Biomarker Discovery from Pancreatic Cancer Secretome Using a Differential Proteomic Approach*Ⓢ

Mads Grønborg‡§, Troels Zakarias Kristiansen‡§, Akiko Iwahori‡, Rubens Chang¶, Raghunath Reddy‡, Norihiro Sato¶, Henrik Molina‡§, Ole Nørregaard Jensen§, Ralph H. Hruban¶‖, Michael G. Goggins¶, Anirban Maitra‡¶‖‡‡, and Akhilesh Pandey‡¶‖§§**

Quantitative proteomics can be used as a screening tool for identification of differentially expressed proteins as potential biomarkers for cancers. Candidate biomarkers from such studies can subsequently be tested using other techniques for use in early detection of cancers. Here we demonstrate the use of stable isotope labeling with amino acids in cell culture (SILAC) method to compare the secreted proteins (secretome) from pancreatic cancer-derived cells with that from non-neoplastic pancreatic ductal cells. We identified 145 differentially secreted proteins (>1.5-fold change), several of which were previously reported as either up-regulated (*e.g.* cathepsin D, macrophage colony stimulation factor, and fibronectin receptor) or down-regulated (*e.g.* profilin 1 and IGFBP-7) proteins in pancreatic cancer, confirming the validity of our approach. In addition, we identified several proteins that have not been correlated previously with pancreatic cancer including perlecan (HSPG2), CD9 antigen, fibronectin receptor (integrin β1), and a novel cytokine designated as predicted osteoblast protein (FAM3C). The differential expression of a subset of these novel proteins was validated by Western blot analysis. In addition, overexpression of several proteins not described previously to be elevated in human pancreatic cancer (CD9, perlecan, SDF4, apoE, and fibronectin receptor) was confirmed by immunohistochemical labeling using pancreatic cancer tissue microarrays suggesting that these could be further pursued as potential biomarkers. Lastly the protein expression data from SILAC were compared with mRNA expression data obtained using gene expression microarrays for the two cell lines (Panc1 and human pancreatic duct epithelial), and a correlation coefficient (*r*) of 0.28 was obtained, confirming previously reported poor associations between RNA and protein expression studies. ***Molecular & Cellular Proteomics 5:157–171, 2006.***

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Pancreatic ductal adenocarcinoma (pancreatic cancer) is the fourth leading cause of cancer death in the United States. This year it is estimated that 31,860 Americans will be diagnosed with pancreatic cancer, and 31,270 will die from it (1). Most patients diagnosed with pancreatic cancer die from their disease within months, and only 4% survive 5 years after diagnosis. The overwhelming majority (>80%) of patients with pancreatic cancer present with locally advanced disease or distant metastases, rendering the cancer surgically inoperable (2). Therefore, detection of pancreatic cancer at an early, and hence potentially resectable stage, offers the best hope for cure. Unfortunately tumor markers that are currently utilized for the detection of pancreatic cancer in clinical practice lack the sensitivity and specificity needed to detect potentially curable lesions. For example, serum CA-19-9 is considered the best test for pancreatic cancer (3); however, CA-19-9 can also be elevated in a myriad of non-neoplastic conditions such as acute and chronic pancreatitis, hepatitis, and biliary obstruction, greatly diminishing its specificity (4). In addition, patients with certain blood group antigen types do not express the CA-19-9 antigen. Therefore, the sensitivity of CA-19-9 approaches ~80%, limiting its use for screening purposes particularly for the diagnosis of localized, resectable pancreatic cancers (5). The dismal prognosis and late presentation of pancreatic cancer in most individuals emphasizes the need for developing an improved early detection strategy. This is especially true as advancements in our understanding of the genetics of pancreatic cancer have helped identify families and individuals who harbor an inherited predisposition to this disease (6). Improved early diagnosis strategies in such at-risk patients could potentially save lives (7).

Global profiling of differentially expressed genes and proteins in cancers *versus* normal tissues has been utilized to identify novel neoplasia-associated biomarkers (8). Over the past 5 years, our group has identified a large number of such

From the ‡McKusick-Nathans Institute of Genetic Medicine and the Department of Biological Chemistry, The Johns Hopkins University, Baltimore, Maryland 21205, the §Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark, and the ¶Department of Pathology and The Sol Goldman Pancreatic Cancer Research Center and the ‖Department of Oncology, The Johns Hopkins University, Baltimore, Maryland 21287

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differentially expressed transcripts in pancreatic cancers using cDNA or oligonucleotide-based microarray platforms (9–11). Although the global transcript profiling of pancreatic cancers has yielded numerous insights into the biology of pancreatic cancers (12, 13) and novel therapeutic and imaging targets (14–16), the results in terms of biomarker discovery have been less striking. There are two potential causes for the small fraction of differentially expressed transcripts in pancreatic cancer that translate into effective biomarkers. *First*, there may be significant dissociation between overexpressed transcripts and overexpressed proteins; indeed such a discordance has been reported in comparisons of global transcriptomic and proteomic analyses of other human cancers (17). *Second*, because most expression techniques use "bulk" homogenates of cell lines and/or tissues, there is no preferential enrichment for the secreted class of proteins (*i.e.* the cancer "secretome" (18)), the portion of the proteome expected to harbor the most promising biomarkers.

To overcome these major limitations of biomarker discovery, we have focused in recent years on newly emerging proteomic approaches for unraveling the pancreatic cancer secretome. Two-dimensional gel electrophoresis is a widely used and robust method for studying differentially expressed proteins in cancers *versus* normal tissues (19, 20). Alternative methods like mRNA profiling have also been used in high throughput experiments but do not discriminate between secreted and non-secreted components. Therefore, we have relied principally on technologies that can be used to directly analyze aberrant proteins within clinically relevant body fluids such as serum or pancreatic juice. For example, SELDI-TOF/MS has been used previously to identify peak patterns in the mass spectrum of serum and pancreatic juice that distinguish neoplastic from non-neoplastic samples (21); a common pitfall of SELDI, however, is the inability to determine the actual proteins or peptides that may be contributing to these differential peak patterns. LC-MS/MS is an alternative approach that couples protein fractionation with mass spectrometry to generate a comprehensive catalog of the proteome of analyzed fluid samples such as pancreatic juice and bile (22, 23).

Although both SELDI and LC-MS/MS are technically capable of secretome analyses, they remain essentially qualitative approaches (*i.e.* peak patterns or peptides are identified solely as present or absent in normal and cancerous specimens). However, small quantities of many proteins are likely to be present in body fluids of normal individuals or those with benign pancreatic disorders, even those that demonstrate significant quantitative differences in cancer patients. *Quantitative* proteomic approaches are therefore required to significantly increase the repertoire of candidate biomarkers, which can then be tested on larger sample sets using traditional ELISA-based approaches. Such newly described technologies include mass tagging (*e.g.* ICAT) (24, 25), ^{18}O labeling (26), and stable isotope labeling with amino acids in cell culture (SILAC)[1] (27, 28), all of which can be combined with mass spectrometry to yield powerful tools to quantitatively analyze cancer secretomes. SILAC has been used previously for relative quantitation in several systems such as differential changes in cells upon epidermal growth factor or insulin simulation and dynamics of lipid raft proteins or phosphorylation events (29–32). Most recently, SILAC has also been used for relative quantitation of lysates from prostate cancer cell lines with varying metastatic potential (33).

In this study we used SILAC to compare the secretomes of two human pancreatic cell lines, an immortalized non-neoplastic human pancreatic duct epithelial (HPDE) cell line and a pancreatic ductal adenocarcinoma cell line (Panc1). Three features of our study design increase our yield of potential biomarkers. First, we directly compare a well established *in vitro* model of normal *ductal* epithelium (*i.e.* HPDE (34–36)) with its neoplastic *ductal* counterpart (*i.e.* Panc1), circumventing abundant acinar and islet proteins that would be present in bulk pancreatic tissue comparisons. Second, by restricting our analyses to quantitating relative differences in proteins in the secreted compartment alone, we maximize the possibility of identifying candidates that have the greatest potential for translation to fluid-based biomarkers for pancreatic cancer detection. Third, we validate our best biomarker candidates from the proteomic study by immunohistochemical labeling using pancreatic cancer tissue microarrays.

MATERIALS AND METHODS

Cell Culture and SILAC Reagents—Custom Dulbecco's modified Eagle's medium lacking arginine and lysine was prepared by Invitrogen. [$^{13}C_6$]Arginine (heavy arginine) and [$^{13}C_6$]lysine (heavy lysine) were purchased from Cambridge Isotopes, Inc. (Andover, MA). Panc1 pancreatic cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were grown in Dulbecco's modified Eagle's medium with a combination of heavy arginine plus lysine with 10% dialyzed fetal bovine serum plus antibiotics. Immortalized non-neoplastic HPDE cells were obtained from Dr. Ming Tsao, Ontario, Canada (35) and grown in defined keratinocyte-serum free medium with supplements (Invitrogen) and antibiotics. We have demonstrated previously that the HPDE cell line represents a valid *in vitro* surrogate for human ductal epithelium (10, 34, 37–39). For harvesting conditioned media, HPDE and Panc1 cells were grown as described above but in the absence of serum.

Sample Preparation—Approximately 3×10^7 HPDE or Panc1 cells were grown to 80% confluence, washed six times in serum-free medium, and incubated for 24 h in serum-free medium. The condi-

[1] The abbreviations used are: SILAC, stable isotope labeling with amino acids in cell culture; HPDE, human pancreatic duct epithelial; SPARC, secreted protein acidic and rich in cysteine; SDF4, stromal cell-derived factor 4; ER, endoplasmic reticulum; Mac-2BP, Mac-2-binding protein; IGFBP, insulin-like growth factor-binding protein; IGFBP-rP, insulin-like growth factor binding protein-related protein; LOX, lysyl oxidase; LOXL, lysyl oxidase-like; apoE, apolipoprotein E; PTK7, protein-tyrosine kinase 7; HSPG2, heparan sulfate proteoglycan 2; IHC, immunohistochemistry; TMA, tissue microarray; MCP-1, monocyte chemotactic protein 1; L1CAM, L1 cell adhesion molecule isoform 1.

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tioned medium containing the secreted proteins was collected and filtered using a 0.45-μm filter (Millipore, Bedford, MA) and subsequently concentrated using a 3,000-Dalton molecular mass cutoff spin column, Centriprep (Millipore). A dilution series of the concentrated media (after Centriprep concentration) from HPDE and Panc1 cell cultures was run on a 4–12% gradient NuPAGE gel (Invitrogen) and silver-stained. To ensure normalization, three different ratios close to 1:1 were mixed, and five bands (different molecular weights) from each sample were cut out and digested by trypsin as described previously (22). The digested bands were analyzed by LC-MS/MS, and the mixed sample in which the most proteins were in a 1:1 ratio was chosen for the large scale experiment. For the initial small scale normalization experiments, ~10 μg (protein concentration measure by DC protein assay, Bio-Rad) of protein from each sample were mixed. For the large scale experiment (used for quantitation and identification), ~50 μg of each sample were mixed and run on a NuPAGE 4–12% gradient gel. The gel was subsequently silver-stained, excised into 25 bands, and digested by trypsin. A replicate of the sample was generated by loading the same amount of sample (50 μg of each sample) on another 4–12% NuPAGE gel and digested as described above.

Liquid Chromatography—Each fraction from the in-gel digestion was analyzed by automated nanoflow LC-MS/MS. An Agilent Technologies 1100 series system was used to deliver a flow of 1.5 μl/min during desalting of the sample and 250 nl/min during elution of the peptides into the mass spectrometer. Each sample was loaded onto an on-line analytical fused silica needle column (Proxion Biosystems, Odense, Denmark) packed with 5-μm Vydac C_{18} resin. Washing and desalting was done with 95% mobile phase A (H_2O with 0.4% acetic acid and 0.005% heptafluorobutyric (v/v)) and 5% mobile phase B (90% acetonitrile, 0.4% acetic acid, 0.005% heptafluorobutyric acid in water). Samples were eluted from the analytical column by a linear gradient of 90% mobile phase A to 60% mobile phase A. A 34-min gradient was used for elution. A potential of 2.8 kV was applied to the emitter (Proxion Biosystems). The spectra were acquired on a Micromass (Manchester, UK) Q-TOF API-US system equipped with an ion source designed by Proxion Biosystems. All data were obtained in positive ion mode. Data-dependent acquisition was used with a ion mass window set to 2.5 Da. MS to MS/MS switch was set to switch on a threshold of 10 counts/s, and MS/MS to MS was set to an intensity below a threshold of 2 counts/s. Charge state recognition was used to estimate the collision energy for the fragmented precursor. Scan time was set to 0.9 s, and interscan time was set to 0.1 s. The number of components (*i.e.* number of MS/MS per MS scan) was set to three resulting in a total cycle time (one MS and three MS/MS spectra) of 10 s. Data analysis was performed using MassLynx 4.0 software.

