Published in final edited form as:

Prostate. 2009 January 1; 69(1): 49–61. doi:10.1002/pros.20853.

Identification of secreted glycoproteins of human prostate and bladder stromal cells by comparative quantitative proteomics

Young Ah Goo^{1,2,3,*}, Alvin Y. Liu^{1,3,4}, Soyoung Ryu², Scott A. Shaffer², Lars Malmström², Laura Page^{1,4}, Liem T. Nguyen², Catalin E. Doneanu⁵, and David R. Goodlett^{2,3,*}

¹Urology (Box 356510), University of Washington, Seattle, WA 98195

²Medicinal Chemistry (Box 357610), University of Washington, Seattle, WA 98195

³Institute for Systems Biology, 1441 N. 34th St. Seattle, WA, 98103

⁴Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98195

⁵Waters Corporation, 34 Maple St. Milford, MA, 01757

Abstract

Background—Functional development of the prostate is governed by stromal mesenchyme induction and epithelial response. Stromal/epithelial signaling can be mediated through direct cell-cell contact and diffusible factors and their cell surface receptors. These inducers are likely secreted or membrane-associated extracellular proteins. Given the importance of intercellular communication, it is possible that diseases like cancer could arise from a loss of this communication. One approach to gain a molecular understanding of stromal cells is to identify, as a first step, secreted stromal signaling factors. We proposed to do this by comparative analysis between bladder and prostate.

Methods—Secreted proteins were identified from cultured normal prostate and bladder stromal mesenchyme cells by glycopeptide-capture method followed by mass spectrometry. Differences in protein abundance between prostate and bladder were quantified from calculated peptide ion current area (PICA) followed by Western validation. Functional and pathway analyses of the proteins were carried out by Gene Ontology (GO) and Teranode software.

Results—This analysis produced a list of 116 prostate and 84 bladder secreted glycoproteins with ProteinProphet probability scores > 0.9. Stromal proteins upregulated in the prostate include cathepsin L, follistatin-related protein, neuroendocrine convertase, tumor necrosis factor receptor, and others that are known to be involved in signal transduction, extracellular matrix interaction, differentiation and transport.

Conclusions—We have identified a number of potential proteins for stromal signaling and bladder or prostate differentiation program. The prostate stromal/epithelial signaling may be accomplished through activation of the ECM-receptor interaction, complement and coagulation cascades, focal adhesion and cell adhesion pathways.

*Corresponding Authors: University of Washington, Medicinal Chemistry, Box 357610, Seattle, WA 98195-7610, USA, Tel: 206-616-4586, Fax: 206-685-3252, goodlett@u.washington.edu, youngah@u.washington.edu.

Keywords

bladder; prostate; stromal cells; secreted proteins

INTRODUCTION

The prostate develops from the urogenital sinus, and this development is governed by stromal induction and epithelial response. In addition to interaction between epithelium and stromal mesenchyme, morphogenesis and functional cytodifferentiation are dependent on interactions between epithelium and basement membrane, and the extracellular matrix (ECM) [1]. Homeostasis of the prostate and bladder is maintained by reciprocal signaling between stromal and epithelial cells [2,3]. Prostate development is also under hormonal control and the influence of androgen is primarily mediated by the stromal cells [4]. During prostatic development, signaling through the stromal androgen receptor (AR) is required to induce prostatic epithelial identity, ductal morphogenesis and glandular differentiation [5]. There is also evidence that stromal-epithelial interaction is involved in the development of the gut [6] and the kidney [7]. In a comparative cDNA microarray analysis of cultured prostate and bladder stromal cells, proenkephalin (PENK) was shown to have the highest fold difference in expression [8]. Prostate-specific expression of PENK was verified by RT-PCR analysis of stromal cells obtained by laser capture microdissection (LCM) and database analysis of prostate cell transcriptomes. RT-PCR analysis of matched prostate cancer (CP) and normal prostate (NP) cDNA showed undetectable or decreased PENK expression in CP. Given the importance of intercellular communication, it is possible that diseases such as benign prostatic hyperplasia and prostate cancer could arise from a loss of or defect in this communication [9]. Investigation into how the normal stromal/epithelial interaction is affected in cancer may lead to a better understanding of the cancer process [10,11].

It is hoped that a molecular understanding of prostate carcinogenesis will in turn lead to effective therapies, and more importantly, preventive measures. One of the keys lies in the identification of stromal cell factors that mediate stromal/epithelial interaction, expression of which is abnormal in cancer. These signaling factors are most likely extracellular proteins, such as secreted or membrane-associated proteins. *N*-linked glycosylation is a common post-translational modification found in extracellular proteins, and the analytical strategy used in this work is targeting this class of proteins. Cultured stromal cells were used to provide enough material for proteomics analysis. It has been shown that epithelial induction can be achieved with stromal cells in co-culture system [12,13]. Therefore, these signaling molecules are still being synthesized when in culture. There are also several reported studies that showed media from cultured stromal cells could stimulate migration of prostate epithelial cells and proliferation of breast epithelial cells [14,15]. In our unpublished work, a pluripotent embryonal carcinoma cell line, NCCIT, was used as an in vitro model to demonstrate the inductive property of prostate stromal cells. Diffusible factors (i.e., present in culture media) made by prostate stromal cells could induce morphologic, cell surface antigen and gene expression changes including loss of stem cell markers in the treated NCCIT cells (Pascal, L. *et al.*, submitted for publication). These cumulative evidences suggest that stromal signaling molecules can be identified from an analysis of culture media.

Conventional quantitative methods of measuring changes in protein expression generally utilize comparison of protein “spot” intensities on 2DE gels [16]. While the resolving power of 2DE-MS (mass spectrometry) is arguably its best feature, it suffers from loading capacity problems which affect the ultimate achievable sensitivity, and its inability to directly analyze membrane proteins. Shotgun proteomic methods circumvent these problems by direct MS analysis of complex protein mixtures [17]. The isotope coded affinity tag (ICAT)-based shotgun proteomic strategy for protein expression profiling is predicated on distinguishing two protein populations that are labeled with two different stable isotope “mass” tags [18]. Statistics-based “label-free” quantification methods provide for analysis of large numbers of samples that circumvents pair-wise or higher experimental design limitations. As such, an enhanced stable isotope-free quantitative proteomic analysis was carried out to identify differentially expressed secreted proteins in the culture media of normal prostate and bladder stromal cells. This comparative analysis was expected to identify many, if not all, differentially expressed proteins that could play a role in organ-specific intercellular communication in normal prostate and bladder development.

