Proteomic Analysis of Conditioned Media from the PC3, LNCaP, and 22Rv1 Prostate Cancer Cell Lines: Discovery and Validation of Candidate Prostate Cancer Biomarkers

Girish Sardana,[†,‡] **Klaus Jung,**[§] **Carsten Stephan,**[§] **and Eleftherios P. Diamandis***[,†,‡,‖]

Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada, Department of Urology, Charité—Universitätsmedizin Berlin, Germany, and Department of Clinical Biochemistry, University Health Network and Toronto Medical Laboratories, Toronto, Ontario, Canada.

Early detection of prostate cancer is problematic due to the lack of a marker that has high diagnostic sensitivity and specificity. The prostate specific antigen (PSA) test, in combination with digital rectal examination, is the gold standard for prostate cancer diagnosis. However, this modality suffers from low specificity. Therefore, specific markers for clinically relevant prostate cancer are needed. Our objective was to proteomically characterize the conditioned media from three human prostate cancer cell lines of differing origin [PC3 (bone metastasis), LNCaP (lymph node metastasis), and 22Rv1 (localized to prostate)] to identify secreted proteins that could serve as novel prostate cancer biomarkers. Each cell line was cultured in triplicate, followed by a bottom-up analysis of the peptides by two-dimensional chromatography and tandem mass spectrometry. Approximately, 12% (329) of the proteins identified were classified as extracellular and 18% (504) as membrane-bound among which were known prostate cancer biomarkers such as PSA and KLK2. To select the most promising candidates for further investigation, tissue specificity, biological function, disease association based on literature searches, and comparison of protein overlap with the proteome of seminal plasma and serum were examined. On the basis of this, four novel candidates, follistatin, chemokine (C-X-C motif) ligand 16, pentraxin 3 and spondin 2, were validated in the serum of patients with and without prostate cancer. The proteins presented in this study represent a comprehensive sampling of the secreted and shed proteins expressed by prostate cancer cells, which may be useful as diagnostic, prognostic or predictive serological markers for prostate cancer.

Keywords: Prostate cancer • biomarkers • proteomics • conditioned media • cell culture • bioinformatics • early detection

Introduction

Currently, serum prostate specific antigen (PSA) levels, combined with digital rectal examination (DRE), are the recommended screening tests for early detection of prostate cancer (CaP) in asymptomatic men over the age of 50.[1] However, there is considerable controversy surrounding the efficacy of the PSA test in reducing the overall mortality of CaP.[2,3] These concerns stem from overdiagnosis and the lack of specificity of PSA in discriminating CaP from benign prostatic hyperplasia (BPH).[3] While PSA has been shown to correlate very well with tumor volume,[4] it is unable to predict with certainty the biological aggressiveness of the disease. Several refinements

of the PSA test have been shown to increase its sensitivity and specificity.[5–9] However, there is still a need to develop noninvasive tests to identify clinically relevant CaP.[10]

Mass spectrometry (MS)-based proteomic technologies are currently in the forefront of cancer biomarker discovery. Serum or plasma is usually the discovery fluid of choice;[11] however, several studies have employed MS for biomarker discovery in various other biological fluids.[12,13] In addition, a number of alternative approaches have been used, such as MS spectra profiling,[14,15] peptide profiling,[16,17] and isotopic labeling.[18] There are many inherent limitations to using MS for biomarker discovery in complex biological mixtures such as serum.[19] The main concern is the suppression of ionization of low-abundance proteins by high-abundance proteins such as albumin and immunoglobulins. Depletion strategies have been used to remove high-abundance proteins which have aided in improving the detection limit of MS.[20,21] One approach to overcome the limitations posed by biological fluids is to study the secretome of cell lines grown in serum-free media (SFM). The proteins identified from the conditioned media (CM) are

* To whom correspondence should be addressed. Dr. E. P. Diamandis, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 60 Murray Street, Room 6-201, Toronto, Ontario, M5T 3L9, Canada. Tel: 416-586-8443. Fax: 416-619-5521. Email: ediamandis@mtsinai.on.ca.
† University of Toronto.
‡ Mount Sinai Hospital.
§ Charité—Universitätsmedizin Berlin.
‖ University Health Network and Toronto Medical Laboratories.

specific to the cell line being cultured as there are no other contaminating proteins, therefore, greatly simplifying MS analysis. Studies analyzing the CM from prostate,[22] colon,[23] endothelial,[24] adipose,[25] nasopharyngeal[26] and retinal epithelial cells[27] have already been conducted, thus, demonstrating the versatility of this approach.

Previously, we have shown the PC3(AR)[6] CaP cell line can be grown in a serum-free environment and the secreted proteins present can be readily identified by MS-based methods.[28] In this study, we performed a detailed proteomic analysis of the conditioned media (CM) of three CaP cell lines: PC3, LNCaP, and 22Rv1. From this analysis, we identified 2124 proteins by using a bottom-up approach, consisting of offline strong cation exchange (SCX) chromatography followed by capillary C-18 reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS). Our extensive lists of proteins, and their cellular and biological classifications, may form the basis for discovering novel CaP biomarkers.

The utility of this approach was examined by validating four candidates by ELISA. Each of them was found elevated in serum of a subset of prostate cancer patients; a positive correlation was also observed with serum PSA levels. Furthermore, our approach identified many known CaP biomarkers including PSA, KLK2, KLK11, prostatic acid phosphatase and prostate specific membrane antigen, further supporting the view that this unbiased approach may aid in new CaP biomarker discovery efforts.

Materials and Methods

Cell Culture. The PC3, LNCaP, and 22Rv1 cell lines were purchased from the American type Culture Collection (Rockville, MD). All cell lines were grown in T-175 culture flasks (Nunc) in RPMI 1640 culture medium (Gibco) supplemented with 8% fetal bovine serum (FBS) (Hyclone). Cells were cultured in a humidified incubator at 37 °C and 5% CO_2. Cells were seeded at varying densities. PC3 and 22Rv1 cells were grown for 2 days in 30 mL of RPMI and 8% FBS. Afterward, the medium was removed, and the flask was gently washed three times with 30 mL of phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). Thirty milliliters of chemically defined Chinese Hamster Ovary (CDCHO) (Gibco) medium supplemented with glutamine (8 mmol/L) (Gibco) was added to the flasks and incubated for 2 days. The LNCaP cell line was grown as above, except that the cells were incubated for 3 days in RPMI and 8% FBS before the media were changed to CDCHO. All cell lines were grown in triplicate and independently processed and analyzed. A negative control was also prepared with the same procedures as above, except no cells were seeded.

After incubation in CDCHO, the CM was collected and spun down (3000g) to remove cellular debris. Aliquots were taken for measurement of total protein, lactate dehydrogenase (a marker of cell death), and kallikreins 3, 5, 6 (internal control proteins). The remainder was frozen at −80 °C until further use.

Measurement of Total Protein, Lactate Dehydrogenase, and Kallikreins 3, 5, 6. The total protein of the CM was measured using the Coomassie (Bradford) assay (Pierce Biotechnology) as recommended by the manufacturer.

Lactate dehydrogenase (LDH) levels in the CM were measured via an enzymatic assay based on conversion of lactate to pyruvate. NADH production from NAD^+ during this reaction
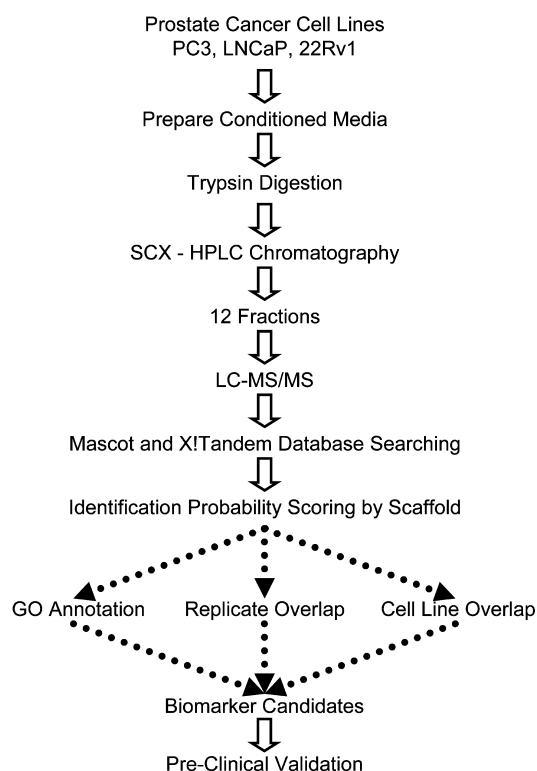

Figure 1. Workflow of proteomic methods used. For additional information, refer to Materials and Methods.

was monitored at 340 mm with an automated method and converted to Units per liter (U/L) (Roche Modular Systems).

