Published in final edited form as:

J Proteome Res. 2011 October 7; 10(10): 4835–4844. doi:10.1021/pr2006318.

Proteomic Analysis of an Immortalized Mouse Pancreatic Stellate Cell Line Identifies Differentially-Expressed Proteins in Activated vs. Non-Proliferating Cell States

Joao A. Paulo, PhD,

Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA, Department of Pathology, Children's Hospital Boston, Boston, MA, Proteomics Center at Children's Hospital Boston, Boston, MA

Raul Urrutia, MD,

Division of Gastroenterology and Hepatology, Gastroenterology Research Unit, Mayo Clinic and Foundation, Rochester, MN

Peter A. Banks, MD,

Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Darwin L. Conwell, MD, MS[#], and

Center for Pancreatic Disease, Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital and Department of Medicine, Harvard Medical School, Boston, MA

Hanno Steen, PhD^{*,#}

Department of Pathology, Children's Hospital Boston and Harvard Medical School, Boston, MA
Proteomics Center at Children's Hospital Boston, Boston, MA

Abstract

Pancreatic stellate cells (PaSC) are mediators in chronic pancreatitis and pancreatic cancer pathogenesis. Proteins regulating the biomolecular pathways involved in the conversion of activated to quiescent PaSC may have a significant influence in the development of chronic pancreatitis. We aim to compare differentially expressed proteins from an immortalized cell line of mouse PaSC in the activated and serum-starved cell states using mass spectrometry-based proteomics. PaSC cultured in media supplemented with fetal bovine serum (FBS) proliferate in the activated state, while serum starvation promotes the cellular transition to a "pseudo-quiescent" state. Using these two cell states, we performed a comparative mass spectrometry (GeLC-MS/MS) proteomic analysis. We identified over 2000 non-redundant proteins in PaSC. Qualitative and label-free quantitative analysis revealed several hundred proteins that were differentially abundant

*Corresponding author Hanno Steen, Ph.D, Children's Hospital Boston, Dept. of Pathology/Enders 1130, 320 Longwood Ave, Boston, MA 02115, USA, Voice: +1-617-919-2629, Fax: +1-617-730-0168, Hanno.Steen@childrens.harvard.edu.

[#]these authors contributed equally to this work

COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

JP carried out the experiments and drafted the original manuscript. JP and DC conceived of the study. HS facilitated the mass spectrometric/proteomics experiments. JP, DC, PB and HS participated in its design and coordination. All authors helped to draft the manuscript and approved the final manuscript.

between the cell states. Proteins that were more abundant in activated PaSC included cytoskeletal proteins and ribosomal proteins, while those more abundant in pseudo-quiescent PaSC included proteins involved in protein degradation-related pathways (lysosome, ubiquitin-mediated proteolysis, and the proteasome). Investigation of the role of PaSC in the pathogenesis of chronic pancreatitis using the mass spectrometry-based proteomics strategy described herein will lead to further insights into the molecular mechanisms associated with the disease.

Keywords

pancreatitis; biomarker; proteomics; fibrosis; pseudo-quiescent

INTRODUCTION

Chronic pancreatitis is a debilitating disorder which can only be detected at a moderate to advanced stage on radiologic and endoscopic imaging, and as such, only symptomatic treatment is possible. One of the hallmarks of chronic pancreatitis is the development of pancreatic fibrosis¹. According to the SAPE hypothesis², an initial insult to acinar and ductal cells (e.g., alcohol) is followed by the invasion of inflammatory cells, which produce cytokines and activate baseline quiescent pancreatic stellate cells (PaSC).

PaSC, which reside in exocrine areas of the pancreas, are myofibroblast-like cells known to transition from the quiescent to activated phenotypes upon insult. These cells are analogous to hepatic stellate cell, as these stellate cells show 99% identity at the transcriptome level^{3,4}. PaSC mediate pancreatic repair, as they migrate to the injured location where their secretions may nucleate cell proliferation, migration, and assembly⁵⁻⁸. Moreover, PaSC are involved in the pathogenesis of chronic pancreatitis and pancreatic cancer. Identification of differentially expressed proteins between activated and quiescent PaSC may provide valuable insight into the development of chronic pancreatitis. In response to pancreatic injury, PaSC undergo both morphologic and functional changes which include the expression of several growth factors and cytokines that are involved in inflammatory and fibrotic processes^{5,9-14}. Understanding the mechanisms that regulate the cellular functions of PaSC could provide potential targets for the treatment or diagnosis of pancreatic fibrosis, which is a precursor to the development of chronic pancreatitis¹⁵.

Pancreatic stellate cells are currently an underexplored resource for potential biomarker proteins. Investigation of the roles of PaSC in pancreatic disease may improve our understanding of the mechanism of fibrosis associated with chronic pancreatitis. Although extensive studies have primarily focused on the proteomic analyses of hepatic stellate cells¹⁶⁻²¹, to date, there have been no analogous large-scale proteomics studies focusing on PaSC.

In this study, we aim to (1) identify the proteome of a mouse PaSC cell line³ in the activated and a non-proliferating pseudo-quiescent cell state using mass spectrometry-based (GeLC-MS/MS) analysis, (2) qualitatively compare proteins between the activated and pseudo-quiescent cell states, (3) quantitatively compare proteins common to activated and pseudo-quiescent cell states using QSPEC, and (4) determine the localization, molecular function, and signaling pathways of the differentially-abundant proteins using gene ontology (GO) and KEGG pathway analysis. The proteins and pathways identified in our work provide a scaffold upon which to build future studies directed at investigating the roles of stellate cells in the fibrotic processes associated with chronic pancreatitis.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's-F12 medium (DMEM/F12; 11330) was purchased from Gibco (Carlsbad, CA). Fetal bovine serum (FBS; F0392) was purchased from Sigma (St. Louis, MO). CellStripper (25-056-CL) was purchased from Mediatech (Manassas, VA). SeeBluePlus2 Pre-Stained standard (LC5925), LDS (lithium dodecyl sulfate) sample buffer (NP0008), NuPAGE 4–12% Bis-Tris polyacrylamide gels (NP0335), SimplyBlue Coomassie stain (LC0665), and MES-SDS (2-(N-morpholino)ethanesulfonic acid-sodium dodecyl sulfate) electrophoresis buffer (NP002) were from Invitrogen (Carlsbad, CA). Other reagents and solvents were from Sigma-Aldrich (St. Louis, MO) and Honeywell, Burdick & Jackson (Morristown, NJ), respectively. The mouse PaSC cell line was a kind gift from Dr. Raul Urrutia, Mayo Clinic, Minneapolis, MN.