MATERIALS AND METHODS

Prostate and bladder tissue preparation and cell culture

Human tissue acquisition was carried out under UW IRB approval. Prostate specimens were obtained from patients undergoing radical prostatectomy for their cancer treatment. Bladder specimens were obtained from patients undergoing cystoprostatectomy for their cancer treatment. Normal prostate and bladder tissues were harvested from non-cancerous part of the resected organs. Prostate tissues were obtained from cancer-free transition zones by our pathologist in the UW tissue acquisition program. Regions of bladder mucosae and wall that appear grossly normal both visually and by palpation were identified as normal urinary bladder. The cellular composition of the normal specimens was determined by histological examination of tissue block sections to ascertain that there was no histopathological evidence of carcinoma [8,9,19]. Three cancer-free prostate and two cancer-free bladder tissues were used for the study. Cell cultures were initiated either by placing cut tissue pieces on tissue culture plates or by plating single cells prepared by tissue digestion with collagenase as described previously [8]. Briefly, minced tissue was placed in RPMI-1640 media supplemented with 5% fetal bovine serum (FBS), gentamicin, and 10^{-8} M dihydrotestosterone (DHT), and digested with type I collagenase (Invitrogen, Carlsbad, CA) at room temperature overnight with gentle stirring. The digested tissue was passed through Falcon 70µm filter (Becton-Dickinson, Franklin Lakes, NJ) and aspirated with 18-gauge needle. The cell suspension was partitioned into epithelial and stromal fractions by centrifugation on discontinuous Percoll density gradient. Prostate or bladder stromal cells taken from the Percoll gradients were cultured in RPMI-1640 supplemented with 10% FBS and DHT. Cells were trypsinized and serially passaged. Light microscopy was used to check cell morphology. Cells were monitored for expression of smooth muscle actin (α SMA) and non-expression of epithelial cell-specific glycoprotein (EGP) by RT-PCR. Primer pairs (and expected product size) used were: α SMA, 5' GCCTCTGGACGCACAACCTGGCATCG and 3' GTTTGCTGATCCACATCTGCTGGAAGG (650 bp); EGP, 5' TGGAGGTGCCG TTGCACTGCTT and 3' CGACTTTTGCCGCAGCTCAGGA (290 bp). For positive EGP

expression, the prostate cancer cell line, LNCaP, was used. Percoll gradients were used additionally to remove any contaminant epithelial cell types, if present, at serial passages.

Variability assessment of cultured stromal cells by transcriptome analysis

Following media collection, the stromal cells were lysed for RNA isolation, and the RNA quality was checked by BioAnalyzer (Agilent Technologies, Santa Clara, CA). The RNA was used to assess gene expression variations, if any, among the 3 prostate or between the 2 bladder cultures by DNA microarray analysis using the Affymetrix UG-133 Plus 2.0 GeneChips, which contain probesets representing 54,675 genes, splice variants, and ESTs. Total RNA was reverse transcribed with oligo(T)/T7 promoter to produce cDNA, and second-strand cDNA was then synthesized. In vitro transcription was performed in the presence of biotinylated ribonucleotides. The labeled cRNA was hybridized to the arrays, washed and stained with streptavidin-Phycoerythrin using Affymetrix FS-450 fluidics station, and data was collected with GeneChip Scanner 3000. The Affymetrix data was analyzed with GeneChip Operating Software (GCOS). Scanned images of the arrays were converted to numerical data by GCOS. The raw Affymetrix data was filtered to mask genes with signal intensities greater the background threshold and normalized by RMA (robust multi-array) to create correlation matrix.

Glycopeptide-capture proteomics

Prostate and bladder stromal cells (10^7 cells) in the third or fourth passage were placed in 10 ml of RPMI-1640 without FBS and cultured for 24 h. The duration chosen was based on our previous experiments that showed no induction of serum deficiency shock proteins within this time period (unpublished data). This would also allow the cells to enter a stationary, non-cycling, or dormant state, and minimize differential protein expression due to cells at various cell cycle phases [9]. The use of glycopeptide-capture precluded analysis of one of the most abundant proteins, albumin, which is not glycosylated. The serum-free media treatment was called for because the FBS supplement would overwhelm proteomic analysis with not only bovine albumin but also with other abundant serum proteins. Media from three different prostate stromal cell cultures (2 plates each at 10 ml/plate) were combined, as were media from two different bladder stromal cell cultures (3 plates each). The 60 ml of media was made cell-free by centrifugation and filtration. Proteins in the media were concentrated into approximately 1 ml using Centricon Plus-20 (NMWL: 5000, Millipore, Billerica, MA). Protein concentration was estimated by BCA protein assay (Thermo Fisher Scientific Inc., Rockford, IL). A portion of each protein preparation was set aside for Western blot analysis.

In the *N*-linked glycoprotein-capture method [20], 1 mg protein from each sample was exchanged into coupling buffer (100 mM NaAc and 150 mM NaCl, pH 5.5) using desalting column (Bio-Rad, Hercules, CA), and oxidized by adding 15 mM sodium periodate at room temperature for 1 h. After removal of the oxidant with desalting column, the sample was conjugated to hydrazide resin (Bio-Rad) at room temperature for 10–24 h. Non-glycosylated proteins were removed by washing the resin three times with 1 ml of 8 M urea, 0.4 M NH_4HCO_3 , pH 8.3. After the wash, proteins were reduced in 8 mM Tris-(2-carboxyethyl) phosphine at room temperature for 30 min and alkylated in 10 mM iodoacetamide for 30 min. The resin was washed twice with 1 ml 0.1 M NH_4HCO_3 , and trypsin was added at 1

mg/200 mg protein for digestion at 37°C overnight. Trypsin cleavage products were removed, and the resin was washed three times, each successively, in the following solutions: 1.5 M NaCl, 80% acetonitrile (ACN), 100% methanol, water, and 0.1 M NH_4HCO_3 . *N*-Linked glycopeptides were released from the resin by 0.3 μl peptide-*N*-glycosidase F (PNGase F, 500 units/ μl , New England Biolabs, Beverly, MA) at 37°C overnight. Released peptides were dried and resuspended in 5% ACN, 0.1% formic acid for MS analysis.

Mass spectrometry analysis

Each sample was analyzed in quadruplicate by using a Michrom Paradigm MS4B high performance liquid chromatography (HPLC) system (Michrom Bioresources, CA) that was coupled via electrospray ionization (ESI) on-line to a linear ion trap (LTQ) Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (i.e., LTQ-FT, ThermoFinnigan, CA). Peptides were trapped on a home-made 100 μm i.d. $\times$ 18 mm precolumn packed with 5 μm , 200 Å-pore size Magic C18 AQ beads (Michrom Bioresources) in a pressure cell (Brechtbuhler, Spring, TX). Subsequent peptide separation was obtained on a home-made 75 μm i.d. $\times$ 150 mm analytical column packed with 5 μm , 100 Å-pore size Magic C18 AQ beads. After injection of 0.5 μg of total sample onto the column, a 15-min wash with 85% buffer A (100% ddH₂O), 5% buffer B (100% acetonitrile), and 10% buffer C (1% v/v formic acid in ddH₂O) was applied. Peptides were eluted using a linear gradient of 5% buffer B to 45% buffer B in 60 min, then to 80% in 5 min where the acetonitrile concentration remained at 80% for 10 min prior to column re-equilibration with 5%. Buffer C remained constant at 10% during gradient operation. The LTQ-FT mass spectrometer was operated in a data-dependent mode such that precursor ion survey scans of 400–1800 m/z were acquired in the FT-ICR with a resolution at $R=100,000$ at m/z 524 and peptide tandem mass spectra in the LTQ ion trap. The five most intense ions were selected in the linear ion trap and subjected to collision induced dissociation (CID) in series using a trap target value of 5,000 and 60 sec of dynamic exclusion. The total MS-MS/MS scan cycle time was ~ 1.5 s. The general mass spectrometric conditions were: ESI voltage at 1.3 kV; ion transfer tube temperature at 200 °C; and normalized collision energy at 30%.