Data Analysis, Quantitation, and Database Searching—Peak lists were generated from the raw data using the following criteria: smooth window, 4.00; number of smooth, 2; smooth mode, Savitzky Golay; and percentage of peak height to calculate centroid spectra, 80% with no base-line subtraction. The generated peak lists (pkl-file) were searched against the RefSeq database (build 33) (www.ncbi.nlm.nih-.gov./RefSeq/) using Mascot version 2.0. with a mass accuracy of 1.1 Da for the parent ion (MS) and 0.3 for the fragment ions (MS/MS). The peptides were constrained to be tryptic with a maximum of two missed cleavages. Carbamidomethylation of cysteines was considered a fixed modification, whereas oxidations of methionine residues, "heavy" arginine (+6 Da), heavy lysine (+6 Da), and formation of pyroglutamic acid for peptides containing an N-terminal glutamine were considered as variable modifications. An initial protein list was generated using the following screening procedure. Only proteins containing at least one unique peptide (we refer to a peptide as being unique for a specific protein if the sequence has not been assigned to a different protein) with a Mascot score over 25 were considered in the dataset. The highest scoring peptide was manually interpreted to confirm the predicted sequence by Mascot and to eliminate potential false positives. For all proteins identified based on a single peptide, the corresponding MS/MS spectra are provided in Supplemental Fig. 1. Quantitation was performed on three to four peptides (if available) by comparing the extracted ion chromatogram of the corresponding light and heavy peptides. In addition, the ratios in the MS spectrum of the correlated "light" and heavy peptides were measured. In cases where the ratio of the light and heavy peptides was difficult to determine because of low signal in one of the states, a conservative value of 20 (for up-regulated proteins) or 0.05 (1⁄20) for down-regulated proteins was used.

Antibodies—Anti-perlecan, (catalog number H1890-92A) and anti-Rac2 antibody (catalog number R0400) were purchased from US Biological). Anti-monocyte chemotactic protein 1 (MCP-1) antibody (catalog number HM2011) was purchased from Cell Sciences (Canton, CA). Anti-L1 cell adhesion molecule isoform 1 (L1CAM) antibody (catalog number ab3200) and anti-transferrin (catalog number ab10208) antibody were purchased from Abcam (Cambridge, MA). Anti-CD9 antibody (catalog number 555370), anti-apolipoprotein E (apoE) antibody (catalog number 610449), and anti-stromal cell-derived factor 4 (SDF4) antibody was purchased from BD Biosciences. Anti-amyloid β A4 antibody (catalog number NE1003) was purchased from Calbiochem. Anti-Mac-2-binding protein (Mac-2BP) antibody (catalog number BMS146) was purchased from Bender MedSystems (Burlingame, CA). Anti-cathepsin B antibody (catalog number AF953), anti-connective tissue growth factor antibody (catalog number MAB660), anti-cathepsin D (catalog number MAB1014), anti-IGFBP-7 antibody (catalog number MAB1334), anti-secreted protein acidic and rich in cysteine (SPARC) antibody (catalog number MAB941), and anti-secretory leukocyte protease inhibitor antibody (catalog number MAB1274) were purchased from R&D Systems (Minneapolis, MN). Anti-fibronectin receptor (integrin β1) antibody (catalog number 42026) was from QED Biosciences (San Diego, CA).

Western Blot Analysis—A subset of proteins determined to be differentially expressed by SILAC was confirmed by Western blot analysis; these proteins were selected using a combination of parameters including level of differential expression in cancer *versus* normal, protein function, and availability of commercial antibodies. Approximately 10 μg of protein form HPDE and Panc1 cells were run on a NuPAGE 4–12% gradient gel (Invitrogen). After gel electrophoresis the resolved proteins were transferred onto nitrocellulose. The membranes were blocked in 5% skimmed milk powder in phosphate-buffered saline containing 0.1% Tween 20 overnight at 4 °C and incubated with the relevant antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated antibody and developed using enhanced chemiluminescence detection according to the manufacturer's instructions (Amersham Biosciences).

Immunohistochemical Labeling of Tissue Microarrays—Immunohistochemical labeling was performed on pancreatic cancer tissue microarrays for the most promising candidates based on biological relevance and/or Western blot analysis; this included six proteins that were confirmed as up-regulated in the Panc1 secretome (CD9, perlecan, SDF4, apoE, fibronectin receptor, and L1CAM). Tissue microarrays of pancreatic cancer were constructed as described previously (40, 41). Briefly archival sections of human pancreatic cancers and adjacent normal pancreata were selected as "donor" blocks from which 1.4-mm cores were retrieved for constructing the arrays. Each case was represented by four cores, which included three cores of each cancer to exclude intratumoral heterogeneity of protein expression and one core of normal pancreatic parenchyma. Immunohistochemical labeling using various antibodies (anti-CD9, anti-perlecan, anti-L1CAM, anti-apoE, anti-SDF4, and anti-fibronectin receptor) was

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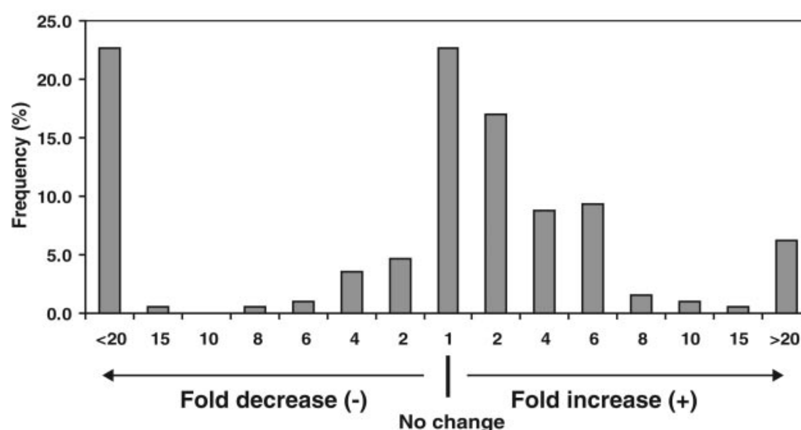

Fig. 1. **Summary of changes in abundance of proteins identified by SILAC.** A total of 195 proteins was identified and quantitated by SILAC. In cases where the abundance of one of the peptide species (light or heavy) was very low or undetectable, a >20-fold change is indicated as shown.

performed as described previously (40, 41). Tissue microarrays were scored by a gastrointestinal pathologist (A. M.) with extensive familiarity in pancreatic cancer morphology and immunohistochemistry. CD9 and fibronectin receptor labeling was scored as positive based on specific membrane localization, whereas perlecan, L1CAM, SDF4, and apoE were scored based on cytoplasmic/membrane localization.

Comparison with Data from DNA Microarrays—To determine concordance between differential expression of proteins determined using SILAC and differential expression of transcripts determined using a DNA microarray-based platform (Affymetrix), we performed a global expression analysis of Panc1 and HPDE lines (42). Total RNA was extracted from cultured Panc1 and HPDE cells using TRIzol reagent (Invitrogen) and was purified using an RNeasy minikit (Qiagen, Valencia, CA). First and second stranded cDNA was synthesized from 10 μg of total RNA using T7-(dT)$_{24}$ primer (Genset Corp., South La Jolla, CA) and the SuperScript Choice system (Invitrogen). Labeled cRNA was synthesized from the purified cDNA by *in vitro* transcription reaction using the BioArray HighYield RNA Transcript Labeling kit (Enzo Diagnostics, Inc., Farmingdale, NY) at 37 °C for 6 h. The cRNA was fragmented at 94 °C for 35 min in a fragmentation buffer (40 mmol/liter Tris acetate (pH 8.1), 100 mmol/liter potassium acetate, 30 mmol/liter magnesium acetate). The fragmented cRNA was then hybridized to the Human Genome U133A chips (Affymetrix, Santa Clara, CA) with 18,462 unique gene/expressed sequence tag transcripts at 45 °C for 16 h. The washing and staining procedure was performed in the Affymetrix Fluidics Station according to the manufacturer's instructions. The probes were then scanned using a laser scanner, and signal intensity for each transcript (background-subtracted and adjusted for noise) was calculated using Microarray Suite Software 5.0 (Affymetrix). -Fold change analysis of signal intensities obtained from DNA microarrays between Panc1 and HPDE lines was performed using Data Mining Tool software (Affymetrix).

RESULTS AND DISCUSSION

A SILAC-based Strategy for Quantitative Secretome Analysis—A pancreatic adenocarcinoma cell line (Panc1) and an immortalized HPDE cell line served as our model system. HPDE cells were grown in normal medium containing light arginine and lysine, whereas Panc1 cells were grown in medium containing heavy arginine plus lysine. Twenty-four hours prior to harvesting of the conditioned medium, the cells were extensively washed in serum-free medium to remove any "contaminants" originating from serum in the medium. Subsequently the conditioned medium was harvested and filtered. The conditioned medium was desalted and concentrated using a 3,000-Dalton molecular mass cutoff spin column. We used a mixture of heavy arginine and lysine amino acids in this study to maximize the number of labeled peptide pairs for quantitation. The mass spectrometric analysis (LC-MS/MS) was done in duplicate and resulted in identification of 195 proteins in total (see Supplemental Fig. 2). Of these, 145 proteins were found to be differentially regulated (>1.5-fold change) in the secretome of Panc1 cells *versus* HPDE cells. Fig. 1 shows the distribution of changes in abundance of these proteins. Of these, a total of 68 proteins were up-regulated >1.5-fold, whereas 77 proteins were down-regulated >1.5-fold in cancer (Panc1) compared with normal (HPDE) cells. Twelve (6%) of the overexpressed proteins were highly up-regulated (defined arbitrarily as ≥20-fold) in cancer compared with normal, whereas 23% of the down-regulated proteins were highly down-regulated (≥20) (Table I and Table II).

It should to be noted that not all the proteins identified in our study are known to be secreted. Some of the proteins are localized to the plasma membrane or endoplasmic reticulum (ER), whereas others are cytosolic proteins. The cytosolic proteins likely originate from the minority of cells that die or are otherwise disrupted during the procedure. To estimate the number of dead cells, we performed Trypan blue staining for both cells lines (HPDE and Panc1) after 24 h of starvation and under normal growth (control). Both cells lines showed less than 3.5% of dead cells with no difference between starved and control cells.

