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Identification of Prostate-Enriched Proteins by In-depth Proteomic Analyses of Expressed Prostatic Secretions in Urine

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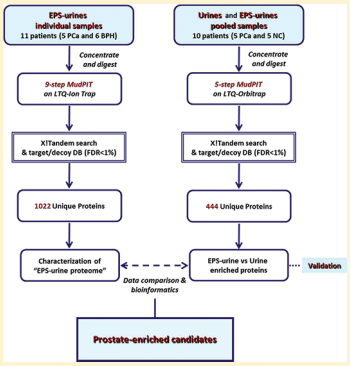
Supporting Information

ABSTRACT: Urinary expressed prostatic secretion or "EPS-urine" is proximal tissue fluid that is collected after a digital rectal exam (DRE). EPS-urine is a rich source of prostate-derived proteins that can be used for biomarker discovery for prostate cancer (PCa) and other prostatic diseases. We previously conducted a comprehensive proteome analysis of direct expressed prostatic secretions (EPS). In the current study, we defined the proteome of EPS-urine employing Multidimensional Protein Identification Technology (MudPIT) and providing a comprehensive catalogue of this body fluid for future biomarker studies. We identified 1022 unique proteins in a heterogeneous cohort of 11 EPS-urines derived from biopsy negative noncancer diagnoses with some benign prostatic diseases (BPH) and low-grade PCa, representative of secreted prostate and immune system-derived proteins in a urine background. We further applied MudPIT-based proteomics to generate and compare the differential proteome from a subset of pooled urines (pre-DRE) and EPS-urines (post-DRE) from noncancer and PCa patients. The direct proteomic comparison of these highly controlled patient sample pools enabled us to define a list of prostate-enriched proteins detectable in EPS-urine and distinguishable from a complex urine protein background. A combinatorial analysis of both proteomics data sets and systematic integration with publicly available proteomics data of related body fluids, human tissue transcriptomic data, and immunohistochemistry images from the Human Protein Atlas database allowed us to demarcate a robust panel of 49 prostate-derived proteins in EPS-urine. Finally, we validated the expression of seven of these proteins using Western blotting, supporting the likelihood that they originate from the prostate. The definition of these prostatic proteins in EPS-urine samples provides a reference for future investigations for prostatic-disease biomarker studies.



KEYWORDS: *Proteomics, prostate cancer, expressed prostatic secretions, urine*

INTRODUCTION

Recent advances in mass spectrometry (MS) instrumentation and sample preparation methods have propelled proteomics forward for the identification of biomarkers from body fluids and tissue extracts.[1] However, widespread clinical adaptation of candidate protein biomarkers has been hampered, in part, by a lack of suitable biological specimens. Serum or plasma samples, for instance, offer accessibility and ease of collection but have a high dynamic range (i.e., 10−12 orders of magnitude),[2] thus complicating the identification and quantification of low abundance proteins and surpassing the capabilities of existing separation and analysis techniques. Direct analysis of tumor tissues potentially provides access to greater concentrations of tumor-specific proteins but, tumor tissues are extremely heterogeneous and often represent a small proportion of the total organ mass, resulting in unsatisfactory yields of tumor-specific proteins.

Organ-proximal fluids, like breast ductal or prostatic secretions, are alternative sources of biological material to identify and quantify proteins that can mirror the physiological state of a given tissue.[3] Proximal fluids have been hypothesized to be a rich source of biomarkers because of their high concentration of shed and secreted proteins.[4] In the current study, we focus on the identification of prostate-derived proteins from expressed prostatic secretions (EPS) obtained via urine collection following digital rectal exam (DRE). These EPS-urine samples are a potentially rich source of informative

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proteins that are secreted/released from the prostate into the extracellular environment. Resolving disease-specific protein expression signatures may be useful as diagnostic/prognostic biomarkers, particularly if they are obtainable in a straightforward, noninvasive fashion. Thus, we have looked to EPS-urine as a potentially informative resource of prostate-secreted proteins and a clinically attractive biofluid that can be used to routinely screen for prostatic disease biomarkers.

The exocrine compartment of the prostate is composed of differentiated epithelial cells, that actively secrete proteins such as prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), prostaglandins, vimentin and keratins, into the glandular lumen.[5] Upon application of DRE, these secretory proteins, as well as immune response derived and cellular proteins, are forced into the urethra and mixed with urine in the urinary tract. These mixed fluids can then be collected in voided urine (EPS-urine). Use of EPS-urine has some key advantages over urine as a source of putative biomarkers of prostatic conditions. Despite the complex urine protein background of EPS-urine, there is increased potential for detection of low abundance prostate-specific proteins in this fluid. Due to the close proximity to the prostate, a higher concentration of prostate-enriched proteins is achieved and normally low abundance proteins are present at concentrations that far exceed those found in serum. Such secreted/released proteins may be involved in key pathways that lead to prostatic disease formation and progression. Methodologies that can selectively target prostate-enriched proteins would facilitate the discovery of highly informative biomarkers and potential drug targets. The value of EPS-urine is further enhanced by its applicability to routine clinical diagnostics, due to its ease of collection, enabling repeat and even longitudinal studies.[6]

The present study aimed to uncover the constituents of EPS-urine by employing Multidimensional Protein Identification Technology (MudPIT), to provide a valuable resource of MS-detectable proteins. A cohort of EPS-urine samples representative of the most common samples collected clinically, from benign prostatic diseases and low-grade PCa, was analyzed to provide a representative proteome catalogue of this fluid. Similar to our recent investigation on direct-EPS, which is obtained under anesthesia by prostate massage just prior to prostatectomy,[7] we have carefully analyzed this protein database for functionally enriched categories. We also combined this analysis using bioinformatics and compared our EPS-proteome to several publicly available proteomics data sets of related body fluids, including direct-EPS[7] and urine.[8] To subselect for proteins likely secreted from the prostate in this complex urine-protein background, we also performed a highly controlled quantitative comparison of pooled samples collected prior to DRE (urine) and post-DRE (EPS-urine) from men. Importantly, these pre- and post- samples were collected from the same patients, enabling direct quantitative intrapatient comparisons to identify proteins that are up-regulated in the post-DRE samples. The combination of these data and their integration with publicly available proteomic,[7,8] microarray data from BioGPS portal (http://biogps.org/),[9] and immunohistochemistry images from the Human Protein Atlas (HPA; http://www.proteinatlas.org/)[10,11] allowed us to generate a panel of 49 proteins likely secreted by the prostate and detectable in EPS-urine. These proteins will be of relevance for future investigations in larger EPS-urine cohorts as potential prostate disease biomarkers as well as compelling candidates for biological analyses.

