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Lipocalin-2 Promotes Pancreatic Ductal Adenocarcinoma by Regulating Inflammation in the Tumor Microenvironment

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Abstract

Lipocalin-2 (LCN2) promotes malignant development in many cancer types. LCN2 is upregulated in patients with pancreatic ductal adenocarcinoma (PDAC) and in obese individuals, but whether it contributes to PDAC development is unclear. In this study, we investigated the effects of Lcn2 depletion on diet-induced obesity, inflammation and PDAC development. Mice with acinar cell-specific expression of KrasG12D were crossed with Lcn2-depleted animals and fed isocaloric diets with varying amounts of fat content. Pancreas were collected and analyzed for inflammation, pancreatic intraepithelial neoplasia (PanIN) and PDAC. We also used a syngeneic orthotopic PDAC mouse model to study tumor growth in the presence or absence of Lcn2 expression. In addition, to understand the mechanistic role of how LCN2 could be mediating PDAC, we studied LCN2 and its specific receptor solute carrier family 22 member 17 (SLC22A17) in human

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pancreatic cancer stellate cells (PSC), key mediators of the PDAC stroma. Depletion of Lcn2 diminished extracellular matrix deposition, immune cell infiltration, PanIN formation and tumor growth. Notably, it also increased survival in both obesity-driven and syngeneic orthotopic PDAC mouse models. LCN2 modulated the secretion of pro-inflammatory cytokines in PSC of the PDAC tumor microenvironment, while downregulation of LCN2-specific receptor SLC22A17 blocked these effects. Our results reveal how LCN2 acts in the tumor microenvironment links obesity, inflammation and PDAC development.

Keywords

Lipocalin 2; PDAC; obesity; inflammation; pancreatic cancer stellate cells

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with a dismal long-term survival [1, 2]. It is projected to become the 2nd leading cause of cancer-related deaths in the United States by 2030 due to both an aging population and the obesity epidemic [1, 3–5]. Epidemiological data indicates that obesity adversely influences PDAC-related mortality in a dose-dependent manner [2]. Moreover, a high BMI is associated with increased risk of PDAC, early age at onset, and decreased overall survival [6]. Therefore, it is essential to elucidate the biology linking obesity and PDAC development. Lipocalin-2 (LCN2) is an adipokine elevated in the serum and visceral adipose tissue of obese individuals [7]. It regulates adipose tissue inflammation, is a marker associated with obesity, and modulates insulin resistance [8–10]. LCN2 is known to be secreted by adipocytes [11, 12], immune (neutrophils and macrophages), and tumor cells [13]. Elevated serum LCN2 levels have been reported in ovarian, breast, colorectal, gastric and pancreas cancers [13–15]. In PDAC, LCN2 expression levels increase in early pancreatic intraepithelial neoplasia lesions and increased expression correlates with progression [16–18]. LCN2 is highly upregulated in serum and serves as a potential biomarker for patients with PDAC compared to those with chronic pancreatitis and normal healthy individuals [19–21]. Efforts to investigate the biological role of LCN2 expression in PDAC have so far been inconclusive. *In vitro* and *in vivo* studies, using orthotopic and/or subcutaneous pre-clinical mouse models, have reported contradictory results with regards to the role of Lcn2 in PDAC growth, invasion and metastasis [22–24]. Therefore, given the mounting evidence linking LCN2 to obesity and inflammation, we hypothesized that LCN2 could link obesity and PDAC development.

Here we investigated the role of Lcn2 in a diet-induced obesity PDAC mouse model in which a genetically engineered mouse model (GEMM) was fed a high fat diet (HFD) [25]. We find that Lcn2 null mice expressing pancreas-specific oncogenic *KRas* developed fewer PanINs, less inflammation and fibrosis, and displayed increased survival when fed a HFD. Moreover, we find that tumor growth was delayed and survival increased in Lcn2 null mice after injection of mouse PDAC cells expressing Lcn2. In addition, we showed that when human pancreatic cancer stellate cells (PSCs), which are important regulators of the PDAC tumor microenvironment, are treated with Lcn2 it induced a pro-inflammatory response.

This response was blocked after expression of the LCN2-specific receptor, solute carrier family 22, member 17 (SLC22A17) was downregulated.

Material and Methods

Ethics Statement

All experiments were performed in compliance with the regulations and ethical guidelines for experimental animal studies of the Institutional Animal Care and Use Committee at the UT M.D. Anderson Cancer Center and/or OSU.

Genetically engineered transgenic mice

KRas^{G12D} mice expressing conditional knock-in mutant *KRas*^{G12D} were obtained from the Mouse Models of Human Cancer Consortium Repository (NIH Bethesda, MD) [26]. *KRas*^{G12D} mice were bred with Ela-CreERT (CRE) mice described previously [27] mice to generate *KRas*^{G12D}/CRE. *Lcn2* whole-body knockout (*Lcn2*^{-/-}) animals were kindly provided by Dr. Ralph Arlinghaus (MD Anderson Cancer Center) [28] and bred with *KRas*^{G12D}/CRE mice to generate (*Lcn2*^{-/-}/*KRas*^{G12D}/CRE).