Experimental Workflow

The experimental workflow was as summarized in Figure 1. Briefly, an immortalized cell line of mouse PaSC³ was grown in DMEM media either with or without fetal bovine serum (FBS). The experiments were performed in triplicate, that is, the following procedure was repeated in parallel for three plates of PaSC in each cell state. Cells were harvested by dislodgment from a 10-cm cell culture dish using non-enzymatic CellStripper reagent. Cells were then lysed and crudely fractionated into soluble and membrane-enriched solubilized protein fractions. The isolated protein fractions were separated by SDS-PAGE and processed using standard GeLC-MS/MS techniques. Finally, the collected mass spectrometric data were analyzed using a series of bioinformatics methods including database searching with ProteinPilot, spectral counting-based quantification with QSpec, gene ontology (GO) classification, and KEGG pathway analysis.

Cell growth and harvesting

An immortalized pancreatic stellate cell line from mouse (*mus musculus*) origin was propagated in Dulbecco's modified Eagle's-F12 medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Upon achieving 90% confluency, the growth media was aspirated and the cells were washed 3 times with ice-cold PBS. For the preparation of pseudo-quiescent cells, DMEM was added without FBS supplementation. In parallel, fresh DMEM/10% FBS media was added to those cells which were slated to remain in the activated state. Twenty-four hours after the addition of fresh media, the cells were dislodged with non-enzymatic CellStripper, harvested with the addition of 10 mL PBS and pelleted by centrifugation at $3,000 \times g$ for 5 min at 4°C, after which the supernatant was removed. Each cell state was investigated in triplicate.

Cell lysis and crude fractionation

One milliliter of TBSp (50 mM Tris, 150 mM NaCl, pH 7.4 supplemented with 1X Roche Complete protease inhibitors) was added per plate of cells, which were then homogenized with 25 strokes of a Potter-Elvehjem glass homogenizer. The homogenate was sedimented by ultracentrifugation at $100,000 \times g$ for 60 minutes at 4°C. The supernatant (s1 fraction) was collected. The pellet was reconstituted in 1 mL ice-cold TBSp plus 1% Triton X-100 and 0.5% SDS. The reconstituted pellet was once again homogenized with 25 strokes of a Potter-Elvehjem glass homogenizer and incubated on ice with gentle agitation for 1 hour. The detergent-solubilized protein (s2 fraction) was separated from the insoluble proteins and cellular debris by ultracentrifugation at $100,000 \times g$ for 60 minutes at 4°C. Protein concentrations for both the s1 and s2 fractions were determined using the bicinchoninic acid (BCA) assay (23225, ThermoFisher Scientific). This procedure was illustrated in Figure 2.

SDS-PAGE analysis of cell lysates

LDS sample buffer (2 μ l) was added at a 1X concentration to 100 μ g of protein. To each sample, 2 μ l of 0.1 M DTT (dithiothreitol) were added, and the sample was then incubated at 56°C for 1 hour. After cooling, samples were alkylated with 2 μ l of 40% acrylamide for 30 minutes at 23°C. SDS-PAGE protein separation was performed at 150 volts in MES buffer for 45 minutes. Gels were rinsed in deionized water for 10 minutes, fixed in 45% methanol/45% water/10% acetic acid for 30 minutes, stained with SimplyBlue Coomassie for 1 hour, and destained overnight in deionized water.

GeLC-MS/MS analysis

In total, six gel lanes (3 for activated cells and 3 for quiescent cells) of cell lysate proteins were subjected to in-gel tryptic digestion followed by reversed-phase liquid chromatography in-line with a tandem mass spectrometer (GeLC-MS/MS). In brief, entire gel lanes were divided into 10 sections and proteins in each gel section were digested with trypsin^{22, 23}. Peptides extracted from each gel section were fractionated and analyzed by a nanoflow reversed-phase ultra-high pressure liquid chromatography system (nanoLC, Eksigent) in-line with a linear trap quadrupole-Fourier transform ion cyclotron mass spectrometer (LTQ-FT Ultra, Thermo Scientific). The reversed-phase liquid chromatography columns (15 cm $\times$ 100 μ m ID) were packed in-house (Magic C18, 5 μ m, 100 Å, Michrom BioResources). Samples were analyzed with a 60-minute linear gradient (0–35% acetonitrile with 0.2% formic acid) and data were acquired in a data-dependent manner, with 6 MS/MS scans for every full scan spectrum.

Bioinformatics and data analysis

LC/MS data were converted into 'mascot generic format' exporting the 200 most abundant fragment ions for each MS/MS spectrum (PMID:19743429). All data were searched against the UniProt human database (20,330 entries, downloaded: November 11, 2010) using the Paragon Algorithm²⁴, which is integrated into the ProteinPilot search engine (v.3; AB SCIEX, Foster City, CA). Search parameters were set as follows: sample type, identification; Cys alkylation, propionamide; Digestion, trypsin; Instrument, Orbitrap/FT (1–3 ppm); Special factors, gel-based ID; ID focus, none; database, UniProt; detection protein threshold, 99.0%; and search effort, thorough ID. We defined an identified protein as one containing at least one peptide of >95% confidence, as determined by the Paragon Algorithm²⁴. As an integral aspect of the ProteinPilot package, the analysis included a decoy database search, which resulted in a false discovery rate of less than 1% at the protein level as determined using the Proteomics System Performance Evaluation Pipeline Software (PSPEP).