Identification of Known Markers of Pancreatic Cancer—Our approach led to the identification of several known as well as novel proteins that were elevated in cancer compared with normal. Among the proteins previously known to be up-regulated in pancreatic cancer were cathepsin D, Mac-2BP, fibronectin receptor (integrin β1), macrophage colony stimulation factor, and cathepsin B. These proteins have been

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TABLE I

Partial list of proteins identified to be up-regulated in pancreatic cancer (Panc1) versus non-neoplastic (HPDE) cells by SILAC

RefSeq accession no.	Protein name	Ratio (Panc1/HPDE)		Localization	Signal peptide	Protein function	Previously reported in pancreatic cancer/Ref.
		SILAC	DNA array				
NP_001141.1	Aminopeptidase N	20[a]	0.7	Extracellular, plasma membrane	Yes	Metalloprotease, protein metabolism	102
NP_002264.1	Keratin 8	20[a]	2.5	Cytoplasm	No	Structural protein, cell growth	10
NP_006175.2	Nucleobindin 1	20[a]	0.3	Extracellular, Golgi	Yes	Calcium-binding protein, signal transduction	103
NP_057631.1	SDF4	20[a]	1	Golgi, ER	Yes	Calcium-binding protein, signal transduction	No
NP_000032.1	Apolipoprotein E	20[a]	0.9	Extracellular	Yes	Transport/cargo protein	No
NP_839946.1	Hyaluronan	20[a]	TNP[b]	Extracellular	Yes	Adhesion molecule, signal transduction	104–106
NP_002973.1	MCP-1	20[a]	270	Extracellular	Yes	Chemokine, immune response	107–109
NP_039268.1	Dimethylarginine dimethylaminohydrolase 2	20[a]	4.2	Cytoplasm	No	Hydrolase, metabolism	No
NP_002309.1	Lysyl oxidase-like 2	20[a]	TND[c]	Extracellular	Yes	Oxidase, cell growth	No
NP_060599.1	Pyridoxine 5′-phosphate oxidase	20[a]	0.5	Cytoplasm	No	Oxidase, metabolism	No
XP_291627.3	Prostate cancer-associated gene 5	20[a]	TNP	Unknown	No	Unknown	37
NP_002769.1	Saposin A	10.7	1	Extracellular	Yes	Integral membrane protein, signal transduction	103
NP_001760.1	CD9 antigen	8.0	0.5	Plasma membrane	No	Immune response	10
NP_001900.1	Cathepsin D	6.7	1.2	Lysosome, extracellular	Yes	Aspartic protease, protein metabolism	10, 100, 110
NP_006817.1	Tryptophan 5, monooxygenase activation protein (14-3-3 θ)	6.2	1.7	Cytoplasm	No	Adapter molecule, signal transduction	No
NP_001279.2	Chloride intracellular channel 1	5.6	2.8	Plasma membrane, nucleus	No	Ligand-gated channel, transport	No
NP_002061.1	Guanine nucleotide-binding protein	5.4	1	Plasma membrane, ER	No	GTPase, signal transduction	No
NP_001869.1	Cellular retinoic acid-binding protein 2	5.3	0.9	Cytoplasm	No	Transcription regulatory protein	No
NP_004915.2	Actinin, α 4	5.2	3.8	Cytoplasm	No	Cytoskeletal protein, cell growth	No
NP_000416.1	L1CAM	5.2	12	Plasma membrane	Yes	Adhesion molecule	No
NP_005520.2	Perlecan (HSPG2)	5.1	0.8	Extracellular	Yes	Extracellular matrix protein, cell growth	No
NP_066953.1	Peptidylprolyl isomerase A (cyclophilin A)	4.9	1	Cytoplasm	No	Chaperone, protein metabolism	100
NP_005558.1	Galectin-3-binding protein (Mac-2BP)	4.9	0.5	Extracellular	Yes	Extracellular matrix protein, immune response	44, 111
NP_004379.1	Di-*N*-acetylchitobiase	4.7	2.8	Lysosome	Yes	Glycosidase, metabolism	No
NP_001366.1	Deoxyribonuclease II	4.7	0.9	Lysosome	Yes	Deoxyribonuclease, metabolism	112
NP_690619.1	PTK7	4.6	0.8	Plasma membrane	Yes	Tyrosine kinase, signal transduction	No
NP_066012.1	KIAA1627	4.5	TNP	Unknown	No	Methyltransferase, metabolism	No
NP_003364.1	Vinculin	4.3	TND	Cytoplasm	No	Cytoskeletal protein, cell growth	No
NP_055703.1	Predicted osteoblast protein (FAM3C)	4.2	1.1	Extracellular	Yes	Cytokine, signal transduction	No
NP_001055.1	Transketolase	4.2	1	Cytoplasm	No	Transketolase, metabolism	No
NP_001091.1	Actin, α 1	4	TND	Cytoplasm	No	Structural protein, cell growth	No
NP_002863.1	Ras-related C3 botulinum toxin substrate 2 (Rac-2)	3.5	TND	Plasma membrane	No	GTPase, signal transduction	No
NP_002202.2	Fibronectin receptor (integrin β1)	3.4	1.2	Plasma membrane, ER, extracellular	Yes	Cell surface receptor, signal transduction	73, 113
NP_000475.1	Amyloid β (A4)	3	0.4	Plasma membrane, ER, extracellular	Yes	Cell surface receptor, signal transduction	114
NP_006073.1	Tubulin, α	3	2	Cytoplasm	No	Structural protein, cell growth	10
NP_478059.1	Phosphoserine aminotransferase isoform 1	3	0.5	Extracellular	No	Aminotransferase, metabolism	No

[a] An arbitrary value of 20 was chosen for proteins where the intensity of the peptides in the MS spectra was either very low or absent in normal (HPDE) compared to cancer (Panc1).

[b] TNP, transcript not present on the chip.

[c] TND, transcript not detected on the chip.

validated by several platforms (9, 38, 43, 44) and were also identified to be up-regulated in our proteomic study (Table I). Table I provides a partial list of proteins identified along with the heavy/light ratios as well as annotations regarding presence of signal peptide, subcellular localization, and protein function mainly obtained from the Human Protein Reference Database (45). Supplemental Table 1 provides detailed information on all proteins identified in this study including the

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TABLE II
Partial list of proteins identified to be down-regulated in pancreatic cancer (Panc1) versus non-neoplastic (HPDE) cells by SILAC

RefSeq accession no.	Protein name	Ratio (Panc1/HPDE)		Localization	Signal peptide present	Protein function
		SILAC	DNA array			
NP_001054	Transferrin	0.05[a]	TND[b]	Extracellular, plasma membrane, ER	Yes	Transport
NP_002017	Fibronectin 1	0.05[a]	0.012	Extracellular, ER	Yes	Extracellular matrix protein, cell growth
NP_036246	Caspase 14	0.05[a]	TNP[c]	Cytoplasm	No	Cysteine protease, protein metabolism
NP_002643	Prolactin-induced protein (PIP)	0.05[a]	TND	Extracellular	Yes	Secreted polypeptide
NP_004985	Matrix metalloproteinase 9 (MMP-9)/gelatinase B	0.05[a]	0.9	Extracellular, plasma membrane	Yes	Metalloprotease, protein metabolism
NP_001176	α_2-Glycoprotein 1	0.05[a]	TND	Extracellular	Yes	Secreted protein, metabolism lipolysis
NP_444513	Dermcidin	0.05[a]	TNP	Extracellular	Yes	Secreted polypeptide, immune response
NP_003371	Vimentin	0.05[a]	104	Cytoplasm	No	Cytoskeletal protein, cell growth
NP_000517	Keratin 14	0.05[a]	0.014	Cytoplasm	No	Cytoskeletal protein, cell growth
NP_001314	Cystatin M	0.05[a]	0.07	Extracellular	Yes	Protease inhibitor, protein metabolism
NP_002288	Lipocalin 1	0.05[a]	TND	Extracellular	Yes	Transport
NP_057436	Carboxypeptidase A4	0.05[a]	0.2	Extracellular	Yes	Metalloprotease, protein metabolism
NP_006542	Lipophilin B	0.05[a]	TND	Extracellular	Yes	Secreted polypeptide
NP_002990	Syndecan 4	0.05[a]	0.17	Plasma membrane	Yes	Cell surface receptor, signal transduction
NP_005597	Legumain	0.05[a]	TND	Lysosome	Yes	Cysteine protease, protein metabolism
NP_001903	cathepsin L	0.05[a]	0.4	Lysosome	Yes	Cysteine protease, protein metabolism
NP_002954	S100 calcium-binding protein A7	0.05[a]	TND	Extracellular, ER	No	Calcium-binding protein, signal transduction
NP_003236	Transglutaminase 3	0.05[a]	TND	Cytoplasm	No	Aminotransferase, metabolism
NP_001544	IGFBP-7	0.05[a]	0.07	Extracellular	Yes	Adhesion molecule, signal transduction
NP_005969	S100 calcium-binding protein A2	0.05[a]	0.02	Cytoplasm	No	Calcium-binding protein, signal transduction
NP_000349	Transforming growth factor-β1	0.05[a]	0.75	Extracellular	Yes	Ligand, signal transduction
NP_002566	Plasminogen activator inhibitor, type 2	0.05[a]	0.01	Extracellular	Yes	Protease inhibitor, protein metabolism
NP_003055	SLPI	0.05[a]	0.2	Extracellular	Yes	Protease inhibitor, protein metabolism
NP_004939	Desmocollin 1	0.05[a]	TND	Extracellular	Yes	Hydrolase, protein metabolism
NP_000230	Lysozyme	0.05[a]	TND	Extracellular	Yes	Hydrolase, protein metabolism
NP_002334	Lactotransferrin	0.05[a]	TND	Extracellular	Yes	Transport/cargo
NP_115997	Myosin 18B	0.05[a]	TNP	Cytoplasm	No	Motor protein, cell growth
NP_062564	Interleukin 1 family, member 9	0.05[a]	TND	Cytoplasm	No	Ligand, signal transduction
NP_001892	Connective tissue growth factor (CTGF)	0.05[a]	0.5	Extracellular, Golgi	Yes	Extracellular matrix protein, cell growth
NP_036529	Protease inhibitor 13	0.05[a]	0.01	Cytoplasm	No	Protease inhibitor, protein metabolism
NP_005204	Cystatin A	0.05[a]	0.006	Extracellular	No	Protease inhibitor, protein metabolism
NP_003625	NIPSNAP 1	0.05[a]	0.5	Unknown	No	Unknown
NP_000198	Proinsulin	0.05[a]	TND	Extracellular	Yes	Growth factor, signal transduction
NP_002955	S100 calcium-binding protein A8	0.05[a]	0.08	Extracellular, cytoplasm, plasma membrane	No	Calcium-binding protein, signal transduction
NP_003237	Thrombospondin 1	0.06	0.13	Extracellular	Yes	Extracellular matrix protein, cell growth
NP_076956	Chromosome 7 open reading frame 24	0.1174	0.68	Unknown	No	Unknown
NP_001145	Annexin 5	0.1371	1.1	Extracellular, plasma membrane	Yes	Calcium-binding protein, signal transduction
NP_065877	Synaptotagmin XIII	0.1449	TND	Plasma membrane, Golgi	No	Membrane transport protein
NP_005130	Annexin A3	0.1961	0.28	Cytoplasm	No	Calcium-binding protein, signal transduction
NP_001419	Enolase 1	0.204	1.7	Lysosome, plasma membrane	Yes	Hydrolase, protein metabolism
NP_000138	Fucosidase, α-L-1	0.2174	0.6	Lysosome, plasma membrane	Yes	Hydrolase, protein metabolism
NP_001677	ATP synthase	0.2222	1.1	Plasma membrane	No	Transport/cargo, metabolism
NP_000090	Cystatin C	0.2222	0.15	Extracellular	Yes	Protease inhibitor, protein metabolism
NP_002037	Glyceraldehyde-3-phosphate dehydrogenase	0.2439	1.1	Cytoplasm	No	Dehydrogenase, metabolism
NP_003775	Megsin	0.25	0.0032	Cytoplasm	No	Protease inhibitor, protein metabolism
NP_005013	Profilin 1	0.2941	1.3	Cytoplasm	No	Cytoskeletal protein, cell growth
NP_006423	Epididymal secretory protein	0.3125	0.16	Lysosome	No	Transport/cargo, metabolism
NP_000934	Cyclophilin C	0.3571	0.6	extracellular, ER	Yes	Chaperone, metabolism
NP_003109	SPARC	0.3846	0.13	Extracellular	Yes	Secreted polypeptide, signal transduction
NP_002071	Aspartate aminotransferase 2	0.4166	0.8	Mitochondrion	Yes	Aminotransferase, metabolism
NP_000909	Prolyl 4-hydroxylase	0.4347	0.8	Extracellular, ER	Yes	Isomerase, metabolism
NP_002988	Syndecan 1	0.4347	0.28	Plasma membrane	Yes	Receptor, signal transduction

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TABLE II—*continued*

RefSeq accession no.	Protein name	Ratio (Panc1/HPDE)		Localization	Signal peptide present	Protein function
		SILAC	DNA array			
NP_705935	Tropomyosin 3	0.4347	TND	Cytoplasm	No	Cytosleletal protein. cell growth
NP_001545	Cysteine-rich, angiogenic inducer, 61	0.5	1	Extracellular, plasma membrane	Yes	Extracellular matrix protein, signal transduction

[a] An arbitrary value of 0.05 (1⁄20) was chosen for proteins where the intensity of the peptides in the MS spectra was either very low or absent in cancer (Panc1) compared to normal (HPDE).
[b] TND, transcript not detectable.
[c] TNP, transcript not present on chip.

peptide sequences, Mascot scores, any post-translational modifications, and heavy/light intensity ratios. Mac-2BP is a secreted glycoprotein that was originally found in human breast carcinoma cells and is a member of the scavenger receptor cysteine-rich domain family of proteins (46–48). Mac-2BP is known to bind galectins, β1 integrins, collagens, and fibronectin and serves as an endogenous ligand for galectin-3 (44, 48, 49). It has been observed to be elevated in serum of patients with different solid tumors, *e.g.* breast, ovarian, lung, and colorectal cancers, and is often associated with poor survival and metastatic spread in these malignancies (46, 50–53). In our study, it was increased 5-fold in cancer cells (Panc1) as compared with normal cells (HPDE). Several of the cathepsins (*e.g.* cathepsin B and cathepsin D) were also identified in our study. Cathepsins are lysosomal proteolytic enzymes that function as cysteine proteinases and are known to be present in almost all mammalian cells (54). Cathepsins B and D are both involved in different cancers and were up-regulated 3- and 7-fold, respectively. The up-regulation of these proteins was also confirmed by Western blotting using the conditioned media from the two cell lines (Fig. 2*A*). Prosaposin, a protein that has been shown to interact with cathepsin D, was also identified in our screen and found to be highly up-regulated (11-fold) in cancer (Panc1) compared with normal (HPDE). Although many proteins known to be elevated in pancreatic cancer were identified in our screen, we still missed a few of the known candidates (*e.g.* hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP) and deleted in malignant brain tumor (DMBT1)).

