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Analysis of Seminal Plasma from Patients with Non-obstructive Azoospermia and Identification of Candidate Biomarkers of Male Infertility

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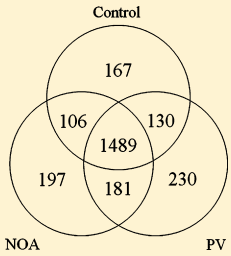
Supporting Information

ABSTRACT: Infertility affects approximately 15% of couples with equivalent male and female contribution. Absence of sperm in semen, referred to as azoospermia, accounts for 5−20% of male infertility cases and can result from pretesticular azoospermia, non-obstructive azoospermia (NOA), and obstructive azoospermia (OA). The current clinical methods of differentiating NOA cases from OA ones are indeterminate and often require surgical intervention for a conclusive diagnosis. We catalogued 2048 proteins in seminal plasma from men presented with NOA. Using spectral-counting, we compared the NOA proteome to our previously published proteomes of fertile control men and postvasectomy (PV) men and identified proteins at differential abundance levels among these clinical groups. To verify spectral counting ratios for candidate proteins, extracted ion current (XIC) intensities were also used to calculate abundance ratios. The Pearson correlation coefficient between spectral counting and XIC ratios for the Control−NOA and NOA−PV data sets is 0.83 and 0.80, respectively. Proteins that showed inconsistent spectral counting and XIC ratios were removed from analysis. There are 34 proteins elevated in Control relative to NOA, 18 decreased in Control relative to NOA, 59 elevated in NOA relative to PV, and 16 decreased in NOA relative to PV. Many of these proteins have expression in the testis and the epididymis and are linked to fertility. Some of these proteins may be useful as noninvasive biomarkers in discriminating NOA cases from OA.



KEYWORDS: *seminal plasma, vasectomy, nonobstructive azoospermia, proteomics, infertility, testis, epididymis*

INTRODUCTION

Nearly 15% of couples are infertile or subfertile with males and females equally contributing to the cause of infertility.[1] Infertility in men could be categorized as a reduced sperm count (oligospermia), reduced sperm motility (asthenospermia), abnormal sperm morphology (teratospermia) or with more severe cases, a complete lack of spermatozoa in semen, referred to as azoospermia. Azoospermia accounts for 5−20% of infertility cases in men and could be further categorized as (a) pretesticular azoospermia, (b) testicular failure or non-obstructive azoospermia (NOA), and (c) obstructive azoospermia (OA) due to congenital bilateral absence or blockage of the vas deferens or epididymis.[2] Nonobstructive azoospermia can result from (a) reduced spermatogenesis, otherwise known as hypospermatogenesis, (b) maturation arrest at early or late stages of spermatogenesis, or (c) Sertoli-cell only syndrome (SCOS) resulting in complete lack of spermatogenesis.[3]

Current clinical methods can readily diagnose patients with pretesticular azoospermia (due to hypothalamic or pituitary dysfunction); however, differentiation of NOA from OA is not simple. Pretesticular azoospermic patients characteristically have low follicle-stimulating hormone (FSH) and luteinizing hormone (LH) serum levels. If patients have high FSH and LH levels, along with small testes bilaterally, they are diagnosed with NOA. However, men with normal FSH and LH levels could present with either NOA or OA, and the only means of differentiating these two groups of men is by testicular biopsy.[4] Protein biomarkers that could be used to diagnose NOA, thereby eliminating the need for biopsies, would benefit many azoospermic men; hence, for this reason, differential gene expression studies by Fox et al., Rockett et al., Feig et al., Okada et al., and Dubé et al. have aimed to identify genes that may be characteristic of NOA.[5−9]

Recently, using mass spectrometry (MS) based proteomic approaches we identified over 2000 proteins in seminal plasma from fertile control men and postvasectomy (PV) men.[10] We believe that the composition of seminal plasma from PV men

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biologically mimics that of men with obstructive azoospermia. Semen from PV men may be different than semen from men with congenital or acquired obstructive azoospermia, but vasectomy is considered the major cause of OA.[11] For this reason, we chose PV men since we were confident that there was complete blockage of vas deferens resulting in sperm-free samples for the purposes of identifying markers of obstruction. In that study, we were able to identify a number of proteins found only in Control and proteins at higher abundance in Control, many of which had their origin in the reproductive tract. A number of proteins unique to Control, or elevated in Control, have been further validated by our group using MS-based selected-reaction monitoring (SRM) assays.[12] In the present study, we delineated the proteome of seminal plasma from NOA men. Upon comparative analysis of the seminal proteomes of NOA, Control and PV utilizing a label-free spectral counting[13] methodology, we identified proteins with different levels among these clinical groups. By measuring peptide peak intensities, also referred to as extracted ion current (XIC),[14] we were able to calculate relative protein abundance ratios and compare them to ratios obtained from spectral counting, thereby increasing stringency on candidate selection. Since little is known about NOA and its effects on the urogenital system, these proteins may have either lower or higher relative abundances in NOA. Some of these proteins may be useful in differentiating NOA from OA, thereby eliminating the need for testicular biopsies.

MATERIALS AND METHODS

Sample Preparation and Mass Spectrometry

Samples were prepared and analyzed as described previously.[10] Briefly, semen from five men diagnosed with NOA was collected after 3 or more days of sexual abstinence. Fertile control men were aged 31, 34, 38, 39 and 51; NOA men were aged 30 (high FSH), 34 (normal FSH, no sperm from testicular sperm extraction (TESE), maturation arrest), 35 (normal FSH, no sperm from TESE, maturation arrest), 36 (high FSH) and 37 (high FSH); post vasectomy men were aged 40, 42, 48, 55, and 57. Samples were left to liquefy at room temperature and centrifuged to remove sperm and obtain seminal plasma (supernatant). NOA pools containing 3 mg of total protein were prepared such that each individual patient contributed 0.6 mg of total protein. Three NOA pools were further reduced with dithiothreitol (DDT), alkylated with iodoacetamide and digested with trypsin overnight. Protein digests were then fractionated using strong-cation exchange (SCX) chromatography into 60 fractions. Out of the 60 fractions, 21 (some fractions were pooled) were desalted and microextracted with C_{18} tips. Forty microliters of sample were then loaded onto an in-house packed 3 cm long 5 μm particle C_{18} trap-column from a 96-well microplate autosampler using the EASY-nLC system (Proxeon Biosystems, now Thermo Fisher Scientific, San Jose, CA) and an increasing concentration of acetonitrile of 55 or 88 min was used to elute peptides from the trap-column onto an in-house packed 5 cm long 3 μm particle C_{18} analytical column. This liquid chromatography setup was coupled online to an LTQ-Orbitrap XL (Thermo Fisher Scientific) mass spectrometer using a nanoelectrospray ionization source (Proxeon Biosystems, now Thermo Fisher Scientific). The full MS^1 scan was acquired in the Orbitrap with subsequent MS^2 scans on the top six parent ions in the linear ion trap (LTQ) in data-dependent mode. Dynamic exclusion, monoisotopic precursor selection and charge state screening were enabled such that only +2 and +3 ions were subjected to MS^2 fragmentation. The resulting XCalibur RAW files were uploaded into Mascot Daemon (v.2.2) and extract_msn was used to generate Mascot Generic Files (MGFs). MGFs were searched with Mascot (Matrix Science, London, U.K.; version 2.2) and X!Tandem (Global Proteome Machine Manager, version 2006.06.01) against the concatenated (75426 forward and 75426 reverse protein sequences) nonredundant IPI.Human v.3.54 database with parent and fragment tolerances of 7 ppm and 0.4 Da, respectively. Searches were resctricted to tryptic peptides with one missed cleavage allowed, fixed carbamidomethylation of cysteines and the following variable modifications: oxidation of methionines, deamidation of asparagines and glutamines, cyclization of N-terminal glutamines and glutamic acids (pyro-Glu), and protein N-terminal acetylation. Scaffold (Proteome Software Inc., Portland, OR; v.2.0) was used to merge Mascot and X!Tandem search result files and filter the data to achieve a false-positive rate (FPR) of 1.2%. ProteinCenter (Proxeon Biosystems, now Thermo Fisher Scientific) was used to retrieve Genome Ontology annotations and pathway analysis from Kyoto Encyclopedia of Genes and Genomes (KEGG). Pathways were also investigated using Igenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood, California). For a more detailed procedure refer to Batruch et al.[10]

