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Title: Investigation of human testis protein heterogeneity using two-dimensional electrophoresis

Running Head: Protein heterogeneity in human testis.

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Abstract

The testis is the male gonad responsible for spermatogenesis and male hormone secretion. The complicated processes of spermatogenesis and steroidogenesis determine the complexity of protein expression control in the testis. In this study, the heterogeneity of human testis proteins was investigated using two-dimensional gel electrophoresis (2-DE). A total of 847 protein spots corresponding to 462 unique proteins were identified successfully by mass spectrometry. Notable heterogeneity was evidenced by the presence of more than one spot with different MW and/or pI values for each of 180 different proteins. Analysis of the detected peptides of these proteins indicated that this heterogeneity was partly the result of alternative splicing and/or proteolysis. SP_PIR_Keywords analysis suggested that alternative initiation sites and various forms of posttranslational modification may also contribute toward this heterogeneity. Using Pro-Q Diamond phosphostain, 68 spots representing 52 proteins were stained, confirming the presence of phosphorylated forms of these proteins in the human testis. These data were used to establish a proteome reference database, which can be accessed over the Internet (http://reprod.njmu.edu.cn/2d). This database provides an initial reference map of the human testis and serves as a useful resource for comparative proteomics studies of the human testis under normal and pathological states. The abundant protein heterogeneity observed in this study and further investigation of its biological significance will contribute toward understanding protein expression regulation in the human testis and will generate insight into the molecular mechanism of spermatogenesis.

Keywords: 2-DE/ Human testis/Protein heterogeneity/Phosphostaining

Introduction

The testis is the primary male sex gland responsible for the production of sperm and the secretion of testosterone. Since germ cell expansion and differentiation involve many cellular changes and regulatory steps, the testis utilizes complex gene regulation mechanisms. Alternative splicing is believed to be an ideal mechanism for controlling gene expression at the transcriptional level in the testis (Walker, et al., 1999). Thirty percent of human expressed sequence tags (ESTs) in the testis were recorded to undergo alternative splicing, a frequency just less than the brain (Yeo, et al., 2004). Genome-wide analysis of ESTs showed that the testis has the greatest enrichment of tissue-specific splice forms (Xu, et al., 2002), which could be involved in various functions, including spermatogenesis and steroidogenesis (Carreau, et al., 2001, Hammes, et al., 2001, Venables, 2002). Our laboratory also used cDNA microarrays to investigate alternative splice variants related to human testis development and identified 74 novel alternatively spliced forms (Huang, et al., 2005). Similar to transcriptional control, translational control is also an important regulation mechanism in germ cells. Most mRNAs in late meiotic and haploid spermatogenic cells display high levels of free messenger ribonucleoprotein particles (mRNPs), indicative of a block against the initiation of translation (Kleene, 1996). Every mRNA is at least partially translationally repressed in meiotic and haploid spermatogenic cells; however, the extent of repression of individual mRNA species is regulated individually and varies greatly between different mRNA species (Kleene, 2003). Further, protein diversity is greatly increased by posttranslational protein modification, including phosphorylation, glycosylation, and acetylation (Phizicky, et al., 2003, Tyers and Mann, 2003). The phosphorylation and dephosphorylation of many proteins, for example, M

phase promoting factor (MPF), cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), protein kinase C (PKC), and protein kinase A (PKA), has been tightly related to the regulation of meiosis and sperm function (Breitbart, 2003, Fix, et al., 2004, Liguori, et al., 2005, Liu, et al., 2000). Therefore, the characterization of the protein expression profile of the
5 testis and the investigation of its protein heterogeneity will provide important clues regarding the process of spermatogenesis and the physiological function of the testis.