Kallikrein 3 (KLK3; PSA), KLK5 and KLK6 were measured with in-house enzyme-linked immunosorbent assays (ELISAs) as described earlier.[29–31]

Conditioned Media Sample Preparation and Trypsin Digestion. Our general protocol is shown in Figure 1. Approximately, 30 mL of CM from each cell line, which corresponded to 1 mg of total protein, was dialyzed overnight using a 3.5 kDa cutoff dialysis tubing (Spectra/Por) at 4 °C in 5L of 1 mM ammonium bicarbonate solution with one buffer exchange. The dialyzed CM was lyophilized overnight to dryness followed by resolubilization with 322 µL of 8 M urea, 25 µL of 200 mM dithiothreitol (DTT), and 25 µL of 1 M ammonium bicarbonate. The sample was vortexed thoroughly and incubated at 50 °C for 30 min. One hundred and twenty five microliters of 500 mM iodoacetamide was added and the sample was incubated in the dark at room temperature for 1 h. The sample was then desalted using a NAP-5 column (GE Healthcare), lyophilized to dryness, and resuspended in 120 µL of 50 mM ammonium bicarbonate, 100 µL of methanol, 150 µL of H_2O and 5 µg of trypsin (Promega), vortexed thoroughly, and incubated for approximately 12 h at 37 °C.

Strong Cation Exchange High Performance Liquid Chromatography. The tryptic digests were lyophilized and resuspended in 120 µL of 0.26 M formic acid in 10% acetonitrile (ACN) (mobile phase A). The sample was fractionated using an Agilent 1100 HPLC system connected to a PolySULFOETHYL A column containing a hydrophilic, anionic polymer (poly-2-sulfethyl aspartamide) with a 200 Å pore size and a diameter of 5 µm (The Nest Group, Inc.). A 1 h linear gradient was used, with 1 M ammonium formate and 0.26 M formic acid in 10% ACN (mobile phase B) at a flow rate of 200 µL/min. Fractions

were collected via a fraction collector every 5 min (12 fractions per run), and frozen at −80 °C for further use. A protein cation exchange standard, consisting of three peptides, was run at the beginning of each day to assess column performance (Bio-Rad).

Online Reversed Phase Liquid Chromatography-Tandem Mass Spectrometry. Each 1 mL fraction was C18-extracted using a Zip TiP$_{C18}$ pipet tip (Millipore) and eluted in 4 μL of 90% ACN, 0.1% formic acid, 10% water, and 0.02% trifluoro-acetic acid (TFA) (Buffer B). To this mixture, 80 μL of 95% water, 0.1% formic acid, 5% ACN, and 0.02% TFA (Buffer A) was added, and half of this volume (40 μL) was injected via an autosampler on an Agilent 1100 HPLC. The peptides were first collected onto a 2 cm C18 trap column (inner diameter 200 μm), then eluted onto a resolving 5 cm analytical C18 column (inner diameter 75 μm) with an 8 μm tip (New Objective). The HPLC was coupled online to an LTQ 2-D Linear Ion Trap (Thermo Inc.). A 120 min gradient was used on the HPLC and peptides were ionized via nanoelectrospray ionization. The peptides were subjected to tandem mass spectrometry (MS/MS) and DTAs were created using the Mascot Daemon v2.16 and extract_msn (Matrix Science). Parameters for DTA creation were min mass 300, max mass 4000, automatic precursor charge selection, min peaks 10 per MS/MS scan for acquisition, and min scans per group of 1.

Database Searching and Bioinformatics. Mascot, v2.1.03 (Matrix Science)[32] and X!Tandem v2.0.0.4 (GPM, Beavis Informatics Ltd.)[33] database search engines were used to search the spectra from the LTQ runs. Each fraction was searched separately against both search engines using the IPI Human database V3.16[34] with trypsin specified as the digestion enzyme. One missed cleavage was allowed; a variable oxidation of methionine residues and a fixed modification of carbamidom-ethylation of cysteines was set with a fragment tolerance of 0.4 Da and a parent tolerance of 3.0 Da.

The resulting DAT files from Mascot and XML files from X!Tandem were put into Scaffold v01_05_19 (Proteome Soft-ware)[35] and searched by allocating all DAT files into one biological sample and all XML files into another biological sample. This was repeated three times for each cell line. The cutoffs in Scaffold were set for 95% peptide identification probability and 80% protein identification probability. Identi-fications not meeting these criteria were not included in the displayed results. The sample reports were exported to Excel, and an in-house developed program was used to extract Gene Ontology (GO) terms for cellular component for each protein and the proportion of each GO term in the data set. Proteins that were not able to be classified by GO terms were checked with Swiss-Prot entries and against the Human Protein Refer-ence Database[36] and Bioinformatic Harvester (http://harves-ter.embl.de) to search for cellular component annotations. Finally, the overlap between proteins identified from each cell line and within each of the replicates for per cell line was determined by in-house built software. Each protein was also searched against the Plasma Proteome Database (www.plas-maproteomedatabase.org). The list of proteins were also com-pared with those found in seminal plasma by Pilch et al.[37] and in breast cancer cell line CM by Kulasingam et al.[38] In addition, we used Ingenuity Pathway Analysis software (Ingenuity Sys-tems) to determine differences in biological networks in the extracellular and membrane proteins of each cell line, as well as overlay molecular functions with respect to disease condi-tions associated with each of the biomarker candidates.

The same set of spectra produced by the LTQ was searched with the same parameters as above, but against a reversed IPI Human database V3.16 which was created using the Reverse.pl script from The Wild Cat Toolbox (Arizona Proteomics). The DAT and XML files from this "reversed" search were input into Scaffold as before and the identified peptides meeting the preset cutoffs were identified. The false positive rate (FPR) was calculated as follows: FPR = no. False peptides/(no. True peptides + no. False peptides).

Validation of Candidates. A preclinical validation was conducted where the concentrations of four candidate proteins (follistatin, chemokine (C-X-C motif) ligand 16, pentraxin 3 and spondin 2) were measured by ELISA in the serum of patients with or without prostate cancer and in the CM of each cell line. These candidates were selected for validation using similar criteria as Kulasingam et al.[38] Follistatin (R&D Systems), chemokine (C-X-C motif) ligand 16 (R&D Systems) and pen-traxin 3 (Alexis Biochemicals) were measured using their respective manufacturer's protocol. Spondin 2 was measured at diaDexus Corporation, San Francisco, CA, using an in-house developed assay. Serum samples from patients were collected at the Toronto Medical Laboratories, Toronto, Canada. The study was carried out after Institutional Review Board approval; median ages of patients were 75 and 67 for the cancer and noncancer groups, respectively.

Results

Cell Culture Optimization. Growth conditions of the three cell lines were optimized in order to reduce cell death and maximize secreted protein levels. Cells were incubated in SFM for 2 days at different seeding densities. Total protein, LDH and the concentration of KLK5 and KLK6 in the CM of PC3, and KLK3 in the CM of LNCaP and 22Rv1 cells were measured. The ratio of KLK3, 5, and 6 concentrations with LDH levels for each culture condition (Supplementary Figures 1, 2, 3) were compared. The optimal seeding concentrations were 7.5×10^6, 22×10^6, and 75×10^6 cells for PC3, LNCaP, and 22Rv1 cell lines, respectively, as these gave the highest ratio of KLK production (indicator of secreted proteins) to LDH (indicator of cell death). The total protein of the CM for the optimized seeding densities was 33, 39, and 39 μg/mL for PC3, LNCaP, and 22Rv1 cells, respectively. Thus, 30 mL of media contained approximately 1 mg of total protein.

Proteins Identified by Mass Spectrometry. A schematic of the sample preparation and bioinformatics utilized in this study is shown in Figure 1. Each cell line was independently cultured in triplicate to determine the reproducibility of our method in identifying proteins between the replicates. After setting a cutoff of 95% peptide probability and 80% protein probability in Scaffold, 2124 proteins were identified that met the criteria from all three cell lines combined (Supplementary Table 1). In total, from the three replicates per cell line, 1157, 1285, and 1116 proteins were found in the PC3, LNCaP, and 22Rv1 cell lines, respectively (Supplementary Table 2). The sequence coverage, peptide sequences identified and observed m/z are listed for each peptide identified (Supplementary Table 3a−c).