Down-regulated Proteins That Are Known to Be Involved in Cancer—Some of the down-regulated proteins identified in our study include members of the insulin-like growth factor-binding protein (IGFBP) superfamily, profilin 1, and one of the most abundant extracellular cysteine proteinase inhibitors, cystatin C. IGFBPs act as both positive and negative regulators in the insulin-like growth factor signaling pathway and play an important role in the development and progression of cancers such as prostate cancer (55). This family has recently been expanded to include insulin-like growth factor binding protein-related proteins (IGFBP-rPs). Two IGFBP-rPs, IGFBP-rP1 and IGFBP-rP2, were identified in our study as strongly down-regulated proteins (>20-fold). IGFBP-rP1, also known as IGFBP-7, has been reported to be significantly down-regulated in malignant leptomeningeal and mammary epithelial cells compared with normal cells (56). A similar down-regulation is seen in the case of prostate cancer, and it has been suggested that this gene might be inactivated by CpG hypermethylation (57). IGFBP-7 was confirmed to be down-regulated by Western blotting (Fig. 2*B*). One other protein that was found to be significantly down-regulated in cancer is profilin 1 (PEN1). Profilin plays an important role in actin filament assembly (58) and interacts with different ligands including actin-related protein 2 (Arp2), phosphatidylinositol 4,5-bisphosphate, and gephyrin (59, 60). Profilin 1 was identified by differential display to be down-regulated in tumorigenic breast cancer cell lines (61). SPARC was also identified in our study and was found to be down-regulated by SILAC (3-fold) and by Western blotting (Fig. 2*B*). SPARC is a glycoprotein shown previously to be involved in diverse biological processes including tissue remodeling, wound repair, morphogenesis, cellular differentiation, cell proliferation, and cell migration (62). SPARC is overexpressed in many human malignancies (63). In addition, we have shown recently that SPARC is overexpressed in the stromal fibroblasts immediately adjacent to the neoplastic epithelium in primary pancreatic cancer but rarely in the cancers themselves (64).

Novel Differentially Expressed Proteins in the Pancreatic Cancer Secretome—A large number of proteins identified in our study are proteins that have not been described previously to be overexpressed in pancreatic cancer (Table I). A partial list of proteins found to be down-regulated in the cancer secretome compared with normal is provided in Table II. These proteins facilitate a range of different biological functions including enzymatic reactions (*e.g.* lysyl oxidase-like 2 (LOXL2), transport (*e.g.* apolipoprotein E), metabolism (*e.g.* biotinidase), cell growth/structure (*e.g.* actinin α 4), and signal transduction (*e.g.* stromal cell-derived factor 4, fibronectin receptor (integrin β1), and predicted osteoblast protein (FAM3C)). Below we discuss proteins not described previously to be involved in pancreatic cancer in more detail.

Lysyl oxidase (LOX) is an amino oxidase that oxidizes primary amines to reactive aldehydes (65). This family of enzymes is involved in regulation of biogenesis of connective tissue, development, cell growth, and tumor suppression. Recently four new proteins designated lysyl oxidase-like proteins have been described (LOXL, LOXL2, LOXL3, and LOXL4) that are functionally similar but genetically distinct from the LOX family of proteins (65). Several members of the LOXL

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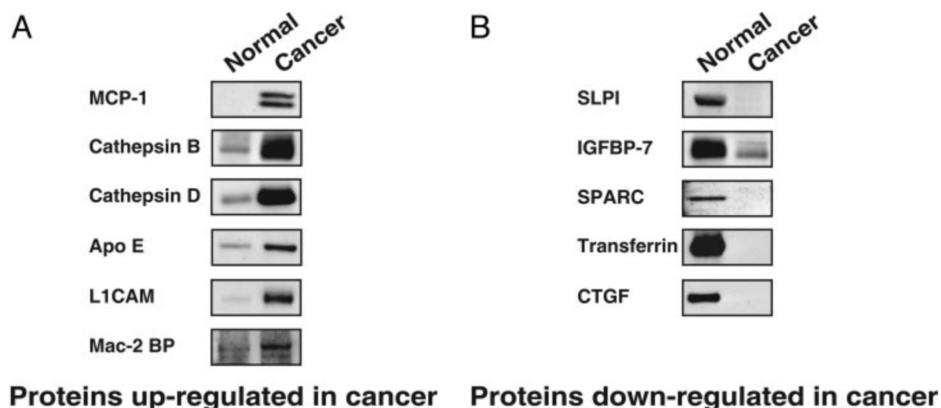

FIG. 2. **Validation of protein expression by Western blotting.** Conditioned medium from HPDE (*Normal*) and Panc1 (*Cancer*) cells was loaded onto SDS-PAGE gels, and the proteins were transferred onto nitrocellulose and probed with the indicated antibodies. *A* shows some of the proteins that were up-regulated, whereas *B* shows down-regulated proteins. *CTGF*, connective tissue growth factor; *SLPI*, secretory leukocyte protease inhibitor.

family have been described to be involved in breast cancer, and LOXL2 has been shown to play an important role in the invasive characteristics of metastatic breast cancer lines (66). We identified LOXL2 to be highly up-regulated (≥20-fold) in the Panc1 secretome.

ApoE was first identified as a protein component of triglyceride-rich low density lipoproteins. It was later shown that apoE serves as a ligand for low density lipoprotein receptors, which are involved in cholesterol transport and metabolism (67). ApoE also facilitates other biological functions such as stimulation of cholesterol efflux from macrophages, prevention of platelet aggregation, and inhibition of proliferation (68). There are three major isoforms of apoE (apoE4, apoE2, and apoE2) that differ from each other by only a single amino acid. Still the different isoforms of apoE have profound effects at the structural and functional level. ApoE is synthesized in several places including liver, brain, spleen, lung, ovary, kidney, and muscle (69). The liver is responsible for ~60–80% of the production of apoE, whereas 20–40% is derived from extrahepatic sources such as macrophages (69). ApoE has been described to be elevated in several cancers including ovarian and prostate cancer (70, 71). In our study, apoE was found to be up-regulated 20-fold in cancer (Panc1) as compared with normal (HPDE).

Several proteins involved in signal transduction were also identified as highly up-regulated in the Panc1 secretome, including fibronectin receptor (integrin β1). Integrins are a family of heterodimeric transmembrane receptors that mediate interactions with components of the extracellular matrix and other plasma membrane proteins. Integrins are composed of an α and β subunit (72), and each of the individual subunits consists of an extracellular domain, a transmembrane region, and a cytosolic domain (73). They are involved in a number of biological functions including reorganization of the actin cytoskeleton and differentiation (74). Altered expression of integrins plays a crucial role in several cancers including pancreatic, prostate, and colon cancer (73, 75, 76). In our study, fibronectin receptor (integrin β1) was up-regulated 3.4-fold in Panc1 cells.

Other signaling molecules identified in our study include protein-tyrosine kinase 7 (PTK7) and Ras-related C3 botulinum toxin substrate 2 (Rac-2). PTK7 and Rac-2 were found to be up-regulated 4.6- and 3.5-fold in cancer secretome, respectively (Table I). A novel member of a cytokine-like gene family, tentatively designated as predicted osteoblast protein in databases (FAM3C), was identified in our study to be up-regulated 4.2-fold in Panc1 secretome (Table I). Northern blot analysis reveals that FAM3C is expressed in several different tissues including pancreas (77).

Perlecan (heparan sulfate proteoglycan 2 (HSPG2)) and CD9 were also identified to be elevated in cancer *versus* normal by a factor of 5.1 and 8.0, respectively (Fig. 3). Perlecan gene is located on chromosome 1 (78) and is a major component of the basement membranes and extracellular matrices (79–81). Perlecan is ~440 kDa, which makes it one of the larger gene products in humans (82). It contains five distinct domains of which only one is unique to perlecan (the heparan sulfate binding region) (80). Perlecan transcription is up-regulated by transforming growth factor-β (83) and is thought to play a role in angiogenesis and growth by acting as a co-receptor for basic fibroblast growth factor (FGF2) (84). Increased levels of perlecan have been observed in breast carcinoma (85) and metastatic melanoma (86). CD9 (originally called motility-related protein 1, MRP-1) is a membrane protein with a molecular mass of 24 kDa and is expressed on the surface of human platelets (87). It was originally identified as a surface antigen of hematopoietic cells (88) but has subsequently been detected in a wide variety of non-hematopoietic cells including central and peripheral nervous system (89, 90). It is a member of the transmembrane 4 superfamily that comprises a group of cell surface proteins characterized by four hydrophobic transmembrane domains (91). CD9 is involved in many cellular functions such as adhesion, migration, signal transduction, and differentiation (92). CD9 protein expression has been shown to be lower in metastatic lymph nodes than the corresponding primary breast tumors (93) and associated with high tumor grade and lymph node metastasis in pancreatic cancer (94). In addition, the level of CD9 has been to be shown to be elevated in the pancreatic stroma (95). Because several of the differentially expressed proteins iden-

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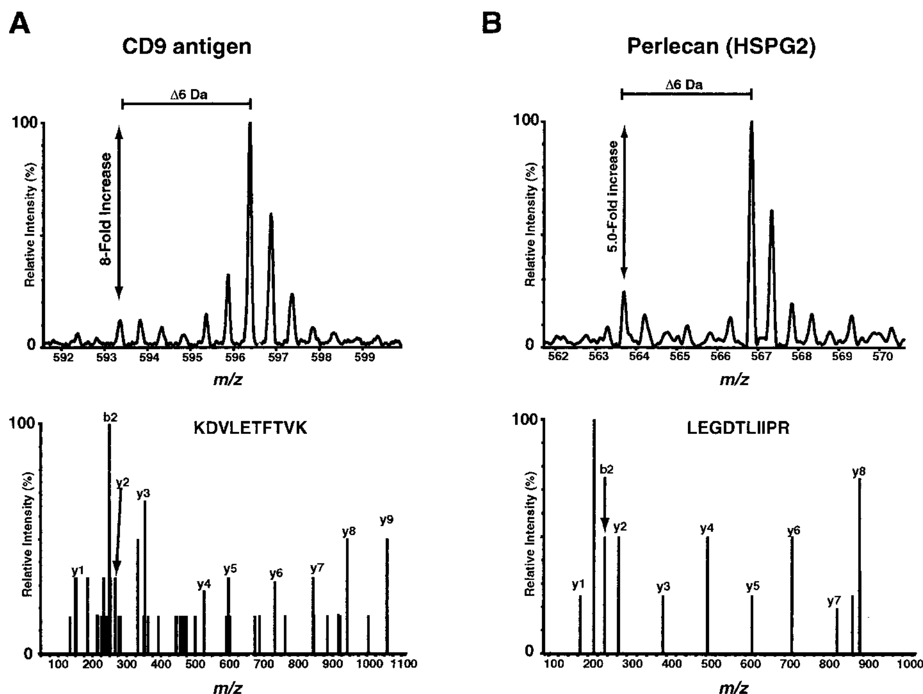

FIG. 3. **Quantitation by SILAC.** *A* and *B* show the MS and MS/MS spectra of one of the peptides derived from CD9 antigen and perlecan (HSPG2), respectively. Both proteins were also validated as up-regulated proteins in cancer *versus* normal tissue by IHC (Fig. 4).

tified in our study were not previously described to be elevated in pancreatic cancer (*e.g.* perlecan, CD9, fibronectin receptor, apoE, and SDF4), we decided to carry out validation studies by immunohistochemistry (IHC) using pancreatic cancer tissue microarrays (TMAs).