Identification of glycoproteins

Tandem mass spectral RAW (ThermoFinnigan) files were converted to mzXML format using the program ReAdW [21]. Acquired MS/MS spectra were searched for sequence matches against the International Protein Index (IPI) human protein database (version 3.01) using SEQUEST. The following modifications were set as search parameters: parent ion and fragment mass tolerance at 1.2, trypsin digestion cleavage after K or R (except when followed by P), 1 allowed missed cleavage site, carboxymethylated cysteines, oxidized methionines, and conversion of Asn (N) to Asp (D) at *N*-glycosites by PNGase F. PeptideProphet and ProteinProphet, which compute a probability likelihood of each identification being correct, were used for statistical analysis of search results [22]. A PeptideProphet probability score of 0.5 was chosen as a filter, which corresponds to an error rate of 3%. For proteins, a ProteinProphet probability score of 0.9 was applied, which corresponds to a $< 0.02\%$ error rate. For proteins identified by only one unique peptide sequence, they were included in the study when multiple observations (≥ 2) of the

same peptide were made. To reduce further the false positive rate, peptides were filtered for the *N*-linked glycosylation motif (N-X-S/T where X is any amino acid except proline).

Isotope-free labeling quantitative analysis of glycoproteins

Samples were analyzed in quadruplicate by LC-MS on the LTQ-FT. Reproducibility of eluting peptide ion map patterns (i.e., m/z vs. time) and relative peptide ion intensity values between runs was tested using The Dragon Visualization tool [23]. Expression levels of proteins were estimated using PICA (peptide ion current area) [24] with a modification of ion intensity areas as previously described [25–27]. Peptide ion intensities were normalized using total ion intensity following background noise subtraction, and retention time shift between runs was adjusted to align identical peptides from separate analyses. All peptides identified for a given protein were collected, and theoretical m/z of the first three isotopic peaks of the peptide were calculated. These three isotopic peaks were smoothed over time using the Savitzky-Golay filter and extracted for further analysis only when they persisted in both accurate m/z (± 0.015) and respective retention time (initially, within ± 3 minutes). Only isotopic peaks with three or more scans to reconstruct the ion chromatogram were extracted in the study. Protein expression was estimated by summing the ion current areas of the corresponding peptides. The protein ratio between bladder and prostate was derived by dividing an average protein expression value of bladder by that of prostate. Differentially expressed proteins were identified by two-sample *t*-test (with unequal variance) and false discovery rate (FDR), which was corrected for multiple testing errors. Proteins with FDR 0.05 were considered to be significant for differential expression and subjected to further analysis.

Western blot analysis

Differentially expressed proteins identified via the label-free quantification method, PICA, were validated by Western blot analysis. Five to 10 μ g of bladder or prostate protein samples (as described in the section “Glycopeptide-capture proteomics”) were resolved on 4–12% NuPAGE® Bis-Tris gel (Invitrogen) and transferred to PVDF or nitrocellulose membrane for incubation with primary antibodies, followed by washes and incubation with HRP-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ). The dilution ranges for primary and secondary antibodies were 1:100–1:1,000 and 1:2,000–1:5,000 respectively. Reactivity was visualized by enhanced chemiluminescence (Amersham Biosciences). Protein molecular weight was estimated using the Dual color protein standard (Bio-Rad Laboratories). The commercial sources of antibodies for the following proteins were Santa Cruz Biotechnology (Santa Cruz, CA): FSTL1 (follistatin related protein), LAMC1 (laminin γ 1), AXL (receptor tyrosine kinase), IGF2R (cation-independent mannose-6-phosphate receptor), STC1 (stanniocalcin); Chemicon (Temecula, CA): TIMP1 (metalloproteinase inhibitor 1), APOH (β -2-glycoprotein I); Aviva Systems Biology (San Diego, CA): TNFRSF11B (tumor necrosis factor receptor superfamily member 11B); Abcam (Cambridge, MA): SPARC (secreted acidic cysteine rich glycoprotein); Biovender (Chandler, NC): TF (serotransferrin); BD PharMingen (San Diego, CA): CTSL (cathepsin L), CD90. The CD90 antibody was used as a control for gel loading of protein. CD90 is a GPI-anchored protein found released into the culture media by stromal cells, and the

expression of CD90 in cultured bladder and prostate stromal cells has been previously reported [28].

RESULTS

Cultured stromal cells

Under our culture conditions, the stromal cultures at harvest contained essentially α SMA stromal fibromuscular cells with no detectable EGP epithelial elements (Fig. 1). EGP is specifically expressed by epithelial cells (e.g., LNCaP). The transcriptome datasets of the different stromal cultures showed that there was no significant gene expression differences between individual prostate or bladder cultures (Fig. 2).

Secreted proteins of human prostate and bladder stromal cells

A total of 116 glycoproteins were identified in the media of prostate stromal cell cultures and, of these, 34 were identified in the prostate but not the bladder. A total of 84 glycoproteins were identified in the media of bladder stromal cell cultures and 2 of these were identified in the bladder but not the prostate. The two secreted proteomes thus shared expression of 82 proteins (Fig. 3a). Caution is needed in these types of comparisons because proteins identified in one sample only may be due to under-sampling by data-dependent ion selection [29], data filtering, or peptides that failed to generate the necessary 3 isotopic peaks for inclusion for quantification. A complete list of the identified proteins is provided in data supplement (Additional Table 1).

Identified glycoproteins were annotated with Gene Ontology (GO) [30], which assigns putative cellular compartmentalization and molecular functions. Not surprisingly, > 75% of the proteins identified by glycopeptide-capture were annotated as secreted or membrane-associated molecules (Fig. 3b). Protein- and ion-binding functions, hydrolase activity (catalytic hydrolysis of C-O, C-N, C-C, phosphoric anhydride bonds, etc.) were the most common ascribed to these secreted proteins (Fig. 3c). Both bladder and prostate proteins exhibited a similar categorization in their GO functions. The binding, signal transduction, and transport functions could all be characteristic for cells engaged in intercellular communication.