A control flask was also prepared that did not contain any cells but were treated with the same procedure. A total of 69 proteins were identified in the negative control flask (Supple-mentary Table 4), which represented FBS-derived proteins from incomplete washing of the tissue culture flasks. These proteins were removed from the list of identified CM proteins of each cell line and were not considered further in the data analysis.

Table 1. Known Prostate Biomarkers Identified in the Conditioned Media of PC3, LNCaP, and 22Rv1 Cell Lines[a]

	PC3 (no. of peptides)		LNCaP (no. of peptides)		22Rv1 (no. of peptides)	
protein	Mascot	X!Tandem	Mascot	X!Tandem	Mascot	X!Tandem
KLK2			6	5	4	4
KLK3(PSA)			9	8	5	5
KLK11					7	6
ACPP			7	7		
Mac-2BP	15	13	7	7		
Zn-α2-GP			7	10	15	11
PSMA			2	2		
NGEP			1	0		

[a] Protein name and number of peptides identified by Mascot and X!Tandem for each cell line are listed. KLK2, human kallikrein 2; PSA, prostate specific antigen; KLK11, human kallikrein 11; ACPP, prostatic acid phosphatase; Mac-2BP, Mac 2 binding protein, Zn-α2-GP, zinc alpha 2 glycoprotein; PSMA, prostate specific membrane antigen; NGEP, new gene expressed in prostate.

Furthermore, to empirically determine FPR of peptide detection, the data set was searched against a reversed IPI Human v3.16 database using the same search parameters and Scaffold cutoffs. A total of 4, 4, and 6 proteins from the PC3, LNCaP, and 22Rv1 cell lines, respectively, were observed (Supplementary Table 5). Each protein was identified by one peptide with Mascot and with no peptides identified by X!Tandem (0% FPR). A FPR of <1% for all 3 cell lines was calculated by Mascot.

Identification of Internal Control Proteins. As an internal control, we sought to identify by LC-MS/MS three proteins that were known to be secreted by these cell lines and were monitored by ELISA during our cell culture optimization. The approximate initial concentrations of these proteins in CM are given in parentheses, below. We confidently identified KLK3 in the CM of LNCaP (∼550 μg/L) and 22Rv1 (∼3 μg/L) with several peptides (Table 1). PC3 cells do not secrete any detectable KLK3 by ELISA, and as expected, this protein was not identified in its CM. One KLK6 peptide from the CM of PC3 (∼1.5 μg/L) was identified, but no peptides from KLK5 were identified by MS. Together, these data suggest that the detection limit of our MS-based method for protein identification is in the low microgram per liter (μg/L) range.

Reproducibility between Replicates. Next, we investigated the reproducibility of our method by culturing each cell line in triplicate. The Mascot and X!Tandem results from the PC3, LNCaP, and 22Rv1 cell lines are shown in Supplementary Figure 4. In general, a 56% overlap of identified proteins in all three replicates from both Mascot and X!Tandem for each of the cell lines was observed. Approximately 20% of proteins were found in two replicates and 24% were exclusive to one replicate. This data highlights the ionization efficiency of the mass spectrometer and, thus, the need for replicate analysis of samples by MS to obtain a more comprehensive list of the secretome of these cell lines.

Differences in Proteins Identified Between Cell Lines. Following this, the proteins identified from each of the three replicates of each cell line were combined to form a nonredundant list per cell line (Supplementary Table 2). These three lists were compared to determine their overlap. The results are shown in Figure 2. About 54% of proteins were unique to one of the cell lines; 21% were common in all 3 cell lines, with 24% were identified in two of the cell lines. This data highlights the heterogeneity of CaP cell lines and the need to investigate multiple cell lines, to obtain a comprehensive picture of the
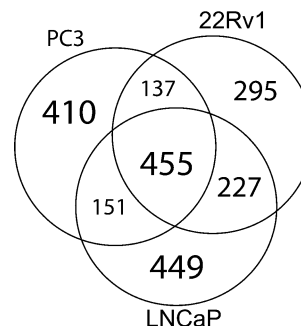

Figure 2. Overlap of proteins identified between each cell line: Each circle represents a cell line.
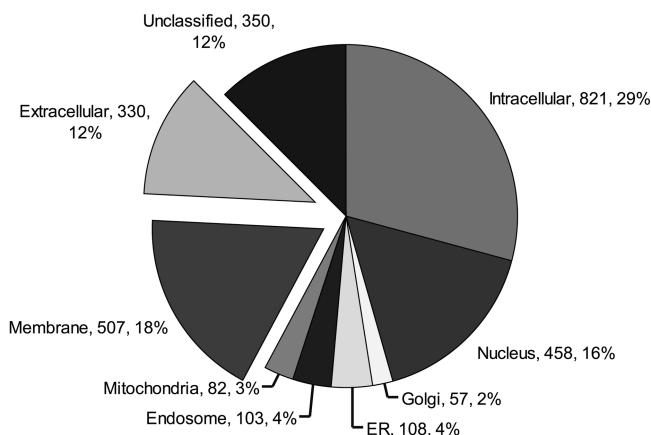

Figure 3. Classification of proteins by cellular location. Each protein identified after MASCOT and X!Tandem searching was classified by its cellular location using Genome Ontology classifiers (www.geneontology.org).

CaP proteome. The overlap among the cell lines for extracellular and membrane proteins, rather than total proteins, yielded similar heterogeneity (Supplementary Figure 6).

Genome Ontology Distributions of Proteins. As shown in Figure 3, 12% of the proteins identified were classified as extracellular, 18% as membrane and 12% as unclassified. The remainder of the proteins identified in the CM were classified as intracellular, nucleus, golgi, endoplasmic reticulum (ER), endosome or mitochondria (58%). Classification by cellular localization is redundant since a protein can be classified in more than one compartment. A similar distribution was found with the cellular component distribution of each cell line (Supplementary Figure 5).

Secreted and Membrane Proteins. After GO annotation, 329 proteins were classified as extracellular, 504 as membranous and 339 as unclassified (Supplementary Table 6). From the list of extracellular and membrane proteins, several previously known CaP biomarkers were identified. These included KLK3 (prostate specific antigen, PSA),[39] KLK2,[5,6] KLK11,[40] prostatic acid phosphatase (acid phosphatase, prostate; ACPP),[41] Mac-2BP,[42] zinc-α-2-glycoprotein,[43] a new gene expressed in the prostate (NGEP)[44] and prostate specific membrane antigen (PSMA)[45] (Table 1). Comparing all extracellular, membrane and unclassified proteins with the Plasma Proteome Database yielded 93 membrane proteins, 98 extracellular proteins, and 27 unclassified proteins as being found previously in serum.