Treatments in Animals

Animals at 40 days old were given tamoxifen orally for 3 days. Mice were fed either a CD (Test Diet DIO 58Y2; Lab Supply) in which 10% of energy was derived from fat (*KRas*^{G12D}/CRE, n=48; *Lcn2*^{-/-}/*KRas*^{G12D}/CRE, n=21; *Lcn2*^{+/+}, n=24; *Lcn2*^{-/-}, n=21) or a HFD (Test Diet DIO 58Y1 van Heek Series; Lab Supply, Fort Worth, TX), in which 60% of energy was derived from fat (*KRas*^{G12D}/CRE, n=42; *Lcn2*^{-/-}/*KRas*^{G12D}/CRE, n=36; *Lcn2*^{+/+}, n=21; *LCN2*^{-/-}, n=20). Two experimental groups were designed to test the effect of *Lcn2* depletion and diet type either short-term after 50 days on diet or a long-term survival study. The pancreas, adipose tissue, and serum were collected to measure serum levels of LCN2 and other secreted molecules as well as for histological and protein analysis. Body weight (BW) was measured weekly.

Syngeneic orthotopic model of pancreatic cancer

C57BL/6 strain mice were injected with cells derived from a pancreatic tumor of a *PDX-1-CRE*, *LSL-KRas*^{G12D}, *LSL-Trp53*^{-/-} (KPC) genetically engineered mouse model (GEMM) and transfected with enhanced firefly luciferase (KPC-LUC) [29, 30]. KPC-LUC cells in single suspension were prepared and mixed in HBSS and 20% Matrigel (BD Biosciences, Bedford, MA, USA). A suspension of 0.5×10⁶ KPC-LUC cells was carefully implanted as previously described [30]. Tumor growth was visualized by injecting mice with D-Luciferin (150 µg/mouse; Caliper Life Sciences) and using the IVIS imaging system (Caliper Life Sciences) [30]. Animals (*Lcn2*^{+/+} n=20 and *Lcn2*^{-/-} n=17) were imaged weekly until euthanasia was required. Bioluminescence was measured using the Living Image® software.

Immunohistochemistry

Pancreatic tissue was fixed with 10% formaldehyde, 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 supplemental table 1. Collagen was detected using a Picro-Sirius Red Stain Kit (Abcam, ab150681).

Cell Culture

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) and tested negative for the presence of mycoplasma. 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 normal epithelial cells (HPNEs) were obtained from Dr. Timothy Eberlein (St. Louis, MO) and Dr. Tsao (Ontario Cancer Institute, Toronto, ON, Canada) respectively. PSC#1 and PSC#2 were obtained from resected human PDAC samples by the outgrowth method and immortalized using lentiviral vectors with human telomerase (hTERT) or SV40 large T antigen (TA_g) [31]. Primary PSCs were isolated by the outgrowth method from fresh patient resected human PDAC samples tissue and characterized by morphology [32]. Cell lines were validated by STR DNA fingerprinting using the Promega 16 High Sensitivity STR Kit (Catalog # DC2100). The STR profiles were compared to online search databases (DSMZ/ATCC/JCRB/RIKEN) of approximately 2500 known profiles; along with the MD Anderson Characterized Cell Line Core (CCLC) database of approximately 2600 known profiles. The STR profiles matched known DNA fingerprints or were unique.

RNA isolation and quantitative RT-PCR

RNA was isolated using TRIzol® Reagent (Life Technologies). The 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 supplemental table 2.

Immunoblotting

Lysates from cultured cells and mouse pancreatic tissue were prepared using RIPA buffer and a cocktail of protease inhibitors (Roche, Indianapolis, IN). Conditioned media was prepared by collecting media from cells in 10mm plates and concentrating the media ten-fold in Ultra-15 centrifugal filter units (EMD Millipore, Billerica, MA). Protein samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% milk in 1X phosphate buffered saline with 1% Tween-20 for one hour and incubated with primary antibodies: monoclonal rat anti-LCN2 (R&D) and polyclonal rabbit anti SLC22A17 (Thermo Scientific) overnight at 4 °C. Membranes were incubated with corresponding secondary antibodies and signal was detected by visualizing near-infrared fluorescent signals with the Odyssey® imaging system (Li-Cor, Lincoln, NE).

Lentivirus-mediated and siRNA SLC22A17 knockdown

Mission short-hairpin RNA (shRNA) bacterial glycerol stock plasmid for SLC22A17 with sequence CCGGGCGATTCTACAGCGAATGATCTCGAGATCATTCGCTGTAGGAATCGCTTTT TG was obtained from Sigma Aldrich (St. Louis, MO). 2.3 µg of the each respective plasmid was co-transfected with the envelope PMD2.G (2 µg) and packaging PsPAX2 (4.7 µg) plasmids (Addgene) into 293FT cells using Lipofectamine 2000 (Life Technologies). PSC#1 were incubated with virus supernatant and polybrene for 24 hours. Stable knockdown cells were selected using 1 µg/mL puromycin. SLC22A17 was also transiently knockdown in PSC#2 using a pool of four siRNAs with the following sequences: 1) CAGCATTATGCTGCTGCCGGA, 2) AGGGAGCCCATCGGACTTCTA 3) CGCACGGTGGCTGATAGTGAA 4) TCGGGAAGACTGAGTAGGGAA. siRNA transfection was performed using 12 µL of HiPerfect transfection reagent (QIAGEN, Valencia, CA) and 12.5 nM of each siSLC22A17 siRNAs for 24 hours.

Recombinant human LCN2 treatment

PSCs with endogenous or knockdown of SLC22A17 were plated at 2×10^5 cells per well in 6-well plates or at 1×10^6 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 or as indicated.

Cytokine/adipokine protein array

Concentrated conditioned media or serum from mice was used to detect the expression of cytokines using the Proteome Profiler human cytokine array panel A/Mouse Adipokine Profiler (R&D Systems, Minneapolis, MN). 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.

Human sera and plasma collection

Serum samples were collected as described before.[33] Plasma was isolated from peripheral venous blood which was obtained in sodium heparin tubes from a cohort of treatment naïve patients with metastatic pancreatic cancer under an IRB approved protocol [34].