Differences between proteins secreted by prostate and bladder stromal cells

A total of 29 proteins with FDR = 0.05 were considered to be statistically significant for differential expression between prostate and bladder (Table 1). Only three of these 29 proteins were annotated in GO as either intracellular or unknown; all others were as secreted or membrane-associated cell surface proteins. When queried for GO biological processes, these proteins were found to be involved predominantly in physiology, cell adhesion, communication, and development (Fig. 4). Of these differentially expressed proteins, quantitative proteomic analysis found that 24 were over-expressed in the prostate and 5 in the bladder. Their differential expression was validated by Western blot analysis for those with available antibodies (Fig. 5). For some proteins, no antibody was available and so could not be similarly validated. Two prostate proteins, serotransferrin (TF) and stanniocalcin (STC1), with FDR > 0.05 were also included for Western. STC1 was detected only in the

prostate by MS but its expression was detected in both prostate and bladder by Western with an increased level in the prostate. It is well known that mass spectrometry based shotgun proteomic profiling as conducted here has a random component to it, which precludes identification of all proteins present in any given sample. Thus, it must be born in mind that absence of a protein by MS does not indicate absence in the sample.

Pathway analysis of the secreted proteins and implication in cell-cell signaling

All of the secreted proteins were analyzed for their pathway involvement by Teranode pathway analysis software [31] through the KEGG *Homo sapiens* database. Table 2 shows the number of pathways identified by two or more secreted proteins found in this study. Identification of such pathways suggested that they might be active in stromal cell biology. The ECM-receptor interaction, complement and coagulation cascades, focal adhesion and cell adhesion were all mapped with the most number of secreted proteins. For example, in ECM-receptor interaction, the upregulation of FN (fibronectin) and LAMC (laminin γ 1) protein family in prostate stromal cells relative to bladder stromal cells was displayed (Fig. 6). In this diagram, grey nodes indicate the component proteins of this pathway, and the 6 identified proteins are given different colors to indicate expression levels in which red is to signify decreased, green increased, and yellow no change. Useful information included for each identified protein is the international protein index (IPI) ID, ProteinProphet probability score, annotation, tissue type expression, prostate/bladder expression ratio, and associated FDR.

DISCUSSION

In this study, we identified a number of secreted stromal proteins that are candidate signaling molecules in tissue homeostasis. Of the prostate proteins, cathepsin L (CTSL) was the most differentially expressed protein in prostate stromal cells. In the literature, this lysosomal cysteine protease is reported to be synthesized in large amounts and secreted by tumor cells in culture. Tumor CTSL activity was proposed to facilitate invasion and metastasis by its degradation of ECM components [32–34]. An elevated CTSL level has been observed in primary cell cultures from tumors [35]. Follistatin related protein (FSTL1) modulates the action of growth factors on cell proliferation and differentiation. It has structural similarity to the bone morphogenetic protein antagonist follistatin, as well as to the ECM-modifying BM-40/SPARC/osteonectin. In mouse development, FSTL1 is strongly expressed in the mesenchyme but not the epithelium [36]. Neuroendocrine convertase 2 (PCSK2) releases hormones and neuropeptides from their precursors. PSG5 (pregnancy-specific β -1-glycoprotein), TNFRSF11B (tumor necrosis factor receptor superfamily member 11B), FBN1 (fibrillin), IGFBP3 (insulin-like growth factor binding protein), and TIMP1 (metalloproteinase inhibitor 1) have been reported to be expressed in carcinoma-associated fibroblasts and normal gland-associated fibroblasts [37,38]. Membrane bound receptors including AXL (receptor tyrosine kinase), PTK7 (protein tyrosine kinase), PTPRK (protein tyrosine phosphatase receptor type K), and IGF2R (Insulin-like growth factor II receptor; also known as cation-independent mannose-6-phosphate receptor) were previously reported to be upregulated in prostate stromal cells. AXL appears to mediate cell growth and survival and to be involved in prostate cancer progression [39]. PTK7 functions as a cell

adhesion molecule and has been implicated in gastric cancer as a candidate oncogene [40]. PTPRK regulates growth hormone signaling in breast cancer cells by interacting with phosphatase receptors [41]. IGF2R transports phosphorylated lysosomal enzymes from the Golgi complex and cell surface to lysosomes and play a key role in signaling networks [42]. CTSC (dipeptidyl-peptidase I) is a thiol protease that activates serine proteases such as elastase and cathepsin G. Dipeptidyl peptidases were implicated as inhibitors of prostate cancer by their blocking of basic fibroblast growth factor signaling pathway [43].

For the bladder proteins, APOH (β -2-glycoprotein I, apolipoprotein H) appears to prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells [44]. SERPING1 (plasma protease C1 inhibitor) was reported to regulate physiological pathways including complement activation, blood coagulation, fibrinolysis and the generation of kinins [45]. Defects in SERPING1 are the cause of hereditary angioneurotic edema. DSG2 (desmoglein) is involved in the interaction of plaque proteins and intermediate filaments in cell-cell adhesion [46]. CADM1 (nectin-like protein 2) is a cell adhesion molecule.

The enhanced label-free quantification method we employed could effectively identify differential expression between samples. The ICAT method is useful for pair-wise comparisons, but is unwieldy and expensive for analysis of large numbers of samples. The label-free quantification provides a major advance for when analysis of tens of clinical samples is needed, particularly as data on each sample is acquired independently of others, and changes in protein expression are calculated between any pairs of samples.

Steroid sex hormones, such as the androgen, regulate epithelial proliferation, secretory protein production, and cellular differentiation in the prostatic development. Androgen regulates epithelial proliferation via paracrine mechanisms requiring the appropriate receptors in the stroma. Androgen receptor (AR) in the epithelium alone is not sufficient for the regulation of epithelial proliferation; stromal AR together with stromal-epithelial interaction are required [47,48]. The future in epithelial-stromal interaction study depends on the identification and characterization of molecules regulating epithelial growth and differentiation [49]. Among the reported candidates of paracrine mediators, fibroblast growth factor 10 (FGF10) and insulin-like growth factor 1 (IGF-1) exhibit growth inhibition of the prostate in mice [50,51]. Androgen ablation is a viable treatment strategy for prostate cancer because androgen regulates prostate cancer growth. Recent studies have reported androgen-regulated genes that are mediators of androgen in both normal and malignant prostate tissues. Most of these studies are aimed to identify underlying tumor cell and host factors that drive development of resistance to hormonal therapy and emergence of androgen-independent prostate cancer [52–55]. A stress responsive protein GRP78 (78-kDa glucose-regulated protein) is one of the factors identified and its expression is associated with the castration-resistant state and greater risk of prostate cancer recurrence [56,57]. Another signaling molecule is ezrin, and androgen-induced prostate cancer cell invasion is mediated by phosphorylated ezrin [58]. In our study, insulin-like growth factor II receptor (IGF2R) and insulin-like growth factor binding protein 3 (IGFBP3) were identified. While neither GRP78 nor ezrin was identified in our study, a 150-kDa oxygen-regulated protein (ORP150) was identified (Supplementary Table 1). ORP150 is a new member of the heat

shock protein family that functions as a molecular chaperone, and its expression was found to be increased in infiltrating prostate cancer cells [59].

Not all of the previously reported signaling factors have been identified in our study. This could be due to those factors present in low-abundance levels beyond the detection limit of current proteomics methods. It could be also due to the proteins were not active or expressed at the time when the media was collected. We note that it is well known that absence of a protein in a list of proteins identified by shotgun proteomics does not indicate absence in the sample. Some proteins are more difficult to capture in the MS analysis methods employed [60].