Label-free Quantitation by Spectral Counting

Normalized spectral counts were used to select proteins at different abundances in NOA relative to Control and PV data sets. The selection criteria was the same as that described earlier,[10] which required proteins to be elevated or decreased by at least 2-fold. Upon normalization of spectral counts using the combined NOA, Control and PV data sets, a small number of proteins had their spectral counts significantly elevated due to protein ambiguity (shared peptides with other proteins). These proteins were removed from the list.

Label-free Quantitation using Extracted Ion Current (XIC)

RAW files corresponding to Control, NOA and PV data sets were uploaded into MaxQuant[15] v. 1.1.1.25 (www.maxquant.org) and searched with Andromeda[16] (built into MaxQuant) against the nonredundant IPI.Human v.3.54 database. Search parameters included a fixed carbamidomethylation of cysteines and variable modifications of oxidation of methionine and N-terminal protein acetylation. Data was initially searched against a "human first search" database with a parent tolerance of 20 ppm and a fragment tolerance of 0.5 Da in order to calculate and adjust the correct parent tolerance to 5 ppm for the search against the IPI.Human fasta file. During the search, the IPI.Human fasta database was randomized and FPR was set to 1% at the peptide and protein levels. Data was analyzed with "Label-free quantification" checked and the "Match between runs" interval set to 2 min. "LFQ Intensity" columns corresponding to each protein in replicate Control, NOA and PV groups were averaged and used to calculate Control/NOA and NOA/PV ratios (Supplementary Table 1, Supporting Information).

Spectral Count and XIC Comparison

Spectral counting fold changes were initially used to select proteins at differential abundances in Control-NOA and NOA-PV data sets. For these same proteins, XIC fold changes were extracted. Spectral counting and XIC fold changes, for Control-

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NOA and NOA-PV data sets, were $\log_{10}$ transformed. The $\log_{10}$ values corresponding to spectral count and XIC were plotted using an $x-y$ scatter plot and the Pearson correlation coefficient was calculated. Proteins that according to spectral counting, were uniquely identified in one clinical group, could not be log transformed and were omitted from the plot. Only proteins with XIC ratios 2 or greater and those that showed consistent up- or down-regulation relative to spectral counting ratios were used for further analysis.

Public Database Searches

Differentially expressed proteins were checked for tissue specificity using UniProtKB, UniGene, BioGPS and Human Protein Atlas databases. Since expression profiles using the above-mentioned databases are represented in different formats, for proteins to be annotated, we used the following criteria: in UniGene, expression had to be "restricted" or the tissue was required to have a dominant/major contribution relative to other tissues; in BioGPS, expression had to be "exclusive" or had to exceed the average expression by at least 3-fold; and in Human Protein Atlas, expression had to be "strong". Furthermore, UniGene and BioGPS do not have epididymis and seminal vesicles in their tissue list; therefore, proteins that were expressed in the epididymis were considered to be originating from the testis.

RESULTS AND DISCUSSION

We identified 2048 proteins in the NOA seminal plasma pool, with 1732, 1756, and 1765 proteins in each replicate (Figure 1,

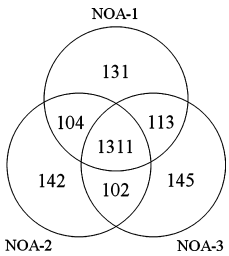

Figure 1. Venn diagram showing overlap of 2048 proteins identified among triplicate NOA seminal plasma samples.

Supplementary Table 2a, Supporting Information). The false-positive rate (FPR) of 1.2% was achieved by adjusting Mascot and X!Tandem peptide thresholds in Scaffold 2.0. FPR was calculated as $2 \times FP/(TP+FP)$, where FP (false positive) is the number of proteins matching the reverse database and TP (true positive) is the number of proteins matching the forward database. Detailed information about peptide sequences, scores and modifications for the NOA data set, can be found in Supplementary Table 2b (Supporting Information). The original Scaffold file "NOA_SP.sfd"containing all NOA proteins can be downloaded from ProteomeCommons Tranche (https://proteomecommons.org/data-search.jsp) using hash code lTZ9iWukjHMsFwijXYTQztdi1+/XBUTtOfVAPv/+uIQ088/r83QeIQO6YXX/btb6NpxjeNDc+4aA/nmKrYFfbFH0B3wAAAAAAAACXA== and opened using a free Scaffold viewer (http://www.proteomesoftware.com/Scaffold/Scaffold_viewer.htm or https://proteomecommons.org/data-search.jsp and hash code PeKjzQ60/EYjyg0pipg/o9b+eCFQ0/M3u6VMX6jEyhhdDexsRwsKxcJeez2PF0rfFKKR4vBM+ZaUKJSKkdk998ybqLkAAAAAAAAB5Q==).

Upon comparison of the NOA proteome to the seminal plasma work by Pilch et al.,[17] we found 667 proteins in common (Supplementary Table 2a, Supporting Information). Since the experimental design of examining NOA samples was identical in every respect to our previous Control and PV work,[10] we directly compared these three data sets with the aim of identifying proteins that may be diagnostic for NOA. To ensure that the mass spectrometer was at the same level of sensitivity with the NOA data set as with previously published Control and PV, we reran and reanalyzed several SCX fractions from the Control and found a similar number of peptides and proteins. Upon merging NOA, Control and PV data sets in Scaffold, we identified 2500 proteins in seminal plasma (at an FPR of 1.2%): 1489 are common in all three, 1595 are shared between NOA and Control, and 1670 between NOA and PV (Figure 2, Supplementary Table 3, Supporting Information).