Histology

Pathologic scores were determined as described previously. [25] Briefly, PanIN lesions frequency was evaluated by counting all foci of PanIN lesions. Each affected lobule, irrespective of the number of duct profile, was scored as 1. Pathologic score of the level of inflammation: 1: non-aggregation, occasional scattered leukocytes; 2: intermediate level of leukocytes; 3: aggregates of leukocytes within the parenchyma in a follicle-like formation. Pathologic score of the degree of fibrosis: 1: occasional periductal and scant perilobular

fibrosis; 2: intermediate level; 3: definite extensive fibrosis, involving 50% of parenchyma with resultant parenchymal (acinar) atrophy.

LCN2 ELISA

Human and mouse LCN2 Quantikine ELISA kits (R&D Systems, Minneapolis, MN) were used to measure LCN2 levels in human serum and plasma as well as mouse serum and fat lysates as per manufacturer instructions. All samples were assayed in duplicate and compared to a standard curve to quantitate expression.

Statistics

Statistical analysis was performed using the Prism 5 software program (GraphPad Software San Diego, CA). Results are expressed as the mean $\pm$ 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 than 0.05 were considered significant.

Results

LCN2 is highly upregulated in human PDAC and in an obesity-driven PDAC genetic mouse model

We confirmed previous reports that LCN2 is elevated in sera from PDAC patients [19, 20]. LCN2 sera levels were compared among patients with normal pancreas, chronic pancreatitis (a benign inflammatory disease of the pancreas) and PDAC using ELISA. (Figure 1A). Our results showed that serum levels of LCN2 are significantly higher in patients with PDAC (223.2 ng/ml): 1.9 fold increase when compared to those with normal pancreas (115.3 ng/ml) and 2.36 fold increase when compared to those with chronic pancreatitis (94.45 ng/ml). Plasma levels of LCN2 in patients with metastatic PDAC were also analyzed and found to be high (160.6 ng/ml) compared to a previous study which has shown LCN2 plasma levels to be around 111 ng/ml in PDAC patients compared to 67 ng/ml in healthy controls [20]. Furthermore, we analyzed mRNA levels of other members of the lipocalin family using microarray data from an earlier study [35] and found that LCN2 is the most up-regulated member of the lipocalin family in human PDAC compared to normal pancreas and pancreatitis tissue samples (Figure 1B).

Because of the reported associations between the expression levels of the adipokine LCN2 in obese individuals and its possible link with cancer [7, 36, 37], we examined if body mass index correlated with plasma levels of LCN2 in our cohort of PDAC patients. No correlation was evident between body mass index (BMI) and plasma levels of LCN2 in PDAC patients. (Supplemental Figure 1) We then examined *Lcn2* levels in serum, pancreas and fat tissue in the obesity-driven PDAC mouse model [25]. We found that *Lcn2* serum levels in the *KRas*^{G12D}/CRE mice increased from 156.7 ng/ml to 316.6 ng/mL, a ~2 fold change, after being fed a HFD. However we did not observe significant changes in the level of *Lcn2* in serum from CRE control mice after feeding a HFD (Figure 1C). In addition, we observed a significant upregulation of *Lcn2* expression in both mRNA (Figure 1D) and protein (Figure 1E) levels in the pancreas of *KRas*^{G12D}/CRE mice fed a HFD, which were susceptible to

PDAC development. We also observed that *KRas*^{G12D}/CRE mice express Lcn2 in the adipose tissue lysates (Figure 1F) and that the stromal vascular fraction from murine adipose tissue expressed Lcn2. (Supplemental Figure 2)

Lack of Lcn2 prevents diet-induced obesity in mice

In order to explore the role of Lcn2 in obesity, Lcn2 wild type (*Lcn2*^{+/+}) and Lcn2 whole-body knockout mice (*Lcn2*^{-/-}) were fed either a control diet (CD) or a HFD over a period of 300 days. During this time, *Lcn2*^{+/+} animals fed a HFD tripled their weight, increasing from ~20g to ~60g (Figure 2A). Although *Lcn2*^{-/-} animals fed a HFD gained weight, their average weight was 20g less than *Lcn2*^{+/+} animals fed the same diet. *Lcn2*^{+/+} animals fed a HFD had a significantly higher weight compared to controls fed a CD after 30 days. In contrast, it took six times longer, (180 days), to observe significant weight changes in *Lcn2*^{-/-} mice fed a HFD compared to CD (Table 1).

Next, we examined whether Lcn2 depletion protected against weight gain in mice susceptible to PDAC development by diet-induced obesity. For this purpose, *Lcn2*^{-/-} mice were bred with *KRas*^{G12D}/CRE mice to generate triple transgenic mice lacking Lcn2 but expressing pancreas specific *KRas*^{G12D} (*Lcn2*^{-/-}/*KRas*^{G12D}/CRE). As expected, *Lcn2*^{-/-}/*KRas*^{G12D}/CRE mice lack serum levels of Lcn2 (Supplemental Figure 3A) and when fed HFD for >50 days, mice developed less volume of visceral adipose tissue and showed significantly lower changes in body weight compared to *KRas*^{G12D}/CRE mice (Figure 2B, Supplemental Figure 3B). These results indicate that lack of Lcn2 prevents weight gain and obesity in the diet-induced PDAC mouse model.