Proteomics research that focuses on the final gene products has the advantage of studying complex biological events and diseases, providing information that cannot be gained from pure gene sequencing. Recently, some studies have used proteomic technology to investigate the
10 process of spermatogenesis (Com, et al., 2003, Guillaume, et al., 2001, Guillaume, et al., 2001). *Huang et al.* also established a two-dimensional polyacrylamide gel electrophoresis (2-DE)-based porcine testis protein database (Huang, et al., 2005). However, with regard to the human testis, there is a lack of 2-DE-based proteome-wide study. 2-DE, as a classical proteomic technology that can separate thousands of proteins simultaneously, provides intact isomeric forms of proteins as a
15 result of alternative splicing and the combination of different posttranslational modifications (PTMs) (Mann and Jensen, 2003, Marko-Varga and Fehniger, 2004). This is an advantage that 2-DE holds over many other proteomic approaches (Marko-Varga and Fehniger, 2004).

In the present study, 2-DE was applied toward the analysis of the testes protein composition, thereby constructing the 2-DE protein reference map for the normal human testis. This map
20 contained 1908 protein spots. Altogether, 847 protein spots were identified by searching against the human International Protein Index (IPI) database, corresponding to 462 unique gene products.

The heterogeneity of proteins expressed in the human testis has been demonstrated by an average of 2 spots for each protein. Bioinformatics analysis and 2-DE following phosphorylation dye staining enabled the determination of the source of this heterogeneity. This database, which has been published on the website http://reprod.njmu.edu.cn/2d, should provide a useful resource
5 for studying the developmental biology and pathology of the human testis.

Materials and methods

Reagents

Urea, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), immobilized pH gradient strips (IPG strips), immobilized pH gradient buffer (IPG buffer), Tris, molecular weight
10 markers, acrylamide, diacrylamide, SDS, ammonium persulphate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED), iodoacetamide, glycerol were from GE Healthcare (Uppsala, Sweden). Thiourea, acetonitrile (ACN), ammonium bicarbonate (NH4HCO3), trifluoracetic acid (TFA), formaldehyde, matrix material (α-cyano-4-hydroxy cinnamic acid, α-HCCA) were from Sigma Chemical (St. Louis, MO, USA). HaltTM Protease Inhibitor
15 Cocktail, EDTA-free was from PIERCE (Rockford, IL, USA). Sequencing grade-modified trypsin (trypsin) was obtained from Promega (Madison, WI, USA). Peptide calibration standards were purchased from Bruker (Bruker Daltonik, Bremen, Germany). Dithiothreitol (DTT) was from Shenggong (Shanghai, China).

Testicular samples collection

20 Testis specimens were collected from three fertile men by biopsy after informed consent. Three healthy Chinese male volunteers between the ages of 28 and 40 yr, married with at least

one child, were recruited. These human studies were ratified by the ethics committees of Nanjing Medical University and were in accordance with national and international guidelines. All subjects gave written consent to participate in the studies after a well explanation of the study's purpose, benefit, and possible risks. The subjects had no significant medical history, and all had the physical examination and semen analysis during recruitment. None of the subjects was undernourished, as determined by history and physical examination. Before entering the experimental grouping, subjects also had three consecutive semen analysis at 2-wk intervals, which confirmed their semen were normal (sperm count >20 million/ml, motility>50%, oval forms>10%) according to the World Health Organization Manual for the Examination of Human Semen (WHO, 1999). For each biopsy, 120mg of tissue was collected and divided into 4 equal portions: one was fixed in Bouin's solution and used for morphological and immunohistochemical examination, another was stored in RNAlater Solution and the other two parts were frozen in liquid nitorgen and prepared for protein extraction.

Protein extraction and two dimensional gel electrophoresis

In this study, 30mg of each biopsy specimen was used. Protein lysates were isolated after crushing the tissues in liquid nitrogen with a mortar, and then were extracted with lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 2% (v/v) IPG Buffer pH3-10, NL and 1% (v/v) inhibitor protease cocktail. The extracts were centrifuged at 40,000Xg for 1hr, and the supernatants were stored at –70 °C. The protein concentration was estimated by the method of Bradford (Bradford, 1976) using BSA as the standard. Protein (120ug) from each sample was loaded by gel rehydration on 24-cm immobilized, pH 3-10, nonlinear gradient strips for 2-DE.