Validation of CD9, Perlecan, SDF4, Fibronectin Receptor, and ApoE Overexpression by IHC Using Labeling of Pancreatic Cancer TMAs—The validation of *in vitro* results using tissue sections of pancreatic cancer is critical as it can avoid artifacts related to passaging of cell lines in culture. For example, we have shown previously (115) that melanoma cell adhesion molecule (also called MUC18) is significantly overexpressed in biliary cancer cell lines that have been passaged *in vitro* but is not expressed in tissue sections. To avoid such potential pitfalls, we selected *six* up-regulated proteins (CD9, up-regulated 8-fold (Fig. 3*A*); perlecan, up-regulated 4.5-fold (Fig. 3*B*); fibronectin receptor (integrin β1), up-regulated 3.4-fold; SDF4, up-regulated 20-fold; apoE, up-regulated 20-fold; and L1CAM, up-regulated 5.2-fold) for validation in tissue sections of pancreatic cancers using TMAs. CD9 was expressed in robust membranous distribution in seven of 18 (39%) pancreatic cancers on the TMA with no expression seen in adjacent normal pancreatic parenchyma (Fig. 4*A*). CD9 labeling demonstrated a pattern of apical luminal accentuation similar to the pattern we have reported previously for other secreted proteins in pancreatic cancers such as prostate stem cell antigen and mesothelin (15, 16). In addition, labeling of intraluminal contents was often seen within neoplastic glandular structures, consistent with CD9 secretion (Fig. 4*B*). Perlecan was expressed in 13 of 18 (72%) cancers demonstrating strong cytoplasmic/membrane labeling (Fig. 4*C*). Similar to CD9, luminal apical labeling was also seen with perlecan, including the presence of labeling in intraluminal contents, consistent with secretion by the neoplastic cells (Fig. 4*D*). Normal pancreatic parenchyma essentially did not express perlecan at detectable levels. Along the same lines, we found robust expression of SDF4 (cytoplasmic) (Fig. 4*E*) in nine of 10 (90%) cancers, membranous fibronectin receptor expression (Fig. 4*F*) in 10 of 18 (56%) cancers, and cytoplasmic apolipoprotein E expression (Fig. 4*G*) in 14 of 18 (78%) cases. The corresponding expression in normal pancreatic ductal epithelium was minimal or absent as illustrated in Fig. 4. In contrast to these five proteins, we did not find evidence of L1CAM expression in any of the cancer tissues or normal pancreata. L1CAM is a neural marker, and the presence of robust labeling of normal nerve sections in multiple TMA cores served as an internal positive control for the antibody (data not shown). The absence of L1CAM overexpression in pancreatic cancer is unlikely to be an artifact because Western blot analysis confirmed L1CAM overexpression in Panc1-conditioned media compared with HPDE (Fig. 2*A*). Thus, it likely represents an artifact of tissue culture conditions that leads to induction of cell adhesion molecules such as L1CAM and melanoma cell adhesion molecule (as previously reported (115)) and underscores the importance of tissue validation in biomarker studies.

Correlation of Protein (SILAC) with mRNA (DNA Microarray) Expression Data—To determine how well the protein expression data correlate with expression data at the transcript level, we compared the protein level quantitation data obtained by the SILAC method with mRNA data obtained by a DNA microarray experiment for these two cell lines (Fig. 5). This was

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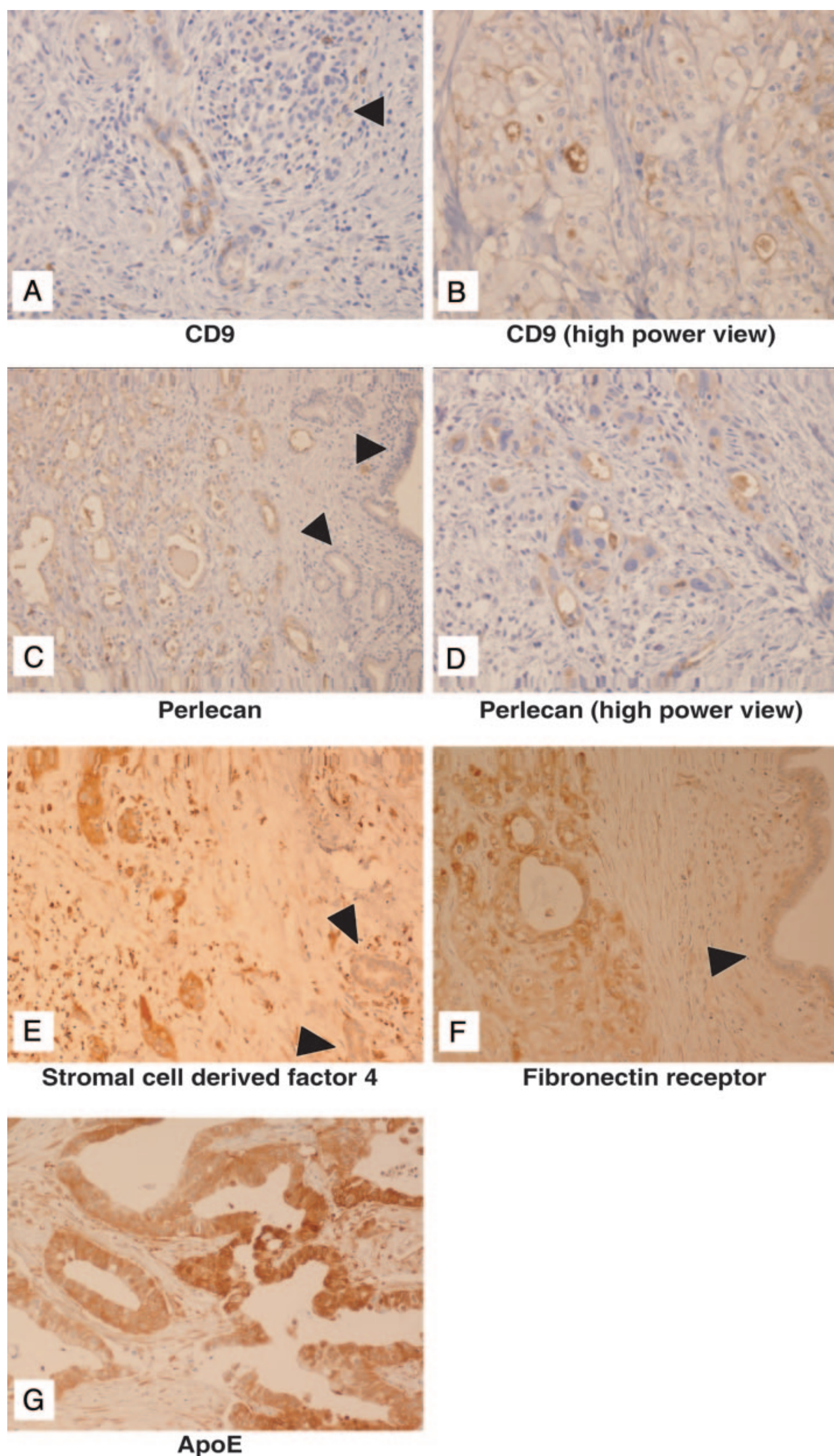

FIG. 4. **Validation of differential expression of five up-regulated SILAC proteins by immunohistochemistry on pancreatic cancer tissue microarrays.** *A*, membranous CD9 expression in an infiltrating pancreatic cancer gland. Adjacent non-neoplastic parenchyma is essentially negative (*arrowhead*) except for interspersed neutrophils. *B*, high power view of a pancreatic cancer with robust membranous CD9 expression including luminal accentuation and labeling of intraluminal contents. *C*, low power view of perlecan expression in a pancreatic cancer with absence of labeling in adjacent normal ducts (*arrowheads*). *D*, high power view demonstrates cytoplasmic/membranous labeling with luminal accentuation and labeling of intraluminal contents. *E*, expression of SDF4 in pancreatic cancer, demonstrating robust cytoplasmic expression in infiltrating nests of cancer cells (*left of field*), whereas normal ducts are essentially negative (*arrowheads*). Note that SDF4 is also expressed in admixed inflammatory cells in the stroma. *F*, membranous expression of fibronectin receptor (integrin β1) in pancreatic cancer, whereas adjacent normal epithelium (*arrowhead*) does not label. *G*, robust cytoplasmic expression of apoE in pancreatic cancer, including occasional labeling of intraluminal secretions.

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done by comparing the differential expression of proteins in the secretome (*i.e.* all proteins where relative quantitation data were available from the SILAC experiments) with that of mRNAs using an Affymetrix DNA microarray (version U133A). Here the comparison was only done on a subset of the proteome of the cell lines as only the secretome was analyzed by

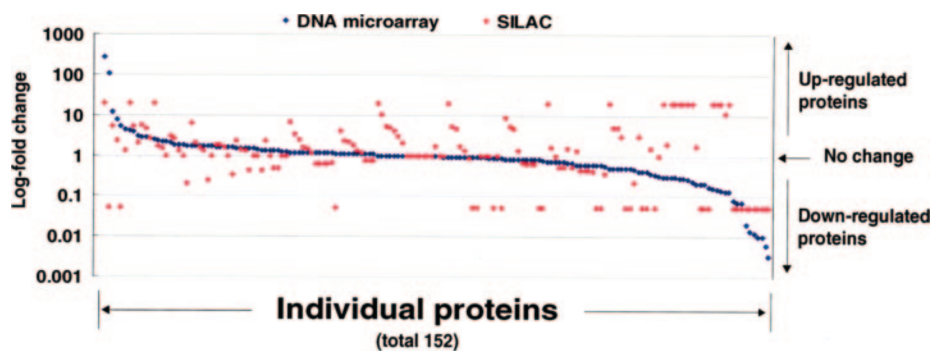

FIG. 5. **Correlation of mRNA and protein expression levels.** The -fold changes obtained by proteomic experiments (SILAC) were compared with mRNA expression data (DNA microarray) for Panc1 *versus* HPDE cells. The -fold changes at the protein level are marked in *red* and at the mRNA level are marked with *blue*. The correlation coefficient (*r*) between the -fold changes at protein and mRNA levels was 0.28.

the proteomic method. We were able to obtain corresponding RNA expression data for 152 of 195 (78%) proteins identified by SILAC. The lack of RNA expression data on some of the proteins identified by SILAC was due to two reasons: 1) the gene encoding the protein identified by SILAC was not represented on the DNA microarray, or 2) the abundance of the transcript (*i.e.* mRNA level) was too low to be detected. In 7% of the cases (14 proteins) the gene was not present on the chip, and in 15% (29 proteins) the gene was present on the chip, but the level of transcript was too low to be detected. A Blast search of the nucleotide sequences of the identified proteins against the probes on the chip was conducted to ensure that no genes were missed. As seen in Fig. 6, 13% of the proteins were found to be up-regulated >1.5-fold, and 21% were found to be down-regulated >1.5-fold at both at the protein and mRNA level, respectively. Sixteen percent were found to have no change in both studies, whereas 50% did not show correlation between mRNA and protein abundance values. To see how well the proteomic data correlated with the transcriptomic data, the correlation coefficient (*r*) was calculated and found to be 0.28. One explanation for the relatively poor correlation between mRNA and protein expression data, as also observed previously (96–98), is that we are comparing protein expression values for the pancreatic secretome with RNA expression data from whole cell lysates. Secreted proteins (*e.g.* cytokines) tend to be regulated strongly at the post-transcriptional level, and this regulation could result in a greater discordance.