Lcn2 depletion reduces inflammation, fibrosis, and the formation of PanIN lesions in an obesity-driven PDAC genetic mouse model

To evaluate the role of LCN2 in modulating the progression of PDAC by diet-induced obesity, we examined *KRas*^{G12D}/CRE and *Lcn2*^{-/-}/*KRas*^{G12D}/CRE animals after 50 days on a HFD. We found that *Lcn2*^{-/-}/*KRas*^{G12D}/CRE mice fed a HFD developed fewer PanIN lesions, less fibrosis, and inflammation compared to *KRas*^{G12D}/CRE controls (Figure 2C-D). Picrosirius red staining, which stains collagens I and III, revealed less collagen remodeling of the extracellular matrix (ECM) in *Lcn2*^{-/-}/*KRas*^{G12D}/CRE mice compared to *KRas*^{G12D}/CRE mice (Figure 2C). *KRas*^{G12D}/CRE animals also exhibited higher levels of alpha smooth muscle actin (alpha-SMA), one of the markers for activated PSCs, which are key regulators of PDAC stroma [38] (Figure 2C). Next, we evaluated the effects of Lcn2 depletion on the inflammatory response in the pancreas of these animals. We assessed the presence of leukocytes by staining for the pan-leukocyte marker CD45. The pancreas of *KRas*^{G12D}/CRE animals displayed a higher level of CD45⁺ cells infiltration compared to *Lcn2*^{-/-}/*KRas*^{G12D}/CRE mice. Further, *KRas*^{G12D}/CRE animals had an increased recruitment of F4/80⁺ macrophages (Figure 2C). Importantly, only 40% of *Lcn2*^{-/-}/*KRas*^{G12D}/CRE animals developed PanIN 2 lesions compared to a significantly higher rate of 90% of the *KRas*^{G12D}/CRE animals. These results suggest that Lcn2 is an important mediator of inflammation, fibrosis and PanIN development in PDAC.

Depletion of *Lcn2* extends survival in an obesity-driven PDAC genetic mouse model

Next, we evaluated the effects of *Lcn2* depletion on the survival of mice with genetic predisposition to develop PDAC. *Lcn2*^{-/-}/*KRas*^{G12D}/CRE survived longer than *KRas*^{G12D}/CRE animals fed either a CD or a HFD (Figure 3A). As expected from our previous findings, [25] feeding of a HFD significantly reduced the median survival of *KRas*^{G12D}/CRE animals (269 days to 195.5 days) (Table 2). In contrast, *Lcn2*^{-/-}/*KRas*^{G12D}/CRE animals fed a HFD displayed a significant increase in median survival (249.5 days), compared to *KRas*^{G12D}/CRE mice fed a HFD. Moreover, *Lcn2* depletion also significantly increased survival of animals fed a CD (296 days). These results indicate that regardless of diet, *Lcn2* depletion allows for increased survival in *KRas*^{G12D}/CRE mice, which were predisposed to develop PDAC by the expression of oncogenic *KRas*^{G12D} in the pancreas. Some *Lcn2*^{-/-}/*KRas*^{G12D}/CRE animals in this survival cohort did develop PanIN lesions and fibrosis (Figure 3B). However, *KRas*^{G12D}/CRE mice fed a CD or HFD experienced dramatic changes in stromal remodeling at around their median survival age, while *Lcn2*^{-/-}/*KRas*^{G12D}/CRE mice demonstrated less prominent ECM changes and immune cell infiltration at that age (Figure 3B-C). These results suggest that *Lcn2* has the potential to alter the expression of ECM components as well as immune cell infiltration 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 and the need to further understand the biological role of LCN2 in cancer, we examined the role of *Lcn2* in a syngeneic orthotopic model. KPC-LUC cells were injected into the pancreas of *Lcn2*^{+/+} and *Lcn2*^{-/-} mice (Figure 4A–B, Supplemental Figure 4). Serum *Lcn2* levels significantly increased in *Lcn2*^{+/+} mice after tumor cell implantation while *Lcn2* serum levels were undetectable in *Lcn2*^{-/-} animals (Figure 4C). Three weeks after tumor cell implantation, most *Lcn2*^{+/+} animals had developed large tumors visualized by a strong bioluminescent signal that was an average of ~15 fold higher than mice lacking *Lcn2* (Figure 4A–B, Supplemental Figure 5). In addition, the median survival of *Lcn2*^{-/-} animals after tumor cell implantation was significantly longer (~3 fold) than *Lcn2*^{+/+} mice (105 days versus 33 days respectively) (Figure 4D). All the *Lcn2*^{+/+} mice became moribund and were humanely euthanized by 104 days after tumor cell implantation, while about 50% of *Lcn2*^{-/-} animals remained healthy at that time. Surviving *Lcn2*^{-/-} animals were sacrificed at 282 days in order to analyze the pancreas. Consistent with the results from the GEMM experiments, we observed that *Lcn2*^{-/-} mice had little to no pancreas ECM remodeling or inflammatory cell infiltration compared to *Lcn2*^{+/+} animals in our orthotopic syngeneic model (Figure 5A). The *Lcn2*^{-/-} animals that develop tumors at a later time (10 out of 17) also had altered stroma and were infiltrated by macrophages and leukocytes. However, the presence of inflammatory, alpha-SMA positive, and proliferative stromal cells (shown by Ki67 staining), was attenuated in the orthotopic tumors of *Lcn2*^{-/-} mice compared to those in *Lcn2*^{+/+} mice (Supplemental Figure 6). We then isolated cells from the peritoneal cavity, a technique that mostly yields macrophages [39]. We showed that depletion of *Lcn2* does not impair cell invasion of peritoneal macrophages (Supplemental Figure 6). Moreover, the serum of *Lcn2*^{-/-} mice contained significantly lower levels of macrophage colony stimulating factor (M-CSF), an essential chemokine for the differentiation, proliferation and