MATERIALS AND METHODS

Materials

Ultrapure-grade urea, ammonium bicarbonate, ammonium acetate, calcium chloride and Tris were from BioShop Canada, Inc. (Burlington, ON, Canada). Ultrapure-grade iodoacetamide, DTT, and formic acid were from Sigma-Aldrich. HPLC-grade solvents (methanol, acetonitrile, and water) were obtained from Thermo Fisher Scientific (San Jose, CA). Trifluoroacetic acid was from J.T. Baker (Phillipsburg, NJ). Mass spectrometry-grade trypsin was from Promega (Madison, WI). Solid-phase extraction C18 MacroSpin Columns were from The Nest Group, Inc. (Southboro, MA).

EPS-Urine Collection and Concentration

All samples were collected from patients and utilized after informed consent following Institutional Review Board-approved protocols at Urology of Virginia and the Eastern Virginia Medical School along with the Research Ethics Board of the University Health Network. All personal information or identifiers beyond diagnosis and lab results were not available to the laboratory investigators. EPS-urine samples were collected by performing a gentle massage of the prostate gland during DRE prior to biopsy, as previously described.[7] The massage consisted of three strokes on each side of the median sulcus of the prostate and the expressed fluid from the glandular network of the prostate was subsequently voided in urine.

To generate sample pools from noncancer and cancer patients, 10−20 mL of urine and EPS-urine were collected from the same individual an hour before the DRE massage, herein denoted as urine, and after DRE, herein denoted as EPS-urine. Urine and EPS-urine from a group of 5 patients with PCa and 5 biopsy negative, noncancer individuals (Table 1), were pooled together to generate a sample panel comprising 4 different conditions: U_NC, urine noncancer; EPS-U_NC, EPS-urine noncancer; U_Ca, urine cancer; EPS-U_Ca, EPS-urine cancer. After collection, samples were stored on ice for no longer than 1 h. Each sample was aliquoted and stored at −80 °C until use.

Table 1. Clinical Information for the Urine and EPS-Urine Pooled Samples (NC and PCa) Analyzed by MudPIT[a]

	age	serum PSA	Gleason	risk
NC	61	3.8		B
	82	10.8		B
	59	5.9		B
	66	3.3		N
	57	6.4		N
Mean ± SD	65 ± 10	6 ± 2.9		
PCa	75	2.6	3 + 3	L
	84	11.7	3 + 3	I
	74	2.3	3 + 4	L
	71	6.4	3 + 3	L
	60	5.7	3 + 3	L
Mean ± SD	72.8 ± 8.6	5.7 ± 3.8		

[a]Urine and EPS-urine sample pairs were collected from the same patient and pooled together as described in Materials and Methods (serum PSA in ng/mL). In the risk column, B denotes patients with BPH, N denotes patients with no identifiable prostatic diseases, L denotes patients with low-risk PCa, and I denotes patients with intermediate-risk PCa.

Individual EPS-urines were obtained from an independent cohort of 11 different patients: 5 with low-grade PCa and 6 with biopsy negative benign conditions (BPH) (Table 2). Following collection, 9 mL of EPS-urine was centrifuged at

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Table 2. Clinical Information for the PCa (5 samples) and BPH (6 samples) Individual EPS-Urines Analyzed by MudPIT[a]

		age	serum PSA	Gleason	stage	treatment
PCa	EPS-U1	59	9.2	3 + 3	T1c	DVP
	EPS-U2	66	1.9	3 + 3	T1c	AS
	EPS-U3	77	2.7	3 + 4	T2b	Cryo
	EPS-U4	73	5.9	3 + 3	T1c	DVP
	EPS-U5	53	6.0	3 + 3	T1c	AS
Mean ± SD		65.6 ± 9.8	5.1 ± 2.9			
BPH	EPS-U6	76	4.5			
	EPS-U7	63	N/A			
	EPS-U8	57	3.2			
	EPS-U9	74	2.8			
	EPS-U10	54	4.3			
	EPS-U11	61	4.8			
Mean ± SD		64.2 ± 8.9	3.9 ± 0.9			

[a]Serum PSA values are from the time of initial diagnosis. The treatment column indicates the clinical course followed for each cancer patient (DVP − Da Vinci prostatectomy; Cryo, cryoablation therapy; AS, active surveillance) (serum PSA in ng/mL).

14000× *g* to remove the cell pellet/sediment. The supernatant was recovered and concentrated using an Amicon Ultra-15 Centrifugal Filter (3 kDa cutoff; Millipore, Billerica, MA) according to the manufacturer's instructions. Approximately 500 μL of each concentrated EPS-urine sample was recovered from the filter device and stored at −80 °C until use.

Protein Digestion and Peptide Preparation

For MS analysis, all samples were first quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, San Jose, CA) and volumes corresponding to 100 μg of total protein for the pools and 150 μg of total protein for the individual EPS-urines were resuspended in 50 μL of 8 M urea, 2 mM DTT, 50 mM Tris-HCl, pH 8.5, and incubated at 37 °C with constant shaking for 30 min. Carbamidomethylation was performed by incubating samples with 8 mM of iodoacetamide for 30 min at 37 °C in the dark. Samples were then diluted to approximately 1.5 M urea using 100 mM ammonium bicarbonate, pH 8.5. Calcium chloride was added to a final concentration of 2 mM and the protein mixture was digested with trypsin (1:40 trypsin to protein ratio) at 37 °C overnight. The digested peptide mixture was purified with C18 MacroSpin columns and concentrated by vacuum centrifugation and reconstituted to a volume of 40 μL with 0.1% formic acid. Samples were stored at −80 °C until used for MudPIT analysis.

MudPIT Analyses

Individual EPS-urines (5 PCa and 6 BPH) were analyzed in triplicate using a fully automated 9-cycle MudPIT procedure as previously described.[12,13] A quaternary HPLC pump was interfaced with a linear ion-trap mass spectrometer (LTQ, Thermo Fisher Scientific, San Jose, CA) equipped with a nanoelectrospray source (Proxeon Biosystems, Odense, Denmark). The pooled urine and EPS-urine samples were analyzed in triplicate on a LTQ Orbitrap XL, using a modified 5-cycle MudPIT, as previously described.[14]

Protein Identification and Data Analysis

Raw data obtained from all MudPIT runs were converted to *m/z* XML using ReAdW and searched by X!Tandem against a locally installed version of the human UniProt complete human proteome (www.uniprot.org) protein sequence database (version 2010_06; number of entries 20295). A target/decoy search was performed to experimentally estimate the number of false-positive identifications (<1% estimated FDR) and an in-house protein grouping algorithm was applied to satisfy the principles of parsimony.[15−17] The following parameters were applied according to the instrument used:

LTQ analyses of individual EPS-urines. The search was performed with a fragment ion mass tolerance of 0.4 Da and a parent ion mass tolerance of 4 Da. Complete tryptic digest was assumed. Carbamidomethylation of cysteine was specified as fixed and oxidation of methionine as a variable modification. Only proteins identified with two unique high-quality peptide identifications per triplicate were considered, as previously reported[15−17] (11 decoy proteins identified; FDR ∼1%). Each sample (n = 11) was examined by 3 technical replicates (33 total MudPIT analyses).