The finding that the majority of the differentially expressed secreted proteins were proteases, kinases, and growth factors involved in protein binding, transport and/or signal transductions suggests their possible involvement in stromal and epithelial communication, and organ-specific development. Stromal secretion of cytokines, growth factors, and proteases is essential for epithelial differentiation and morphogenesis. Some of these factors are also involved in reciprocal stromal/epithelial interaction in prostate cancer [61]. Thus, stromal/epithelial signaling may be accomplished through transmission via activation of the ECM-receptor and focal adhesion pathways. With their identification, one can begin to study their biological function and we are developing an in vitro assay system to test the functional property of these identified proteins. In addition, expression of these organ-specific stromal genes in tumor-associated stroma is being investigated.

CONCLUSIONS

A number of secreted stromal proteins that are candidate signaling molecules in tissue homeostasis were identified by glycopeptide-capture proteomics coupled with a novel label-free proteomics quantification method. Organ-specific secreted proteins are potential paracrine signaling molecules for the induction of epithelial differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Contract grant acknowledgment: This work was supported in full by Department of Army grant W81XWH-06-1-0108; and in part by National Institutes of Health grants: CA101052, DK63630, CA111244; UW Ecogenetics and Environmental Health (NIEHS P30ES07033); and NW RCE for Biodefense and Emerging Infectious Diseases (1U54 AI57141).

REFERENCES

1. Bissell MJ, Ram TG. Regulation of functional cytodifferentiation and histogenesis in mammary epithelial cells: role of the extracellular matrix. *Environ Health Perspect.* 1989; 80:61–70. [PubMed: 2647485]
2. Peehl DM. Human prostatic epithelial and stromal cell lines and strains. *Urol Oncol.* 1996; 2(4): 100–103. [PubMed: 21224147]

3. Staack A, Hayward SW, Baskin LS, Cunha GR. Molecular, cellular and developmental biology of urothelium as a basis of bladder regeneration. *Differentiation*. 2005; 73(4):121–133. [PubMed: 15901280]
4. Lee C. Role of androgen in prostate growth and regression: stromal-epithelial interaction. *Prostate Suppl*. 1996; 6:52–56. [PubMed: 8630230]
5. Cunha GR, Hayward SW, Wang YZ, Ricke WA. Role of the stromal microenvironment in carcinogenesis of the prostate. *Int J Cancer*. 2003; 107(1):1–10. [PubMed: 12925950]
6. Kedinger M, Lefebvre O, Duluc I, Freund JN, Simon-Assmann P. Cellular and molecular partners involved in gut morphogenesis and differentiation. *Philos Trans R Soc Lond B Biol Sci*. 1998; 353(1370):847–856. [PubMed: 9684282]
7. Saxen L. What is needed for kidney differentiation and how do we find it? *Int J Dev Biol*. 1999; 43(5):377–380. [PubMed: 10535313]
8. Goo YA, Goodlett DR, Pascal LE, Worthington KD, Vessella RL, True LD, Liu AY. Stromal mesenchyme cell genes of the human prostate and bladder. *BMC Urol*. 2005; 5:17. [PubMed: 16343351]
9. Zhao H, Ramos CF, Brooks JD, Peehl DM. Distinctive gene expression of prostatic stromal cells cultured from diseased versus normal tissues. *J Cell Physiol*. 2007; 210(1):111–121. [PubMed: 17044071]
10. Ayala G, Tuxhorn JA, Wheeler TM, Frolov A, Scardino PT, Otori M, Wheeler M, Spitler J, Rowley DR. Reactive stroma as a predictor of biochemical-free recurrence in prostate cancer. *Clin Cancer Res*. 2003; 9(13):4792–4801. [PubMed: 14581350]
11. Dawson LA, Maitland NJ, Turner AJ, Usmani BA. Stromal-epithelial interactions influence prostate cancer cell invasion by altering the balance of metalloproteinase expression. *Br J Cancer*. 2004; 90(8):1577–1582. [PubMed: 15083188]
12. Daly-Burns B, Alam TN, Mackay A, Clark J, Shepherd CJ, Rizzo S, Tatoud R, O'Hare MJ, Masters JR, Hudson DL. A conditionally immortalized cell line model for the study of human prostatic epithelial cell differentiation. *Differentiation*. 2007; 75(1):35–48. [PubMed: 17244020]
13. Lang SH, Smith J, Hyde C, Macintosh C, Stower M, Maitland NJ. Differentiation of prostate epithelial cell cultures by Matrigel/stromal cell glandular reconstruction. *In Vitro Cell Dev Biol Anim*. 2006; 42(8–9):273–280. [PubMed: 17163777]
14. You X, Yu HM, Cohen-Gould L, Cao B, Symons M, Vande Woude GF, Knudsen BS. Regulation of migration of primary prostate epithelial cells by secreted factors from prostate stromal cells. *Exp Cell Res*. 2003; 288(2):246–256. [PubMed: 12915116]
15. Dong-Le Bourhis X, Berthois Y, Millot G, Degeorges A, Sylvi M, Martin PM, Calvo F. Effect of stromal and epithelial cells derived from normal and tumorous breast tissue on the proliferation of human breast cancer cell lines in co-culture. *Int J Cancer*. 1997; 71(1):42–48. [PubMed: 9096664]
16. Yan JX, Sanchez JC, Tonella L, Williams KL, Hochstrasser DF. Studies of quantitative analysis of protein expression in *Saccharomyces cerevisiae*. *Electrophoresis*. 1999; 20(4–5):738–742. [PubMed: 10344242]
17. Goodlett DR, Keller A, Watts JD, Newitt R, Yi EC, Purvine S, Eng JK, von Haller P, Aebersold R, Kolker E. Differential stable isotope labeling of peptides for quantitation and de novo sequence derivation. *Rapid Commun Mass Spectrom*. 2001; 15(14):1214–1221. [PubMed: 11445905]
18. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol*. 1999; 17(10):994–999. [PubMed: 10504701]
19. Pascal LE, True LD, Campbell DS, Deutsch EW, Risk M, Coleman IM, Eichner LJ, Nelson PS, Liu AY. Correlation of mRNA and protein levels: cell type-specific gene expression of cluster designation antigens in the prostate. *BMC Genomics*. 2008; 9:246. [PubMed: 18501003]
20. Zhang H, Li XJ, Martin DB, Aebersold R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. *Nat Biotechnol*. 2003; 21(6):660–666. [PubMed: 12754519]
21. Pedrioli PG, Eng JK, Hubley R, Vogelzang M, Deutsch EW, Raught B, Pratt B, Nilsson E, Angeletti RH, Apweiler R, Cheung K, Costello CE, Hermjakob H, Huang S, Julian RK, Kapp E, McComb ME, Oliver SG, Omenn G, Paton NW, Simpson R, Smith R, Taylor CF, Zhu W,