survival of macrophage precursor cells (Figure 5B). Further analysis of the serum of these animals also revealed reduced levels of putative PDAC biomarkers such as, intercellular adhesion molecule-1 (ICAM-1), insulin-like growth factor binding protein 1 (IGFBP-1) and tissue inhibitor of metalloproteinases 1 (TIMP-1) [40–42] (Figure 5B). Subsequent, analysis of bulk pancreas tissue revealed that *Lcn2*^{+/+} animals injected with KPC-LUC cells had significantly higher expression levels of the pro-inflammatory cytokines interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and interleukin-1B (IL-1B) and had higher expression of matrix metalloproteinase 9 (MMP-9) (Figure 5C). These results recapitulate results obtained in our genetic mouse model experiments, supporting the role of *Lcn2* in altering the stromal composition and inflammatory response in the PDAC tumor microenvironment.

LCN2 stimulates a pro-inflammatory response in PSCs

To further elucidate a mechanism of how LCN2 could mediate the stromal changes observed in the mouse models, we examined the role of LCN2 and the LCN2-specific receptor, SLC22A17 in modulating the production of pro-inflammatory mediators by PSCs. We showed for the first time that PSCs expressed significantly higher levels of the SLC22A17 compared to PDAC cell lines and normal pancreatic epithelial cells (Figure 6A). Moreover we observed that LCN2 is not expressed by PSCs (Figure 6A-B). In both our genetic and orthotopic syngeneic mouse models, *Lcn2* null animals showed a significant reduction in the mRNA expression levels of SLC22A17 in the pancreas tissue (Supplemental Figure 7).

To determine whether LCN2 may promote a pro-inflammatory response in PSCs, we first treated PSCs with different doses of exogenous recombinant human LCN2 (rhLCN2) (Supplemental Figure 8) and found that treatment with either 100 ng/mL or 500 ng/mL rhLCN2 resulted in the induction of the pro-inflammatory cytokine IL-6. We next evaluated a time-course of LCN2 mediated effects and observed a maximal mRNA expression induction of various cytokines after 48 hours of treatment (Supplemental Figure 8). At this time, exogenous LCN2 induced PSCs to secrete higher levels of the pro-inflammatory molecules complement component 5a (C5a), IL-6, stromal cell-derived factor 1 (SDF-1), interleukin-8 (IL-8) and ICAM-1 as assessed by a human cytokine array (Figure 6C, Supplemental Figure 8).

To determine if these effects were LCN2 receptor-mediated, we examined the effects of downregulating the expression levels of the LCN2-specific receptor SLC22A17 in PSCs (Supplemental Figure 8). LCN2 treatment of PSC#1 cells expressing control shRNA vector increased the mRNA expression levels of the pro-inflammatory cytokines IL-6, MCP-1, IL-8, and IL-1B, as well as the matrix metalloproteinases MMP-1, MMP-3 and MMP-9, the complement factor C5/C5a, C5aR1 and alpha-SMA (Figures 6D, Supplemental Figure 8). However, upon expressing shRNA against SLC22A17 in PSCs, the ability of LCN2 to induce the expression of all these molecules was significantly diminished (Figures 6D). These results were recapitulated in a second PSC line, PSC#2 using siRNA against SLC22A17 to downregulated expression levels (Supplemental Figure 8). Thus, these results suggest a previously unidentified role for the LCN2-specific SLC22A17 receptor in PDAC malignancy mediated by the modulation of PSCs in the tumor microenvironment.

Discussion

Some studies suggest that LCN2 serves as a tumor suppressor, while others proposed that it is a tumor promoting factor. [22–24] Therefore, the need of using GEMMs to study the role of Lcn2 in PDAC was warranted and addressed in the current study. While it is well known that LCN2 is upregulated in various types of cancer and in obese patients, studies linking LCN2 to both PDAC and obesity are lacking [13]. Here we confirmed that LCN2 is the highest overexpressed member of the lipocalin family in PDAC. However, we did not observe any correlation between body mass index (BMI) and plasma levels of LCN2 in patients with metastatic PDAC. Most PDAC patients at time of diagnosis present with advanced disease in which substantial weight loss had already occurred. Based on our findings and prior reports on LCN2 as a biomarker of PDAC, we suspect that LCN2 may contribute at earlier stages, such as inflammatory processes that contribute to tumor progression. Thus, possibly due to timing, we were unable to capture any potential relationships in this patient cohort.

Moreover, in this study, we have revealed for the first time a possible mechanism through which Lcn2 depletion attenuated stromal changes, decreased immune cell infiltration and prolonged survival in a PDAC genetic mouse model and an orthotopic syngeneic mouse model. We also showed that PSCs mediated the LCN2-mediated secretion of pro-inflammatory cytokines. It has been demonstrated that LCN2 binds to two cell-surface proteins: 1) SLC22A17 and 2) low density lipoprotein receptor related protein (LRP2 or megalin) [13]. Both SLC22A17 and LRP2 have been reported to bind and mediate the cellular uptake of LCN2 [43–45]. While SLC22A17 has been shown to bind specifically to LCN2, LRP2 also binds numerous structurally unrelated molecules including aprotinin, vitamin D3, transferrin and lactoferrin [46, 47]. 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 [47–49]. Moreover, we observed that Lcn2 is also upregulated in the serum after HFD consumption in animals that express pancreas-specific mutant *KRas*. To the best of our knowledge, these data are the first to link increased expression of Lcn2 to diet-induced obesity in a GEMM of PDAC. In addition, we showed that Lcn2 deficient animals, exhibit attenuated weight gain and adiposity, suggesting a role for LCN2 in obesity preventative efforts.