LTQ-Orbitrap XL analyses of pooled urines and EPS-urines. The search was performed with a fragment ion mass tolerance of 0.4 Da and a parent ion mass tolerance of ±10 ppm. Complete tryptic digest was assumed. Carbamidomethylation of cysteine was specified as fixed and oxidation of methionine as variable modification. Only proteins identified with two unique high quality peptide identifications per analyzed sample were considered, as previously reported[14,18,19] (2 decoy proteins identified; FDR ∼0.5%). Each sample pool was analyzed by ≥3 technical replicates (13 total MudPIT analyses).

Protein relative abundance was calculated using the QSpec algorithm.[20] Proteins were considered to be up-regulated in the pooled EPS-urine samples versus the urine samples if they complied with the following parameters: false discovery rate (FDR) <0.05 and fold change (FC) ≥ 2, based on the QSpec algorithm.[20]

Gene Ontology Annotation and Data Comparison

Functional annotations (Gene Ontology terms, KEGG pathways, and Swiss Prot entries) were assigned using the Database for Annotation, Visualization and Integrated Discovery (DAVID, bioinformatics resources v6.7; http://david.abcc.ncifcrf.gov/).[21] Unique proteins detected in the EPS-urine data set were compared to the UniProt database and the top five significantly over-represented categories were reported (p-value <0.001). Comparisons of the present EPS-urine data set to urine[8] and direct-EPS[7] data sets was accomplished using ProteinCenter (Proxeon Biosystems, Odense, Denmark). Proteins were sequence-aligned against each other and only proteins with at least 95% sequence identity were considered to match (i.e., protein clusters).

Prostate-Enriched Proteins Characterization

The BioGPS portal (http://biogps.org/)[9] was used to map identified proteins against available mRNA microarray data sets. We selected 25 major organ systems among those available in BioGPS and linked our proteins via gene accessions. The expression level for each gene was based on averaged probe intensities, and the significant enrichment in prostate tissue (>2-fold change) was calculated as a $\log_2$ ratio compared to the other selected tissues. The random sampling analysis was carried out using the unpaired one-tailed Students t test. A p-value ≤ 0.05 was considered statistically significant. The alphabetical roster of the selected organs is as follows: bone marrow, colon, heart, hypothalamus, kidney, liver, lung, lymph node, ovary, pancreas, placenta, prostate, salivary gland, skeletal muscle, skin, small intestine, smooth muscle, spinal cord, testis, thalamus, thymus, thyroid, uterus, whole blood, and whole brain.

The UniProt database (http://www.uniprot.org/)[22] was used to assign subcellular localization to the 49 proteins enriched in EPS-urine pooled samples. We manually reported annotations and grouped the 49 proteins into three main categories: secreted, membrane, and intracellular (which includes cytoplasmic, nuclear, and lysosomal). Identified proteins were also screened against the Human Protein Atlas database (HPA; http://www.proteinatlas.org/)[10,11] for availability of antibodies and to examine their prostate tissue expression patterns.

SDS-PAGE and Western blot analysis on urine and EPS-urine pools

For Western blotting, 40 μg of total proteins were separated on 8 or 10% SDS-PAGE gels and blotted on PVDF membranes (0.2 μm; Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% milk in TBS-Tween (0.2%) for 1 h at room temperature and subsequently incubated overnight at 4 °C with the following primary antibodies: anti-Lactoferrin (1:1000 #ab10110; Abcam, Cambridge, U.K.), anti-CD10 (MME 1:1000 #ab951; Abcam, Cambridge, U.K.), anti-TIMP1 (1:2000 #RP1-TIMP1; Triple Point Biologics, Forest Grove, OR), anti-CD13 (ANPEP 1:500 #ab7417; Abcam, Cambridge, U.K.), anti-TGM4 (1:500 #sc55791; Santa Cruz Biotechnology, Santa Cruz, CA), anti-14-3-3σ (1:250 #ab14123; Abcam, Cambridge, U.K.), and anti−PARK7 (1:1000 #ab11251; Abcam, Cambridge, U.K.). After three 10-min washes with TBS-Tween (0.2%), membranes were incubated with antimouse/antirabbit/antigoat IgG-HRP secondary antibody (Invitrogen, Carlsbad, CA) at a dilution of 1:25000 for 1 h at room temperature, washed and visualized with the SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, San Jose, CA).

RESULTS AND DISCUSSION

Proteome Profiling of EPS-Urine

One goal of the current study was to provide a high-quality, well-annotated resource of proteins present in EPS-urine. This could improve our understanding of general prostate biology in the aging male and guide the discovery of novel PCa biomarkers. While commercial genetic assays have been developed using EPS-urines as a source of prostate-derived genetic material,[23−25] an in-depth proteomic analysis of this fluid has not been reported. In contrast to direct-EPS,[7] which is likely to contain prostate-secreted proteins at a higher concentration, EPS-urine is highly diluted by a dynamic and variable urine background. Nevertheless, EPS-urine samples are clinically more relevant since they can be obtained by routine DRE examination, and can be collected repeatedly for longitudinal sample collection relevant to active surveillance monitoring of men with PCa.

There were two interconnecting aims to our current study (Figure 1). The first aim was to provide the first in-depth proteome catalogue of this clinically useful proximal tissue fluid (Figure 1, Left panels), in order to standardize the proteome of EPS-urine based on clinical collection procedures that can be performed in a routine clinical setting. In particular, individual EPS-urine samples from men diagnosed with low-grade PCa (n = 5; Gleason total 6−7) and biopsy negative benign conditions (n = 6, BPH) were selected for MudPIT-based proteomic analysis (Table 2). The proteomic characterization of the EPS-urine was performed on this heterogeneous group of 11 samples as a representation of the different types of patient origin, reflective of the most common benign and prostate cancer conditions presenting in urology clinics. Each sample of 150 μg total protein was directly digested in-solution and analyzed in triplicate by a 9-step MudPIT on a LTQ-Ion Trap mass spectrometer, as previously described.[12,15] A total of 1022 unique proteins were identified in the EPS-urines (Supplemental Table 1, Supporting Information) by at least two unique peptides (Figure 1, Left panels of flowchart), ranging from 178 to 667 unique proteins determined per individual MudPIT run (Figure 2A). Although the same amount of total protein was digested and each sample was analyzed in triplicate with a relatively low overall standard deviation (average standard deviation of 27), there was still a large variation in total proteins detected for each sample. This high variability is reflective of the biological intersample variations among human specimens, which complicates data profiling analyses, as well as of the dynamic metabolic changes within each individual that are manifested in urine protein content,[26] and highlights some of the general problems of proteomic analyses of proximal body fluids.[27] These results could also reflect variation in sample collection, as the DRE procedure required to "express" the prostatic fluids will be different for each individual.[6] Other known limitations are related to the DRE-collection procedure and can be attributed to the physician (e.g., size of hand, ability to reach to the prostate, etc.) or to some patient physical parameters (e.g., orientation on table during examination, overweight status, etc.). Therefore, standard collection protocols as well as internal standards are required to ensure proper collection and to circumvent the introduction of sample variability resulting from the collection procedure.[6]