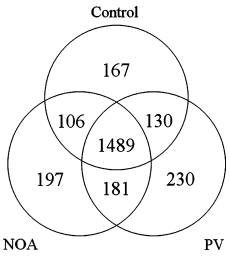

Figure 2. Venn diagram showing overlap of proteins between NOA, PV and Control pools. A total of 2500 proteins have been identified.

The original Scaffold file "Control-NOA-PV_SP.sfd"containing all Control-NOA-PV proteins can be retrieved from ProteomeCommons-Tranche (https://proteomecommons.org/data-search.jsp) using hash code M/mmTHUpXe8PXZAViYbG5qCL8+JsjLq7vliaPEsEJww8ydTnBKoyHf+6tloP5m8+BbsN4Xi1IOAu0G4/0RuHIuoBIWUAAAAAAAAClA==.

The majority of the proteins are cytoplasmic, membrane and extracellular (Figure 3a). This is very similar to our previous findings on Control and PV samples.[10] In terms of function, the majority of the proteins are involved in catalytic activity, followed by protein binding (Figure 3b). Four-hundred thirty eight proteins are categorized as enzymes. Relative to the referance human IPI database containing 86719 proteins, glycosidases, hydrolases and glycosylales are overrepresented with 28, 365, and 30 proteins respectively. This finding is consistent with the knowledge that seminal plasma liquefaction requires many enzymes acting in cascades and agrees with work by Pilch et al.[17] The underrepresented enzyme categories include transferases consisting of 156 proteins, transferring phosphorus-containing group proteins with 59 members, and 30 serine/threonine kinases. In terms of biological processes, the top categories are metabolism, regulation, cell organization and biogenesis, response to stimulus, cell communication and transport (Figure 3c). The latter of which has been shown to be important in successful capacitation of spermatozoa.[18] There are also 198 proteins involved in reproduction.

In this study, we used spectral counting to identify proteins that were elevated or decreased among Control−NOA and NOA−PV data sets to have an analogous comparison to previously published Control−PV work. Since spectral counting is a semiquantitative method that is not remarkably sensitive to small abundance changes, especially with low abundance proteins identified by few spectra, we implemented

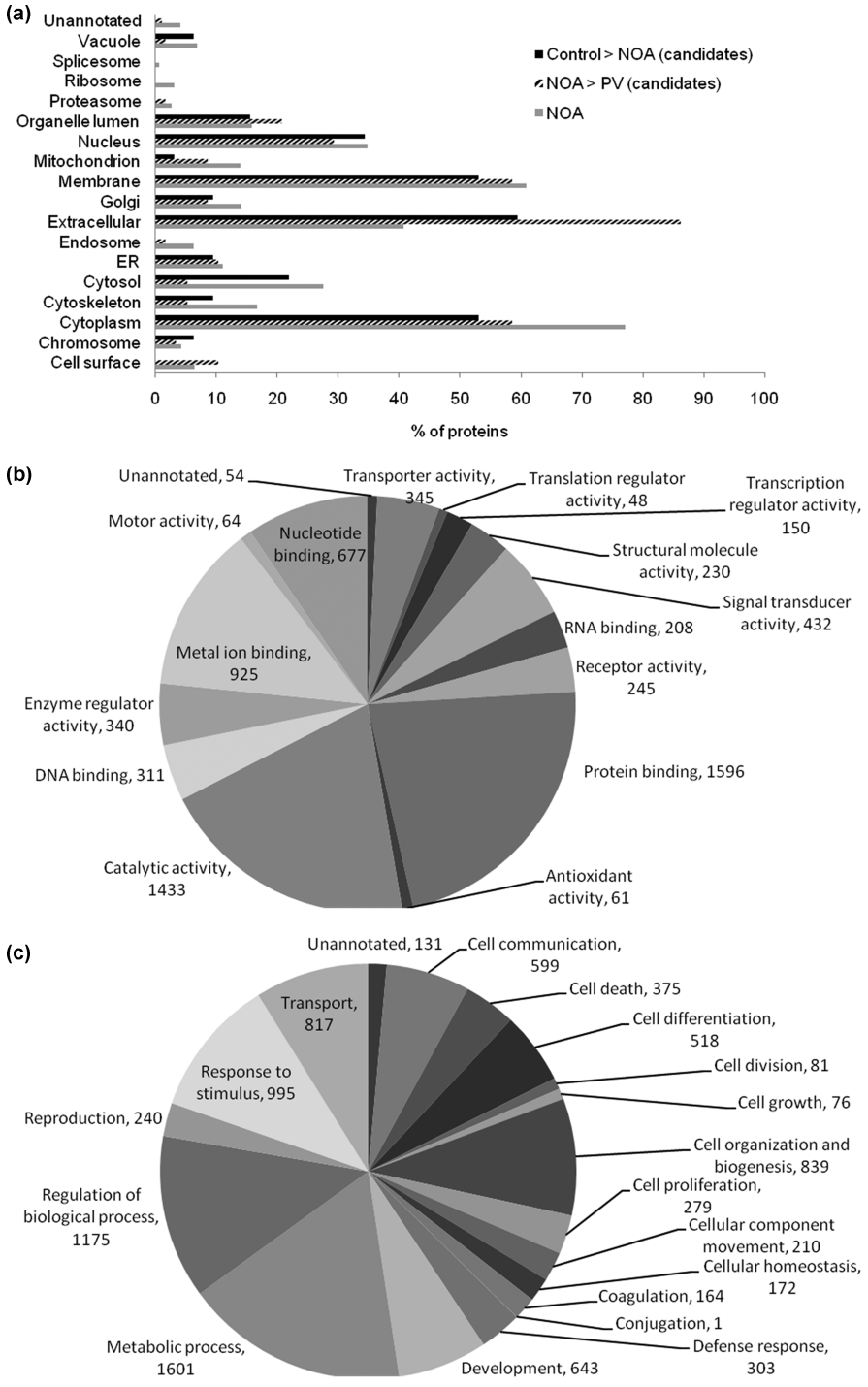

Figure 3. (a) Distribution of cellular component of proteins in NOA seminal plasma samples, proteins elevated in Control relative to NOA (Control > NOA), and proteins elevated in NOA relative to PV (NOA > PV). Proteins belonging to Control > NOA and NOA > PV groups are enriched for extracellular component while the cytoplasmic component is decreased. Distribution of (b) molecular function and (c) biological process of proteins identified in NOA seminal plasma samples.

another label-free approach that utilizes extracted-ion current (XIC) intensities to verify the spectral counting ratios. Spectral counting and XIC are label-free strategies, and both present certain challenges;[19−21] however, by combining both quantitative results, we were able to generate a more reliable protein candidate list. The resulting MaxQuant "proteinGroups" file contained 2700 identified proteins and their corresponding XIC intensities, of which 32 proteins were reverse hits and 39 contaminant protein hits (Supplementary Table 1, Supporting Information).