Lcn2 deficiency also reduced pancreatic stromal fibrosis in animals expressing mutant *KRas* fed either a CD or a HFD. The ability of Lcn2 to stabilize MMP9 activity has been implicated to promote mammary tumor growth and metastasis [50]. Here we showed that MMP9 expression is significantly reduced in Lcn2 null animals, suggesting that MMP9 may play a role in Lcn2-mediated PDAC growth. Further, increased collagen expression has been shown to support ECM and facilitate tumor cell proliferation, migration and survival [51]. Moreover, the expression of alpha-SMA is prominently increased in mutant *KRas* animals expressing Lcn2, compared to Lcn2 null mice. This observation supports the ability of LCN2 to activate PSCs. These cells are major contributors of ECM proteins, which likely explain the increased levels of collagens in these animals.

In addition, we observed an increase in the numbers of leukocytes and macrophages in the animals expressing *Lcn2* and mutant *KRas* compared to those with deleted *Lcn2*. Tumor-associated macrophages (TAMs) have been shown to infiltrate the PDAC stroma at an early stage and can be either pro- or anti-tumorigenic [51, 52]. Some studies suggest that early presence of TAMs aids in the acinar to ductal metaplasia, a key early process in PDAC development [52]. In fact, we observed that, in *Lcn2*^{-/-}/*KRas*^{G12D}/CRE animals fed a HFD for 50 days, there were lower levels of leukocyte and macrophage infiltration which correlated with a decreased presence of early PanIN lesions. We also observed attenuated collagen deposition and immune cell infiltration, regardless of diet, in both genetic and syngeneic PDAC mouse models that do not express LCN2. These results suggest that *Lcn2* expression promotes stromal remodeling and development of PDAC. *Lcn2* was dispensable for macrophage migration to sites of inflammation in mice [53] which is consistent with our data showing recruitment of macrophages to inflammatory areas on the *Lcn2* null mice. Moreover, we showed that invasion of peritoneal macrophages derived from *Lcn2* null mice was not impaired.

In this study we also observed increased PanIN development and reduced median survival of *KRas*^{G12D}/CRE mice compared to the *Lcn2*^{-/-}/*KRas*^{G12D}/CRE mice fed a HFD. It is possible that with the increased adiposity, cells within the adipose tissue are contributing to the secretion of pro-inflammatory factors, such as LCN2 that could promote PDAC development. LCN2 is known to be expressed by adipose tissue [11, 12] and here we showed expression of LCN2 and the LCN2 receptor SLC22A17 (Supplemental Figure 9) in adipose tissue-derived from PDAC mice. Moreover, LCN2 expression levels in adipose tissue have been positively correlated with expression of other proinflammatory cytokines. [11, 12] Adipose tissue is composed of a variety of cell types, among these are the adipose stromal cells (ASCs) which are components of the stromal vascular fraction (SVF). ASCs have been demonstrated to promote tumor growth [54–57] and visceral fat from obese individuals was shown to increase ASCs population and trafficking to the tumor microenvironment [58, 59]. Here we showed that cells from the SVF of murine adipose tissue expressed *Lcn2* and the *Lcn2* receptor SLC22A17. Therefore, there remains a possibility that the adipose tissue via ASCs and/or other cells may be mediating the secretion of proinflammatory molecules such as LCN2 that could contribute to tumor growth. It is also possible that ASCs could be trafficking to areas of PDAC and contributing to PDAC fibrosis for which future studies are necessary. Other groups have also shown that diet-induced obesity increases inflammation of peripancreatic fat in mouse models of PDAC. [60] This further suggests an essential role of adiposity and the risk for PDAC development and complement our current findings. Interestingly, we found that more of the *Lcn2*^{-/-}/*KRas*^{G12D}/CRE mice treated with caerulein developed inflammation and PanIN lesions compared to those treated with vehicle alone. (Supplemental Figure 3) This data suggests that caerulein-induced inflammation and diet-induced obesity inflammation might be acting via different mechanisms that merit further investigation.

Our results indicate that re-expression of *Lcn2* in the pancreas, via orthotopic injection of tumor cells expressing *Lcn2*, is not sufficient to allow for tumor growth and progression in *Lcn2* null animals. This suggests that other sources of *Lcn2*, perhaps secreted from inflammatory cells and/or adipose tissue as discussed above, are needed to create a suitable

tumor microenvironment for disease progression [7, 13]. Since Lcn2 has been shown to be expressed by immune cells, it is possible that Lcn2 derived from the immune system could also contribute to PDAC. It has been previously shown that Lcn2-depleted marrow cells prevent the development of BCR-ABL-induced leukemia, indicating the possible role of inflammatory cell derived Lcn2 in tumor development [61]. The cross-talk between tumor cells and stromal components in PDAC is essential for establishing this environment. PSCs are stromal cells that play a key role in secreting molecules such as cytokines and growth factors that aid in the remodeling of the PDAC tumor microenvironment [52, 62, 63]. In this study we show for the first time that pancreatic stromal cells don't synthesize LCN2 but do express LCN2-specific receptors. Moreover, exogenous LCN2 induces PSCs to secrete pro-inflammatory factors, matrix metalloproteinases, and the complement component C5a. We also observed elevated levels of IL-6, MCP-1, IL-1B and MMP-9 in the pancreas of Lcn2 expressing animals after tumor cell implantation. Because MCP-1, IL-8 and C5a are potent immune cell chemoattractants, these results provide a possible explanation as to why we observed a lack of immune cell presence *in vivo* in Lcn2 null mice.