To obtain a systematic overview of the functional categories of proteins expressed in EPS-urine, we performed Gene Ontology (GO)[28] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analyses[29] on the 1022 identified proteins (Figure 3). We limited the comparison to several representative GO terms in order to obtain a high-level functional overview. In the biological processes category, a large proportion of identified proteins had functional roles involved in proteolytic activity, cellular adhesion and motion, and immune responses (Figure 3A). In the cellular component category (Figure 3B), GO terms with an annotation to the extracellular region were overrepresented; this was further supported by the evidence that ~50% of the detected proteins were classified in the Swiss-Prot database as proteins with signal peptide sequences, and more than 30% were classified as

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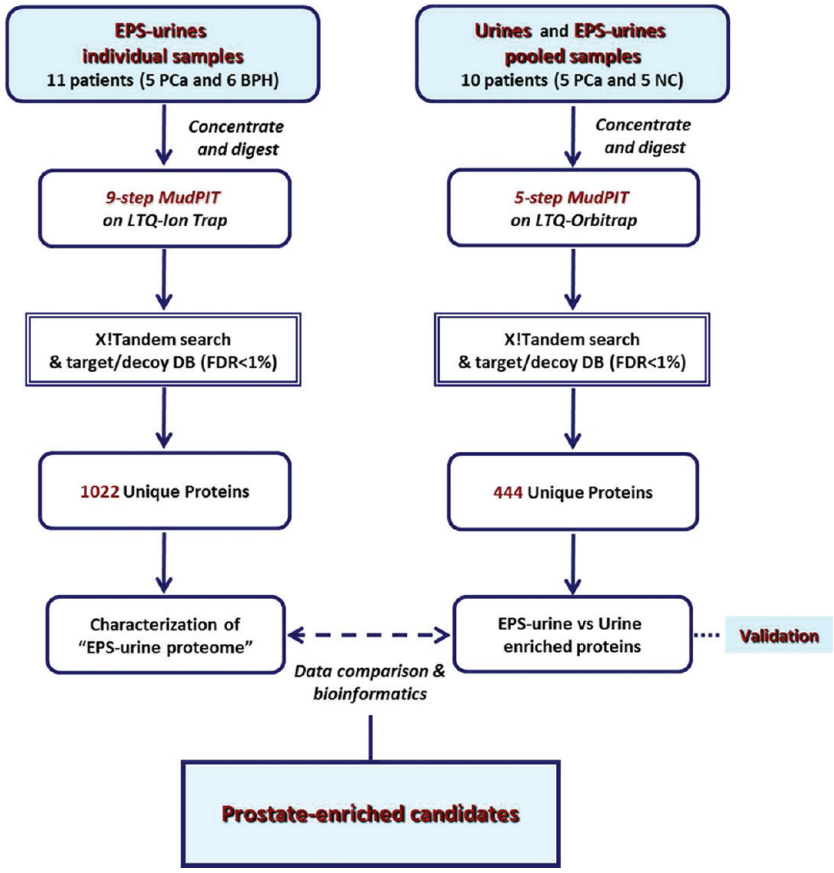

Figure 1. Study workflow. Proteomic analysis of EPS-urine and urine samples. The EPS-urine proteome was defined by MudPIT analyses of 11 individual heterogeneous samples (PCa and BPH) of EPS-urine. A similar analysis was performed on pooled urine and EPS-urine samples (PCa and NC). The comparison of the two data sets using bioinformatics data mining lead to the identification of some putative prostate-enriched candidates within the complex EPS-urine proteome.

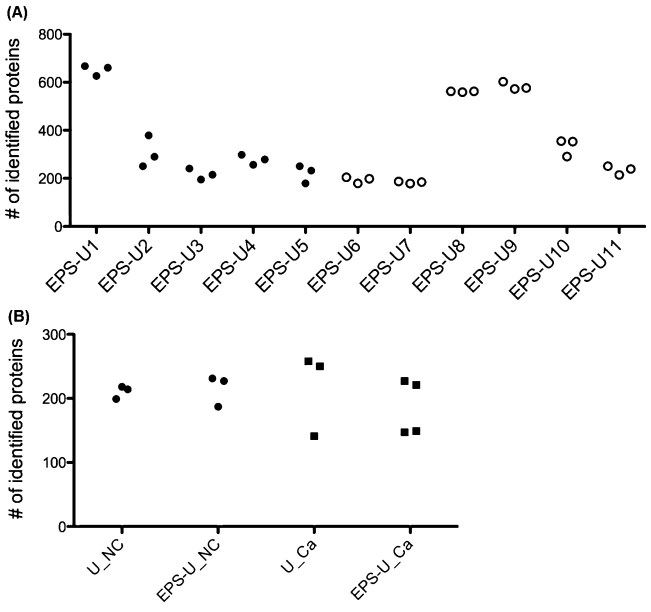

Figure 2. Characterization of the EPS-urine proteome. Number of unique proteins detected per MudPIT run respectively in the (A) individual EPS-urine samples and in the (B) pooled urine and EPS-urines. The black dots in A (from EPS-U1 to EPS-U5) represent the EPS-urines from individual PCa patients and the white dots (from EPS-U6 to EPS-U11) represent the EPS-urines from individual BPH patients. Every sample was analyzed in triplicate. (EPS-U, EPS-urine; U, urine; Ca, prostate cancer; NC, noncancer).

secreted proteins (Figure 3D). Notably, 12% of the EPS-urine proteins contained the annotation of vesicle localization by GO (Figure 3B); this could explain why the lysosomal pathway is the most highly over-represented among the KEGG pathways in our EPS-urine data set (Figure 3E).

In the molecular function category, a large proportion of detected proteins had calcium ion binding activity and were involved in the regulation of peptidase activity (Figure 3C). On the basis of the global GO-term analyses, EPS-urine proteins belong to a large variety of functional categories and, as expected from a secreted fluid fraction, a large number of these proteins are extracellular and contain a signal peptide sequence.