- Aebersold R. A common open representation of mass spectrometry data and its application to proteomics research. *Nat Biotechnol.* 2004; 22(11):1459–1466. [PubMed: 15529173]
22. Goo YA, Yi EC, Baliga NS, Tao WA, Pan M, Aebersold R, Goodlett DR, Hood L, Ng WV. Proteomic analysis of an extreme halophilic archaeon, *Halobacterium* sp. NRC-1. *Mol Cell Proteomics.* 2003; 2(8):506–524. [PubMed: 12872007]
 23. Radulovic D, Jelveh S, Ryu S, Hamilton TG, Foss E, Mao Y, Emili A. Informatics platform for global proteomic profiling and biomarker discovery using liquid chromatography-tandem mass spectrometry. *Mol Cell Proteomics.* 2004; 3(10):984–997. [PubMed: 15269249]
 24. Ryu S, Gallis B, Goo YA, Shaffer A, Radulovic D, Goodlett DR. Comparison of a label-free quantitative proteomic method based on peptide ion current area to the isotope coded affinity tag method. *Cancer Informatics.* 2008; 4:243–255. [PubMed: 19259412]
 25. Colinge J, Chiappe D, Lagache S, Moniatte M, Bougueleret L. Differential proteomics via probabilistic peptide identification scores. *Anal Chem.* 2005; 77(2):596–606. [PubMed: 15649059]
 26. Liu H, Sadygov RG, Yates JR 3rd. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal Chem.* 2004; 76(14):4193–4201. [PubMed: 15253663]
 27. Wiener MC, Sachs JR, Deyanova EG, Yates NA. Differential mass spectrometry: a label-free LC-MS method for finding significant differences in complex peptide and protein mixtures. *Anal Chem.* 2004; 76(20):6085–6096. [PubMed: 15481957]
 28. Liu AY, LaTray L, van Den Engh G. Changes in cell surface molecules associated with in vitro culture of prostatic stromal cells. *Prostate.* 2000; 44(4):303–312. [PubMed: 10951495]
 29. Yi EC, Marelli M, Lee H, Purvine SO, Aebersold R, Aitchison JD, Goodlett DR. Approaching complete peroxisome characterization by gas-phase fractionation. *Electrophoresis.* 2002; 23(18):3205–3216. [PubMed: 12298092]
 30. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet.* 2000; 25(1):25–29. [PubMed: 10802651]
 31. Goo YA, Pajkovic N, Shaffer S, Goodlett DR, van Breemen R. Systematic investigation of lycopene effects in LNCaP cells by use of novel large-scale proteomic analysis software. *Proteomics Clin Appl.* 2007; 1(5):513–523. [PubMed: 20740054]
 32. Chauhan SS, Goldstein LJ, Gottesman MM. Expression of cathepsin L in human tumors. *Cancer Res.* 1991; 51(5):1478–1481. [PubMed: 1997186]
 33. Staack A, Tolic D, Kristiansen G, Schnorr D, Loening SA, Jung K. Expression of cathepsins B, H, and L and their inhibitors as markers of transitional cell carcinoma of the bladder. *Urology.* 2004; 63(6):1089–1094. [PubMed: 15183956]
 34. Shuja S, Sheahan K, Murnane MJ. Cysteine endopeptidase activity levels in normal human tissues, colorectal adenomas and carcinomas. *Int J Cancer.* 1991; 49(3):341–346. [PubMed: 1917131]
 35. Friedrich B, Jung K, Lein M, Turk I, Rudolph B, Hampel G, Schnorr D, Loening SA. Cathepsins B, H, L and cysteine protease inhibitors in malignant prostate cell lines, primary cultured prostatic cells and prostatic tissue. *Eur J Cancer.* 1999; 35(1):138–144. [PubMed: 10211102]
 36. Adams D, Larman B, Oxburgh L. Developmental expression of mouse follistatin-like 1 (Fstl1): Dynamic regulation during organogenesis of the kidney and lung. *Gene Expr Patterns.* 2007; 7(4):491–500. [PubMed: 17129766]
 37. Joesting MS, Perrin S, Elenbaas B, Fawell SE, Rubin JS, Franco OE, Hayward SW, Cunha GR, Marker PC. Identification of SFRP1 as a candidate mediator of stromal-to-epithelial signaling in prostate cancer. *Cancer Res.* 2005; 65(22):10423–10430. [PubMed: 16288033]
 38. Cunha GR, Cooke PS, Kurita T. Role of stromal-epithelial interactions in hormonal responses. *Arch Histol Cytol.* 2004; 67(5):417–434. [PubMed: 15781983]
 39. Sainaghi PP, Castello L, Bergamasco L, Galletti M, Bellosta P, Avanzi GC. Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor. *J Cell Physiol.* 2005; 204(1):36–44. [PubMed: 15605394]
 40. Gorringe KL, Chin SF, Pharoah P, Staines JM, Oliveira C, Edwards PA, Caldas C. Evidence that both genetic instability and selection contribute to the accumulation of chromosome alterations in cancer. *Carcinogenesis.* 2005; 26(5):923–930. [PubMed: 15677628]