Separation was performed as previously described (Huo, et al., 2004). Isoelectric focusing was carried out in an IPGphor apparatus. Second dimension separation was done in 12% polyacrylamide gels using an Ettan-Dalt six system (GE Healthcare, San Francisco, CA, USA). 2-DE of each sample was performed once, gels were visualized by silver-staining according to the published procedure (Shevchenko, et al., 1996) except that glutaraldehyde was omitted in the sensitizing solution. Silver-stained gels were scanned and analyzed using 2D Elite Image Master software (GE Healthcare, San Francisco, CA, USA).

Phosphoprotein separation and detection

For phosphoprotein detection, 40ug of protein extract was separated by 2-DE and stained with Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR, USA). Briefly, the gel was fixed in 50% methanol / 10% acetic acid overnight, washed three times with deionized water, incubated in Pro-Q Diamond phosphoprotein gel stain for 90 min, and destained with three washes of 20% ACN in 50 mM sodium acetate (pH 4.0) and two washes with deionized water. Images were acquired using Typhoon Variable Model Imagels 9400 (GE Healthcare, San Francisco, CA, USA) with excitation at 532 nm and a 580 nm band pass emission filter. After scanning, the same gel was silver-stained to detect total protein spots and match to the micropreparative 2-DE gels.

Enzymatic in-gel tryptic digestion

Protein spots were excised, dehydrated in acetonitrile, and dried at room temperature. Proteins were reduced with 10mM DTT / 25 mM NH4HCO3 at 56°C for 1 h and alkylated with 55 mM iodoacetamide / 25mM NH4HCO3 in the dark at room temperature for 45 min in situ. Gel

pieces were thoroughly washed with 25mM NH4HCO3, 50% ACN, 100% ACN, and dried in a Speedvac. Dried gel pieces were reswollen with 2-3ul of trypsin (Promega, Madison, WI, USA) solution (10 ng/ul in 25 mM ammonium bicarbonate) at 4°C for 30min. Excess liquid was discarded and gel plugs were incubated at 37°C for 12 h. TFA was added to a final concentration of 0.1% to stop the digestive reaction.

Mass spectrometry

Digests were immediately spotted onto 400m anchorchips (Bruker Daltonics, Bremen, Germany). Spotting was achieved by pipetting 1 ul of analyte onto the MALDI target plate in duplicate and then adding 0.05ul of 2mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% TFA/ 33% ACN which contained 2mM ammonium phosphate.Bruker Peptide Calibration Mixture (containing Angiotensin_II, MH+1046.542; Angiotensin_I, MH+1296.685;Substance_P, MH+1347.735; Bombesin, MH+1619.822; Renin_Substate, MH+1758.932; ACTH 1-17 clip,MH+2093.086; ACTH 18-39 clip, MH+2465.198; Somatostatin 28 clip, MH+3147.471) was spotted down for external calibration. All samples were allowed to air dry at room temperature, and 0.1% TFA was used for on-target washing. All samples were analyzed in a time-of-flight Biflex IV mass spectrometer (Bruker Daltonics, Bremen, Germany), and the spectrometer was run in positive ion reflectron mode with the accelerating voltage of 19KV. Tandem mass spectra were given by an Ultraflex II mass spectrometer (Bruker Daltonics, Bremen,Germany) in LIFT mode.