Overlap with Previous Data. Given that we had previously analyzed the CM of the PC3(AR)[6] cell line,[28] the overlap between this set of data and our previous one was examined (Supplementary Table 7). In brief, the current data yielded
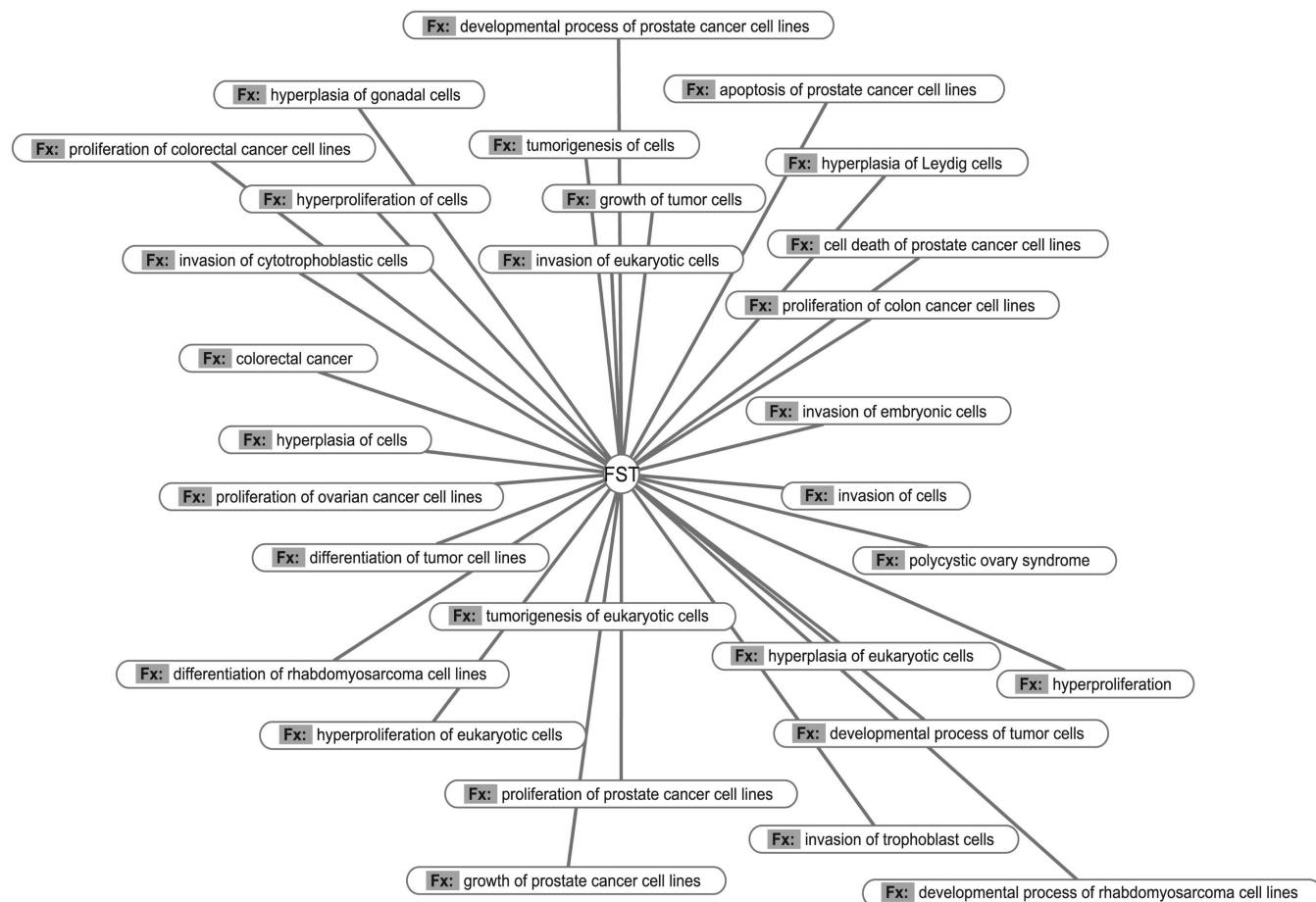
Figure 4. Molecular functions related to diseases associated with Follistatin. Web diagram depicting the biological functions that follistatin is associated with, in the context of disease. Diagram generated through Ingenuity Pathway Analysis software (Ingenuity Systems).

5-fold more proteins owing in part to the improvements in sample preparation, the type of mass spectrometer used (more sensitive) and the powerful bioinformatics (higher confidence of protein probability).

Overlap with Seminal Plasma Proteins. Recently, Pilch and Mann[37] conducted an in-depth proteomic survey of the seminal plasma proteome and identified 932 unique proteins. Comparing our list of extracellular, membrane and unclassified proteins found in the CM of prostate cancer cell lines with those reported in a biological fluid such as seminal plasma[37] resulted in 108 extracellular, 120 membranous, and 40 unclassified proteins as being common. These are highlighted in Supplementary Table 8.

Biological Network Analysis. The extracellular and membrane proteins from each cell line were analyzed using Ingenuity Pathway Analysis software v5.51 (Ingenuity Systems) and functional networks were developed (Supplementary Table 9). Cancer, cellular movement and pathways involved in tissue development were listed among the top networks for each of the cell lines. However, in the case of 22Rv1, these were listed secondary to the "housekeeping" functional networks. Next, the functions and disease association of each of the four candidates selected were examined. Follistatin displayed the most direct connections to cancer such as prostate,[46,47] colon,[48] and ovarian cancer[49] (Figure 4). Chemokine (C-X-C motif) ligand 16 displayed connections to inflammation and chemotaxis[50] (Supplementary Figure 7A). Pentraxin 3 showed multiple connections to tissue and embryonic development[51] (Supple-

mentary Figure 7B). Few studies have been conducted on spondin 2; it was shown to be involved in neuronal guidance and lung cancer[52] (Supplementary Figure 7C).

Overlap with Breast Cancer Secretome. The proteins identified by Kulasingam et al.[38] in the CM of two breast cancer cell lines were compared with those identified in our analysis of CaP CM. There was an overlap of 256 proteins that were present in the BT474, MDA468, PC3, LNCaP and 22Rv1 cell line CM (Supplementary Table 10). These proteins were then subjected to biological network analysis (Supplementary Table 11) in a similar manner as stated above. Pathways related to cancer and cell growth were among the top identified.

Validation of Follistatin, Chemokine (C-X-C Motif) Ligand 16, Pentraxin 3, and Spondin 2. From the list of proteins identified, preclinical validation of four candidates were performed using 42 serum samples from patients with or without CaP (Figure 5) and the presence of these proteins in the CM of PC3, LNCaP and 22Rv1 cell lines was examined (Supplementary Figure 8). The concentration of each candidate in the CM correlates semiquantitatively with the number of unique spectra (shown in parentheses) identified from each peptide of each candidate after database searching by Mascot/ X!Tandem: Follistatin (PC3, 100/128; 22Rv1, 0/1); CXCL16 (LNCaP, 3/4, 22Rv1, 2/2); PTX3 (PC3, 45/52); SPON2 (LNCaP, 152/149, 22Rv1, 2/2). A significant difference (Kruskal−Wallis Test) in sera of patients with or without CaP in all four candidates (Figure 5A,C,E,G) by ELISA was observed. In addition, the correlation between PSA levels and candidate levels
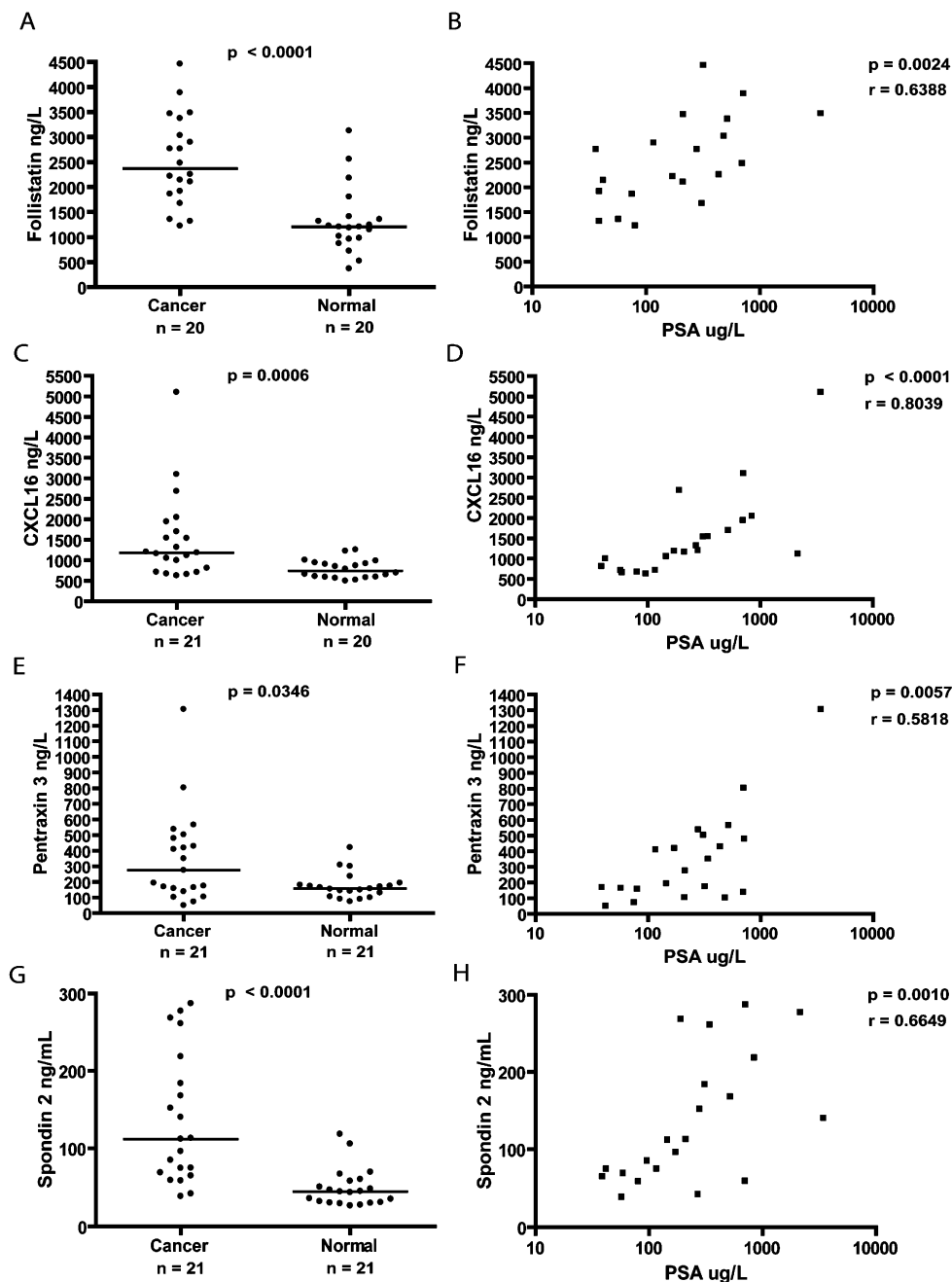
Figure 5. Validation of follistatin, chemokine (C-X-C motif) ligand 16, pentraxin 3 and spondin 2 in serum. Levels of follistatin, CXCL16, PTX3 and SPON2 measured in serum of patients with or without CaP (A, C, E, G, respectively). Median values are shown by a horizontal line. *P*-values were calculated using a Kruskal–Wallis test. Correlations of each candidate with PSA levels (B, D, F, H), *p*-values calculated by the Spearman correlation (r = Spearman correlation coefficient).