The magnitude of some of the RNA-protein discrepancies was striking. For example, cathepsin D, which was found to be up-regulated 6.7-fold at the protein level, showed no significant change at the mRNA level (1.2-fold). As previously noted, cathepsin D is a proteolytic enzyme that is present in almost all mammalian cells and has been shown be involved in several cancers (99, 100). Another example is Mac-2BP, which was up-regulated 5-fold at the protein level but was down-regulated 2-fold at the mRNA level. The overexpression of Mac-2BP in a variety of solid cancers is well known, and we have demonstrated previously the up-regulation of Mac-2BP in the secretome of biliary cancers (101). Finally CD9 antigen, which SILAC demonstrated to be differentially overexpressed in the pancreatic cancer secretome and was confirmed as

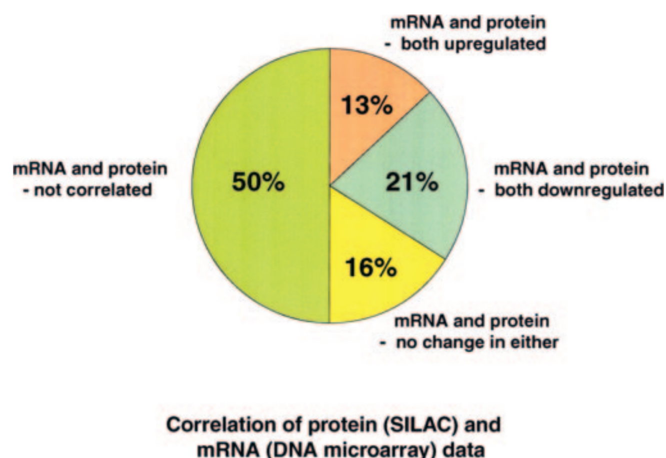

FIG. 6. **Distribution of the proteins on the basis of correlation between protein expression changes (SILAC) and mRNA expression changes (DNA microarray).** As seen in the pie chart 13% of the entries were found to be up-regulated at both the mRNA and protein levels, whereas 21% were found to be down-regulated at the mRNA and protein levels. Sixteen percent of the entries did not show any change at either the mRNA or protein expression level. Many entries (50%) did not correlate at the mRNA and the protein level. These include (*a*) discordant entries, (*b*) no change in mRNA level but up- (≥1.5) or down-regulation (≤1.5) at the protein level, and (*c*) no change in proteins level but up- (≥1.5) or down-regulation (≥1.5) at the mRNA level.

being overexpressed at the protein level in pancreatic cancer tissues (see above) was also down-regulated 2-fold in Panc1 *versus* HPDE cells based on DNA microarray data. Thus, we would not have carried out additional proteomic studies on CD9 if we relied solely on the DNA microarray data. Our data reinforce the importance of assessing both the transcriptome and the proteome of human cancers.

Conclusions—The use of mass spectrometry and quantitative proteomics has proven to be a promising strategy for identifying potential biomarkers for a number of cancers. In this study we used SILAC as an initial screening tool to identify proteins that are differentially expressed in the pancreatic cancer secretome. By restricting our analysis to quantitating relative differences in the secreted compartment alone, we increased our chances of identifying a fluid-based biomarker for pancreatic cancer. In total, we identified 195

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proteins of which 68 proteins were up-regulated >1.5-fold in cancer. Several of these proteins have been described previously to be elevated in pancreatic cancer (*e.g.* Mac-2BP, MCP-1, aminopeptidase N, lysyl oxidase-like, and cathepsin D), validating our SILAC approach as a screening tool to identify novel biomarkers for pancreatic cancer. A large set (40%) of proteins were also found to be down-regulated >1.5-fold in cancer of which several were described previously to be down-regulated in pancreatic cancer (*e.g.* IG-FBP-7, profilin 1, and SPARC). The expression profile for a subset of the up- and down-regulated proteins (11 proteins) was also verified by Western blot analysis (Fig. 2) validating the quantitation data obtained by SILAC. Several proteins not described previously to be elevated in pancreatic cancer were also identified in our study including CD9, perlecan, apoE, SDF4, and fibronectin receptor. These proteins were subsequently validated by immunohistochemical labeling on pancreatic cancer microarrays. CD9 and fibronectin receptor showed robust expression in the membranes in 39 and 56%, respectively, of the cancers tested by TMA, whereas perlecan, SDF4, and apoE showed cytoplasmic/membrane labeling in 72, 90, and 78%, respectively, of the cancers tested by TMA. By using a rational combination of quantitative proteomic profiling and antibody-based validation techniques, we identified a number of potential clinically useful biomarkers for pancreatic cancer that could be pursued in larger studies for validation purposes. This approach could be extended to most cancers for which cell line models are available.

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REFERENCES

1. Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M. (2001) Cancer statistics, 2001. *CA - Cancer J. Clin.* **51,** 15–36
2. Yeo, T. P., Hruban, R. H., Leach, S. D., Wilentz, R. E., Sohn, T. A., Kern, S. E., Iacobuzio-Donahue, C. A., Maitra, A., Goggins, M., Canto, M. I., Abrams, R. A., Laheru, D., Jaffee, E. M., Hidalgo, M., and Yeo, C. J. (2002) Pancreatic cancer. *Curr. Probl. Cancer* **26,** 176–275
3. Pleskow, D. K., Berger, H. J., Gyves, J., Allen, E., McLean, A., and Podolsky, D. K. (1989) Evaluation of a serologic marker, CA19-9, in the diagnosis of pancreatic cancer. *Ann. Intern. Med.* **110,** 704–709
4. Akdogan, M., Sasmaz, N., Kayhan, B., Biyikoglu, I., Disibeyaz, S., and Sahin, B. (2001) Extraordinarily elevated CA19-9 in benign conditions: a case report and review of the literature. *Tumori* **87,** 337–339
5. Ni, X. G., Bai, X. F., Mao, Y. L., Shao, Y. F., Wu, J. X., Shan, Y., Wang, C. F., Wang, J., Tian, Y. T., Liu, Q., Xu, D. K., and Zhao, P. (2005) The clinical value of serum CEA, CA19-9, and CA242 in the diagnosis and prognosis of pancreatic cancer. *Eur. J. Surg. Oncol.* **31,** 164–169
6. Petersen, G. M., and Hruban, R. H. (2003) Familial pancreatic cancer: where are we in 2003? *J. Natl. Cancer Inst.* **95,** 180–181
7. Goggins, M., Canto, M., and Hruban, R. (2000) Can we screen high-risk individuals to detect early pancreatic carcinoma? *J. Surg. Oncol.* **74,** 243–248
8. Rosty, C., and Goggins, M. (2002) Early detection of pancreatic carcinoma. *Hematol. Oncol. Clin. N. Am.* **16,** 37–52
9. Iacobuzio-Donahue, C. A., Ashfaq, R., Maitra, A., Adsay, N. V., Shen-Ong, G. L., Berg, K., Hollingsworth, M. A., Cameron, J. L., Yeo, C. J., Kern, S. E., Goggins, M., and Hruban, R. H. (2003) Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. *Cancer Res.* **63,** 8614–8622
10. Iacobuzio-Donahue, C. A., Maitra, A., Olsen, M., Lowe, A. W., van Heek, N. T., Rosty, C., Walter, K., Sato, N., Parker, A., Ashfaq, R., Jaffee, E., Ryu, B., Jones, J., Eshleman, J. R., Yeo, C. J., Cameron, J. L., Kern, S. E., Hruban, R. H., Brown, P. O., and Goggins, M. (2003) Exploration of global gene expression patterns in pancreatic adenocarcinoma using cDNA microarrays. *Am. J. Pathol.* **162,** 1151–1162
11. Iacobuzio-Donahue, C. A., Argani, P., Hempen, P. M., Jones, J., and Kern, S. E. (2002) The desmoplastic response to infiltrating breast carcinoma: gene expression at the site of primary invasion and implications for comparisons between tumor types. *Cancer Res.* **62,** 5351–5357
12. Berman, D. M., Karhadkar, S. S., Maitra, A., Montes De Oca, R., Gerstenblith, M. R., Briggs, K., Parker, A. R., Shimada, Y., Eshleman, J. R., Watkins, D. N., and Beachy, P. A. (2003) Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* **425,** 846–851
13. Miyamoto, Y., Maitra, A., Ghosh, B., Zechner, U., Argani, P., Iacobuzio-Donahue, C. A., Sriuranpong, V., Iso, T., Meszoely, I. M., Wolfe, M. S., Hruban, R. H., Ball, D. W., Schmid, R. M., and Leach, S. D. (2003) Notch mediates TGFα-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* **3,** 565–576
14. Nichols, L. S., Ashfaq, R., and Iacobuzio-Donahue, C. A. (2004) Claudin 4 protein expression in primary and metastatic pancreatic cancer: support for use as a therapeutic target. *Am. J. Clin. Pathol.* **121,** 226–230
15. Argani, P., Iacobuzio-Donahue, C., Ryu, B., Rosty, C., Goggins, M., Wilentz, R. E., Murugesan, S. R., Leach, S. D., Jaffee, E., Yeo, C. J., Cameron, J. L., Kern, S. E., and Hruban, R. H. (2001) Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin. Cancer Res.* **7,** 3862–3868
16. Argani, P., Rosty, C., Reiter, R. E., Wilentz, R. E., Murugesan, S. R., Leach, S. D., Ryu, B., Skinner, H. G., Goggins, M., Jaffee, E. M., Yeo, C. J., Cameron, J. L., Kern, S. E., and Hruban, R. H. (2001) Discovery of new markers of cancer through serial analysis of gene expression: prostate stem cell antigen is overexpressed in pancreatic adenocarcinoma. *Cancer Res.* **61,** 4320–4324
17. Wulfkuhle, J. D., Sgroi, D. C., Krutzsch, H., McLean, K., McGarvey, K., Knowlton, M., Chen, S., Shu, H., Sahin, A., Kurek, R., Wallwiener, D., Merino, M. J., Petricoin, E. F., III, Zhao, Y., and Steeg, P. S. (2002) Proteomics of human breast ductal carcinoma in situ. *Cancer Res.* **62,** 6740–6749
18. Greenbaum, D., Luscombe, N. M., Jansen, R., Qian, J., and Gerstein, M. (2001) Interrelating different types of genomic data, from proteome to secretome: 'oming in on function. *Genome Res.* **11,** 1463–1468
19. Hanash, S. (2001) 2-D or not 2-D—is there a future for 2-D gels in proteomics? Insights from the York proteomics meeting. *Proteomics* **1,** 635–637
20. Hanash, S. (2001) Mining the cancer proteome. *Proteomics* **1,** 1189–1190
21. Koopmann, J., Zhang, Z., White, N., Rosenzweig, J., Fedarko, N., Jagannath, S., Canto, M. I., Yeo, C. J., Chan, D. W., and Goggins, M. (2004) Serum diagnosis of pancreatic adenocarcinoma using surface-enhanced laser desorption and ionization mass spectrometry. *Clin. Cancer Res.* **10,** 860–868
22. Gronborg, M., Bunkenborg, J., Kristiansen, T. Z., Jensen, O. N., Yeo, C. J., Hruban, R. H., Maitra, A., Goggins, M. G., and Pandey, A. (2004)