We specifically showed for the first time that the LCN2 receptor SLC22A17 plays a significant role in the LCN2-mediated pro-inflammatory responses observed in PSCs. We suggest a novel role of LCN2 and its specific receptor SLC22A17 in mediating the synthesis of molecules important for remodeling the PDAC stroma leading to increased tumor growth and decreased survival. In conclusion, our results showed that Lcn2 depletion delayed weight gain, adiposity and tumor growth and increased survival in PDAC mouse models. LCN2 also modulated the production of pro-inflammatory factors in PSCs and reduced the infiltration of macrophages in the stroma of both obesity and non-obesity mouse models of PDAC. Therefore, this study indicates that LCN2 may be an important link between inflammation, obesity and PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. LCN2 is highly upregulated in patients with PDAC and in an obesity-driven PDAC genetic mouse model

(A) LCN2 in human sera from patients with normal pancreas controls (n=10), chronic pancreatitis (n=10), or PDAC (n=10) as well as plasma from patients with metastatic PDAC (n=69), as measured by ELISA *p<0.05; **p<0.01 (B) mRNA expression levels of lipocalin family member molecules in normal (n=10), pancreatitis (n=16) and PDAC (n=14) tissue samples. ***p<0.001 (C) Lcn2 in serum from mice with and without *KRas*^{G12D} expression before and after being fed a HFD for 7 weeks as measured by ELISA (n=8). *p<0.05 (D)

Pancreas mRNA levels of Lcn2 in mice fed a CD vs a HFD for 7 weeks (n=3). *p<0.05 **(E)** Representative immunohistochemical staining of Lcn2 from mice expressing *KRas*^{G12D} fed a CD or a HFD for 7 weeks. **(F)** Lcn2 in fat lysates from mice with and without *KRas*^{G12D} expression in mice fed a CD vs a HFD for 7 weeks (n=3–5). *p<0.05 and p=0.0512 when compared to CRE in CD. See also supplemental figure 1, 2, and 9

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Figure 2. Depletion of Lcn2 delays weight gain, decreases pancreas inflammation, fibrosis, and formation of PanIN lesions in an obesity-driven PDAC genetic mouse model
 (A) Body weight over time after long-term consumption of CD or a HFD from mice with or without *Lcn2* expression. **p*<0.05 (B) Representative images from mice expressing pancreas specific *KRas*^{G12D} with or without *Lcn2* expression fed a HFD >50 days, visceral adipose tissue, traced with white line. (C) Representative images of H & E, Picrosirius Red (collagen stain) and immunohistochemical staining for alpha-SMA, CD45 and F4/80, from mouse pancreas with endogenous *Lcn2* (top panel) or *Lcn2* null (bottom panel) expressing pancreas

specific *KRas*^{G12D}. **(D)** Pathologist scoring of pancreas inflammation, fibrosis, and PanIN lesions from mice expressing pancreas specific *KRas*^{G12D}, with (n=10) and without (n=10) *Lcn2* expression fed a HFD for 7 weeks. *p<0.05, **p<0.01 See also Table 1 and supplemental figure 3.

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Figure 3. Depletion of Lcn2 extends survival in an obesity-driven PDAC genetic mouse model
(A) Survival curves of mice fed a long-term CD ($KRas^{G12D}/CRE$, n=48; $Lcn2^{-/-}/KRas^{G12D}/CRE$, n=21) vs a HFD ($KRas^{G12D}/CRE$, n=32; $Lcn2^{-/-}/KRas^{G12D}/CRE$, n=26) expressing pancreas specific $KRas^{G12D}$, with endogenous Lcn2 (shown in black) or without whole body expression of Lcn2 (shown in gray). Corresponding p-values are shown in table 2. **(B)** Representative mouse pancreas H & E staining at median survival age. **(C)** Representative Picrosirius Red (collagen stain) and immunohistochemical staining for F4/80, CD45 and alpha-SMA of mouse pancreas from A were euthanized at around the medium survival age.

Animals fed a CD are shown in the top two rows and animals fed a HFD are shown in the bottom two rows.

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Figure 4. Depletion of Lcn2 delays PDAC tumor growth and extends survival in a syngeneic orthotopic PDAC model

(A–B) Bioluminescent signal over time, indicating tumor growth from a syngeneic orthotopic model of PDAC using luciferase-labeled mouse PDAC cells (derived from a PDAC genetic mouse model; *KRas*^{G12D}/p53^{-/-}/pdx1-CRE) (KPC-LUC) implanted in *Lcn2*^{+/+} (n=20) and *Lcn2*^{-/-} mice (n=17). ***p<0.001 (C) LCN2 expression as measured by ELISA in serum from mice with or without Lcn2 expression and with (n=7) or without (n=4) tumor cell implantation in the pancreas after 3 weeks. (D) Survival curve of *Lcn2*^{+/+}

and *Lcn2*^{-/-} mice from A after tumor cell implantation. ****p<0.0001 See also supplemental figure 4 and 5.