in serum of patients with CaP was significant and positive by Spearman analysis (Figure 5B,D,F,H).

Discussion

LC-MS/MS analysis allows the elucidation of the identity of thousands of proteins in complex mixtures, in a high-throughput fashion. This technology has been applied to cancer biomarker discovery, where biological fluids, tissues or cell cultures have been analyzed for differences in protein expression.[22,23,53–55] However, the dynamic range of current LC-MS/MS methods is not adequate to identify all proteins in a complex mixture such as serum. Even with depletion of highly abundant proteins and extensive fractionation, this is a major challenge still faced today.[19]

Prostate cancer, when diagnosed early, is associated with favorable clinical outcomes.[56] Our objective was to identify proteins secreted by three tumorigenic CaP cell lines of differing origin and phenotype. The purpose of examining the secretome of cell lines of differing origin was to obtain a more complete picture of the proteome of CaP since CaP is a heterogeneous disease[57,58] and it requires a diverse model system for biomarker discovery. Thus, we chose to focus on shed and secreted proteins from three CaP cell lines, since this approach is amenable to MS and these proteins will most likely be produced by the tumor in measurable amounts to be detected via a blood test.

In our previous study of the CM of the PC3(AR)[6] cell line,[28] we found a large number of intracellular proteins. In another

study, similar data were seen with the LNCaP cell line.[22] We sought to reduce the amount of intracellular proteins by optimizing the cell culture (Supplementary Figures 1, 2, 3). Yet, our current data (Figure 3) revealed a similar distribution of proteins by cellular component. From this, we deduce that cell death during culture is an unavoidable contaminant when analyzing CM by proteomics. However, previous studies from our laboratory utilizing a similar cell culture-based approach found that the proteins identified in cell lysates do not contain as many extracellular proteins as the conditioned media for that cell line.[38] Furthermore, the extracellular proteins identified in the cell lysate displayed minimal overlap with the proteins identified in the CM illustrating that analyzing the CM, despite the amount of cell death occurring in SFM, leads to a significant enrichment of secreted proteins which may be novel serological markers. We also believe that our method may include microsomal proteins that were not removed during centrifugation of the CM after harvesting.

From our previous work, we determined that replicate analysis expanded coverage and increased the number of identified proteins.[28] As can be seen in Supplementary Figure 4, there are proteins that are uniquely identified by only one replicate. This is most likely due to the incomplete ionization of certain peptides during an LC-MS/MS run. Many studies have shown that cell lines do represent the tumor from which they originated. Hence, the proteins that they secrete should reflect the genetic alterations that they harbor. Given that the biological triplicates yielded a more complete coverage of the secretome for a cell line, it is highly probable that the differences in the proteins identified among the 3 cell lines (Figure 2) indeed reflects the heterogeneity of that cell line.

We confirmed the presence of two internal control proteins (PSA and KLK6) in the CM at microgram per liter (μg/L) concentrations as measured by ELISA and identified by MS (Supplementary Figures 1, 2, 3). We were not able to identify by MS KLK5 which was also present in PC3 at about the same concentration as KLK6 (2 μg/L). It is possible that the stringent peptide and protein probability cutoffs utilized in this study were too strict to allow identification of KLK5 by MS. Alternatively, it is possible that a concentration of 2 μg/L is close to the detection limit of our methodology, and hence, it was not identified. We thus conclude that our method can identify proteins in CM of approximately low microgram per liter (μg/L) or higher, a detection limit which nevertheless is 2−3 orders of magnitude better than the ones achieved by using serum.[59] Furthermore, based on the currently used biomarkers in the clinic, this is the expected concentration range that potential tumor markers should be observed in serum.

The use of multiple search engines has been shown to increase confidence, as well as expand coverage.[60] In this study, Mascot and X!Tandem were used since these search engines use different algorithms to determine if a mass spectrum matches an entry in the database. The use of both search engines served to provide an independent confirmation of the results. The use of peptide[61] and protein prophet algorithms[62] contained within Scaffold allowed increased confidence of the protein identification probabilities. To further increase confidence, we performed a search against a reversed IPI human database[63] and obtained FPR of <1% for the cell lines by Mascot (Supplementary Table 5). The low FPR highlights the fidelity of the search approach used. In addition, to eliminate contaminants left over from the FBS, we processed a control

flask that did not contain any cells and deleted from the list of proteins identified from the CaP cell lines the FBS-derived proteins.

Moreover, from the extracellular, membrane and unclassified proteins identified in this study (shortened list of candidates), 98, 93, and 26 proteins in extracellular, membrane, and unclassified protein lists, respectively, were found in the plasma proteome. It is possible that the other proteins are of low abundance and have not yet been identified in circulation. The shortened list was also compared against the list of proteins found in seminal plasma.[37] We reasoned that if a protein identified from our CaP cell lines is also detected in seminal plasma, it is likely to be secreted or shed at relatively high concentration by prostate cells. We found 108 extracellular, 120 membranous and 40 unclassified proteins in our CM, as well as in the seminal plasma proteome (Supplementary Table 8).

To discern functional differences between the "secretome" of the three cell lines, we performed a biological analysis of the extracellular and membrane proteins identified from each cell line's CM. First, we compared the extracellular and membrane proteins identified between each cell line (Supplementary Figure 6). We found 18% and 20% of proteins identified as extracellular and membrane, respectively, were common to all three cell lines. This is a significantly lower overlap than the overall number of proteins which was common between the cell lines, indicating that there are significant differences specifically within the "secretome" from each cell line. We further investigated functional differences between the cell lines using the Ingenuity Pathway Analysis. Biological interaction networks are listed in Supplementary Table 9. The top ranked networks were those involved with cell movement, signaling, cancer and the cell cycle. Furthermore, 22Rv1 was not represented as having any extracellular proteins involved with cellular movement, whereas PC3 and LNCaP did. This is interesting and relevant since 22Rv1 was derived from an organ-confined prostate tumor, while PC3 and LNCaP were derived from metastatic tumors. In addition, each cell line differs with its sensitivity to androgens, a major component to prostate development: PC3 does not express the androgen receptor (AR) and is a model for androgen insensitive tumors, 22Rv1 expresses the AR but is considered AR insensitive, and LNCaP, which expresses AR, is considered androgen dependent. In addition, we also investigated the common proteins in breast CM identified by Kulasingam et al.[38] with those identified in this study to look for similarities between these two hormonally regulated cancers. Here, we see that cancer and cell proliferation networks are among the top pathways identified to be common (Supplementary Table 11). This commonality between the cell lines highlights that these tumors utilize similar processes during carcinogenesis and warrants further study to delineate biomarkers that could be useful for diagnosis.