To further characterize the EPS-urine proteome in the context of other prostate-related fluids, the current data set was integrated with normal human urine[8] and direct-EPS,[7] which showed that 455 proteins are shared between these fluids by proteomics analyses (Figure 3F). Present among these shared proteins were several previously proposed PCa biomarkers (i.e., PSA, PAP, MSMB, PSMA, TMPRSS2), strengthening the rationale of using *in vivo*-obtainable fluids to study the prostate and PCa biology. On the other hand, these candidates were detected in all three fluids, which demonstrates the dilemma in detecting prostate-enriched proteins in a complex protein background of general urine proteins.

Focusing on prostatic secretions, we found that 181 proteins were shared between EPS-urine and direct-EPS (Figure 3F), suggesting that these proteins are specifically enriched in

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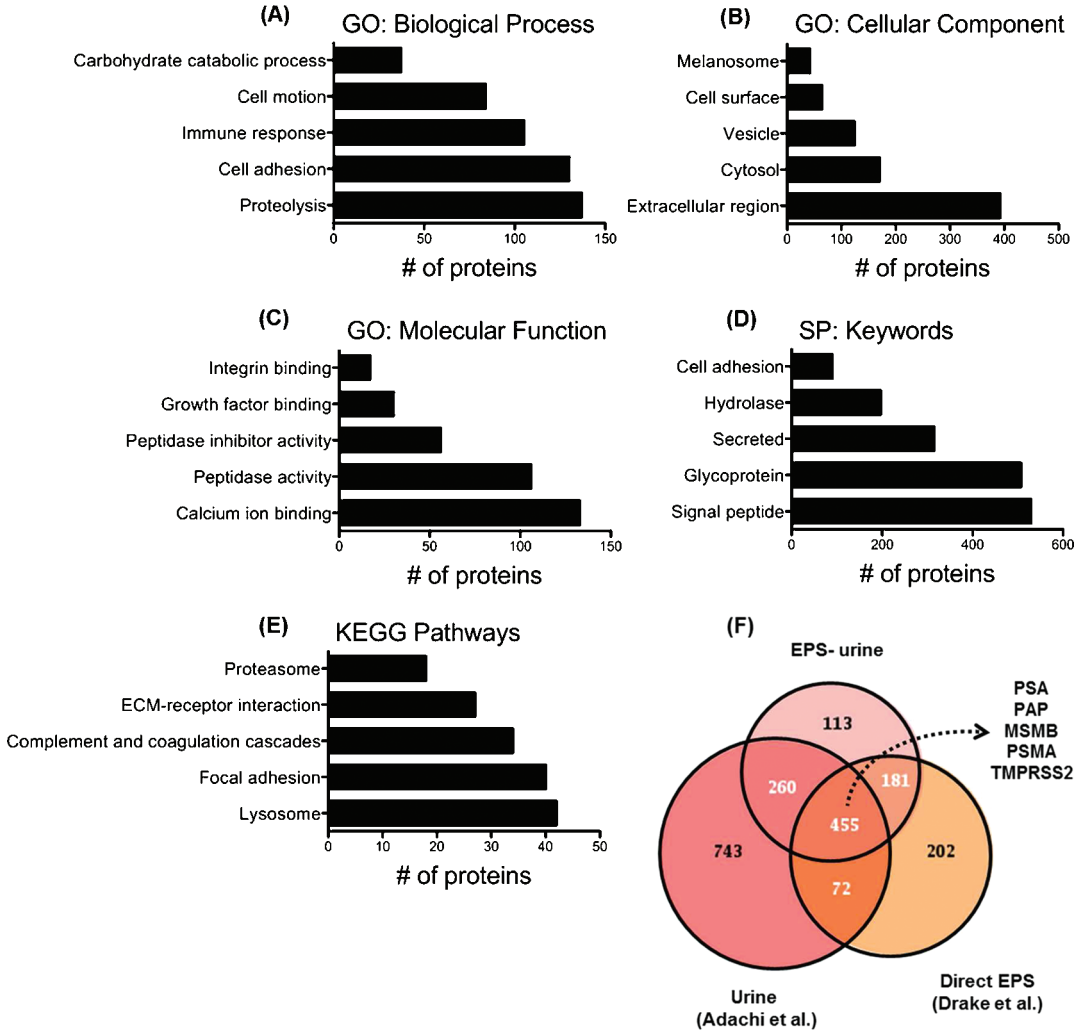

Figure 3. Functional enrichment of proteins detected in EPS-urine and comparison of the EPS-urine proteome to other related body fluids. The list of the 1022 unique proteins identified in the EPS-urine samples was compared to the human UniProt Database. The reported graphs show significantly over-represented ($p < 0.001$) Gene Ontology (GO) terms (A–C), Swiss-Prot entries (D), and KEGG pathways (E) in the EPS-urine data set (several representative annotation terms are shown). (F) Current EPS-urine data set was compared to previously published urine[8] and direct-EPS[7] proteomic data sets. Proteins are clustered in homogeneous groups based on 95% similarity (i.e., cluster anchors).

prostatic secretions and absent or at a very low concentration in urine. This could represent a useful protein data set for selecting new prostate-specific biomarkers with diagnostic and prognostic capacities for prostatic diseases. Interestingly, 113 proteins were unique to EPS-urine (absent from direct-EPS and urine). This could be explained by different MS conditions used between our group and the study by Adachi et al.,[8] as well the phenomenon of random sampling[30] and biological variability, since neither study was likely successful in detecting the entire body fluid proteome and a different, nonoverlapping number of false negatives are likely present.

The present study provides a detailed description of EPS-urine and extensively expands the knowledge of EPS-urine proteome. Our data set of 1022 proteins (Supplemental Table 1, Supporting Information) can be used to implement diagnostic test platforms and improve current screening procedures for prostatic diseases.

Identification of Prostate-Enriched Proteins

A second aim of this current study was to provide the first direct comparison of urine samples pre- and post-DRE using pooled samples from both normal and PCa patients (Figure 1, right). These valuable samples enabled us to identify proteins likely released by the prostate as a result of the DRE. We identified prostate-enriched proteins derived from EPS-urines in a complex protein background of general urine proteins. For this purpose, five patients with PCa and five noncancer individuals were screened once prior to DRE and once after DRE, in order to obtain internally controlled urines and EPS-urines, respectively. Each sample pool was analyzed by a 5-step MudPIT on a LTQ-Orbitrap, leading to the identification of 444 unique proteins (Supplemental Table 1, Supporting Information) (Figure 1, right), with a range of 141 to 258 unique proteins determined per individual MudPIT run (Figure 2B).