To determine the origin of differentially expressed proteins at the tissue level, gene names were checked against gene or protein databases and those that had restricted, exclusive, dominant or above average expression in the testis, epididymis, seminal vesicle and the prostate were marked as originating from the above-mentioned tissues (Supplementary Table 4a, 4b, 5a and 5b, Supporting Information). Proteins that had

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expression marked as "restricted" to a tissue in UniGene or that were "exclusive" only to one tissue in BioGPS are marked with an asterisk in Table 1, Table 2, Supplementary Table 4a, Supplementary Table 4b, Supplementary Table 5a and Supplementary Table 5b (Supporting Information).

Table 1. Proteins at Different Abundances in NOA Relative to Control Seminal Plasma According to Spectral Counting[a]

gene name	control/NOA[b] ratios SC[c]	XIC[d]	protein origin[e]
	Unique to NOA[b]		
SORD	NOA≫Control	NOA≫Control	
GGT7	NOA≫Control	0.2	
	Unique to control		
HIST1H4H	NOA≪Control	13.6	
DPEP3	NOA≪Control	NOA≪Control	testis* (2)
TEX101	NOA≪Control	20220.0	testis* (3)
CEL	NOA≪Control	189.1	
PGK2	NOA≪Control	533.7	testis* (3)
PRKACA	NOA≪Control	2.8	
PGAM2	NOA≪Control	3509.5	
SLC2A14	NOA≪Control	17.0	testis* (3)
CDH2	NOA≪Control	NOA≪Control	
ASRGL1	NOA≪Control	6.6	testis (3)
	↓ in NOA[b]		
STOM	15.0	6.8	
OVCH2	13.5	14.2	
PTGDS	11.7	15.3	testis (2), epididymis (2), prostate (2)
CRISP2	10.0	16.8	testis* (3)
LIPI	7.7	7.2	
LDHC	7.4	112.7	
SERPINA6	7.0	3.6	
CA4	5.3	3.4	
HIST1H2BA	4.2	8.3	testis (3)
MPO	3.4	3.8	
	↑ in NOA[b]		
VAV2	0.04	0.4	
TGM2	0.04	0.4	
SPARC	0.05	0.2	testis (2)
KIAA0368	0.05	0.2	
EPS8L2	0.1	0.4	
SPARCL1	0.1	0.4	
COL6A2	0.2	0.3	
DDX1	0.2	0.5	
CST2	0.2	0.1	
CST4	0.2	0.4	

[a]Respective ratios from extracted ion current (XIC) intensities are also provided. Only up to top 10 proteins for each category are listed. For a full list proteins and additional information refer to Supplementary Table 4b (Supporting Information). * indicates restricted (UniGene) and exclusive (BioGPS) expression. [b]NOA, nonobstructive azoospermia. [c]SC, spectral counts. Fold changes may be overestimated especially in cases where only one out of three replicates contains spectral counts (refer to Supplementary Table 4b for all information, Supporting Information). [d]XIC, extracted ion current ratios. XICs were calculated using MaxQuant (refer to Supplementary Table 1 for all information, Supporting Information). [e]For protein origin to be annotated, the protein needed to be found in testis, epididymis or prostate in at least two out of four databases. The number in bracket indicates number of databases showing above average expression in the tissue.

Table 2. Proteins at Different Abundances in NOA Relative to Postvasectomy (PV) Seminal Plasma According to Spectral Counting[a]

gene name	NOA[b]/PV[c] ratios SC[d]	XIC[e]	protein origin[f]
	Unique to PV[c]		
HIST1H4H	PV≫NOA	0.02	
ELA2	PV≫NOA	0.01	
MMP9	PV≫NOA	0.002	
CORO1A	PV≫NOA	0.01	
SAA2;SAA1	PV≫NOA	0.02	
	Unique to NOA[b]		
LDHC	PV≪NOA	55.2	testis* (2)
BSPH1	PV≪NOA	761.2	
ADAM7	PV≪NOA	56.9	testis* (2)
MFGE8	PV≪NOA	10.0	
REG3G	PV≪NOA	7.4	
MFAP4	PV≪NOA	18.6	
AKAP4	PV≪NOA	18.2	testis* (3)
SORD	PV≪NOA	PV≪NOA	
	↑ in NOA[b]		
MUC5B	83.0	50.2	
CPVL	20.0	3.9	
CRIM2	16.0	6.4	
SLC2A5	14.4	2.3	
ELSPBP1	13.5	11.4	testis* (3)
PATE4	13.0	112.6	prostate* (3)
LOC642103	12.6	7.5	
SPINT3	12.0	11.8	testis* (3)
COL18A1	9.6	4.6	
BGN	9.0	3.5	
	↓ in NOA[b]		
HIST1H2BL	0.04	0.06	
FGG	0.05	0.2	
AZU1	0.07	0.005	
MPO	0.07	0.07	
GSTM2	0.07	0.5	
PRELP	0.1	0.5	
ORM1	0.2	0.1	
FLJ11151	0.2	0.5	
FGB	0.3	0.1	
PAEP	0.5	0.5	

[a]Respective ratios from extracted ion current (XIC) intensities are also provided. Only up to top 10 proteins for each category are listed. For a full list proteins and additional information refer to Supplementary Table 5b (Supporting Information). * indicates restricted (UniGene) and exclusive (BioGPS) expression. [b]NOA, non-obstructive azoospermia. [c]PV, postvasectomy. [d]Fold changes may be overestimated especially in cases where only one out of three replicates contains spectral counts (refer to Supplementary Table 5b for all information, Supporting Information). [e]XIC, extracted ion current ratios. XICs were calculated using MaxQuant (refer to Supplementary Table 1 for all information, Supporting Information). [f]For protein origin to be annotated, the protein needed to be found in testis, epididymis or prostate in at least two out of four databases. The number in bracket indicates number of databases showing above average expression in the tissue.

Control and NOA Comparison

Using the spectral count cut-offs, we identified 98 proteins at differential abundance levels between NOA and Control data sets (Supplementary Table 4a, Supporting Information). For

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proteins to be selected, they needed to be elevated or decreased by at least 2- fold, or be uniquely identified in one clinical group. Upon comparing these 98 proteins to their XIC ratios, the Pearson correlation coefficient is 0.83 (95% confidence interval 0.75−0.89) (Figure 4a). There are 13 proteins that do