Spectral counting

Relative protein quantitation was accomplished using a label-free technique, spectral counting, which compared the number of identified tandem MS spectra for the same protein across multiple data sets. To search for differences in the protein profile among data sets, spectral counts were normalized based on the total spectral counts, as previously suggested²⁵. More specifically, spectral counts of each protein were divided first by the total spectral counts of all proteins from the same sample, and then multiplied by the total spectral counts of the sample with the maximum total number of spectral counts. Significance analysis of our spectral count data was performed using QSPEC, a recently published algorithm for determining the statistical significance of differences in spectral counting data from two sample sets²⁶. According to convention, a Bayes factor greater than 10 suggests strong evidence that a particular protein was differentially expressed between the two cell states, thus a value of 10 was used as our significance threshold²⁷.

Gene Ontology (GO) Analysis

We performed GO analysis²⁸ using the GoFact online tool^{29, 30} and verified manually using the UniProt³¹ database. Annotation categories used were as follows: *functions*: enzyme regulator activity, ion binding, kinase activity, lipid binding, nucleic acid binding, nucleotide binding, oxygen binding, peptidase activity, protein binding, signal transducer activity, structural molecule activity, transcription regulator activity, and transporter activity; *cellular component*: cytoskeleton, cytosol, endoplasmic reticulum (ER), endosome, extracellular matrix, extracellular region, Golgi complex, lysosomes, membrane, mitochondrion, nucleus, ribonucleoprotein complex, and vacuole.

KEGG Pathway Analysis

Using the DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Database (<http://david.abcc.ncifcrf.gov/>) interface^{32, 33}, we analyzed our lists of proteins with KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis^{34–36}.

BioBase ExPlain 3.0 for comparison of proteomics data with gene expression data for pancreatic disease

ExPlain integrates genomic information with biological knowledge databases and computational analysis methods^{37–39}. This database was manually curated from microarray, \miRNA and ChIP-chip/Seq experiments. ExPlain applies an upstream analysis approach based on the implementation of machine learning and graph topological analysis algorithms to identify causality key-nodes in the network of pancreatic disease. We used ExPlain 3.0 to compare our proteomics data to that of previously identified biomarkers of pancreatic disease, which includes pancreatitis and pancreatic neoplasms.

RESULTS

PaSC displayed morphological differences after growth for 24 hr in serum-free media compared to FBS-containing media

An immortalized mouse PaSC cell line was successfully grown in DMEM media with and without 10% FBS. The cells grown in serum-free media for 24 hr, remained attached to the culture dish, but acquired a rounded morphology and stopped proliferating to become what we have termed “pseudo-quiescent” (Figure 3).

Protein patterns between PaSC lysates of activated and pseudo-quiescent PaSC were similar as determined by SDS-PAGE

After 24 hr of growth in the selected media, both activated and pseudo-quiescent cells were lysed and processed as depicted in Figure 2. SDS-PAGE fractionation was performed on the s1 and s2 fractions for both cell states (Figure 4). Similar protein banding patterns were obtained of replicates both within and between the two cell states. It was also worth noting that the s1 and s2 fractions vary significantly in their protein banding patterns. As expected, heavily-stained protein bands were particularly prevalent in the lower molecular weight range of the s2 lanes as these protein bands are indicative of the presence of histone proteins from the nucleus. For GeLC-MS/MS analysis, each gel lane was subdivided into 10 sections that were individually processed and analyzed. The data were merged in ProteinPilot so that each replicate included proteins from an s1 fraction and the corresponding s2 fraction, as was described further in the methods section.

Centrifugation of cell lysate successfully fractionated proteins

When examining the fractions individually, some proteins were identified exclusively in one particular fraction. For the activated PaSC, 26% of the proteins were identified exclusively in the s1 fraction, 36% were exclusive to the s2 fraction, and 38% were identified in both fractions. Similarly, for the pseudo-quiescent PaSCs, 28% of the proteins were identified exclusively in the s1 fraction, 48% were exclusive to the s2 fraction, and 29% were identified in both fractions.

We identified proteins exclusive to either activated or pseudo-quiescent PaSC using our GeLC-MS/MS protein identification strategy

We identified a total of 1925 non-redundant proteins in activated PaSC and 2142 proteins in pseudo-quiescent PaSC. Qualitatively, of these proteins, 374 were identified exclusively in activated PaSC (Supplementary Table 1) and 591 proteins were identified exclusively in pseudo-activated PaSC (Supplementary Table 2). Proteins identified exclusively in activated PaSC included various ribosomal proteins, collagen alpha-1(V) and alpha-1(XII), matrix metalloproteinase-14, and Ras-related protein Rab-13. Similarly, proteins identified exclusively in pseudo-quiescent PaSC included proteasome subunits beta and 8, cathepsin Z, and lysosome membrane protein 2.

Certain proteins common to both activated and pseudo-quiescent PaSC were determined to have statistically significant abundance differences between the two cell states

Statistically significant differences between the 1551 proteins that were identified in both activated and pseudo-quiescent PaSC were assessed using the QSPEC algorithm. Of these proteins that were identified in both cell states, 164 were determined to be of statistically significant higher abundance in activated PaSC (Supplementary Table 3) and likewise, 233 were of higher abundance in pseudo-quiescent PaSC (Supplementary Table 4). In addition, the majority of proteins, 1154, were determined to have no statistically significant difference between the two cell states (Supplementary Table 5). We illustrate these differences in protein identifications by two Venn diagrams (Figure 6) and Table 1.

Proteins determined to be of higher abundance via QSPEC analysis in activated PaSC included plectin, vimentin, fibronectin, actin isotypes (smooth muscle, cytoplasmic 1 and 2) and collagen isotypes (alpha-1 and alpha-2). Similarly, proteins determined to be of higher abundance via QSPEC analysis in pseudo-quiescent PaSC included cathepsin D, calmodulin, proteasome related proteins (26S proteasome regulatory subunits (3, 4, 6A, and 9), proteasome activator complex subunit 1 and 2, proteasome subunit alpha types (2, 5, and 6) and beta-type (3 and 4)), as well as ubiquitin carboxyl-terminal hydrolases (5, 7, and 14). Of the proteins for which differences were not statistically significant in activated and pseudo-quiescent PaSC, among those with the highest number of combined spectral counts included filamin-A, heat shock proteins, tubulin isotypes (beta-5, alpha-1A, beta-2A), talin-1, alpha-actinin-1, myosin-10 and the common housekeeping protein, glyceraldehyde-3-phosphate dehydrogenase. Such proteins are among a core set which may be useful for normalization purposes in future biochemical assays.