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Figure 5. *Lcn2* null mice implanted with tumor cells display diminished stromal remodeling, immune cell infiltration, tumor markers and inflammatory molecules

(A) Representative mouse pancreas with endogenous *Lcn2* expression (top panel) and *Lcn2* null (bottom panel) after tumor cell implantation (H & E staining), Picrosirius Red (collagen stain) and immunohistochemical staining for F4/80, CD45, alpha-SMA and Ki67. See also supplemental figure 6 for staining of a representative *Lcn2* null mice implanted with KPC-LUC cells which formed a tumor. (B) Serum levels of putative PDAC serum biomarkers in animals with (n=6) or without (n=6) endogenous *Lcn2* expression after tumor cell

implantation as measured by densitometry analysis of a cytokine array assay. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (C) mRNA expression of pro-inflammatory and ECM molecules in the pancreas of animals with (n=6) or without (n=6) endogenous Lcn2 expression after tumor cell implantation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Figure 6. LCN2 induces a pro-inflammatory response in PSCs that is blocked after downregulation of the SLC22A17 receptor

(A) mRNA expression of LCN2 and its two receptors SLC22A17 and LRP2 in normal pancreas cells (HPDE and HPNE), PDAC cells (BxPC3, HPAC, CAPAN1, CAPAN2, MIAPACA2, MPANC96) and two immortalized PSC#1 and PSC#2. (B) LCN2 expression as measured by ELISA in supernatants from various primary pancreas PSCs (n=10) (cultured to reach 70% confluency, at passage 3–4) and western blot (insert) for LCN2 of BxPC3, PSC#1 and PSC#1 treated with recombinant human LCN2 cell lysates.

Recombinant human LCN2 (rhLCN2) was used as positive control. **(C)** Levels of pro-inflammatory molecules secreted into the media from PSC#1 after 48hrs of treatment with 500ng/ml of rhLCN2 as measured by densitometry analysis of a cytokine array assay. * $p < 0.05$, *** $p < 0.001$. **(D)** 100 ng/mL of rhLCN2 was ectopically added to PSC#1 expressing shRNA vector control or shRNA SLC22A17 and incubated for 24 and 48 hrs with 100 ng/mL of rhLCN2. The resulting effects on the mRNA expression of IL-6, MCP-1, IL-8, IL-1B, MMP-1, MMP-3, MMP-9, C5a, C5aR1, and alpha SMA were examined. rhLCN2 induced a significant upregulation of all molecules after rhLCN2 treatment. This effect is diminished upon downregulation of SLC22A17 expression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (student t-test). See also supplemental figure 7 and 8.

Table 1**Timeline changes in mouse body weight**

The weight of *Lcn2^{+/+}* and *Lcn2^{-/-}* animals fed either a CD or HFD was recorded over time. The mean weight of each group was calculated and CD weights were compared with HFD weights for each group to determine differences in weight gain over time. * *Lcn2^{+/+}* CD vs *LCN2^{-/-}* CD: $p=0.0203$ (240 days), $p=0.0157$ (270 days), ° *Lcn2^{+/+}* HFD vs *LCN2^{-/-}* HFD: $p<0.001$, *Lcn2^{+/+}* CD vs *LCN2^{-/-}* HFD: $p>0.05$, All values are expressed as median and standard deviation (SD)

Days on diet	<i>Lcn2^{+/+}</i> CD	<i>Lcn2^{+/+}</i> HFD	P value <i>Lcn2^{+/+}</i> CD vs HFD	<i>Lcn2^{-/-}</i> CD	<i>Lcn2^{-/-}</i> HFD	P value <i>Lcn2^{-/-}</i> CD vs HFD
0	19.55 (2.74)	22.1 (8.07)	0.1527	19.33 (2.47)	20.18 (2.39)	0.7478
30	23.11 (3.94)	30.68 (6.72)°	<0.0001	24.62 (3.99)	22.77 (3.98)°	0.1451
60	25.57 (4.36)	36.45 (6.90)°	<0.0001	26.15 (4.48)	25.63 (5.29)°	0.7344
90	28.79 (5.86)	44.18 (8.09)°	<0.0001	27.75 (4.46)	29.16 (6.96)°	0.4415
120	30.12 (6.32)	46.12 (8.00)°	<0.0001	27.19 (7.93)	31.22 (7.80)°	0.1093
150	32.53 (7.07)	47.79 (8.43)°	<0.0001	29.93 (6.09)	33.28 (8.35)°	0.1492
180	37.18 (15.5)	51.20 (7.31)°	<0.0001	30.41 (6.44)	35.95 (9.27)°	0.0317
210	34.97 (7.35)	58.41 (22.66)°	<0.0001	30.64 (6.99)	37.56 (9.37)°	0.0106
240	36.81 (7.39)*	57.13 (8.12)°	<0.0001	30.48 (9.93)*	39.28 (9.84)°	0.0070
270	38.33 (8.12)*	60.28 (8.70)°	<0.0001	32.40 (7.43)*	40.51 (10.32)°	0.0061
300	38.84 (7.32)	60.15 (8.68)°	<0.0001	34.49 (7.86)	43.82 (10.21)°	0.0025

Table 2
Lcn2 depletion results in increased survival

The median survival of *KRas*^{G12D}/CRE and *Lcn2*^{-/-}/*KRas*^{G12D}/CRE animals fed either a CD or HFD long term was calculated and assessed for significant changes in survival. (Mantel-Cox test)

		P values Log-rank (Mantel-Cox) test		
		1	2	3
Mice Genotype	Median Survival	<i>KRas</i> ^{G12D} /CRE CD	<i>KRas</i> ^{G12D} /CRE HFD	<i>Lcn2</i> ^{-/-} / <i>KRas</i> ^{G12D} /CRE CD
<i>KRas</i> ^{G12D} /CRE CD	269	NA		
<i>KRas</i> ^{G12D} /CRE HFD	195.5	p=0.0003	NA	
<i>Lcn2</i> ^{-/-} / <i>KRas</i> ^{G12D} /CRE CD	296	p=0.0303	p<0.0001	NA
<i>Lcn2</i> ^{-/-} / <i>KRas</i> ^{G12D} /CRE HFD	249.5	p=0.8794	p=0.0046	p=0.0719