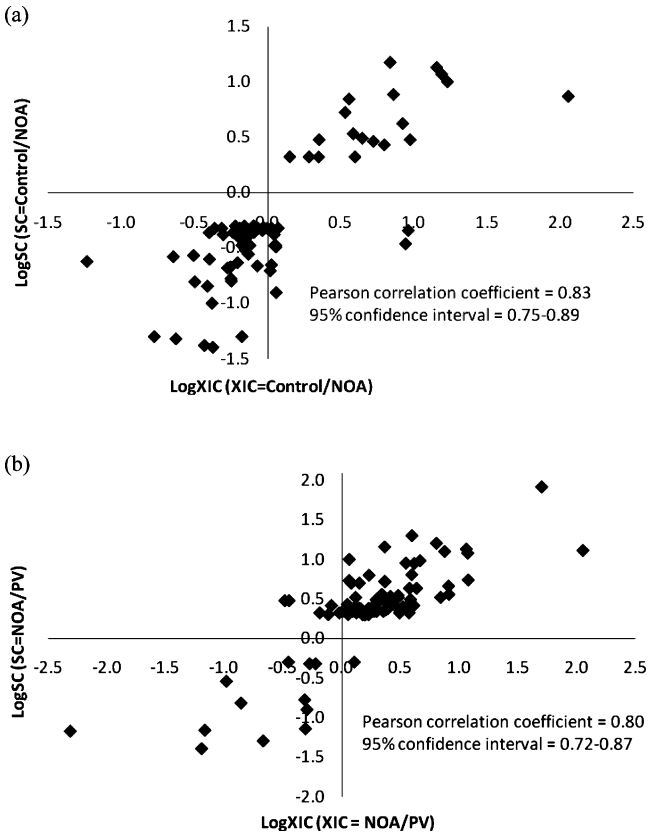

Figure 4. Correlation between LogSC (SC ratios) and LogXIC (XIC ratios) in (a) Control/NOA and (b) NOA/PV data sets.

not correlate as their XIC ratios are opposite of the spectral counting ratios and 33 proteins with XIC ratios that do not satisfy the criteria of being elevated or decreased by at least 2-fold (or Control/NOA < 0.5) in one clinical group relative to the other (Supplementary Table 4a, Supporting Information, Figure 4a). The 13 proteins that do not correlate and 30 out of the 33 belong to the list of proteins at higher concentration in NOA relative to Control and have Control/NOA XIC ratios >0.5. Out of these 43 proteins, 34 had spectra- counting Control/NOA ratios between 0.3 and 0.5. These 13 and 33 proteins are highlighted in blue in Supplementary Table 4a. XIC values for all identified proteins are found in Supplementary Table 1 (Supporting Information). By comparing spectral count ratios to XIC ratios, the original list of 98 proteins has been reduced to 52. Out of 52 proteins that correlate and according to spectral counting, 15 are found only in Control and 19 are at a lower concentration in NOA relative to Control, 2 are found only in NOA and 16 are found at a higher concentration in NOA relative to Control (Supplementary Table 4b, Supporting Information). A short list of proteins, up to 10 from each category based on spectral-count are presented in Table 1. Thirty-one proteins are in common with the earlier Control-PV work[10] (Supplementary Table 4b and 5b, Supporting Information) and are omitted from further discussion. Twenty-nine that are at higher concentration in Control relative to NOA were also elevated in Control relative to PV.

There are 13 proteins showing expression in testis/epididymis in at least 2 databases, DPEP3, TEX101, PGK2, SLC2A14, ASRGL1, SPACA3, SLC1A1, ZPBP, PTGDS, CRISP2, HIST1H2BA, GPR64 and SPARC (Supplementary Table 4b, Supporting Information). Similarly, PTGDS, SEMG1 and SEMG2 are produced by the prostate (Supplementary Table 4b). Of these, ASRGL1 and SPARC have not been identified in the earlier Control-PV study.[10] ASRGL1 is localized to the midpiece region of sperm and its antibodies are found in postvasectomy sera.[22] SPARC is a matricellular protein with a significant role in testis development.[23] SPARC expression is elevated in fetal testis cords, it is also abundantly found in Sertoli and Leydig cells.[23−25]

In our previous study of Control and PV, we found 81 proteins that were elevated (based on spectral counting) in Control[10] and 30 of these have been further verified quantitatively in Control, PV and NOA seminal plasma using selective-reaction monitoring (SRM) approaches by our group (Drabovich et al.).[12] The original goal was to measure all 81 proteins; however, SRM assays could not be developed for all analytes due to poor peptide sensitivity or interferences. For the majority of these proteins, SRM data correlated well with the semiquantitative spectral-counting results (Supplementary Tables 4a, 4b, 5a, 5b, Supporting Information). Of the proteins verified by Drabovich et al. across all three clinical groups of pooled samples, SRM and spectral-counting Control/NOA fold changes did not correlate for MGAM, ECM1, CD177, ABP1, NPC2, CRISP1, SERPINA1, PTGDS, LDHC, GPR64 and CA4. Note, that of these, only PTGDS, LDHC, CA4 and GPR64 have been selected in this study to have differential expression between Control and NOA. In the case of PTGDS, LDHC and CA4, the SRM Control/NOA ratio was 3.5, 1728 and 1.3, respectively, versus 11.7, 7.4, and 5.3 for spectral-counting data and 15.3, 112.7, and 3.4 for XIC. For the remaining proteins, SRM and spectral-counting fold changes were in the opposite direction, however, since these ratios fall in the range 1−2, they are of little statistical significance. PTGDS has previously been studied in our lab on two other occasions using ELISA methods to determine protein levels in normal and azoospermic patients.[26,27] These studies have shown that the median PTGDS concentration in 10 normal men and 14 men diagnosed with NOA was 800 μg/L and 18.5 μg/L, respectively, a 40-fold decrease in NOA. TEX101, which is unique to Control according to spectral counting and elevated over 20000× in Control relative to NOA using XIC, was elevated over 300× in Control according to SRM. Upon close examination of RAW files for TEX101 and manual integration of peptide peaks, we believe that the XIC ratio of 20000 may be an overestimate, since the majority of the peptides in NOA data set are of low intensity and appear within the baseline. The reason we decided to set the lower fold-change limit of at least 2 and not a greater cutoff for candidate selections is because we were concerned about eliminating proteins that are expressed in the testis or epididymis. Testis/epididymis only contribute about 10% of the fluid to the total seminal plasma volume, rendering many of these proteins to be of low abundance with few spectra and difficult to detect.[28]

Despite SRM assays being performed on the same Control, PV and NOA seminal plasma pools, samples were subjected to a different trypsin-digestion protocol and SCX fractionation was not performed. Due to these sample preparation

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differences as well as due to the inherent semiquantitative nature of spectral-counting methodology,[13,19,20] disagreement between spectral-counting, XIC and SRM data for some proteins was expected.