Using information from this study, we developed criteria to narrow down our list of candidate biomarkers: (a) we considered proteins that showed relatively prostate-specific mRNA expression by searching through the UniGene expressed sequence tag online database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene); (b) we selected proteins that were classified as extracellular or membrane and have been identified in seminal plasma;[37] (c) we performed literature searches to ensure that these proteins have not been validated before as CaP biomarkers and highlighted proteins that have shown biological links to CaP and other cancers; (d) we then selected candidates that had commercially available immu-

noassays. The narrowed down candidates are listed in Supplementary Table 12.

From these, follistatin, CXCL16, PTX3 and SPON2 were chosen for further validation based on the above criteria. Each candidate showed several links to CaP[46,64–66] or carcinogenesis.[52,67–72] Each candidate showed a significant difference between patients with or without CaP. In addition, a positive correlation with increasing PSA was also observed (Figure 5B,D,F,H). From these results, we speculate that these candidates show an association with CaP progression. Future studies will determine if in combination they can improve the specificity of the PSA test.

To determine the biological association with CaP, we profiled each of the candidate's links to functions and diseases (Figure 4, Supplementary Figure 7). With the exception of SPON2, each of the candidates displayed several links to cancer development, tissue development, inflammation or chemotaxis. All of these processes have shown to play a role in the malignant development of tumors. However, the involvement of each candidate with respect to CaP pathobiology will need to be further studied to elucidate their role during progression.

In summary, we present a robust method of proteomic analysis of cell culture CM and bioinformatics for new biomarker discovery. The four candidates validated have not been previously shown to be serum markers for CaP but require further study to fully elucidate their roles in CaP progression. Additional candidates from this large database are worth validating in the future.

Abbreviations: ACN, acetonitrile; BPH, benign prostate hyperplasia; CaP, prostate cancer; CDCHO, chemically defined chinese hamster ovary medium; CM, conditioned medium; CXCL16, chemokine (C-X-C motif) ligand 16; DRE, digital-rectal examination; FBS, fetal bovine serum; FPR, false positive rate; GO, gene ontology; KLK2, human kallikrein 2; KLK3, human kallikrein 3; KLK5, human kallikrein 5; KLK6, human kallikrein 6; KLK11, human kallikrein 11; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDH, lactate dehydrogenase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NGEP, new gene expressed in the prostate (also known as transmembrane protein 16G, TMEM16G); PSMA, prostate specific membrane antigen; PTX3, pentraxin 3; SFM, serum free media; SCX, strong cation exchange; SPON2, spondin 2; TFA, trifluoroacetic acid.

Supporting Information Available: Figures of optimization of seeding density for 22Rv1, LNCaP, and PC3; overlap of the 3 replicates from PC3, LNCaP, and 22Rv1 conditioned media; classification of proteins by cellular location; overlap of the extracellular and membrane proteins identified in each cell line; molecular functions related to diseases associated with Chemokine (C-X-C motif) ligand 16, Pentraxin 3 and Spondin 2; and concentrations of each candidate in the conditioned media of each cell line and the control flask. Tables of listing proteins identified from all three cell lines from Mascot and GPM searching; number of unique peptides and total spectra identified per protein in each cell line; proteins identified in control flask; proteins identified in control flask; proteins identified for extracellular, membrane and unclassified components from all three cell lines; comparison of previous and current methods and results of proteomics workflow; proteins identified from our data set and found in Pilch et al. seminal plasma paper; extracellular and membrane proteins (listed by gene name) from each cell line organized into functional networks; proteins identified in all the prostate and breast cancer cell lines; proteins overlapping between prostate and breast cancer cell lines (listed by gene name) organized into functional networks; narrowed down candidates for validation; and peptides and proteins identified in 22Rv1, LNCaP, and PC3 cell line conditioned media. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Barry, M. J. Clinical practice. Prostate-specific-antigen testing for early diagnosis of prostate cancer. *N. Engl. J. Med.* **2001**, *344*, 1373–1377.

(2) Ryan, C. J.; Small, E. J. Prostate cancer update: 2005. *Curr. Opin. Oncol.* **2006**, *18*, 284–288.

(3) Constantinou, J.; Feneley, M. R. PSA testing: an evolving relationship with prostate cancer screening. *Prostate Cancer Prostatic Dis.* **2006**, *9*, 6–13.

(4) Kabalin, J. N.; McNeal, J. E.; Johnstone, I. M.; Stamey, T. A. Serum prostate-specific antigen and the biologic progression of prostate cancer. *Urology.* **1995**, *46*, 65–70.

(5) Martin, B. J.; Finlay, J. A.; Sterling, K.; Ward, M.; Lifsey, D.; Mercante, D.; Jainto, J. M.; Martin, L.; Rayford, W. Early detection of prostate cancer in African-American men through use of multiple biomarkers: human kallikrein 2 (hK2), prostate-specific antigen (PSA), and free PSA (fPSA). *Prostate Cancer Prostatic Dis.* **2004**, *7*, 132–137.

(6) Stenman, U. H. New ultrasensitive assays facilitate studies on the role of human glandular kallikrein (hK2) as a marker for prostatic disease. *Clin. Chem.* **1999**, *45*, 753–754.

(7) Catalona, W. J.; Bartsch, G.; Rittenhouse, H. G.; Evans, C. L.; Linton, H. J.; Horninger, W.; Klocker, H.; Mikolajczyk, S. D. Serum pro-prostate specific antigen preferentially detects aggressive prostate cancers in men with 2 to 4 ng/ml prostate specific antigen. *J. Urol.* **2004**, *171*, 2239–2244.

(8) Haese, A.; Vaisanen, V.; Lilja, H.; Kattan, M. W.; Rittenhouse, H. G.; Pettersson, K.; Chan, D. W.; Huland, H.; Sokoll, L. J.; Partin, A. W. Comparison of predictive accuracy for pathologically organ confined clinical stage T1c prostate cancer using human glandular kallikrein 2 and prostate specific antigen combined with clinical stage and Gleason grade. *J. Urol.* **2005**, *173*, 752–756.

(9) Stephan, C.; Jung, K.; Diamandis, E. P.; Rittenhouse, H. G.; Lein, M.; Loening, S. A. Prostate-specific antigen, its molecular forms, and other kallikrein markers for detection of prostate cancer. *Urology* **2002**, *59*, 2–8.

(10) Ciatto, S.; Bonardi, R.; Mazzotta, A.; Lombardi, C.; Santoni, R.; Cardini, S.; Zappa, M. Comparing two modalities of screening for prostate cancer: digital rectal examination + transrectal ultrasonography vs. prostate-specific antigen. *Tumori* **1995**, *81*, 225–229.

(11) Anderson, N. L.; Anderson, N. G. The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* **2002**, *1*, 845–867.

(12) Alexander, H.; Stegner, A. L.; Wagner-Mann, C.; Du Bois, G. C.; Alexander, S.; Sauter, E. R. Proteomic analysis to identify breast cancer biomarkers in nipple aspirate fluid. *Clin. Cancer Res.* **2004**, *10*, 7500–7510.

(13) Rogers, M. A.; Clarke, P.; Noble, J.; Munro, N. P.; Paul, A.; Selby, P. J.; Banks, R. E. Proteomic profiling of urinary proteins in renal cancer by surface enhanced laser desorption ionization and neural-network analysis: identification of key issues affecting potential clinical utility. *Cancer Res.* **2003**, *63*, 6971–6983.

(14) Petricoin, E. F., III; Ornstein, D. K.; Paweletz, C. P.; Ardekani, A.; Hackett, P. S.; Hitt, B. A.; Velassco, A.; Trucco, C.; Wiegand, L.; Wood, K.; Simone, C. B.; Levine, P. J.; Linehan, W. M.; Emmert-Buck, M. R.; Steinberg, S. M.; Kohn, E. C.; Liotta, L. A. Serum proteomic patterns for detection of prostate cancer. *J. Natl. Cancer Inst.* **2002**, *94*, 1576–1578.

(15) Petricoin, E. F.; Ardekani, A. M.; Hitt, B. A.; Levine, P. J.; Fusaro, V. A.; Steinberg, S. M.; Mills, G. B.; Simone, C.; Fishman, D. A.;

Kohn, E. C.; Liotta, L. A. Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* **2002**, *359*, 572–577.