Database queries and protein identifications

Mass spectra (m/z range 700-4000, resolution 10000-20000) were processed using the FlexAnalysis software (version 2.4, Bruker Daltonics). The parameters used were Peak detection

algorithm: SNAP (Sort Neaten Assign and Place), S/N threshold: 3.0, Quality Factor threshold: 50, and internal calibration: trypsin autodigestion peptides (trypsin_[108-115], MH+842.509; trypsin_[58-77], MH+2211.104). The masses detected frequently that arose from the matrix, trypsin, or known contaminants (e.g., keratins) were removed. The data were searched against
5 human protein IPI database (ipi.HUMAN.v3.30, downloaded from ftp.ebi.ac.uk/pub/databases/IPI, containing 67,922 entries) by an in-house MASCOT (version 2.1, Matrix Science) search engine. For PMF, search conditions included: mass accuracy set as 100 ppm, one missed cleavage allowed, alkylation of cysteine by carbamidomethylation as a fixed modification, and oxidation of methionine as a variable modification. Proteins with a confidence of > 95% and the number of
10 peptides to match ≥ 4 were considered to be identified. Wherever more than one protein identified as a match, they could be distinguished by: first, the pI and MW of the identified proteins could be compared to the gel location to confirm identity; second, a comparison of the peptides identified could show that one matched protein contains a greater number of peptides, or an extra peptide sequence not contained within the other. When the multiple matched proteins cannot be
15 distinguished by any of these methods, they would be recorded as physical redundancy or bioinformatics redundancy. If more than one protein were identified from the spectrum and marked as mixture (confidence value >95%) by Mascot Sever (detailed principle could be found at http://www.matrixscience.com/pdf/2003WKSHP2.pdf), they were considered as physical redundancy (co-migrating proteins). And if more than one protein with the same shared peptides
20 detected has the same Entrez gene ID, they were treated as bioinformatics redundancy (proteins not able to be distinguished). Additionally, the Mascot Score and expect of the first

non-homologous protein to the highest ranked hit was checked. Samples that cannot be unambiguously identified by PMF were then sequenced by tandem mass spectrometry. And some matched peaks chosen randomly from PMF were additionally sequenced by tandem mass spectrometry for verification. Each acquired MS/MS spectra were also processed using the
5 software FlexAnalysis v2.4 using a SNAP method set at a signal to noise ratio threshold of 3.0. For MS/MS spectra searching, the peak lists were submitted by Biotools (version 3.0, Bruker Daltonics) to Mascot Server for searching against human IPI database. Search parameters for MS/MS data: 100 ppm for the precursor ion and 0.3 Da for the fragment ions. Cleavage specificity and covalent modifications were considered the same as described for PMF spectrum analysis. And the
10 confidence greater than 95% was considered significant. All significant MS/MS identifications by Mascot were manually verified for spectral quality, and y and b ion series matches.

Bioinformatics analysis

To access the cause of protein heterogeneity, analysis of the proteins corresponding to two or more than two spots were performed using the program: Database for Annotation, Visualization,
15 and Integrated Discovery (DAVID) 2007 (Dennis, et al., 2003). DAVID is a Web-based, client/server application that allows users to access a relational database of functional annotation. Entrez gene IDs were submitted to DAVID for analysis. The parameters used were SP_PIR_Keywords in the Functional Categories section.

An analysis of diseases associated with proteins that were identified in our human testis
20 proteome was performed using Pathway Studio (v5.0) software (Ariadne Genomics, Inc. Rockville, MA, U.S.A.). The text-mining software uses a database assembled from scientific abstracts and a

manually curated dictionary of synonyms to recognize biological terms (Nikitin, et al., 2003). The Entrez Gene IDs of the identified proteins were imported to Pathway Studio software, and each identified relationship was confirmed manually using the relevant PubMed/Medline hyperlinked texts.

5 ***Construction of an online database***

To construct a human testis 2-DE database, we used the web-based system, Make2D-DB II Package (ver.2.50.1), an HTML generator for 2D images through ExPASy (http://www.expasy.org/ch2d/make2ddb.html). We stored the identified protein spots in a relational database that was made accessible online via a common gateway interface (cgi) script on a
10 webserver (http://reprod.njmu.edu.cn/2d). We hyperlinked the individual protein entries to the relevant spots on the silver-stained and phosphorylated image maps created from the reference gel.