Differences in subcellular localization and molecular function according to gene ontology (GO) were revealed between the activated and pseudo-quiescent PaSC cell states

As shown in Figure 6, we determined 538 proteins as exclusive to (374) or of significantly higher abundance (164) in activated PaSC and 824 as exclusive to (591) or of significantly higher abundance (233) in activated PaSC. These two sets of differentially abundant proteins (538 for activated PaSC and 844 for pseudo-quiescent PaSC) were used for GO classification and KEGG pathway analyses. Using the GoFact online tool [28, 29], we

investigated the subcellular localization and molecular function of these proteins. Examining the subcellular localization, we observed that activated PaSC showed a greater percentage of proteins as being of cytoskeletal, endoplasmic reticulum (ER), membrane, or ribonucleic protein complex origin. However, pseudo-quiescent PaSC showed a greater percentage of proteins as being of extracellular, from the Golgi complex, or of nuclear origin (Figure 7A). Similarly, when examining the molecular function of the differently abundant proteins, we noted that activated PaSC showed a greater percentage of proteins that were enzyme regulating, nucleic acid binding, nucleotide binding, protein binding, or transporters. However, pseudo-quiescent PaSC showed a greater percentage of proteins that were ion binding, kinases, or peptidases (Figure 7B).

Differences in biological pathways (KEGG) were observed between the activated and pseudo-quiescent PaSC cell states

We used the DAVID interface to investigate the biomolecular pathways in which proteins that we determined to be differentially expressed between the two cell states were present^{32, 33}. The proteins used for the KEGG analysis were the same as those used in the GO analysis above. It is important to note that not all proteins identified were associated with a KEGG pathway, thus we are limited to those proteins annotated in the KEGG pathway database. Tables 2 and 3 list the pathways and the number of proteins determined to be in that particular pathway for activated and pseudo-quiescent PaSC cell states, respectively. For activated PaSC (Table 2), the pathways which were comprised by the most number of differentially expressed proteins included the ribosome, focal adhesion, leukocyte migration and oxidative phosphorylation pathways. Similarly, for the pseudo-quiescent PaSC (Table 3), the pathways which were comprised by the greatest number of differentially expressed proteins included regulation of actin cytoskeleton, protein degradation-related pathways (lysosome, ubiquitin-mediated proteolysis, and proteasome), and several sugar and amino acid metabolism pathways. As we observed, differentially-abundant proteins from both activated and pseudo-quiescent PaSC may be expected in common pathways, as these pathways are comprised of a complex network of proteins with certain proteins activating or inhibiting others through different mechanisms.

Comparison of our proteomics data with gene expression data for pancreatic disease revealed some overlapping proteins

The BioBase ExPlain 3.0 database lists 95 protein-encoding genes that have been associated previously with pancreatitis. In addition, 508 protein-encoding genes are correlated with pancreatic neoplasms. Upon submission of our complete list of identified PaSC proteins (a total of 2516 non-redundant proteins), we noted 3 proteins involved in pancreatitis and 21 proteins associated with pancreatic neoplasms and were classified as biomarkers of pancreatic disease (Table 4). These 24 proteins may serve as starting points for future investigations aiming to elucidate the role of PaSC in pancreatic disease.

DISCUSSION

We have successfully identified differentially-expressed proteins from an immortalized mouse PaSC cell line in the activated and pseudo-quiescent states using a GeLC-MS/MS-based strategy. In total, 1925 proteins were identified in activated PaSC and 2142 are identified in pseudo-quiescent PaSC. Of these, 538 proteins are determined to be of higher abundance with statistical significance or are identified exclusively in the activated cells. Analogously, for the pseudo-quiescent cells, 824 proteins are determined to be of higher abundance with statistical significance or are exclusive to this cell state. Using GO classification and KEGG pathway analysis, we determine the presence of a greater number of differentially abundant proteins implicated with cell proliferation in activated PaSC and

with protein degradation in pseudo-quiescent cells. Such a result specifies the significance of protein synthesis in the proliferating, activated PaSC, relative to the non-proliferating, pseudo-quiescent cells.

Supporting our conclusions, GO analysis reveals that the differentially abundant proteins between the activated and quiescent cell states had cellular origins and molecular functions which reflect their actively proliferating and non-proliferating states. In terms of cellular origin, the most striking difference is the increase of proteins from membrane, ribonucleic complexes, and endoplasmic reticulum in activated PaSC, which indicates the need for cellular growth and protein production. Conversely, pseudo-quiescent cells show an abundance of differentially-expressed proteins in the lysosome, a major component of protein turnover in the cell. In terms of molecular function, activated cells have a larger proportion of differentially abundant proteins classified as transporters, as well as nucleotide and nucleic acid binding proteins, again functions of proliferating cells. However, peptidases and ion binding proteins are the predominant protein functions among those of higher abundance in the pseudo-quiescent cells. We further investigate the differentially expressed proteins by KEGG pathway analysis to gain insight into the dissimilarity in GO classifications between the two cell states.

KEGG pathway analysis explores the biomolecular pathways in which certain proteins have been previously determined to have a role. The results of our KEGG pathway analysis correlate well with that of the GO analysis. In activated cells, the pathways with the greatest number of proteins are the ribosome/protein synthesis and focal adhesion pathways. With respect to the ribosomes, this result is in agreement with the relatively greater number of proteins in ribonucleic complexes, as well as the nucleotide and nucleic acid binding function, as determined via GO analysis. Such a result suggests the importance and prevalence of protein synthesis in the proliferating, activated PaSC, relative to the non-proliferating pseudo-quiescent cells. In addition, there is concordance between the proteins involved in the focal adhesion pathway and the higher abundance of membrane proteins as determined via GO analysis, as focal adhesions have a role in cell surface contact and regulatory signaling⁴⁰.