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Comprehensive proteomic analysis of human pancreatic juice. *J. Proteome Res.* **3,** 1042–1055
23. Kristiansen, T. Z., Bunkenborg, J., Gronborg, M., Molina, H., Thuluvath, P. J., Argani, P., Goggins, M. G., Maitra, A., and Pandey, A. (2004) A proteomic analysis of human bile. *Mol. Cell. Proteomics* **3,** 715–728
24. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17,** 994–999
25. Gygi, S. P., Rist, B., Griffin, T. J., Eng, J., and Aebersold, R. (2002) Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags. *J. Proteome Res.* **1,** 47–54
26. Mirgorodskaya, O. A., Kozmin, Y. P., Titov, M. I., Korner, R., Sonksen, C. P., and Roepstorff, P. (2000) Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using ^{18}O-labeled internal standards. *Rapid Commun. Mass Spectrom.* **14,** 1226–1232
27. Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **1,** 376–386
28. Amanchy, R., Kalume, D. E., and Pandey, A. (2005) Stable isotope labeling with amino acids in cell culture (SILAC) for studying dynamics of protein abundance and posttranslational modifications. *Sci. STKE* **2005,** pl2
29. Blagoev, B., Kratchmarova, I., Ong, S. E., Nielsen, M., Foster, L. J., and Mann, M. (2003) A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat. Biotechnol.* **21,** 315–318
30. Ibarrola, N., Kalume, D. E., Gronborg, M., Iwahori, A., and Pandey, A. (2003) A proteomic approach for quantitation of phosphorylation using stable isotope labeling in cell culture. *Anal. Chem.* **75,** 6043–6049
31. Ibarrola, N., Molina, H., Iwahori, A., and Pandey, A. (2004) A novel proteomic approach for specific identification of tyrosine kinase substrates using [^{13}C]tyrosine. *J. Biol. Chem.* **279,** 15805–15813
32. Foster, L. J., De Hoog, C. L., and Mann, M. (2003) Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5813–5818
33. Everley, P. A., Krijgsveld, J., Zetter, B. R., and Gygi, S. P. (2004) Quantitative cancer proteomics: stable isotope labeling with amino acids in cell culture (SILAC) as a tool for prostate cancer research. *Mol. Cell. Proteomics* **3,** 729–735
34. Ueki, T., Walter, K. M., Skinner, H., Jaffee, E., Hruban, R. H., and Goggins, M. (2002) Aberrant CpG island methylation in cancer cell lines arises in the primary cancers from which they were derived. *Oncogene* **21,** 2114–2117
35. Liu, N., Furukawa, T., Kobari, M., and Tsao, M. S. (1998) Comparative phenotypic studies of duct epithelial cell lines derived from normal human pancreas and pancreatic carcinoma. *Am. J. Pathol.* **153,** 263–269
36. Li, M., Yang, H., Chai, H., Fisher, W. E., Wang, X., Brunicardi, F. C., Yao, Q., and Chen, C. (2004) Pancreatic carcinoma cells express neuropilins and vascular endothelial growth factor, but not vascular endothelial growth factor receptors. *Cancer* **101,** 2341–2350
37. Iacobuzio-Donahue, C. A., Maitra, A., Shen-Ong, G. L., van Heek, T., Ashfaq, R., Meyer, R., Walter, K., Berg, K., Hollingsworth, M. A., Cameron, J. L., Yeo, C. J., Kern, S. E., Goggins, M., and Hruban, R. H. (2002) Discovery of novel tumor markers of pancreatic cancer using global gene expression technology. *Am. J. Pathol.* **160,** 1239–1249
38. Hustinx, S. R., Cao, D., Maitra, A., Sato, N., Martin, S. T., Sudhir, D., Iacobuzio-Donahue, C., Cameron, J. L., Yeo, C. J., Kern, S. E., Goggins, M., Mollenhauer, J., Pandey, A., and Hruban, R. H. (2004) Differentially expressed genes in pancreatic ductal adenocarcinomas identified through serial analysis of gene expression. *Cancer Biol. Ther.* **3,** 1254–1261
39. Cao, D., Hustinx, S. R., Sui, G., Bala, P., Sato, N., Martin, S., Maitra, A., Murphy, K. M., Cameron, J. L., Yeo, C. J., Kern, S. E., Goggins, M., Pandey, A., and Hruban, R. H. (2004) Identification of novel highly expressed genes in pancreatic ductal adenocarcinomas through a bioinformatics analysis of expressed sequence tags. *Cancer Biol. Ther.* **3,** 1081–1091
40. Cao, D., Maitra, A., Saavedra, J. A., Klimstra, D. S., Adsay, N. V., and Hruban, R. H. (2005) Expression of novel markers of pancreatic ductal adenocarcinoma in pancreatic nonductal neoplasms: additional evidence of different genetic pathways. *Mod. Pathol.* **18,** 752–761
41. Swierczynski, S. L., Maitra, A., Abraham, S. C., Iacobuzio-Donahue, C. A., Ashfaq, R., Cameron, J. L., Schulick, R. D., Yeo, C. J., Rahman, A., Hinkle, D. A., Hruban, R. H., and Argani, P. (2004) Analysis of novel tumor markers in pancreatic and biliary carcinomas using tissue microarrays. *Hum. Pathol.* **35,** 357–366
42. Sato, N., Fukushima, N., Maitra, A., Matsubayashi, H., Yeo, C. J., Cameron, J. L., Hruban, R. H., and Goggins, M. (2003) Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays. *Cancer Res.* **63,** 3735–3742
43. Mroczko, B., Szmitkowski, M., Wereszczynska-Siemiatkowska, U., and Jurkowska, G. (2004) Stem cell factor and macrophage-colony stimulating factor in patients with pancreatic cancer. *Clin. Chem. Lab. Med.* **42,** 256–260
44. Kunzli, B. M., Berberat, P. O., Zhu, Z. W., Martignoni, M., Kleeff, J., Tempia-Caliera, A. A., Fukuda, M., Zimmermann, A., Friess, H., and Buchler, M. W. (2002) Influences of the lysosomal associated membrane proteins (Lamp-1, Lamp-2) and Mac-2 binding protein (Mac-2-BP) on the prognosis of pancreatic carcinoma. *Cancer* **94,** 228–239
45. Peri, S., Navarro, J. D., Amanchy, R., Kristiansen, T. Z., Jonnalagadda, C. K., Surendranath, V., Niranjan, V., Muthusamy, B., Gandhi, T. K., Gronborg, M., Ibarrola, N., Deshpande, N., Shanker, K., Shivashankar, H. N., Rashmi, B. P., Ramya, M. A., Zhao, Z., Chandrika, K. N., Padma, N., Harsha, H. C., Yatish, A. J., Kavitha, M. P., Menezes, M., Choudhury, D. R., Suresh, S., Ghosh, N., Saravana, R., Chandran, S., Krishna, S., Joy, M., Anand, S. K., Madavan, V., Joseph, A., Wong, G. W., Schiemann, W. P., Constantinescu, S. N., Huang, L., Khosravi-Far, R., Steen, H., Tewari, M., Ghaffari, S., Blobe, G. C., Dang, C. V., Garcia, J. G., Pevsner, J., Jensen, O. N., Roepstorff, P., Deshpande, K. S., Chinnaiyan, A. M., Hamosh, A., Chakravarti, A., and Pandey, A. (2003) Development of human protein reference database as an initial platform for approaching systems biology in humans. *Genome Res.* **13,** 2363–2371
46. Iacobelli, S., Arno, E., D'Orazio, A., and Coletti, G. (1986) Detection of antigens recognized by a novel monoclonal antibody in tissue and serum from patients with breast cancer. *Cancer Res.* **46,** 3005–3010
47. Ullrich, A., Sures, I., D'Egidio, M., Jallal, B., Powell, T. J., Herbst, R., Dreps, A., Azam, M., Rubinstein, M., and Natoli, C. (1994) The secreted tumor-associated antigen 90K is a potent immune stimulator. *J. Biol. Chem.* **269,** 18401–18407
48. Koths, K., Taylor, E., Halenbeck, R., Casipit, C., and Wang, A. (1993) Cloning and characterization of a human Mac-2-binding protein, a new member of the superfamily defined by the macrophage scavenger receptor cysteine-rich domain. *J. Biol. Chem.* **268,** 14245–14249
49. Inohara, H., Akahani, S., Koths, K., and Raz, A. (1996) Interactions between galectin-3 and Mac-2-binding protein mediate cell-cell adhesion. *Cancer Res.* **56,** 4530–4534
50. Marchetti, A., Tinari, N., Buttitta, F., Chella, A., Angeletti, C. A., Sacco, R., Mucilli, F., Ullrich, A., and Iacobelli, S. (2002) Expression of 90K (Mac-2 BP) correlates with distant metastasis and predicts survival in stage I non-small cell lung cancer patients. *Cancer Res.* **62,** 2535–2539
51. Fusco, O., Querzoli, P., Nenci, I., Natoli, C., Brakebush, C., Ullrich, A., and Iacobelli, S. (1998) 90K (MAC-2 BP) gene expression in breast cancer and evidence for the production of 90K by peripheral-blood mononuclear cells. *Int. J. Cancer* **79,** 23–26
52. Scambia, G., Panici, P. B., Baiocchi, G., Perrone, L., Iacobelli, S., and Mancuso, S. (1988) Measurement of a monoclonal-antibody-defined antigen (90K) in the sera of patients with ovarian cancer. *Anticancer Res.* **8,** 761–764
53. D'Ostilio, N., Natoli, C., Grassadonia, A., Rossi, N., Di Stefano, P., Amatetti, C., Tinari, N., and Iacobelli, S. (1996) Prognostic value of a novel interferon-inducible 90K tumor antigen. *Ann. N. Y. Acad. Sci.* **784,** 288–293
54. Staack, A., Tolic, D., Kristiansen, G., Schnorr, D., Loening, S. A., and Jung, K. (2004) Expression of cathepsins B, H, and L and their inhibitors as markers of transitional cell carcinoma of the bladder. *Urology* **63,** 1089–1094
55. Peehl, D. M., Cohen, P., and Rosenfeld, R. G. (1996) The role of insulin-like growth factors in prostate biology. *J. Androl.* **17,** 2–4