Results

15 ***Separation of testis proteins***

Human testes from three men were examined by HE staining and all had normal spermatogenesis (Fig. 1). The protein was extracted from these human testis samples, and separated by 2-D gels individually. The protein spots were visualized following silver staining. IPG strips with a nonlinear gradient from pH 3 to 10 were used. These strips contain a shallow pH
20 gradient between pH 5–7, thereby improving the resolution in that pI range. The combination of these strips with large format SDS gels for second-dimension analysis provided an overview of the

distribution of the human testis proteins. To address sample similarity, all 3 gels were analyzed using Imagemaster. Abundant and visible spots were obtained on all the gels. And 1542 spots were consistently detected in all the 3 gels. The correlation coefficient of these 1542 spots between the gel replicates was 0.87. One 2-D map (Fig. 2) was chosen to be representative of the high resolution and highly reproducible 2-D pattern of the human testis proteome, and was used as a reference map. A total of 1908 spots were detected on the sample map.

Protein identification

Due to variation of some protein spots among gels from different human testis, only the 1542 protein spots that were consistently detected in all 3 gels were excised and processed for identification. In total, 847 spots were identified (indicated in Fig. 2). The corresponding identified spots on the two other gels were shown in Supplementary Fig. 1A-B. The identification information for each spot, including the spot ID, protein name and IPI ID, corresponding Entrez gene ID and gene symbol, theoretical and observed pI and MW values, matched peptide numbers, sequence coverage, and the root mean square error of matched peptide masses, are listed in Supplementary Table I. Since one protein may have multiple IPI IDs, we count the proteins by their corresponding Entrez gene IDs, all the identified spots correspond to 462 distinct gene products. In many cases, multiple IDs were assigned for one protein due to the presence of protein isoforms which cannot be distinguished based on shared peptides detected. They were treated as bioinformatics redundancy, and were indicated in Supplementary Table I. Protein that has one IPI ID was inspected manually, one specific protein isoform was reported only when at least one unique peptide was identified.

Protein heterogeneity

A large number of testis proteins showed heterogeneity, with each protein represented by more than one spot. Protein heterogeneity was observed at regions where multiple spots representing the migration of one protein to different MW regions and/or pI. For 181 proteins, more than 2 spots were detected. And 24 proteins were identified to have more than 5 spots. For example, the spots 149, 200, 218, 219, 223, 245, 337, 357, 1535, and 1569 all matched the HSPA5 protein (GRP78); the spots 149, 200, 218, 219, 223, 245, 337, and 357 had similar MW that corresponded with the theoretical MW of HSPA5, and the peptides detected by MALDI-TOF MS were distributed throughout the protein sequence (the peptides detected and the representative spectrum are shown in Fig. 3A and the upper panel of Fig. 3B). The other 2 spots (spot 1535 and 1569) had MW lower than the theroretical value of HSPA5. The peptides detected in spot 1535 had a V51 residue closest to the N-terminal and an R215 residue closest to the C-terminal (Fig. 3A and the central panel of Fig. 3B). For spot 1569, the residue closest to the N-terminal was N48, while the residue closest to the C-terminal was K353 (Fig. 3A and the lower panel of Fig. 3B). Protein heterogeneity was also observed in the case of α-enolase. It was distributed to 8 different locations on the 2D gel (spots 859, 879, 883, 899, 925, 928, 935, and 956) with similar MW and pI ranging from 6.3 to 7.4.

An SP_PIR_Keywords analysis was performed for testis proteins with at least two spots identified on the gel to investigate the potential cause of the heterogeneity. Among them, 5 proteins were annotated to have at least 2 isoforms due to the usage of alternative initiation codons. Besides mRNA structure regulation, the PTMs of proteins can also cause protein

heterogeneity. The SP_PIR_Keywords analysis showed that 39 of these proteins can be acetylated, and 50 proteins have the potential to be phosphorylated (see Table I). Several proteins can have multiple PTMs, suggesting the complexity of their function regulation.