(16) Villanueva, J.; Philip, J.; Entenberg, D.; Chaparro, C. A.; Tanwar, M. K.; Holland, E. C.; Tempst, P. Serum peptide profiling by magnetic particle-assisted, automated sample processing and MALDI-TOF mass spectrometry. *Anal. Chem.* **2004**, *76*, 1560–1570.

(17) Villanueva, J.; Shaffer, D. R.; Philip, J.; Chaparro, C. A.; Erdjument-Bromage, H.; Olshen, A. B.; Fleisher, M.; Lilja, H.; Brogi, E.; Boyd, J.; Sanchez-Carbayo, M.; Holland, E. C.; Cordon-Cardo, C.; Scher, H. I.; Tempst, P. Differential exoprotease activities confer tumor-specific serum peptidome patterns. *J. Clin. Invest.* **2006**, *116*, 271–284.

(18) Anderson, N. L.; Anderson, N. G.; Haines, L. R.; Hardie, D. B.; Olafson, R. W.; Pearson, T. W. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J. Proteome Res.* **2004**, *3*, 235–244.

(19) Qian, W. J.; Jacobs, J. M.; Liu, T.; Camp, D. G.; Smith, R. D. Advances and challenges in liquid chromatography-mass spectrometry-based proteomics profiling for clinical applications. *Mol. Cell. Proteomics* **2006**, *5*, 1727–1744.

(20) Ramstrom, M.; Hagman, C.; Mitchell, J. K.; Derrick, P. J.; Hakansson, P.; Bergquist, J. Depletion of high-abundant proteins in body fluids prior to liquid chromatography fourier transform ion cyclotron resonance mass spectrometry. *J. Proteome Res.* **2005**, *4*, 410–416.

(21) Fu, Q.; Bovenkamp, D. E.; Van Eyk, J. E. A rapid, economical, and reproducible method for human serum delipidation and albumin and IgG removal for proteomic analysis. *Methods Mol. Biol.* **2007**, *357*, 365–371.

(22) Martin, D. B.; Gifford, D. R.; Wright, M. E.; Keller, A.; Yi, E.; Goodlett, D. R.; Aebersold, R.; Nelson, P. S. Quantitative proteomic analysis of proteins released by neoplastic prostate epithelium. *Cancer Res.* **2004**, *64*, 347–355.

(23) Volmer, M. W.; Stuhler, K.; Zapatka, M.; Schoneck, A.; Klein-Scory, S.; Schmiegel, W.; Meyer, H. E.; Schwarte-Waldhoff, I. Differential proteome analysis of conditioned media to detect Smad4 regulated secreted biomarkers in colon cancer. *Proteomics* **2005**, *5*, 2587–2601.

(24) Pellitteri-Hahn, M. C.; Warren, M. C.; Didier, D. N.; Winkler, E. L.; Mirza, S. P.; Greene, A. S.; Olivier, M. Improved mass spectrometric proteomic profiling of the secretome of rat vascular endothelial cells. *J. Proteome Res.* **2006**, *5*, 2861–2864.

(25) Chen, X.; Cushman, S. W.; Pannell, L. K.; Hess, S. Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach. *J. Proteome Res.* **2005**, *4*, 570–577.

(26) Wu, C. C.; Chien, K. Y.; Tsang, N. M.; Chang, K. P.; Hao, S. P.; Tsao, C. H.; Chang, Y. S.; Yu, J. S. Cancer cell-secreted proteomes as a basis for searching potential tumor markers: nasopharyngeal carcinoma as a model. *Proteomics* **2005**, *5*, 3173–3182.

(27) An, E.; Lu, X.; Flippin, J.; Devaney, J. M.; Halligan, B.; Hoffman, E.; Csaky, K.; Hathout, Y. Secreted proteome profiling in human RPE cell cultures derived from donors with age related macular degeneration and age matched healthy donors. *J. Proteome. Res.* **2006**, *5*, 2599–2610.

(28) Sardana, G.; Marshall, J.; Diamandis, E. P. Discovery of candidate tumor markers for prostate cancer via proteomic analysis of cell culture-conditioned medium. *Clin. Chem.* **2007**, *53*, 429–437.

(29) Yousef, G. M.; Polymeris, M. E.; Grass, L.; Soosaipillai, A.; Chan, P. C.; Scorilas, A.; Borgono, C.; Harbeck, N.; Schmalfeldt, B.; Dorn, J.; Schmitt, M.; Diamandis, E. P. Human kallikrein 5: a potential novel serum biomarker for breast and ovarian cancer. *Cancer Res.* **2003**, *63*, 3958–3965.

(30) Diamandis, E. P.; Scorilas, A.; Fracchioli, S.; Van Gramberen, M.; De Bruijn, H.; Henrik, A.; Soosaipillai, A.; Grass, L.; Yousef, G. M.; Stenman, U. H.; Massobrio, M.; Van Der Zee, A. G.; Vergote, I.; Katsaros, D. Human kallikrein 6 (hK6): a new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. *J. Clin. Oncol.* **2003**, *21*, 1035–1043.

(31) Ferguson, R. A.; Yu, H.; Kalyvas, M.; Zammit, S.; Diamandis, E. P. Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the Immulite immunochemiluminescent third-generation assay: potential applications in prostate and breast cancers. *Clin. Chem.* **1996**, *42*, 675–684.

(32) Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20*, 3551–3567.

(33) Craig, R.; Beavis, R. C. A method for reducing the time required to match protein sequences with tandem mass spectra. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2310–2316.

(34) Kersey, P. J.; Duarte, J.; Williams, A.; Karavidopoulou, Y.; Birney, E.; Apweiler, R. The International Protein Index: an integrated database for proteomics experiments. *Proteomics* **2004**, *4*, 1985–1988.

(35) Searle, B. C.; Brundege, J. M.; Turner, M. *Improving Sensitivity by Combining Results from Multiple MS/MS Search Methodologies with the Scaffold Computer Algorithm*; Human Proteome Organization (HUPO), 4th Annual World Congress, Munich, Germany, 2006.

(36) 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.; Pandey, A. Development of human protein reference database as an initial platform for approaching systems biology in humans. *Genome Res.* **2003**, *13*, 2363–2371.

(37) Pilch, B.; Mann, M. Large-scale and high-confidence proteomic analysis of human seminal plasma. *GenomeBiology* **2006**, *7*, R40.

(38) Kulasingam, V.; Diamandis, E. P. Proteomics analysis of conditioned media from three breast cancer cell lines: a mine for biomarkers and therapeutic targets. *Mol. Cell. Proteomics* **2007**, *6*, 1997–2011.

(39) Makarov, D. V.; Carter, H. B. The discovery of prostate specific antigen as a biomarker for the early detection of adenocarcinoma of the prostate. *J. Urol.* **2006**, *176*, 2383–2385.

(40) Diamandis, E. P.; Okui, A.; Mitsui, S.; Luo, L. Y.; Soosaipillai, A.; Grass, L.; Nakamura, T.; Howarth, D. J.; Yamaguchi, N. Human kallikrein 11: a new biomarker of prostate and ovarian carcinoma. *Cancer Res.* **2002**, *62*, 295–300.

(41) Afzal, S.; Ahmad, M.; Mushtaq, S.; Mubarik, A.; Qureshi, A. H.; Khan, S. A. Morphological features correlation with serum tumour markers in prostatic carcinoma. *J. Coll. Physicians Surg. Pak.* **2003**, *13*, 511–514.

(42) Bair, E. L.; Nagle, R. B.; Ulmer, T. A.; Laferte, S.; Bowden, G. T. 90K/Mac-2 binding protein is expressed in prostate cancer and induces promatrilysin expression. *Prostate* **2006**, *66*, 283–293.

(43) Hale, L. P.; Price, D. T.; Sanchez, L. M.; Demark-Wahnefried, W.; Madden, J. F. Zinc alpha-2-glycoprotein is expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer. *Clin. Cancer Res.* **2001**, *7*, 846–853.

(44) Das, S.; Hahn, Y.; Nagata, S.; Willingham, M. C.; Bera, T. K.; Lee, B.; Pastan, I. NGEP, a prostate-specific plasma membrane protein that promotes the association of LNCaP cells. *Cancer Res.* **2007**, *67*, 1594–1601.

(45) Elsasser-Beile, U.; Wolf, P.; Gierschner, D.; Buhler, P.; Schultze-Seemann, W.; Wetterauer, U. A new generation of monoclonal and recombinant antibodies against cell-adherent prostate specific membrane antigen for diagnostic and therapeutic targeting of prostate cancer. *Prostate* **2006**, *66*, 1359–1370.