When evaluating the GO and KEGG pathway analyses for the pseudo-quiescent cells, there is also correlation, albeit less apparent than for the activated cells. The lysosome (proteolytic) and regulation of actin cytoskeleton pathways have the greatest number of proteins that are of higher abundance in the pseudo-quiescent cells. The morphological changes alone in the conversion of PaSC from activated to pseudo-quiescent are a result of significant alterations in the cellular mechanism, which may explain the higher abundance of proteins involved in the regulation of actin cytoskeleton. It cannot be concluded, without further supporting evidence, that the pathways under consideration are being up- or down-regulated by the identified proteins, but rather that these proteins are present in the pathway in some capacity.

Protein quantitation is an essential aspect of our analysis, as the absence or presence of a protein does not consider the effects of differential protein expression. It follows that one or more proteins present at a lower or higher level relative to another state could alter certain functions of the cellular machinery. To quantify protein abundance in our study, we use the spectral counting approach that allows for label-free quantitation. This method compares the number of MS/MS spectra for the same protein among several data sets. We choose to analyze our data using spectral counting, as we had not performed *a priori* labeling. In addition, studies have shown that there is a strong linear correlation between relative protein abundance and sequence coverage, with a dynamic range of over two orders of magnitude⁴¹. Furthermore, spectral counting quantitation has been shown to be more

reproducible and as having a higher dynamic range than peptide ion chromatogram-based quantitation⁴², and is particularly useful if no *a priori* labeling has been performed, as in the case of this study.

Although we used the well-established and accepted spectral counting method for relative protein quantitation, other methods are available which can exploit multiplexing capabilities and allow for more robust protein quantitation. iTRAQ (isobaric tag for relative and absolute quantification) or TMT (tandem mass tag) labeling strategies^{43, 44} may offer further advantages as all N-terminus and side-chain amines of peptides are labeled and, thus, are quantifiable. Here peptide samples are covalently labeled with isobaric isotope-coded tags that fragment during the MS/MS process producing reporter ions of known masses for which intensities correlate to the abundance of the precursor peptides, and by inference, proteins, in a given sample. Currently, the multiplexing capability of these commercially-available approaches allow for comparison among up to 8 different cell states and/or growth conditions, which has the added benefit of conserving valuable instrument time. Another alternative labeling strategy, metabolic labeling of cell cultures, ensures that all proteins in the culture are labeled prior to digestion. SILAC (stable isotope labeling by/with amino acids in cell culture), for example, is the most commonly used of such methods and can quantitatively compare up to three⁴⁵ or even five⁴⁶ cell states in a single set of mass spectrometric analyses. Such strategies should be considered in future studies comparing PaSC cell states.

We are limited by the unavailability of a truly quiescent PaSC cell line for comparison with the activated mouse PaSC cell line, as PaSC become activated upon culturing. This lack of immortalized quiescent PaSC necessitates the use of the pseudo-quiescent cells. Future studies may analyze freshly-isolated PaSC from normal pancreata (providing quiescent PaSC) and pancreata of individuals with chronic pancreatitis (providing activated PaSC)^{47, 48}. Further verification would be required to ensure that the isolated cells are indeed activated or quiescent, possibly by the use of cell surface markers and/or fluorescence-activated cell sorting (FACS). To this end, we recognize the utility of isolating and comparing the proteomes of the immortalized PaSC cell lines and those of freshly isolated PaSC for future analyses. In addition, the serum deprivation may result in the activation or deactivation of a variety of other pathways. As such, further investigation of the proteomes of other cell types upon serum deprivation may be merited to show that proteins exclusively present in ‘pseudo-quiescent’ PaSC are indeed specific to this cell type and are not a common event in all mammalian cells resulting from serum deprivation. Such experiments will confirm that the cell line can indeed be a surrogate for freshly isolated PaSC and that our findings can be directly translated to *in vivo* studies.

Prior to the completion of this study, we were unaware of the availability of an immortalized human PaSC cell line. As the overarching goal of understanding the molecular mechanisms of chronic pancreatitis is to develop a means of alleviating the burdens of the disease in humans, differences in the biomolecular mechanisms among species may confound certain findings. A cross-species proteomic comparison between the mouse and human PaSC cell lines may be beneficial to alleviate such concerns. Nonetheless, the study of mouse models, with stringently controlled genetics and experimental parameters, provides a conduit from which pharmacological studies can emerge and develop. Furthermore, the methodology which we utilize herein is a valuable resource for further investigations of PaSC.

In summary, we have identified differentially expressed proteins in an activated and pseudo-quiescent mouse PaSC cell line. We show evidence of an imbalance of proteins involved in protein synthesis and protein degradation, which have relatively more differentially abundant proteins in activated and pseudo-quiescent cells, respectively. Such an imbalance

specifies the importance of ribosomal protein synthesis in the proliferating, activated PaSC, relative to the non-proliferating pseudo-quiescent cells where protein degradation is predominant and synthesis is lagging. Validation using an orthogonal methodology, such as western blotting and/or ELISA, may be performed to validate our findings at the individual protein level. In addition, our study has identified the largest number of proteins from PaSC to date. The propagation of cells in the presence and absence of fetal bovine serum (FBS) in tandem with GeLC-MS/MS analysis has progressed our knowledge of the molecular mechanisms which may have a role in PaSC function. In conclusion, we have established a workflow that resulted in the identification of proteins and associated pathways which are potential targets for further studies investigating the transition of activated to quiescent PaSC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funds were provided by the following NIH grants: 1 F32 DK085835-01A1) (JP), 1 R21 DK081703-01A2 (DC) and 5 P30 DK034854-24 (Harvard Digestive Diseases Center; DC). We would also like to thank members of the Steen Lab at Children's Hospital Boston, in particular John FK Sauld and Dominic Winter for their technical assistance and critical reading of the manuscript.