In order to highlight signatures of proteins enriched in the EPS-urine, we next performed a semiquantitative comparative analysis of the EPS-urine and urine data, based on the QSpec algorithm.[20] Proteins were considered to be prostate-enriched if they had a ≥2-fold change in spectral abundance factors[20,30] with a FDR ≤0.05 by QSpec analysis. Applying these criteria to the entire list of 444 proteins, a panel of 49 significantly enriched proteins was generated (Supplemental Table 2, Supporting Information). Interestingly, direct comparison of

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both proteomic data sets (1022 versus 444) showed an overlap of 406 proteins that included the 49 protein signature of "prostate-enriched proteins" (Figure 4).

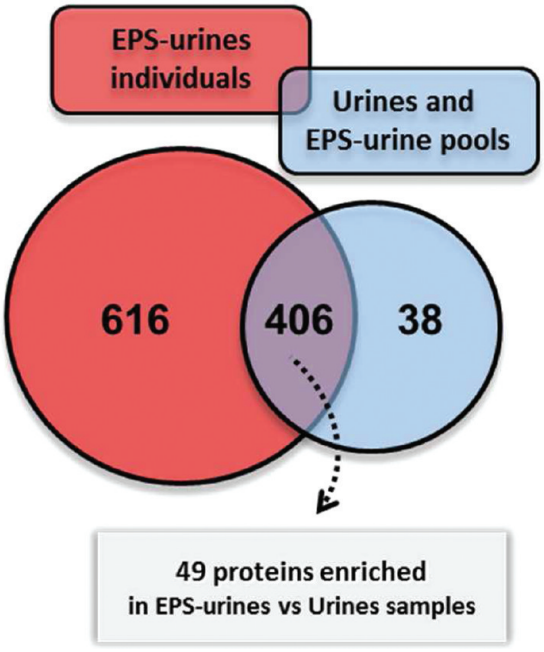

Figure 4. Comparison between the two proteomics data sets of the current study. The Venn diagram shows a comparison between the EPS-urine data set (1022 proteins) and the data set containing proteins identified in both pooled urine and EPS-urine samples (444 proteins). The overlapping area of 406 proteins includes a short-list of proteins enriched in the EPS-urine samples compared to the urine samples (termed prostate-enriched proteins).

The prostate tissue selectivity of the 49 identified protein signature was also evaluated at the transcriptome level (Figure 5A). The 49 proteins were mapped in the BioGPS database, and the gene expression profiles for normal human prostate tissue were compared to 24 other major normal human tissues, under the assumption that transcript levels measured by microarrays can be correlated with protein abundance.[12] Although it is well-known that expression levels of both biomolecules are not always concordant,[15,31,32] our own experience suggests reasonable correlation if the data is locked on the protein level (i.e., using only transcripts with available proteins evidence).[15] This type of comparison can provide additional evidence for prostate-enriched expression levels, similar to our previous investigations.[7] Genes with prostate expression levels 2-fold above the median across all 25 tissues were considered to be prostate-enriched. The distribution of this analysis is shown in Figure 5A and represents the expression level of each gene coding for the 49 selected proteins in the prostate tissue. Among the 49 selected gene products we identified several well-known prostate biomarkers, such as PSA (KLK3), MSMB and ACPP (highlighted in green), supporting our hypothesis that direct comparison of urine and EPS-urine by semiquantitative proteomics results in the identification of prostate-enriched proteins suitable for further investigation in biomarker studies.

According to this analysis, 32 of the 49 informative genes are enriched in the prostatic tissue. The genes indicated with red bars in the graph correspond to the seven proteins for which we obtained antibodies and were able to verify the MS-based proteomics data. Only 5 of 7 of these prostate-enriched genes have high expression levels in prostate. Incidentally, we decided to include all the seven candidates in our following analyses, mainly based on manual annotations and comparisons with other in-house proteomic data sets (unpublished data), that we believe may be more accurate to capture relevant information.

We next examined a random sampling of our data set to address the question of chance selection in arriving at our 49-protein list. We randomly selected (10000 times) proteins from the entire list of 1022 proteins we detected in EPS-urine, to evaluate if a random selection from a larger data set of EPS-urine proteins gave us the same probability to select prostate-enriched proteins. The boxplot shown in Figure 5B reports the comparison between the two data sets. As expected, our short-list of 49 selected proteins is enriched in prostate-specific proteins when compared with a random assortment of 10000 selections from the same EPS-urine database. The median values of the two distributions tend to be lower in the random sampling analysis (median =0.6) compared to our selection (median =2.2) with a highly significant P-value of 0.000114.

Characterization of Prostate-Enriched Proteins

To better characterize our list of prostate-enriched proteins we annotated information available at the HPA[10,11] and UniProt databases.[22] As shown in Figure 6A, 38 proteins (78%) of all 49 have at least one antibody ID (Supplemental Table 2, Supporting Information) available at the HPA,[10,11] which was validated and used to generate tissue expression profiles. In Figure 6A, we further indicated the protein expression patterns based on the immunohistochemistry images available in the database. The evaluation of these images, based on staining intensity and protein distribution in the human normal prostate tissue, suggested that 45% of the proteins positivity was found in the glandular epithelium, only 13% in the stromal cells and 29% showed equal staining between the two compartments. As reported, most of the selected proteins are secreted and hence preferentially expressed in glandular epithelial cells (poorly expressed or absent by stromal cells) along with the physiological function of the prostate gland.

Together protein tissue distribution and cellular localization are parameters that can provide important insight into the function of a protein. As expected, the respective subcellular patterns reported from UniProt database[22] were correlated (Figure 6B) with other additional information collected from other available resources (i.e., ProteinCenter, signal peptide annotations) (Supplemental Table 2, Supporting Information). The majority (33%) of the proteins in the analyzed EPS-urine samples were secreted (without overlap with the other categories) (Figure 6B). This localization is consistent with the biological expectation that urine contains, by definition, many extracellular proteins. Another significant percentage of proteins (22.5%) were localized to the membrane compartment.

Interestingly, although the EPS-urine is not enriched in intracellular proteins (18.4%), almost half (44%) of this category consist of lysosomal proteins. This probably points out that exosome formation is the dominant excretion pathway in urine and reflects the biological and physiological role of these proximal fluids, through the presence of specific transport pathways for lysosomal proteins.

Generation of Candidate Short-list

Our analyses suggested an enrichment of prostate-specific proteins in the EPS-urines compared to the urine samples.