Proteins found only in Control and those more abundant in Control relative to NOA are enriched for extracellular localization, whereas the cytoplasmic component was reduced, according to Genome Ontology analysis within ProteinCenter which uses 86719 human proteins in IPI database as the reference data set (Supplementary Table 4b, Supporting Information, Figure 3a). This is in contrast to cellular localization of complete NOA and Control proteomes, where the percentage of cytoplasmic proteins is greater than those of extracellular origin by nearly 2-fold. According to Protein-Center, several biological processes that are over-represented by this set of proteins involve reproduction. The proteins belonging to these processes include ZPBP, ELSPBP1, PGAM2, SPACA3, GPR64 and SLC2A14. SLC2A14 (GLUT3) for example, having strong expression in the testis, belongs to the sugar transporter family and is involved in glucose transport.[29] Furthermore, in Protein Center the carbohydrate catabolic process and glycolytic pathway according to Kyoto Encyclopedia of Genes and Genomes (KEGG) are over-representedy by LDHC, PGK2, PGAM2, SPACA3 and CALR. These glycolytic proteins are involved in the development of spermatozoa and generation of ATP molecules required for movement of sperm flagellum. According to Ingenuity Pathway Analysis, A2M and CDH2 belong to Germ Cell-Sertoli Cell Junction Signaling. Sertoli cells are known to provide a supportive and nourishing environment in the testis for the development of sperm from primordial germ cells. Absence or decreased levels of these proteins could potentially lead to breakdown of the signaling cascade between the Sertoli cell and the maturing germ cell, and have an effect on production of spermatozoa. In cases of Sertoli-cell only syndrome (SCOS), absence of germ cells could potentially lead to lowered production of these proteins. A2M is synthesized by the Sertoli cell and complexed by PSA, resulting in a conformational change thereby allowing it to bind spermatozoa.[30,31] There is positive correlation between A2M and sperm motility and seminal plasma levels from vasectomised men shows a 12-fold reduction relative to controls.[32]

There are 18 proteins out of 52 that show lower concentration (including unique to NOA) in Control relative to NOA (Supplementary Table 4b, Supporting Information). Of these, only COL6A2 has been previously identified to be at lower abundance in Control relative to PV. GGT7, according to spectral counting is found uniquely in NOA and is part of the glutathione metabolism pathway. Along with PTGDS, glutathione is required for conversion of prostaglandin H_2 (PGH2) to prostaglandin D_2 (PGD2).[33] Furthermore, glutathione plays a role in spermatogenesis and sperm maturation and presence of glutathione transferases in human Leydig and Sertoli cells suggests they may serve as anticarcinogens in germ cells .[34,35] Another protein involved in the glycolytic mechanism and only identified in NOA is SORD. It is an enzyme that converts sorbitol to fructose and increases tyrosine phosphorylation in sperm proteins, suggesting that it may also be involved in sperm capacitation.[36] SORD is found on sperm and can originate from progenitor germ cells or be carried by epididymosomes, membraneous vesicles secreted by the epididymis,[36,37] but it is also expressed in the prostate.[38] Overexpression of these proteins in NOA that are involved in fructose metabolism reinforces the possibility that upregulation of this pathway could be a result of events associated with hypospermatogenesis or maturation arrest. Since spermatogenesis is a complex process and the exact cause of NOA is unknown, it is possible that certain proteins in seminal plasma become elevated due to aberrant cellular processes.

Gene expression studies on tissues from normal men and men with spermatogenic defects, including NOA, by Dubé et al., Feig et al., Fox et al., Okada et al. and Wang et al. identified genes that were over- and under-expressed.[8,7,5,9,39] Genes showing differential expression levels and common to list of proteins differentially expressed between Control and NOA in our study include CRISP1, AKAP4, DPEP3, PGAM2, ASRGL1, SPACA3, ZPBP, CRISP2, LDHC, TEX101, HIST1H2BA, A2M, MPO and ORM1.

NOA and PV Comparison

The group of proteins found to have a variation in expression between PV and NOA are of great interest as these may be useful in differentiating the two cases of azoospermia. Comparison of NOA and PV data sets utilizing spectral counts identified 110 proteins to be differentially expressed (Supplementary Table 5a, Supporting Information) with at least a 2 fold difference in spectral counts. The Pearson correlation coefficient of spectral counting and XIC ratios is 0.80 (95% confidence interval 0.72−0.87) (Figure 4b). There are 7 proteins that do not correlate as their ratios are opposite to those from spectral counting and 28 proteins that are not elevated or decreased by at least 2-fold (Supplementary Table 5a). Initially there were 82 proteins elevated in NOA relative to PV (NOA/PV $\geq$ 2) according to spectral counts, 31 of which had XIC ratios <2 and were eliminated. Twenty-five out of 31 proteins belonged to those at higher abundance in NOA and had spectral-counting NOA/PV ratios between 2 and 3. The 35 proteins that did not correlate are highlighted in blue in Supplementary Table 5a. There are a total of 75 proteins that satisfy the criteria of a difference of at least 2 fold found by both spectral counting and XIC: based on spectral counts, 8 proteins are uniquely found in NOA, 51 are at lower levels in PV relative to NOA, 5 are found only in PV and 11 are found at higher levels in PV (Supplementary Table 5b, Supporting Information). XIC values for all proteins are found in Supplementary Table 1 (Supporting Information).

Proteins LDHC, ADAM7, AKAP4, ELSPBP1, SPINT3, FAM12B, RNASE1, NPC2 and CRISP1 have been previously identified to have expression in the testis/epididymis according to two or more gene or protein databases (Supplementary Table 5b, Supporting Information). In addition to these, PATE4 and OR51E2 are found in the prostate (Supplementary Table 5b). According to Genome Ontology analysis within Protein Center, proteins unique to NOA and those at higher abundance in NOA relative to PV are also mostly of extracellular origin (Supplementary Table 5b, Figure 3a). Proteins short-listed down to 10 in each category according to spectral-count fold change are shown in Table 2. Out of 75 differentially expressed proteins, 47 are in common with candidates identified in the Control-PV study (Supplementary Table 5b).[10]

Many of the proteins in this category have been shown to be involved in male fertility and several have already been discussed in Batruch et al.[10] BSPH1 for example, being a sperm binding protein expressed in the epididymis, is involved

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in sperm capacitation.[40] SORD is the only protein that is uniquely found in NOA and not in Control or PV and has been discussed earlier. In a study of obstructive azoospermia patients with congenital bilateral absence of vas deferens, several genes have been shown to have genomic copy number variations.[41] One such gene that is also elevated in NOA relative to PV is COL18A1. GAS6 protein expressed in Leydig and Sertoli cells acts as a ligand in activating protein-tyrosine kinases, TYRO3 family, AXL and MER.[42] Generally, tyrosine kinases are involved in intracellular signaling and this specific group of proteins is implicated in regulation of phagycytotic activity of Sertoli cells. It has been reported that Sertoli cells have the ability to degrade apoptotic spermatogenic cells, a requirement for proper spermatogenesis.[43−46] In this process, GAS6 promotes binding of Sertoli cells to apoptotic spermatogenic cells.[47] Maturation arrest of spermatogenic cells in NOA may result in elevated numbers of apoptotic cells. This in turn could induce production of GAS6 at higher levels in NOA than in PV in order to signal degradation of these cells.

Proteins in common with infertility studies by Wang et al. and Feig et al. include CD177, MXRA5, ABP1, WFDC2, MPO, ORM1 LDHC, AKAP4, and MGAM.[7,39]

Control, NOA and PV Comparison

There are a number of proteins according to spectral counts and XIC showing differential expression in Control-NOA comparison as well as NOA-PV comparison. Comparative analysis of three data sets reveals several proteins in NOA having spectral counts and XIC values intermediate to Control and PV (Supplementary Table 4b and 5b, Supporting Information). These proteins include LDHC, ELSPBP1, CES7, A2M, OVCH2, PTGDS, GPR64 and ALDH1A1. Many of these are known to have a significant role in male infertility and have been discussed in detail elsewhere.[10] According to a verification study by Drabovich et al.,[12] LDHC and PTGDS concur with our findings while GPR64 and ALDHA1 show a minor decrease in NOA relative to PV. SRM-based verification of ELSPBP1, A2M and OVCH2 has not been performed. Proteins HIST1H4H, LDHC, SORD, ELSPBP1, GMPR, CTS2 and A2M show differential expression in Control-NOA data set as well as in NOA−PV comparison.