LIST OF ABBREVIATIONS

FBS	fetal bovine serum
GeLC-MS/MS	SDS-PAGE (gel) coupled with liquid chromatography tandem mass spectrometry
GO	gene ontology
KEGG	Kyoto encyclopedia of genes and genomics
PaSC	pancreatic stellate cells

References

1. Kloppel G, Detlefsen S, Feyerabend B. Fibrosis of the pancreas: the initial tissue damage and the resulting pattern. *Virchows Arch.* 2004; 445(1):1–8. [PubMed: 15138818]
2. Schneider A, Whitcomb DC. Hereditary pancreatitis: a model for inflammatory diseases of the pancreas. *Best Pract Res Clin Gastroenterol.* 2002; 16(3):347–63. [PubMed: 12079262]
3. Mathison A, Liebl A, Bharucha J, Mukhopadhyay D, Lomberg G, Shah V, Urrutia R. Pancreatic stellate cell models for transcriptional studies of desmoplasia-associated genes. *Pancreatol.* 2010; 10(4):505–16. [PubMed: 20847583]
4. Omary MB, Lugea A, Lowe AW, Pandol SJ. The pancreatic stellate cell: a star on the rise in pancreatic diseases. *J Clin Invest.* 2007; 117(1):50–9. [PubMed: 17200706]
5. Shimizu K. Mechanisms of pancreatic fibrosis and applications to the treatment of chronic pancreatitis. *J Gastroenterol.* 2008; 43(11):823–32. [PubMed: 19012035]
6. Patel M, Fine DR. Fibrogenesis in the pancreas after acinar cell injury. *Scand J Surg.* 2005; 94(2): 108–11. [PubMed: 16111091]
7. Ellenrieder V, Schneiderhan W, Bachem M, Adler G. Fibrogenesis in the pancreas. *Rocz Akad Med Bialymst.* 2004; 49:40–6. [PubMed: 15631312]
8. Apte MV, Wilson JS. Mechanisms of pancreatic fibrosis. *Dig Dis.* 2004; 22(3):273–9. [PubMed: 15753610]

9. Masamune A, Kikuta K, Watanabe T, Satoh K, Hirota M, Hamada S, Shimosegawa T. Fibrinogen induces cytokine and collagen production in pancreatic stellate cells. *Gut*. 2009; 58(4):550–9. [PubMed: 19052021]
10. Farrow B, Albo D, Berger DH. The role of the tumor microenvironment in the progression of pancreatic cancer. *J Surg Res*. 2008; 149(2):319–28. [PubMed: 18639248]
11. Aust S, Jager W, Kirschner H, Klimpfinger M, Thalhammer T. Pancreatic stellate/myofibroblast cells express G-protein-coupled melatonin receptor 1. *Wien Med Wochenschr*. 2008; 158(19–20): 575–8. [PubMed: 18998076]
12. Vonlaufen A, Apte MV, Imhof BA, Frossard JL. The role of inflammatory and parenchymal cells in acute pancreatitis. *J Pathol*. 2007; 213(3):239–48. [PubMed: 17893879]
13. Mews P, Phillips P, Fahmy R, Korsten M, Pirola R, Wilson J, Apte M. Pancreatic stellate cells respond to inflammatory cytokines: potential role in chronic pancreatitis. *Gut*. 2002; 50(4):535–41. [PubMed: 11889076]
14. Apte MV, Haber PS, Darby SJ, Rodgers SC, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Pancreatic stellate cells are activated by proinflammatory cytokines: implications for pancreatic fibrogenesis. *Gut*. 1999; 44(4):534–41. [PubMed: 10075961]
15. Masamune A, Shimosegawa T. Signal transduction in pancreatic stellate cells. *J Gastroenterol*. 2009; 44(4):249–60. [PubMed: 19271115]
16. Deng X, Liang J, Lin ZX, Wu FS, Zhang YP, Zhang ZW. Natural taurine promotes apoptosis of human hepatic stellate cells in proteomics analysis. *World J Gastroenterol*. 2010; 16(15):1916–23. [PubMed: 20397272]
17. Bosselut N, Housset C, Marcelo P, Rey C, Burmester T, Vinh J, Vaubourdolle M, Cadoret A, Baudin B. Distinct proteomic features of two fibrogenic liver cell populations: hepatic stellate cells and portal myofibroblasts. *Proteomics*. 2010; 10(5):1017–28. [PubMed: 20049859]
18. Molleken C, Sitek B, Henkel C, Poschmann G, Sipos B, Wiese S, Warscheid B, Broelsch C, Reiser M, Friedman SL, Tornøe I, Schlosser A, Kloppel G, Schmiegel W, Meyer HE, Holmskov U, Stuhler K. Detection of novel biomarkers of liver cirrhosis by proteomic analysis. *Hepatology*. 2009; 49(4):1257–66. [PubMed: 19177598]
19. Kim PK, Kim MR, Kim HJ, Yoo HS, Kim JS, Cho EH, Kim CW. Proteome analysis of the rat hepatic stellate cells under high concentrations of glucose. *Proteomics*. 2007; 7(13):2184–8. [PubMed: 17549797]
20. Gressner OA, Weiskirchen R, Gressner AM. Biomarkers of liver fibrosis: clinical translation of molecular pathogenesis or based on liver-dependent malfunction tests. *Clin Chim Acta*. 2007; 381(2):107–13. [PubMed: 17399697]
21. Kawada N. Analysis of proteins dominantly expressed in hepatic stellate cells of activated phenotype. *Methods Mol Med*. 2005; 117:371–9. [PubMed: 16118463]
22. Neubauer G, Mann M. Mapping of phosphorylation sites of gel-isolated proteins by nanoelectrospray tandem mass spectrometry: potentials and limitations. *Anal Chem*. 1999; 71(1): 235–42. [PubMed: 9921130]
23. Steen H, Kuster B, Fernandez M, Pandey A, Mann M. Detection of tyrosine phosphorylated peptides by precursor ion scanning quadrupole TOF mass spectrometry in positive ion mode. *Anal Chem*. 2001; 73(7):1440–8. [PubMed: 11321292]
24. Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, Keating SP, Hunter CL, Nuwaysir LM, Schaeffer DA. The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Mol Cell Proteomics*. 2007; 6(9):1638–55. [PubMed: 17533153]
25. Dong MQ, Venable JD, Au N, Xu T, Park SK, Cociorva D, Johnson JR, Dillin A, Yates JR 3rd. Quantitative mass spectrometry identifies insulin signaling targets in *C. elegans*. *Science*. 2007; 317(5838):660–3. [PubMed: 17673661]
26. Choi H, Nesvizhskii AI. False discovery rates and related statistical concepts in mass spectrometry-based proteomics. *J Proteome Res*. 2008; 7(1):47–50. [PubMed: 18067251]
27. Jeffreys, H. *Theory of probability*. 3. Clarendon Press; Oxford: 1961. p. 447
28. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson

- JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology, The Gene Ontology Consortium. *Nat Genet.* 2000; 25(1):25–9. [PubMed: 10802651]
29. Li D, Li JQ, Ouyang SG, Wu SF, Wang J, Xu XJ, Zhu YP, He FC. An integrated strategy for functional analysis in large-scale proteomic research by gene ontology. *Progress in Biochemistry and Biophysics.* 2005; 32(11):1026–1029.
30. Dong L, Jianqi L, Shuguang O, Songfeng W, Jian W, Yunping Z, Fuchu H. An integrated strategy for functional analysis in large scale proteomic research by gene ontology. *Molecular & Cellular Proteomics.* 2005; 4(8):S34–S34.
31. Bairoch A, Apweiler R, Wu CH, Barker WC, Boeckmann B, Ferro S, Gasteiger E, Huang H, Lopez R, Magrane M, Martin MJ, Natale DA, O'Donovan C, Redaschi N, Yeh L-SL. The Universal Protein Resource (UniProt). *Nucl Acids Res.* 2005; 33(suppl_1):D154–159. [PubMed: 15608167]
32. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 2003; 4(5):P3. [PubMed: 12734009]
33. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009; 4(1):44–57. [PubMed: 19131956]
34. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 2000; 28(1):27–30. [PubMed: 10592173]
35. Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* 1999; 27(1):29–34. [PubMed: 9847135]
36. Wixon J, Kell D. The Kyoto encyclopedia of genes and genomes--KEGG. *Yeast.* 2000; 17(1):48–55. [PubMed: 10928937]
37. Kel A, Konovalova T, Waleev T, Cheremushkin E, Kel-Margoulis O, Wingender E. Composite Module Analyst: a fitness-based tool for identification of transcription factor binding site combinations. *Bioinformatics.* 2006; 22(10):1190–7. [PubMed: 16473870]
38. Kel A, Voss N, Jauregui R, Kel-Margoulis O, Wingender E. Beyond microarrays: find key transcription factors controlling signal transduction pathways. *BMC Bioinformatics.* 2006; 7 (Suppl 2):S13. [PubMed: 17118134]
39. Kel A, Voss N, Valeev T, Stegmaier P, Kel-Margoulis O, Wingender E. Explain: finding upstream drug targets in disease gene regulatory networks. *SAR QSAR Environ Res.* 2008; 19(5–6):481–94. [PubMed: 18853298]
40. Chen CS, Alonso JL, Ostuni E, Whitesides GM, Ingber DE. Cell shape provides global control of focal adhesion assembly. *Biochem Biophys Res Commun.* 2003; 307(2):355–61. [PubMed: 12859964]
41. Liu H, Sadygov RG, Yates JR 3rd. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem.* 2004; 76(14):4193–201. [PubMed: 15253663]
42. Zybailov B, Mosley AL, Sardi ME, Coleman MK, Florens L, Washburn MP. Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. *J Proteome Res.* 2006; 5(9):2339–47. [PubMed: 16944946]
43. Zieske LR. A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. *J Exp Bot.* 2006; 57(7):1501–8. [PubMed: 16574745]
44. Shadforth IP, Dunkley TP, Lilley KS, Bessant C. i-Tracker: for quantitative proteomics using iTRAQ. *BMC Genomics.* 2005; 6:145. [PubMed: 16242023]
45. Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. *Mol Cell Proteomics.* 2002; 1(5):376–386. [PubMed: 12118079]
46. Molina H, Yang Y, Ruch T, Kim JW, Mortensen P, Otto T, Nalli A, Tang QQ, Lane MD, Chaerkady R, Pandey A. Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy. *J Proteome Res.* 2009; 8(1):48–58. [PubMed: 18947249]
47. Bachem MG, Schneider E, Gross H, Weidenbach H, Schmid RM, Menke A, Siech M, Beger H, Grunert A, Adler G. Identification, culture, and characterization of pancreatic stellate cells in rats and humans. *Gastroenterology.* 1998; 115(2):421–32. [PubMed: 9679048]

48. Vonlaufen A, Phillips PA, Yang L, Xu Z, Fiala-Beer E, Zhang X, Pirola RC, Wilson JS, Apte MV. Isolation of quiescent human pancreatic stellate cells: a promising in vitro tool for studies of human pancreatic stellate cell biology. *Pancreatology*. 2010; 10(4):434–43. [PubMed: 20733342]

Figure 1. General workflow

The general workflow is as follows: a. Mouse PaSC cell line was grown in DMEM media either with or without fetal bovine serum. b. Cells were lysed and crudely fractionated into cytoplasmic and membrane-rich protein fractions. c. The isolated protein fractions were separated by SDS-PAGE and GeLC-MS/MS was performed. d. The mass spectrometric data was analyzed using a series of bioinformatics methods.

Figure 2. PaSC sample processing for GeLC-MS/MS analysis

Cells, which were grown in the absence or presence of 10% FBS, were dislodged from the culture dish with CellStripper, washed with PBS, and homogenized. Following ultracentrifugation, the soluble protein (s1 fraction) was collected and the insoluble fraction was incubated with detergent-containing buffer. The insoluble fraction was once again subjected to ultracentrifugation to isolate the solubilized fraction (s2). The two isolated fractions were subjected to GeLC-MS/MS analysis.

Figure 3. Activated and pseudo-quiescent PaSC

Light micrograph depicting PaSC that have been grown in A) DMEM media plus 10% FBS and B) serum-free DMEM.