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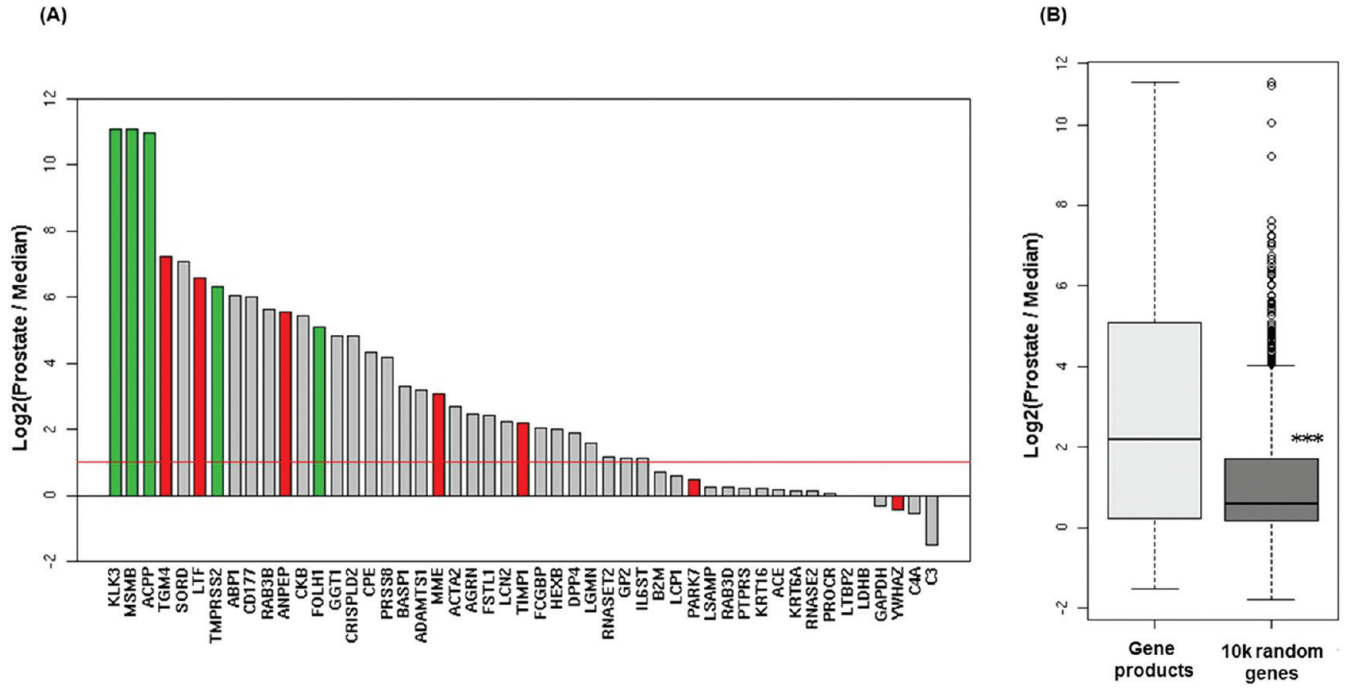

Figure 5. Data mining to identify prostate-enriched proteins. (A) Comparison of the 49 prostate-enriched proteins to published human tissue transcriptomic data from the BioGPS gene portal.[9] On the *X*-axis gene names are shown and the *Y*-axis represents a scale indicating the gene expression level in prostate as compared to 24 additional human tissues. The Y-values were obtained as $\log_2$ [fold change] ratio of prostate gene expression level versus the median value calculated for 25 major organs (listed in Materials and Methods). Green bars highlight protein biomarkers already suggested as candidates for PCa prognosis and diagnosis (used as internal positive controls). Red bars highlight the 7 proteins that have been validated in this study. Red line demarcates the 2-fold increased expression level in normal prostate as compared to all other tissues. (B) Box plot shows prostate-enriched proteins in the 49 selected data set versus a random sampling analysis on EPS-urine current data set. The *Y*-axis has the same $\log_2$ scale reported in A, rectangles are bounded by the lower and upper quartiles, the solid lines in the rectangles are the medians, the box whiskers extend to the minimum to the maximum data point of the rectangle, and the circles represent outliers beyond this range. *** *P*-value < 0.001.

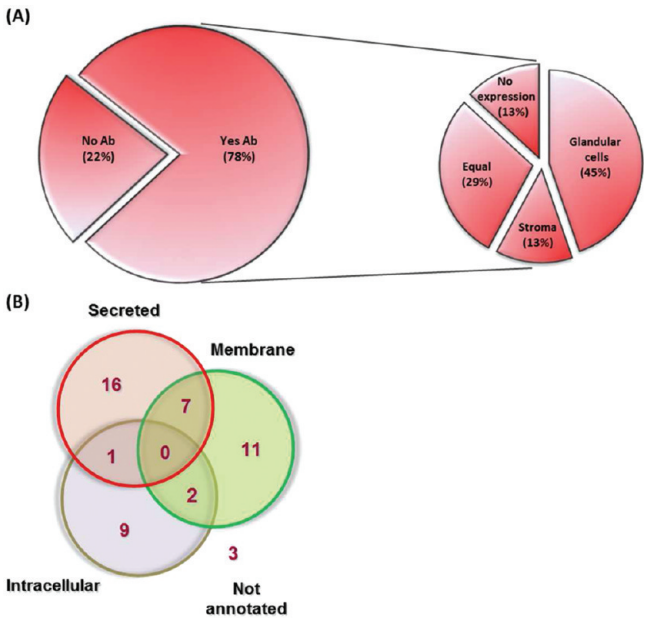

Figure 6. Additional annotations for prostate-enriched proteins. (A) Availability of antibodies reported in the Human Protein Atlas database, for the 49 selected proteins. The IHC images were manually screened and protein distribution was annotated (glandular cells or stroma). (B) Subcellular location of the 49 prostate-enriched according to UniProt database (red, protein number).

In particular, the list of 49 proteins (Supplemental Table 2, Supporting Information) encompasses a number of prostate-specific markers that are currently used or have been previously considered as potential candidates for PCa screening; among these are PRSP or MSMB (Microseminoprotein-β),[33] KLK3 or PSA (Prostate-specific antigen),[34,35] ACPP or PAP (Prostatic acid phosphatase) reported in 1938 as the first serum biomarker for PCa,[36,37] TMPRSS2 (Transmembrane protease serine 2),[38] FOLH1 or PSMA (Prostate-specific membrane antigen).[39]

Each of the 49 differentially expressed proteins were manually inspected for selection as part of a small verification set aimed at validating the findings from our proteomic investigations. Selection was largely based on antibody availability; but other unbiased considerations were tissue expression patterns of candidates across PCa and normal prostate tissues available through the Human Protein Atlas,[10,11] tissue specificity based on mRNA microarray data (see above), subcellular localization by Gene Ontology analysis, and in-house proteomic data sets generated from urines and prostatic fluids from various conditions (unpublished data). This led to a short-list of 7 proteins that were assayed in the verification stage.