41. Leung KC, Brce J, Doyle N, Lee HJ, Leong GM, Sjogren K, Ho KK. Regulation of growth hormone signaling by selective estrogen receptor modulators occurs through suppression of protein tyrosine phosphatases. *Endocrinology*. 2007; 148(5):2417–2423. [PubMed: 17272397]
42. Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell*. 2006; 127(3):635–648. [PubMed: 17081983]
43. Wesley UV, McGroarty M, Homoyouni A. Dipeptidyl peptidase inhibits malignant phenotype of prostate cancer cells by blocking basic fibroblast growth factor signaling pathway. *Cancer Res*. 2005; 65(4):1325–1334. [PubMed: 15735018]
44. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci U S A*. 1990; 87(11):4120–4124. [PubMed: 2349221]
45. Kang HR, Yim EY, Oh SY, Chang YS, Kim YK, Cho SH, Min KU, Kim YY. Normal C1 inhibitor mRNA expression level in type I hereditary angioedema patients: newly found C1 inhibitor gene mutations. *Allergy*. 2006; 61(2):260–264. [PubMed: 16409206]
46. Trojan L, Schaaf A, Steidler A, Haak M, Thalmann G, Knoll T, Gretz N, Alken P, Michel MS. Identification of metastasis-associated genes in prostate cancer by genetic profiling of human prostate cancer cell lines. *Anticancer Res*. 2005; 25(1A):183–191. [PubMed: 15816537]
47. Sugimura Y, Cunha GR, Bigsby RM. Androgenic induction of DNA synthesis in prostatic glands induced in the urothelium of testicular feminized (Tfm/Y) mice. *Prostate*. 1986; 9(3):217–225. [PubMed: 2946028]
48. Simanainen U, Allan CM, Lim P, McPherson S, Jimenez M, Zajac JD, Davey RA, Handelsman DJ. Disruption of prostate epithelial androgen receptor impedes prostate lobe-specific growth and function. *Endocrinology*. 2007; 148(5):2264–2272. [PubMed: 17317769]
49. Cunha GR. Mesenchymal-epithelial interactions: past, present, and future. *Differentiation*. 2008
50. Ruan W, Powell-Braxton L, Kopchick JJ, Kleinberg DL. Evidence that insulin-like growth factor I and growth hormone are required for prostate gland development. *Endocrinology*. 1999; 140(5):1984–1989. [PubMed: 10218945]
51. Donjacour AA, Thomson AA, Cunha GR. FGF-10 plays an essential role in the growth of the fetal prostate. *Dev Biol*. 2003; 261(1):39–54. [PubMed: 12941620]
52. Isaacs JT. The biology of hormone refractory prostate cancer. Why does it develop? *Urol Clin North Am*. 1999; 26(2):263–273. [PubMed: 10361549]
53. Liu AY, Brubaker KD, Goo YA, Quinn JE, Kral S, Sorensen CM, Vessella RL, Belldegrun AS, Hood LE. Lineage relationship between LNCaP and LNCaP-derived prostate cancer cell lines. *Prostate*. 2004; 60(2):98–108. [PubMed: 15162376]
54. Lin B, White JT, Lu W, Xie T, Utleg AG, Yan X, Yi EC, Shannon P, Khrebukova I, Lange PH, Goodlett DR, Zhou D, Vasicek TJ, Hood L. Evidence for the presence of disease-perturbed networks in prostate cancer cells by genomic and proteomic analyses: a systems approach to disease. *Cancer Res*. 2005; 65(8):3081–3091. [PubMed: 15833837]
55. Dehm SM, Tindall DJ. Molecular regulation of androgen action in prostate cancer. *J Cell Biochem*. 2006; 99(2):333–344. [PubMed: 16518832]
56. Misra UK, Deedwania R, Pizzo SV. Activation and cross-talk between Akt, NF-kappaB, and unfolded protein response signaling in 1-LN prostate cancer cells consequent to ligation of cell surface-associated GRP78. *J Biol Chem*. 2006; 281(19):13694–13707. [PubMed: 16543232]
57. Pootrakul L, Datar RH, Shi SR, Cai J, Hawes D, Groshen SG, Lee AS, Cote RJ. Expression of stress response protein Grp78 is associated with the development of castration-resistant prostate cancer. *Clin Cancer Res*. 2006; 12(20 Pt 1):5987–5993. [PubMed: 17062670]
58. Chuan YC, Pang ST, Cedazo-Minguez A, Norstedt G, Pousette A, Flores-Morales A. Androgen induction of prostate cancer cell invasion is mediated by ezrin. *J Biol Chem*. 2006; 281(40):29938–29948. [PubMed: 16873375]
59. Miyagi T, Hori O, Koshida K, Egawa M, Kato H, Kitagawa Y, Ozawa K, Ogawa S, Namiki M. Antitumor effect of reduction of 150-kDa oxygen-regulated protein expression on human prostate cancer cells. *Int J Urol*. 2002; 9(10):577–585. [PubMed: 12445237]

60. Scherl A, Shaffer SA, Taylor GK, Kulasekara HD, Miller SI, Goodlett DR. Genome-specific gas-phase fractionation strategy for improved shotgun proteomic profiling of proteotypic peptides. *Anal Chem.* 2008; 80(4):1182–1191. [PubMed: 18211032]
61. Chung LW, Huang WC, Sung SY, Wu D, Odero-Marah V, Nomura T, Shigemura K, Miyagi T, Seo S, Shi C, Moliterno J, Elmore J, Anderson C, Isotani S, Edlund M, Hsieh CL, Wang R, Shehata B, Zhau HE. Stromal-epithelial interaction in prostate cancer progression. *Clin Genitourin Cancer.* 2006; 5(2):162–170. [PubMed: 17026806]

Figure 1. Expression of stromal cell-specific marker by RT-PCR

Cultured prostate and bladder stromal cells were harvested and analyzed by RT-PCR for expression of smooth muscle actin (α SMA) – positive, and epithelial cell-specific glycoprotein (EGP) - negative. EGP expression was detected in LNCaP cells.

(a) Prostate stromal cells

Correlation Matrix (normalized)
 Grey: $R=0.99$, White: $R=1$

(b) Bladdere stromal cells

Correlation Matrix (normalized)
 Grey: $R=0.99$, White: $R=1$

Figure 2. Correlation matrix of cultured stromal cell transcriptomes

Variation among cultures was found to be insignificant based on correlation efficiency ($R=0.99$); a culture is always perfectly correlated with itself ($R=1$).

(a) Number of identified secreted proteins

(b) Gene Ontology subcellular categorization

(c) Gene Ontology molecular function distribution

Figure 3. Secreted proteomes of cultured prostate and bladder stromal cells

(a) The Venn diagram shows the overlap of the two proteomes, PS = prostate, BL = bladder. Altogether, 118 different proteins were identified. (b) GO subcellular categorization of the proteomes. Most of the identified secreted proteins, 76%, are categorized as extracellular or membrane-associated. NA = not available. (c) GO molecular function distribution. Overall, a similar pattern is seen for the two proteomes but with protein binding and hydrolase activity being more prominent in the prostate.

Figure 4. Gene Ontology biological process distribution of differentially expressed secreted proteins of prostate and bladder stromal cells

Prominent among the processes identified are physiology and communication.

Figure 5. Western blot analysis of identified differentially expressed proteins

Protein preparations of cultured cells were resolved by gel electrophoresis and probed by antibodies to gene products listed on the right. CD90 served as the control for sample loading. The expression level as indicated by the band intensity correlated well with the PICA quantification method. Shown are the results for CTSL (cathepsin L), FSTL1 (follistatin related protein), LAMC1 (laminin γ 1), AXL (receptor tyrosine kinase), TNFRSF11B (tumor necrosis factor receptor superfamily member 11B), SPARC (secreted acidic cysteine rich glycoprotein), TIMP1 (metalloproteinase inhibitor 1), IGFIIR (cation-

independent mannose-6-phosphate receptor), TF (serotransferrin), STC1 (stanniocalcin), APOH (β -2-glycoprotein I), and CD90 (Thy-1).

Figure 6. ECM-receptor interaction pathway

Six secreted proteins in this pathway were identified by our study. The pathway was visualized through Teranode software. Protein expression is indicated by the relative expression level of prostate over bladder. LAMC1 (laminin γ 1) and FN1 (fibronectin1), with increased expression in prostate stromal cells, are colored green; LAMB1 (laminin β 1) and LAMA4 (laminin α 4), with similar levels of expression in the two cell types, are colored yellowish green and yellow respectively; THBS1 (thrombospondin-1) and AGRN (agrin), with no expression ratio as they were found only in the prostate, are colored turquoise.

Differentially expressed secreted proteins identified from the cultured prostate and bladder stromal cells**Table 1**

The protein IPI ID, ProteinProphet probability (PB) score, annotation, expression ratio (prostate/bladder), false discovery rate (FDR), peptide sequence (with putative glycosylation site underlined), GO biological process, GO molecular process, and GO cellular compartment information are tabulated. The list is sorted by FDR.