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56. Hwa, V., Oh, Y., and Rosenfeld, R. G. (1999) The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr. Rev.* **20,** 761–787
57. Mutaguchi, K., Yasumoto, H., Mita, K., Matsubara, A., Shiina, H., Igawa, M., Dahiya, R., and Usui, T. (2003) Restoration of insulin-like growth factor binding protein-related protein 1 has a tumor-suppressive activity through induction of apoptosis in human prostate cancer. *Cancer Res.* **63,** 7717–7723
58. Kang, F., Purich, D. L., and Southwick, F. S. (1999) Profilin promotes barbed-end actin filament assembly without lowering the critical concentration. *J. Biol. Chem.* **274,** 36963–36972
59. Lassing, I., and Lindberg, U. (1985) Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature* **314,** 472–474
60. Giesemann, T., Schwarz, G., Nawrotzki, R., Berhorster, K., Rothkegel, M., Schluter, K., Schrader, N., Schindelin, H., Mendel, R. R., Kirsch, J., and Jockusch, B. M. (2003) Complex formation between the postsynaptic scaffolding protein gephyrin, profilin, and Mena: a possible link to the microfilament system. *J. Neurosci.* **23,** 8330–8339
61. Janke, J., Schluter, K., Jandrig, B., Theile, M., Kolble, K., Arnold, W., Grinstein, E., Schwartz, A., Estevez-Schwarz, L., Schlag, P. M., Jockusch, B. M., and Scherneck, S. (2000) Suppression of tumorigenicity in breast cancer cells by the microfilament protein profilin 1. *J. Exp. Med.* **191,** 1675–1686
62. Brekken, R. A., and Sage, E. H. (2001) SPARC, a matricellular protein: at the crossroads of cell-matrix communication. *Matrix Biol.* **19,** 816–827
63. Porter, P. L., Sage, E. H., Lane, T. F., Funk, S. E., and Gown, A. M. (1995) Distribution of SPARC in normal and neoplastic human tissue. *J. Histochem. Cytochem.* **43,** 791–800
64. Sato, N., Fukushima, N., Maehara, N., Matsubayashi, H., Koopmann, J., Su, G. H., Hruban, R. H., and Goggins, M. (2003) SPARC/osteonectin is a frequent target for aberrant methylation in pancreatic adenocarcinoma and a mediator of tumor-stromal interactions. *Oncogene* **22,** 5021–5030
65. Molnar, J., Fong, K. S., He, Q. P., Hayashi, K., Kim, Y., Fong, S. F., Fogelgren, B., Szauter, K. M., Mink, M., and Csiszar, K. (2003) Structural and functional diversity of lysyl oxidase and the LOX-like proteins. *Biochim. Biophys. Acta* **1647,** 220–224
66. Kirschmann, D. A., Seftor, E. A., Fong, S. F., Nieva, D. R., Sullivan, C. M., Edwards, E. M., Sommer, P., Csiszar, K., and Hendrix, M. J. (2002) A molecular role for lysyl oxidase in breast cancer invasion. *Cancer Res.* **62,** 4478–4483
67. Mahley, R. W. (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* **240,** 622–630
68. Greenow, K., Pearce, N. J., and Ramji, D. P. (2005) The key role of apolipoprotein E in atherosclerosis. *J. Mol. Med.* **83,** 329–342
69. Elshourbagy, N. A., Liao, W. S., Mahley, R. W., and Taylor, J. M. (1985) Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. *Proc. Natl. Acad. Sci. U. S. A.* **82,** 203–207
70. Chen, Y. C., Pohl, G., Wang, T. L., Morin, P. J., Risberg, B., Kristensen, G. B., Yu, A., Davidson, B., and Shih, I.-M. (2005) Apolipoprotein E is required for cell proliferation and survival in ovarian cancer. *Cancer Res.* **65,** 331–337
71. Venanzoni, M. C., Giunta, S., Muraro, G. B., Storari, L., Crescini, C., Mazzucchelli, R., Montironi, R., and Seth, A. (2003) Apolipoprotein E expression in localized prostate cancers. *Int. J. Oncol.* **22,** 779–786
72. Hynes, R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69,** 11–25
73. Arao, S., Masumoto, A., and Otsuki, M. (2000) β1 integrins play an essential role in adhesion and invasion of pancreatic carcinoma cells. *Pancreas* **20,** 129–137
74. Giancotti, F. G., and Ruoslahti, E. (1999) Integrin signaling. *Science* **285,** 1028–1032
75. Moro, L., Perlino, E., Marra, E., Languino, L. R., and Greco, M. (2004) Regulation of β1C and β1A integrin expression in prostate carcinoma cells. *J. Biol. Chem.* **279,** 1692–1702
76. Kuwada, S. K., Kuang, J., and Li, X. (2005) Integrin $\alpha5/\beta1$ expression mediates HER-2 down-regulation in colon cancer cells. *J. Biol. Chem.* **280,** 19027–19035
77. Zhu, Y., Xu, G., Patel, A., McLaughlin, M. M., Silverman, C., Knecht, K., Sweitzer, S., Li, X., McDonnell, P., Mirabile, R., Zimmerman, D., Boyce, R., Tierney, L. A., Hu, E., Livi, G. P., Wolf, B., Abdel-Meguid, S. S., Rose, G. D., Aurora, R., Hensley, P., Briggs, M., and Young, P. R. (2002) Cloning, expression, and initial characterization of a novel cytokine-like gene family. *Genomics* **80,** 144–150
78. Dodge, G. R., Kovalszky, I., Chu, M. L., Hassell, J. R., McBride, O. W., Yi, H. F., and Iozzo, R. V. (1991) Heparan sulfate proteoglycan of human colon: partial molecular cloning, cellular expression, and mapping of the gene (HSPG2) to the short arm of human chromosome 1. *Genomics* **10,** 673–680
79. Noonan, D. M., Fulle, A., Valente, P., Cai, S., Horigan, E., Sasaki, M., Yamada, Y., and Hassell, J. R. (1991) The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. *J. Biol. Chem.* **266,** 22939–22947
80. Murdoch, A. D., Dodge, G. R., Cohen, I., Tuan, R. S., and Iozzo, R. V. (1992) Primary structure of the human heparan sulfate proteoglycan from basement membrane (HSPG2/perlecan). A chimeric molecule with multiple domains homologous to the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J. Biol. Chem.* **267,** 8544–8557
81. Kallunki, P., and Tryggvason, K. (1992) Human basement membrane heparan sulfate proteoglycan core protein: a 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J. Cell Biol.* **116,** 559–571
82. Cohen, I. R., Grassel, S., Murdoch, A. D., and Iozzo, R. V. (1993) Structural characterization of the complete human perlecan gene and its promoter. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10404–10408
83. Iozzo, R. V., Pillarisetti, J., Sharma, B., Murdoch, A. D., Danielson, K. G., Uitto, J., and Mauviel, A. (1997) Structural and functional characterization of the human perlecan gene promoter. Transcriptional activation by transforming growth factor-β via a nuclear factor 1-binding element. *J. Biol. Chem.* **272,** 5219–5228
84. Sharma, B., Handler, M., Eichstetter, I., Whitelock, J. M., Nugent, M. A., and Iozzo, R. V. (1998) Antisense targeting of perlecan blocks tumor growth and angiogenesis in vivo. *J. Clin. Investig.* **102,** 1599–1608
85. Iozzo, R. V., Cohen, I. R., Grassel, S., and Murdoch, A. D. (1994) The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. *Biochem. J.* **302,** 625–639
86. Cohen, I. R., Murdoch, A. D., Naso, M. F., Marchetti, D., Berd, D., and Iozzo, R. V. (1994) Abnormal expression of perlecan proteoglycan in metastatic melanomas. *Cancer Res.* **54,** 5771–5774
87. Boucheix, C., Benoit, P., Frachet, P., Billard, M., Worthington, R. E., Gagnon, J., and Uzan, G. (1991) Molecular cloning of the CD9 antigen. A new family of cell surface proteins. *J. Biol. Chem.* **266,** 117–122
88. Boucheix, C., and Benoit, P. (1988) CD9 antigen: will platelet physiology help to explain the function of a surface molecule during hemopoietic differentiation? *Nouv. Rev. Fr. Hematol.* **30,** 201–202
89. Nakamura, K., Iwamoto, R., and Mekada, E. (1995) Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin $\alpha3\beta1$ at cell-cell contact sites. *J. Cell Biol.* **129,** 1691–1705
90. Kagawa, T., Mekada, E., Shishido, Y., and Ikenaka, K. (1997) Immune system-related CD9 is expressed in mouse central nervous system myelin at a very late stage of myelination. *J. Neurosci. Res.* **50,** 312–320
91. Wright, M. D., and Tomlinson, M. G. (1994) The ins and outs of the transmembrane 4 superfamily. *Immunol. Today* **15,** 588–594
92. Maecker, H. T., Todd, S. C., and Levy, S. (1997) The tetraspanin superfamily: molecular facilitators. *FASEB J.* **11,** 428–442
93. Miyake, M., Nakano, K., Itoi, S. I., Koh, T., and Taki, T. (1996) Motility-related protein-1 (MRP-1/CD9) reduction as a factor of poor prognosis in breast cancer. *Cancer Res.* **56,** 1244–1249
94. Sho, M., Adachi, M., Taki, T., Hashida, H., Konishi, T., Huang, C. L., Ikeda, N., Nakajima, Y., Kanehiro, H., Hisanaga, M., Nakano, H., and Miyake, M. (1998) Transmembrane 4 superfamily as a prognostic factor in pancreatic cancer. *Int. J. Cancer* **79,** 509–516
95. Crnogorac-Jurcevic, T., Efthimiou, E., Capelli, P., Blaveri, E., Baron, A., Terris, B., Jones, M., Tyson, K., Bassi, C., Scarpa, A., and Lemoine, N. R. (2001) Gene expression profiles of pancreatic cancer and stromal desmoplasia. *Oncogene* **20,** 7437–7446
96. Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999) Correlation

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between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* **19,** 1720–1730

97. Chen, G., Gharib, T. G., Huang, C. C., Taylor, J. M., Misek, D. E., Kardia, S. L., Giordano, T. J., Iannettoni, M. D., Orringer, M. B., Hanash, S. M., and Beer, D. G. (2002) Discordant protein and mRNA expression in lung adenocarcinomas. *Mol. Cell. Proteomics* **1,** 304–313
98. Griffin, T. J., Gygi, S. P., Ideker, T., Rist, B., Eng, J., Hood, L., and Aebersold, R. (2002) Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. *Mol. Cell. Proteomics* **1,** 323–333
99. Iacobuzio-Donahue, C., Shuja, S., Cai, J., Peng, P., Willett, J., and Murnane, M. J. (2004) Cathepsin D protein levels in colorectal tumors: divergent expression patterns suggest complex regulation and function. *Int. J. Oncol.* **24,** 473–485
100. Shen, J., Person, M. D., Zhu, J., Abbruzzese, J. L., and Li, D. (2004) Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry. *Cancer Res.* **64,** 9018–9026
101. Koopmann, J., Thuluvath, P. J., Zahurak, M. L., Kristiansen, T. Z., Pandey, A., Schulick, R., Argani, P., Hidalgo, M., Iacobelli, S., Goggins, M., and Maitra, A. (2004) Mac-2-binding protein is a diagnostic marker for biliary tract carcinoma. *Cancer* **101,** 1609–1615
102. Ikeda, N., Nakajima, Y., Tokuhara, T., Hattori, N., Sho, M., Kanehiro, H., and Miyake, M. (2003) Clinical significance of aminopeptidase N/CD13 expression in human pancreatic carcinoma. *Clin. Cancer Res.* **9,** 1503–1508
103. Sato, N., Fukushima, N., Maitra, A., Iacobuzio-Donahue, C. A., van Heek, N. T., Cameron, J. L., Yeo, C. J., Hruban, R. H., and Goggins, M. (2004) Gene expression profiling identifies genes associated with invasive intraductal papillary mucinous neoplasms of the pancreas. *Am. J. Pathol.* **164,** 903–914
104. Mahlbacher, V., Sewing, A., Elsasser, H. P., and Kern, H. F. (1992) Hyaluronan is a secretory product of human pancreatic adenocarcinoma cells. *Eur. J. Cell Biol.* **58,** 28–34
105. Ringel, J., Rychly, J., Nebe, B., Schmidt, C., Muller, P., Emmrich, J., Liebe, S., and Lohr, M. (1999) CD44, bFGF and hyaluronan in human pancreatic cancer cell lines. *Ann. N. Y. Acad. Sci.* **880,** 238–242
106. Theocharis, A. D., Tsara, M. E., Papageorgacopoulou, N., Karavias, D. D., and Theocharis, D. A. (2000) Pancreatic carcinoma is characterized by elevated content of hyaluronan and chondroitin sulfate with altered disaccharide composition. *Biochim. Biophys. Acta* **1502,** 201–206
107. Takaya, H., Andoh, A., Shimada, M., Hata, K., Fujiyama, Y., and Bamba, T. (2000) The expression of chemokine genes correlates with nuclear factor-κB activation in human pancreatic cancer cell lines. *Pancreas* **21,** 32–40
108. Shimada, M., Andoh, A., Araki, Y., Fujiyama, Y., and Bamba, T. (2001) Ligation of the Fas antigen stimulates chemokine secretion in pancreatic cancer cell line PANC-1. *J. Gastroenterol. Hepatol.* **16,** 1060–1067
109. Monti, P., Leone, B. E., Marchesi, F., Balzano, G., Zerbi, A., Scaltrini, F., Pasquali, C., Calori, G., Pessi, F., Sperti, C., Di Carlo, V., Allavena, P., and Piemonti, L. (2003) The CC chemokine MCP-1/CCL2 in pancreatic cancer progression: regulation of expression and potential mechanisms of antimalignant activity. *Cancer Res.* **63,** 7451–7461
110. Tumminello, F. M., Leto, G., Pizzolanti, G., Candiloro, V., Crescimanno, M., Crosta, L., Flandina, C., Montalto, G., Soresi, M., Carroccio, A., Bascone, F., Ruggeri, I., Ippolito, S., and Gebbia, N. (1996) Cathepsin D, B and L circulating levels as prognostic markers of malignant progression. *Anticancer Res.* **16,** 2315–2319
111. Gentiloni, N., Caradonna, P., Costamagna, G., D'Ostilio, N., Perri, V., Mutignani, M., Febbraro, S., Tinari, N., Iacobelli, S., and Natoli, C. (1995) Pancreatic juice 90K and serum CA 19-9 combined determination can discriminate between pancreatic cancer and chronic pancreatitis. *Am. J. Gastroenterol.* **90,** 1069–1072
112. Funakoshi, A., Wakasugi, H., and Ibayashi, H. (1979) Clinical investigation of serum deoxyribonuclease: II. clinical studies of serum deoxyribonuclease activity in pancreatic disease. *Gastroenterol. Jpn.* **14,** 436–440
113. Funahashi, H., Takeyama, H., Sawai, H., Furuta, A., Sato, M., Okada, Y., Hayakawa, T., Tanaka, M., and Manabe, T. (2003) Alteration of integrin expression by glial cell line-derived neurotrophic factor (GDNF) in human pancreatic cancer cells. *Pancreas* **27,** 190–196
114. Hansel, D. E., Rahman, A., Wehner, S., Herzog, V., Yeo, C. J., and Maitra, A. (2003) Increased expression and processing of the Alzheimer amyloid precursor protein in pancreatic cancer may influence cellular proliferation. *Cancer Res.* **63,** 7032–7037
115. Maitra, A., Hansel, D. E., Argani, P., Ashfaq, R., Rahman, A., Naji̇̈, A., Deng, S., Geradts, J., Hawthorne, L., House, M. G., and Yeo, C. J. (2003) Global expression analysis of well-differentiated pancreatic endocrine neoplasms using oligonucleotide microarrays. *Clin. Cancer Res.* **9,** 5988–5995

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