Verification of Proteomic Data

To verify our proteomic data, Western blot analyses were carried out for each of the 7 candidates in pooled EPS-urines and urines from PCa and noncancer patients. The panel in Figure 7A shows that each of our short-listed candidates is more abundant in EPS-urine samples compared to the matching urines, supporting results from our MudPIT analyses. In particular, by comparing noncancer and PCa EPS-urines, we

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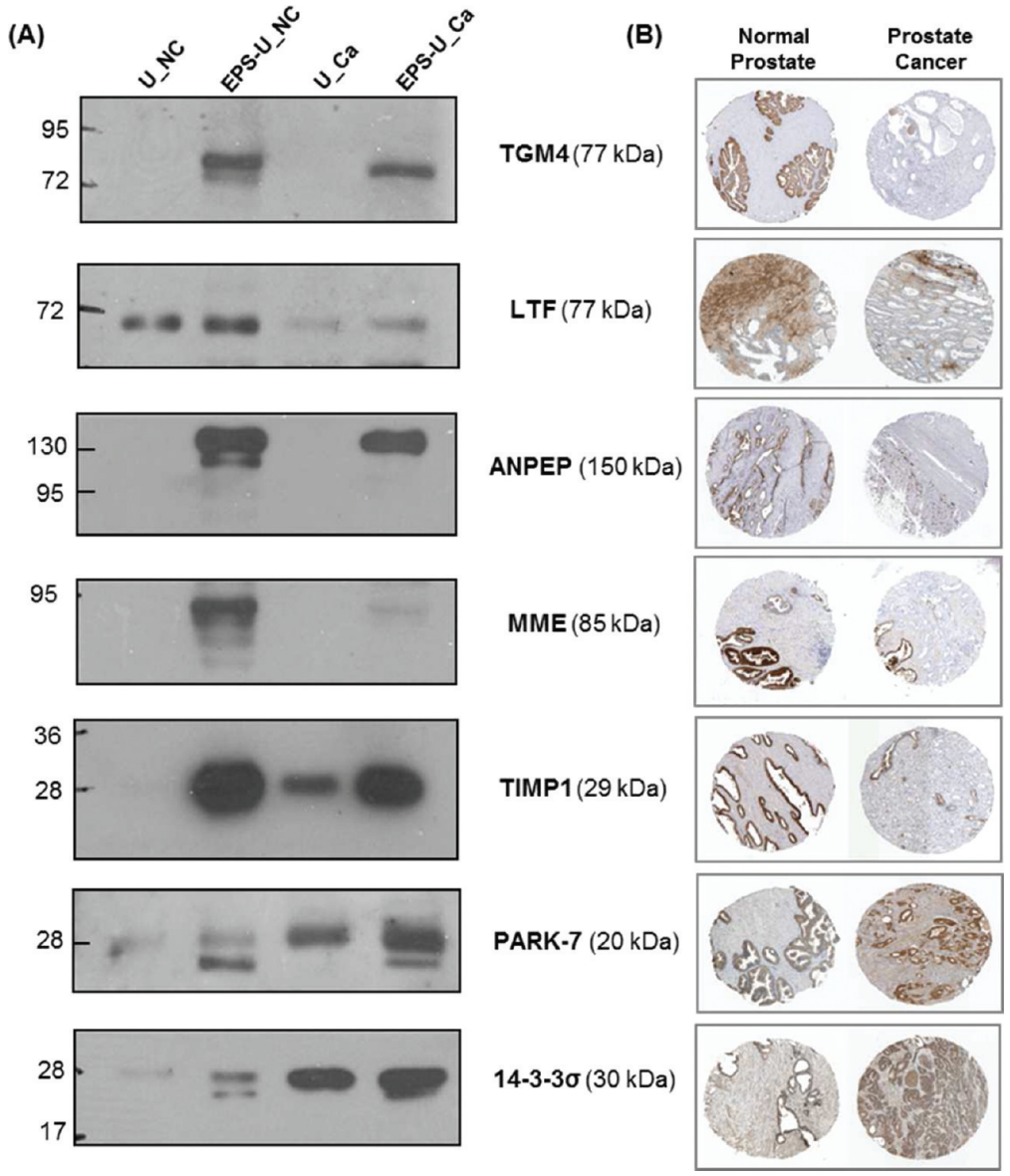

Figure 7. Validation of MS data for seven selected proteins. (A) Western blot analyses of the seven candidate proteins confirmed the proteomic data (based on spectral counts) showing an enrichment of each selected protein in the EPS-urine compared to the urine samples. (B) Immunohistochemistry images obtained from Human Protein Atlas database showing the differential expression in normal and cancerous prostate glandular tissue of the seven selected proteins. The same trend was obtained by MS and Western blot analysis. (U_NC, urine noncancer; EPS-U_NC, EPS-urine noncancer; U_Ca, urine cancer; EPS-U_Ca, EPS-urine cancer).

observed the down-regulation of TGM4, LTF, ANPEP, MME and TIMP1, and up-regulation of PARK7 and 14-3-3σ (Figure 7A), which correlated with our proteomic data analyses. These preliminary results also follow a similar trend when looking at immunohistochemical staining patterns in normal and neoplastic prostatic tissues via Human Protein Atlas,[10,11] but will require further verification in large unrelated EPS-urine cohorts in the future (Figure 7B).

To the best of our knowledge, the current study represents the most comprehensive catalogue of the EPS-urine proteome, and can serve as a resource of MS-detectable prostate-enriched proteins in this proximal tissue fluid. The proteomic characterization of EPS-urine provides a valuable reference for future studies as an in-depth view of potential signatures that may be indicative of various prostatic conditions. A better understanding of this fluid may facilitate the development of highly sensitive and rapid assays that are specific for a given prostatic disease. Evidently, greater numbers of samples need to be assayed to better define the differences between cancer and noncancerous states, and laying the groundwork for the discovery of putative PCa biomarkers. In this proof-of-concept study, we have demonstrated the value of EPS-urine as a rich source of MS-detectable prostate-enriched proteins that can be used for future investigation in biomarker discovery studies.

■ SUMMARY

We employed EPS-urine as a relevant and easily collectable organ-proximal fluid for the identification of prostate-secreted proteins *in vivo*. Our proteomic analyses provide a global characterization of the most prevalent components of EPS-urine and contribute to a better understanding of this fluid. From this wide protein background, we narrowed our

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investigation to select proteins likely secreted from the prostate, derived from the EPS component. We delineate a prostate-enriched protein signature in the complex urine-protein background, performing a highly controlled quantitative comparison of urine and EPS-urine data sets. Our final data demonstrate the value of EPS-urine as a rich source for protein biomarker discovery and provide an in-depth view of proteins that may be used as potential prostate-specific biomarkers for prostatic diseases. The identification of new biomarkers in proximal fluids may facilitate the development of highly sensitive and rapid assays, specific for a given prostatic disease, including PCa screening.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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