In future studies, we plan on quantitatively measuring candidate proteins with large fold changes or those uniquely identified in one clinical group, such as those presented in Table 1 and Table 2 in Control, PV and the three subgroups of NOA. Proteins of greatest interest for example would be those such as SORD and DPEP3 which are found only in NOA and Control, respectively, and also those that have restricted expression to testis or epididymis and known function in reproduction. Proteins with NOA spectral count and XIC values greater than in those in PV but smaller than in Control are of interest as they may have concentration intervals in different clinical groups. These quantitative assays would either involve development of SRM methods or ELISA.

CONCLUSION

We have identified proteins present in NOA seminal plasma. Upon comparative analysis of NOA proteome to Control and PV proteomes using two label-free approaches, spectral counting and XIC, we identified proteins in NOA at differential abundances relative to these other clinical groups. Several of these proteins in NOA were found to have spectral-counts and XICs at levels intermediate to Control and PV. A number of proteins with differential levels in Control and PV samples by spectral counting have been validated by SRM, thereby corroborating our approach. Many of the proteins identified are linked to fertility and are expressed in the urogenital tract. We believe that upon further validation, these proteins may be useful in clinical differentiation of NOA and OA patients.

ASSOCIATED CONTENT

Supporting Information

Supplementary Tables 1, 2a, 2b, 3, 4a, 4b, 5a, 5b and supplementary Scaffold files NOA_SP.sfd and Control-NOA-PV.sfd. This material is available free of charge via the Internet at http://pubs.acs.org. It is also available at ProteomeCommons Tranche (http://proteomecommons.org/tranche).

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ABBREVIATIONS:

PV, postvasectomy; OA, obstructive azoospermia; NOA, non-obstructive azoospermia; SCOS, Sertoli-cell only syndrome; MS, mass-spectrometry; SCX, strong-cation exchange; LC, liquid-chromatography; SRM, single-reaction monitoring; FPR, false-positive rate; AS, asthenoazoospermia

REFERENCES

(1) Nieschlag, E. Scope and Goals of Andrology. In *Andrology: Male Reproductive Health and Dysfunction*, 3rd ed.; Neschlag, E., Behre, M. B., Neschlag, S., Eds.; Springer-Verlag and Goals of Andrology: Berlin, 2010; pp 1−10.

(2) Jarow, J. P.; Espeland, M. A.; Lipshultz, L. I. Evaluation of the azoospermic patient. *J. Urol.* **1989**, *142*, 62−65.

(3) Sigman, M.; Jarow, J. P. Male Infertility. In *Campbell's urology*, 8th ed.; Walsh, P. C., Retic, A. B., Vaughan, E. D., Wein, A. J., Eds.; Saunders: Philadelphia, 2002; pp 1475−1531.

(4) Tournaye, H.; Camus, M.; Goossens, A.; Liu, J.; Nagy, P.; Silber, S.; Van Steirteghem, A. C.; Devroey, P. Recent concepts in the management of infertility because of non-obstructive azoospermia. *Hum. Reprod.* **1995**, *10* (Suppl 1), 115−119.

(5) Fox, M. S.; Ares, V. X.; Turek, P. J.; Haqq, C.; Reijo Pera, R. A. Feasibility of global gene expression analysis in testicular biopsies from infertile men. *Mol. Reprod. Dev.* **2003**, *66*, 403−421.

(6) Rockett, J. C.; Patrizio, P.; Schmid, J. E.; Hecht, N. B.; Dix, D. J. Gene expression patterns associated with infertility in humans and rodent models. *Mutat. Res.* **2004**, *549*, 225−240.

(7) Feig, C.; Kirchhoff, C.; Ivell, R.; Naether, O.; Schulze, W.; Spiess, A. N. A new paradigm for profiling testicular gene expression during normal and disturbed human spermatogenesis. *Mol. Hum. Reprod.* **2007**, *13*, 33−43.

(8) Dube, E.; Hermo, L.; Chan, P. T.; Cyr, D. G. Alterations in gene expression in the caput epididymides of nonobstructive azoospermic men. *Biol. Reprod.* **2008**, *78*, 342−351.