(46) McPherson, S. J.; Mellor, S. L.; Wang, H.; Evans, L. W.; Groome, N. P.; Risbridger, G. P. Expression of activin A and follistatin core proteins by human prostate tumor cell lines. *Endocrinology* **1999**, *140*, 5303–5309.

(47) Wang, Q. F.; Tilly, K. I.; Tilly, J. L.; Preffer, F.; Schneyer, A. L.; Crowley, W. F., Jr.; Sluss, P. M. Activin inhibits basal and androgen-stimulated proliferation and induces apoptosis in the human prostatic cancer cell line, LNCaP. *Endocrinology* **1996**, *137*, 5476–5483.

(48) Nakagawa, H.; Liyanarachchi, S.; Davuluri, R. V.; Auer, H.; Martin, E. W., Jr.; de la, C. A.; Frankel, W. L. Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. *Oncogene* **2004**, *23*, 7366–7377.

(49) Di Simone, N.; Crowley, W. F., Jr.; Wang, Q. F.; Sluss, P. M.; Schneyer, A. L. Characterization of inhibin/activin subunit, follistatin, and activin type II receptors in human ovarian cancer cell lines: a potential role in autocrine growth regulation. *Endocrinology* **1996**, *137*, 486–494.

(50) Hase, K.; Murakami, T.; Takatsu, H.; Shimaoka, T.; Iimura, M.; Hamura, K.; Kawano, K.; Ohshima, S.; Chihara, R.; Itoh, K.; Yonehara, S.; Ohno, H. The membrane-bound chemokine CXCL16 expressed on follicle-associated epithelium and M cells mediates lympho-epithelial interaction in GALT. *J. Immunol.* **2006**, *176*, 43–51.

(51) Camozzi, M.; Rusnati, M.; Bugatti, A.; Bottazzi, B.; Mantovani, A.; Bastone, A.; Inforzato, A.; Vincenti, S.; Bracci, L.; Mastroianni, D.; Presta, M. Identification of an antiangiogenic FGF2-binding site in the N terminus of the soluble pattern recognition receptor PTX3. *J. Biol. Chem.* **2006**, *281*, 22605–22613.

(52) Manda, R.; Kohno, T.; Matsuno, Y.; Takenoshita, S.; Kuwano, H.; Yokota, J. Identification of genes (SPON2 and C20orf2) differentially expressed between cancerous and noncancerous lung cells by mRNA differential display. *Genomics* **1999**, *61*, 5–14.

(53) Jacobs, J. M.; Adkins, J. N.; Qian, W. J.; Liu, T.; Shen, Y.; Camp, D. G.; Smith, R. D. Utilizing human blood plasma for proteomic biomarker discovery. *J. Proteome. Res.* **2005**, *4*, 1073–1085.

(54) Leak, L. V.; Liotta, L. A.; Krutzsch, H.; Jones, M.; Fusaro, V. A.; Ross, S. J.; Zhao, Y.; Petricoin, E. F., III. Proteomic analysis of lymph. *Proteomics* **2004**, *4*, 753–765.

(55) Drake, R. R.; Cazare, L. H.; Semmes, O. J.; Wadsworth, J. T. Serum, salivary and tissue proteomics for discovery of biomarkers for head and neck cancers. *Expert Rev. Mol. Diagn.* **2005**, *5*, 93–100.

(56) Etzioni, R.; Urban, N.; Ramsey, S.; McIntosh, M.; Schwartz, S.; Reid, B.; Radich, J.; Anderson, G.; Hartwell, L. The case for early detection. *Nat. Rev. Cancer* **2003**, *3*, 243–252.

(57) Hill, R.; Song, Y.; Cardiff, R. D.; Van Dyke, T. Heterogeneous tumor evolution initiated by loss of pRb function in a preclinical prostate cancer model. *Cancer Res.* **2005**, *65*, 10243–10254.

(58) Shah, R. B.; Mehra, R.; Chinnaiyan, A. M.; Shen, R.; Ghosh, D.; Zhou, M.; Macvicar, G. R.; Varambally, S.; Harwood, J.; Bismar, T. A.; Kim, R.; Rubin, M. A.; Pienta, K. J. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* **2004**, *64*, 9209–9216.

(59) Barnidge, D. R.; Goodmanson, M. K.; Klee, G. G.; Muddiman, D. C. Absolute quantification of the model biomarker prostate-specific antigen in serum by LC-Ms/MS using protein cleavage and isotope dilution mass spectrometry. *J. Proteome Res.* **2004**, *3*, 644–652.

(60) Kapp, E. A.; Schutz, F.; Connolly, L. M.; Chakel, J. A.; Meza, J. E.; Miller, C. A.; Fenyo, D.; Eng, J. K.; Adkins, J. N.; Omenn, G. S.; Simpson, R. J. An evaluation, comparison, and accurate benchmarking of several publicly available MS/MS search algorithms: sensitivity and specificity analysis. *Proteomics* **2005**, *5*, 3475–3490.

(61) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* **2002**, *74*, 5383–5392.

(62) Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **2003**, *75*, 4646–4658.

(63) Higdon, R.; Hogan, J. M.; Van Belle, G.; Kolker, E. Randomized sequence databases for tandem mass spectrometry peptide and protein identification. *OMICS* **2005**, *9*, 364–379.

(64) Fujii, Y.; Kawakami, S.; Okada, Y.; Kageyama, Y.; Kihara, K. Regulation of prostate-specific antigen by activin A in prostate cancer LNCaP cells. *Am. J. Physiol Endocrinol. Metab.* **2004**, *286*, E927−E931.

(65) Thomas, T. Z.; Wang, H.; Niclasen, P.; O'Bryan, M. K.; Evans, L. W.; Groome, N. P.; Pedersen, J.; Risbridger, G. P. Expression and localization of activin subunits and follistatins in tissues from men with high grade prostate cancer. *J. Clin. Endocrinol. Metab.* **1997**, *82*, 3851–3858.

(66) Risbridger, G. P.; Mellor, S. L.; McPherson, S. J.; Schmitt, J. F. The contribution of inhibins and activins to malignant prostate disease. *Mol. Cell. Endocrinol.* **2001**, *180*, 149–153.

(67) Heydtmann, M.; Lalor, P. F.; Eksteen, J. A.; Hubscher, S. G.; Briskin, M.; Adams, D. H. CXC chemokine ligand 16 promotes integrin-mediated adhesion of liver-infiltrating lymphocytes to cholangiocytes and hepatocytes within the inflamed human liver. *J. Immunol.* **2005**, *174*, 1055–1062.

(68) Hojo, S.; Koizumi, K.; Tsuneyama, K.; Arita, Y.; Cui, Z.; Shinohara, K.; Minami, T.; Hashimoto, I.; Nakayama, T.; Sakurai, H.; Takano, Y.; Yoshie, O.; Tsukada, K.; Saiki, I. High-level expression of chemokine CXCL16 by tumor cells correlates with a good prognosis and increased tumor-infiltrating lymphocytes in colorectal cancer. *Cancer Res.* **2007**, *67*, 4725–4731.

(69) Wagsater, D.; Hugander, A.; Dimberg, J. Expression of CXCL16 in human rectal cancer. *Int. J. Mol. Med.* **2004**, *14*, 65–69.

(70) Willeke, F.; Assad, A.; Findeisen, P.; Schromm, E.; Grobholz, R.; von Gerstenbergk, B.; Mantovani, A.; Peri, S.; Friess, H. H.; Post, S.; von Knebel, D. M.; Schwarzbach, M. H. Overexpression of a member of the pentraxin family (PTX3) in human soft tissue liposarcoma. *Eur. J. Cancer.* **2006**, *42*, 2639–2646.

(71) Klouche, M.; Brockmeyer, N.; Knabbe, C.; Rose-John, S. Human herpesvirus 8-derived viral IL-6 induces PTX3 expression in Kaposi's sarcoma cells. *AIDS* **2002**, *16*, F9–18.

(72) Simon, I.; Liu, Y.; Krall, K. L.; Urban, N.; Wolfert, R. L.; Kim, N. W.; McIntosh, M. W. Evaluation of the novel serum markers B7-H4, Spondin 2, and DcR3 for diagnosis and early detection of ovarian cancer. *Gynecol. Oncol.* **2007**, *106*, 112–118.

PR8003216