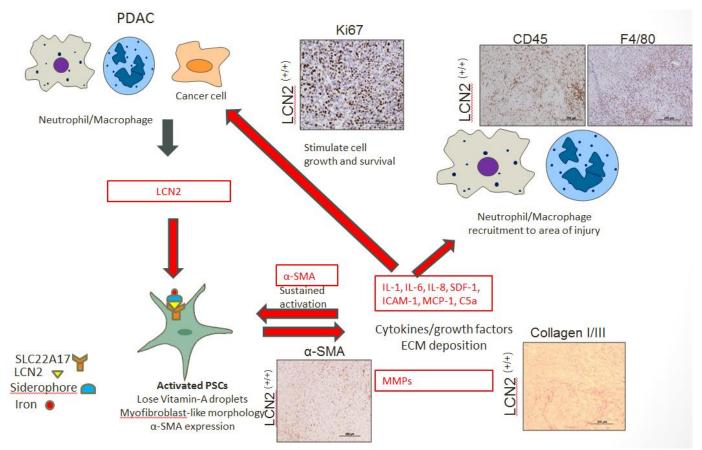


Figure 27. LCN2 participates in the feed-forward loop created by the crosstalk between tumor cells, inflammatory cells and hPSCs. LCN2 is secreted by cancer cells and by neutrophils and macrophages that infiltrate the PDAC stroma. LCN2 can activate hPSCs inducing ECM deposition and the production of proinflammatory mediators that can further recruit neutrophils and macrophages to the PDAC stroma. These mediators can also lead to sustained hPSC activation as well as promote cancer cell growth. Thus, LCN2 participates in a feed-forward loop that helps establish and maintain the PDAC stroma. Given that many have proposed the use of LCN2 as a marker of pancreatic malignancy, here we confirm LCN2's role in promoting the establishment of a protumorigenic microenvironment. In addition, we have shown that LCN2 induces the secretion of molecules including IL-6, IL-8, ICAM-1, SDF-1, TIMP-1, IGFBP-1 and M-CSF that are known to be significantly and differentially upregulated in PDAC. Because LCN2 alone is not sufficient to detect PDAC on its own, we propose the use of LCN2 in a panel with some of these molecules in order to increase the efficiency of early PDAC detention.

Although we've reported novel findings regarding LCN2's biological functions in PDAC, future studies can further examine LCN2's role in the immune cells that infiltrate the PDAC stroma. Because LCN2 is also produced by inflammatory cells, it would be interesting to examine the effect of LCN2 deficient immune cells on tumor growth and survival. In order to address this, a future study could perform a bone marrow transplant from LCN2 null animals to animals with endogenous LCN2 expression, after lethal irradiation to eliminate hematopoietic LCN2 production. In this setting, only immune cells would lack LCN2 and their response to an effect on tumor growth can be assessed. Additionally, because iron regulation is essential for survival, further studies can be performed to understand the role of LCN2 iron regulation in vivo. This study suggested that LCN2 depletion reduced iron levels in bulk pancreas. Within the scope of this study however, we did not examine which cells exhibited lower iron levels. It is possible that LCN2 mediated the increase of iron levels in tumor cells leading to their increased growth and malignancy in LCN2^(+/+) mice. In order to confirm this, iron levels and the expression of iron-

responsive genes in individual compartments within the pancreas can be examined. Nevertheless, this study elucidates and furthers the understanding of LCN2 biology in pancreatic cancer.

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<u>Vita</u>

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