Figure 4. SDS-PAGE protein fractionation of PaSC lysates

Each gel lane represented approximately 100 μ g of proteins from either A) activated PaSC (grown in DMEM and 10% FBS) or B) pseudo-quiescent PaSC (grown in serum-free media). We indicate that the gel lane represents either the soluble protein (s1) or the detergent solubilized protein (s2).

Figure 5. Percent contribution of s1 and s2 fractions to total identified proteins

For both the activated and the pseudo-quiescent cell states, the graph displays the percent contribution of proteins that are exclusive to each fraction or which were present in both fractions.

Figure 6. Venn diagram of the total number of non-redundant proteins identified by GeLC-MS/MS in the two cell states (activated and pseudo-quiescent)

In total, 1925 non-redundant proteins were identified in activated PaSC and 2142 proteins were identified in pseudo-quiescent PaSC. A total of 374 and 591 proteins were identified exclusively in activated and pseudo-activated PaSC, respectively. Statistically significant differences between the 1551 proteins identified in both activated and pseudo-quiescent PaSC were examined using QSPEC. This analysis revealed that an additional 164 proteins that were of statistically significant higher abundance in activated PaSC and 233 proteins which were of higher abundance in pseudo-quiescent PaSC.

Figure 7. Gene ontology analysis of A) subcellular localization and B) molecular function for proteins that were differentially expressed in activated and pseudo-quiescent PaSC

Gene ontology classification using the categories listed of the proteins exclusive to or having statistically significant differences was performed manually with the UniProt ³¹ database or using the GoFact online tool ^{29, 30}.

Table 1

Summary of proteins identified for each cell state.

cell state	replicate #	proteins identified in each replicate	non-redundant proteins identified in each cell state	proteins exclusive to each cell state	proteins statistically more abundant in each cell state	total number of differentially expressed proteins between cell states
activated	1	1581	1925	374	164	538
	2	1626				
	3	1395				
pseudo-quiescent	1	1677	2142	591	233	824
	2	1771				
	3	1859				

total number of differentially expressed proteins between cell states= proteins exclusive to each cell state + proteins statistically more abundant in each cell state

Table 2

KEGG pathways analysis of proteins that were present exclusively, or with statistically significant higher abundance, in activated PaSC.

Pathway	# of proteins
Ribosome	28
Focal adhesion	16
Leukocyte migration	14
Oxidative phosphorylation	14
Endocytosis	13
Spliceosome	12
Tight junction	11
Adherens junction	9
ECM-receptor interaction	7
Steroid biosynthesis	6

Table 3

KEGG pathways analysis of proteins that were present exclusively, or with statistically significant higher abundance, in pseudo-quiescent PaSC.

Pathway	# of proteins
Lysosome	31
Regulation of actin cytoskeleton	23
Glycolysis/Gluconeogenesis	20
Spliceosome	20
Purine metabolism	20
Cell cycle	18
Neurotrophin signaling pathway	18
Amino sugar and nucleotide sugar metabolism	15
Ubiquitin mediated proteolysis	15
Proteasome	13
ErbB signaling pathway	12
Antigen processing and presentation	12
Galactose metabolism	11
Fructose and mannose metabolism	11
Pyruvate metabolism	11
Glioma	11
Other glycan degradation	10
Pentose phosphate pathway	9
DNA replication	9
Starch and sucrose metabolism	9
Glutathione metabolism	9
Citrate cycle (TCA cycle)	8
Arginine and proline metabolism	8
RNA degradation	8
Propanoate metabolism	7
Porphyrin and chlorophyll metabolism	7
Cysteine and methionine metabolism	7
Tyrosine metabolism	7
Fatty acid metabolism	7
Valine, leucine and isoleucine degradation	7
Glycerolipid metabolism	7
Phenylalanine metabolism	6

Table 4

Overlap of proteomics data with gene expression data for pancreatic disease using BioBase Explain 3.0.

Accession	Name	Pancreatic disease	Association
AKT1	RAC-alpha serine/threonine-protein kinase	Pancreatic Neoplasms	Causality
ANXA2	Annexin A2	Pancreatic Neoplasms	Causality
EGFR	Epidermal growth factor receptor	Pancreatic Neoplasms	Causality
HMGA1	High mobility group protein HMG-I/HMG-Y	Pancreatic Neoplasms	Causality
MTAP	S-methyl-5'-thioadenosine phosphorylase	Pancreatic Neoplasms	Causality
POSTN	Periostin	Pancreatic Neoplasms	Causality
ROCK1	Rho-associated protein kinase 1	Pancreatic Neoplasms	Causality
ACTB	Actin, cytoplasmic 1	Pancreatic Neoplasms	Correlation
ALDOA	Fructose-bisphosphate aldolase A	Pancreatic Neoplasms	Correlation
ANXA4	Annexin A4	Pancreatic Neoplasms	Correlation
BID	BH3-interacting domain death agonist	Pancreatic Neoplasms	Correlation
CAV1	Caveolin-1	Pancreatic Neoplasms	Correlation
CD44	CD44 antigen	Pancreatic Neoplasms	Correlation
CDK2	Cell division protein kinase 2	Pancreatic Neoplasms	Correlation
GSTM1	Glutathione S-transferase Mu 1	Pancreatic Neoplasms	Correlation
NCAM1	Neural cell adhesion molecule 1	Pancreatic Neoplasms	Correlation
PCNA	Proliferating cell nuclear antigen	Pancreatic Neoplasms	Correlation
RAN	GTP-binding nuclear protein Ran	Pancreatic Neoplasms	Correlation
RHEB	GTP-binding protein Rheb	Pancreatic Neoplasms	Correlation
RHOC	Rho-related GTP-binding protein RhoC	Pancreatic Neoplasms	Correlation
STAT3	Signal transducer and activator of transcription 3	Pancreatic Neoplasms	Correlation
A2M	Alpha-2-macroglobulin	Pancreatitis	Correlation
ALDH2	Aldehyde dehydrogenase, mitochondrial	Pancreatitis	Correlation
FAS	Fatty acid synthase	Pancreatitis	Causality