Protein	PB	Annotation	PS/BL	FDR	Peptides	Biological process	Molecular function	Cellular location
IP100012887	1	Cathepsin L	4.9	0.001	YSVANDTGFVDIPKQEK YSVANDTGFVDIPK	physiological process	hydrolase activity	extracellular
IP100029723	1	Follistatin-related protein 1	11.0	0.001	GSNYSEILDK FVEQNETAINITYPDQENNK VEQNETAINITYPDQENNK	NA	carbohydrate binding	extracellular
IP100297160	1	Hypothetical protein DKFZp451K1918	3.7	0.001	AFNSTLPTMAQMEK	cell adhesion	carbohydrate binding	membrane associated
IP100029131	1	Neuroendocrine convertase 2	3.0	0.001	RGDLNINMTSPMGTK RNPAGVATTDLYGNCTLR NPEAGVATTDLYGNCTLR	physiological process	hydrolase activity	extracellular
IP100015657	1	Pregnancy-specific beta-1-glycoprotein 5	9.9	0.001	ILILPSVTRNETGYPECEIR	physiological process	NA	extracellular
IP100298281	1	Laminin gamma-1 chain	7.6	0.001	LLNNLTSIK VNNLTSSOISR TANDTSTEAYNLLR	positive regulation of biological process	extracellular matrix structural	membrane associated
IP100020986	1	Lumican	2.6	0.001	LHINHNNLTESVGPLPK KLHINHNNLTESVGPLPK	physiological process	protein binding	extracellular
IP100339227	1	Splice Isoform 7 Of Fibronectin	7.5	0.002	DQCIVDDITYNVNDTFHK WTPLNSSITIGYR HEEGHMLNCTCFGQGR	cell adhesion	protein binding	extracellular
IP100296992	0.99	AXL receptor tyrosine kinase, isoform 1	7.0	0.002	EESPFVGNPGNITGAR	physiological process	transferase activity	membrane associated
IP100298362	1	Tumor necrosis factor receptor superfamily member 11B	3.9	0.002	HIGHANLTFEQLR KHTNCVFGLLLTQK CPDGFTSNETSSK	development	protein binding	extracellular
IP100014572	1	SPARC	7.2	0.002	VCSNDNKTFFDSSCHFFATK	development	ion binding	extracellular
IP100009198	1	Tissue factor pathway inhibitor 2	4.0	0.002	DEGLCSANVTR YFNLSSMTCEK	coagulation	extracellular matrix structural	extracellular
IP100328113	1	Fibrillin 1	7.4	0.002	TAIFAFAFNIHVSINK	development	ion binding	extracellular

Protein	PB	Annotation	PS/BL	FDR	Peptides	Biological process	Molecular function	Cellular location
IP100018305	0.99	Insulin-like growth factor binding protein 3	10.9	0.003	GLCVNASAVSR	positive regulation of biological process	enzyme	extracellular
IP100419941	1	PTK7 protein tyrosine kinase 7, isoform a	1.7	0.003	MHIFQNGSLVIHDAVEDSGR	physiological process	transferase activity	membrane associated
IP1000021081	1	Splice Isoform 1 Of Follistatin	2.2	0.003	SDEPVCASDNATYASECAMK	development	protein binding	extracellular
IP100032292	1	Metalloproteinase inhibitor 1	2.1	0.004	SHNRSEELIAGK FVGTPENVQTLYQR AKFVGTPENVQTLYQR VGTPEVNTLYQR	positive regulation of biological process	enzyme	extracellular
IP100298828	1	Beta-2-glycoprotein I	0.2	0.006	VYKPSAGNNSLYR LGWWSAMPCK	cell communication	carbohydrate binding	extracellular
IP100291866	1	Plasma protease C1 inhibitor	0.5	0.006	DTFVNASR NPNAISSSSQDPESLQDR	physiological process	enzyme	extracellular
IP100470937	1	Protein tyrosine phosphatase, receptor type, K	3.8	0.006	GPLANPIWNVGTGTGR	physiological process	hydrolase activity	membrane associated
IP100289819	1	Cation-independent mannose-6-phosphate receptor	1.5	0.008	DAGVGFPYQEEDNSTYNFR	physiological process	transferase activity	membrane associated
IP100022810	1	Dipeptidyl-peptidase I	63.9	0.008	DVNCVVMGPQEK VTTCNEITMTGWVHDVLGR	cell communication	hydrolase activity	intracellular
IP100169285	1	Hypothetical protein LOC196463	0.1	0.021	HPDAVAWANLTNAIR	NA	NA	NA
IP100023673	1	Galectin-3 binding protein	3.5	0.024	YKGLNLTEDTYKPR DAGVVCTNETR AAIPSAIDTNSK ALGFENATQALGR	cell communication	signal transducer	extracellular
IP100218019	0.99	Splice Isoform 1 Of Basigin	1.8	0.038	ILLTCSLNDSEATEVTGHR	cell communication	carbohydrate binding	membrane associated
IP100016112	1	Melanoma associated gene	11.7	0.045	QGEHLSNSTSAFSTR ILCDNADNTR LSTTECVDAAGESHANNTK	cell communication	protein binding	NA
IP100339223	1	Splice Isoform 3 Of Fibronectin	6.4	0.046	HEEGHMLNCTCFGQGR RHEEGHMLNCTCFGQGR DQCIVDDITYNVNDTFHK	cell adhesion	protein binding	extracellular
IP100028931	0.98	Desmoglein 2	0.7	0.050	INATDADEPNTLSNK	cell adhesion	ion binding	membrane associated

Protein	PB	Annotation	PS/BL	FDR	Peptides	Biological process	Molecular function	Cellular location
IP100003813	1	Nectin-like protein 2	0.7	0.050	VSLTNVSIISDEGR FOLLNFSSELK DTAVEGEEIEV <u>NCT</u> AMASK	cell adhesion	protein binding	membrane associated

Table 2
Pathway distribution of identified secreted proteins

KEGG pathway ID number, pathway name, number of identified proteins in the pathway, and gene ID are listed in columns 1 to 4 respectively.

KEGG_ID	Pathway (<i>Homo sapiens</i>)	Protein number	GENE ID
KEGG_hsa04512	ECM-receptor interaction	6	LAMA4, LAMB1, LAMC1, FN1, THBS1, AGRN
KEGG_hsa04610	Complement and coagulation cascades	6	A2M, PLA1, SERPINC1, MASP2, CD59, SERPING1
KEGG_hsa04510	Focal adhesion	6	LAMA4, LAMB1, LAMC2, FN1, THBS1, HGF
KEGG_hsa04514	Cell adhesion molecules (CAMs)	5	ALCAM, PVR, NEO1, CNTN1, NRCAM
KEGG_hsa04360	Axon guidance	4	EFNA1, EFNA5, SEMA7A, PLXNB2
KEGG_hsa04810	Regulation of actin cytoskeleton	2	CD14, FN1
KEGG_hsa05060	Prion disease	2	LAMB1, LAMC1
KEGG_hsa05010	Alzheimer's disease	2	LRP1, A2M
KEGG_hsa04060	Cytokine-cytokine receptor interaction	2	TNFRSF11B, HGF