(9) Okada, H.; Tajima, A.; Shichiri, K.; Tanaka, A.; Tanaka, K.; Inoue, I. Genome-wide expression of azoospermia testes demonstrates a specific profile and implicates ART3 in genetic susceptibility. *PLoS Genet.* **2008**, *4*, e26.
(10) Batruch, I.; Lecker, I.; Kagedan, D.; Smith, C. R.; Mullen, B. J.; Grober, E.; Lo, K. C.; Diamandis, E. P.; Jarvi, K. A. Proteomic analysis of seminal plasma from normal volunteers and post-vasectomy patients identifies over 2000 proteins and candidate biomarkers of the urogenital system. *J. Proteome Res.* **2011**, *10*, 941−953.
(11) The management of infertility due to obstructive azoospermia. *Fertil. Steril.* **2008**, *90*, S121-S124.
(12) Drabovich, A. P.; Jarvi, K.; Diamandis, E. P. Differential diagnosis of azoospermia with absolutely specific and sensitive biomarkers verified by selected reaction monitoring assay. *Mol. Cell. Proteomics* **2011**, *10*, M110.
(13) Liu, H.; Sadygov, R. G.; Yates, J. R. III A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **2004**, *76*, 4193−4201.
(14) Bondarenko, P. V.; Chelius, D.; Shaler, T. A. Identification and relative quantitation of protein mixtures by enzymatic digestion followed by capillary reversed-phase liquid chromatography-tandem mass spectrometry. *Anal. Chem.* **2002**, *74*, 4741−4749.
(15) Cox, J.; Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **2008**, *26*, 1367−1372.
(16) Cox, J.; Neuhauser, N.; Michalski, A.; Scheltema, R. A.; Olsen, J. V.; Mann, M. Andromeda: A Peptide Search Engine Integrated into the MaxQuant Environment. *J. Proteome Res.* **2011**, *10* (4), 1794−1805.
(17) Pilch, B.; Mann, M. Large-scale and high-confidence proteomic analysis of human seminal plasma. *Genome Biol.* **2006**, *7*, R40.
(18) Baldi, E.; Luconi, M.; Bonaccorsi, L.; Forti, G. Signal transduction pathways in human spermatozoa. *J. Reprod. Immunol.* **2002**, *53*, 121−131.
(19) Duncan, M. W.; Aebersold, R.; Caprioli, R. M. The pros and cons of peptide-centric proteomics. *Nat. Biotechnol.* **2010**, *28*, 659−664.
(20) Mitchell, P. Proteomics retrenches. *Nat. Biotechnol.* **2010**, *28*, 665−670.
(21) Mallick, P.; Kuster, B. Proteomics: a pragmatic perspective. *Nat. Biotechnol.* **2010**, *28*, 695−709.
(22) Bush, L. A.; Herr, J. C.; Wolkowicz, M.; Sherman, N. E.; Shore, A.; Flickinger, C. J. A novel asparaginase-like protein is a sperm autoantigen in rats. *Mol. Reprod. Dev.* **2002**, *62*, 233−247.
(23) Wilson, M. J.; Bowles, J.; Koopman, P. The matricellular protein SPARC is internalized in Sertoli, Leydig, and germ cells during testis differentiation. *Mol. Reprod. Dev.* **2006**, *73*, 531−539.
(24) Howe, C. C.; Overton, G. C.; Sawicki, J.; Solter, D.; Stein, P.; Strickland, S. Expression of SPARC/osteonectin transcript in murine embryos and gonads. *Differentiation* **1988**, *37*, 20−25.
(25) Vernon, R. B.; Sage, H. The calcium-binding protein SPARC is secreted by Leydig and Sertoli cells of the adult mouse testis. *Biol. Reprod.* **1989**, *40*, 1329−1340.
(26) Diamandis, E. P.; Arnett, W. P.; Foussias, G.; Pappas, H.; Ghandi, S.; Melegos, D. N.; Mullen, B.; Yu, H.; Srigley, J.; Jarvi, K. Seminal plasma biochemical markers and their association with semen analysis findings. *Urology* **1999**, *53*, 596−603.
(27) Heshmat, S. M.; Mullen, J. B.; Jarvi, K. A.; Soosaipillai, A.; Diamandis, E. P.; Hamilton, R. J.; Lo, K. C. Seminal plasma lipocalin-type prostaglandin D synthase: a potential new marker for the diagnosis of obstructive azoospermia. *J. Urol.* **2008**, *179*, 1077−1080.
(28) Robert, M.; Gagnon, C. Sperm motility inhibitor from human seminal plasma: presence of a precursor molecule in seminal vesicle fluid and its molecular processing after ejaculation. *Int. J. Androl.* **1994**, *17*, 232−240.
(29) Wu, X.; Freeze, H. H. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* **2002**, *80*, 553−557.
(30) Cheng, C. Y.; Grima, J.; Stahler, M. S.; Guglielmotti, A.; Silvestrini, B.; Bardin, C. W. Sertoli cell synthesizes and secretes a protease inhibitor, alpha 2-macroglobulin. *Biochemistry* **1990**, *29*, 1063−1068.
(31) Birkenmeier, G.; Usbeck, E.; Schafer, A.; Otto, A.; Glander, H. J. Prostate-specific antigen triggers transformation of seminal alpha2-macroglobulin (alpha2-M) and its binding to alpha2-macroglobulin receptor/low-density lipoprotein receptor-related protein (alpha2-M-R/LRP) on human spermatozoa. *Prostate* **1998**, *36*, 219−225.
(32) Glander, H. J.; Kratzsch, J.; Weisbrich, C.; Birkenmeier, G. Insulin-like growth factor-I and alpha 2-macroglobulin in seminal plasma correlate with semen quality. *Hum. Reprod.* **1996**, *11*, 2454−2460.
(33) Lu, S. C. Regulation of glutathione synthesis. *Curr. Top. Cell Regul.* **2000**, *36*, 95−116.
(34) Sies, H. Glutathione and its role in cellular functions. *Free Radic. Biol. Med.* **1999**, *27*, 916−921.
(35) Klys, H. S.; Whillis, D.; Howard, G.; Harrison, D. J. Glutathione S-transferase expression in the human testis and testicular germ cell neoplasia. *Br. J. Cancer* **1992**, *66*, 589−593.
(36) Cao, W.; Aghajanian, H. K.; Haig-Ladewig, L. A.; Gerton, G. L. Sorbitol can fuel mouse sperm motility and protein tyrosine phosphorylation via sorbitol dehydrogenase. *Biol. Reprod.* **2009**, *80*, 124−133.
(37) Frenette, G.; Lessard, C.; Sullivan, R. Polyol pathway along the bovine epididymis. *Mol. Reprod. Dev.* **2004**, *69*, 448−456.
(38) Szabo, Z.; Hamalainen, J.; Loikkanen, I.; Moilanen, A. M.; Hirvikoski, P.; Vaisanen, T.; Paavonen, T. K.; Vaarala, M. H. Sorbitol dehydrogenase expression is regulated by androgens in the human prostate. *Oncol. Rep.* **2010**, *23*, 1233−1239.
(39) Wang, J.; Wang, J.; Zhang, H. R.; Shi, H. J.; Ma, D.; Zhao, H. X.; Lin, B.; Li, R. S. Proteomic analysis of seminal plasma from asthenozoospermia patients reveals proteins that affect oxidative stress responses and semen quality. *Asian J. Androl.* **2009**, *11*, 484−491.
(40) Lefebvre, J.; Boileau, G.; Manjunath, P. Recombinant expression and affinity purification of a novel epididymal human sperm-binding protein, BSPH1. *Mol. Hum. Reprod.* **2009**, *15*, 105−114.
(41) Lee, C. H.; Wu, C. C.; Wu, Y. N.; Chiang, H. S. Gene copy number variations in Asian patients with congenital bilateral absence of the vas deferens. *Hum. Reprod.* **2009**, *24*, 748−755.
(42) Lu, Q.; Gore, M.; Zhang, Q.; Camenisch, T.; Boast, S.; Casagranda, F.; Lai, C.; Skinner, M. K.; Klein, R.; Matsushima, G. K.; Earp, H. S.; Goff, S. P.; Lemke, G. Tyro-3 family receptors are essential regulators of mammalian spermatogenesis. *Nature* **1999**, *398*, 723−728.
(43) Russell, L. D.; Clermont, Y. Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. *Anat. Rec.* **1977**, *187*, 347−366.
(44) Chemes, H. The phagocytic function of Sertoli cells: a morphological, biochemical, and endocrinological study of lysosomes and acid phosphatase localization in the rat testis. *Endocrinology* **1986**, *119*, 1673−1681.
(45) Pineau, C.; Le Magueresse, B.; Courtens, J. L.; Jegou, B. Study in vitro of the phagocytic function of Sertoli cells in the rat. *Cell Tissue Res.* **1991**, *264*, 589−598.
(46) Miething, A. Germ-cell death during prespermatogenesis in the testis of the golden hamster. *Cell Tissue Res.* **1992**, *267*, 583−590.
(47) Xiong, W.; Chen, Y.; Wang, H.; Wang, H.; Wu, H.; Lu, Q.; Han, D. Gas6 and the Tyro 3 receptor tyrosine kinase subfamily regulate the phagocytic function of Sertoli cells. *Reproduction.* **2008**, *135*, 77−87.