Phosphoproteome map of human testis

5 Based on the unexpected protein heterogeneity of the human testis and the result of the Keywords analysis that indicated that phosphorylation was a common protein modification, the Pro-Q Diamond phosphoprotein gel stain was used to construct the phosphoproteome map of the human testis, as shown in Fig. 4. Mapping to the silver-stained entire testis proteome, 68 identified spots representing 52 unique proteins were positively stained with the Pro-Q Diamond dye
10 (indicated in Supplementary Table I). It is to be noted that 9 of these proteins had more than 1 phosphorylated spots on the gel. An example is lamin A/C. A total of 15 spots matching lamin A/C were identified at 2 MW levels and at differing orders of pI. Among them, as shown in Fig. 5, 9 spots with higher MW were stained by the phosphoprotein stain. Additionally, we analyzed the 181 proteins which had more than one spots on the silver-stained gel each and thus had heterogeneity,
15 and found that 35 of these proteins had corresponding spots stained to be phosphorylated by Pro-Q Diamond dye.

Creation of 2-D reference database

The protein identification data were used to construct a web-based reference database of the human testis. The database can be found at our laboratory's website (http://reprod.njmu.edu.cn/2d)
20 or can be linked from the WORLD-2D PAGE index (http://us.expasy.org/ch2d/2d-index.html). The database fulfils the federated 2-DE reference database criteria. Instructions for accessing the

testis database are provided on the home page. The protein spots can be accessed by means of a hyperlinked gel image. Each protein entry includes the accession number, protein name, description, spot ID number, organism, pI, molecular weight, and mass spectrum data as well as links to relevant entries in other online databases.

5 ***Intersection between human testis and human sperm proteome***

The proteins identified in this profile were compared with recently published proteins that are expressed in human sperm (Baker, et al., 2007, Martinez-Heredia, et al., 2006, Yoshii, et al., 2005). The comparison identified 202 proteins common in human testis and human sperm (annotated in Supplementary Table I), accounted for 43.7% of our human testis proteome. Among them, 92

10 proteins had more than one spots on the 2-D gel of human testis.

Analysis of the involvement of proteins identified in the human testis proteome in diseases

To get a better understanding of the human testis proteome, a detailed analysis of the diseases associated with these proteins was performed using Pathway Studio software. The results showed that 27 proteins were related to infertility, 5 proteins were related to azoospermia,

15 and 4 proteins were related to testis cancer (Fig. 6).

Additionally, we compared our human testis proteome with other proteomics studies. Proteomics has already been used to study male fertility. *Huo et al.* (Huo, et al., 2008) compared the protein expressional profiles of human testes of fertile men and azoospermic patients using a proteomic approach by combining 2D-E analyses and mass spectrometry. 10 proteins were

20 identified to be differentially expressed between the normal fertile human testes and those of azoospermic patients. Comparing with our human testis proteome, we found that all the ten

differentially expressed proteins were identified in our proteome. Additionally, *Zhao et al.* (Zhao, et al., 2007) compared sperm protein expression profiles in asthenozoospermic patients with that of normozoospermic donors, and identified 10 differentially expressed proteins, of which 7 were also identified in our testis proteome.

5 **Discussion**

In the present study, a large percentage of the human testis proteins demonstrated heterogeneity and were represented by more than one spot on the 2D gel. Based on our data, we estimate that, on average, one human testis gene product is represented by approximately 2 spots. In fact, the heterogeneity observed in the 2D gel is probably higher because not all possible spots 10 that represent the various proteins were identified successfully. Heterogeneity may partly be due to artifacts resulting from experimental techniques, such as the carbamylation of proteins upon prolonged contact of the sample with urea (McCarthy, et al., 2003). The visualization of multiple spots representing one gene product also suggests the ability of 2D gels to detect different splicing, processing, and PTMs, which result in an alteration of the MW or pI of the polypeptides and 15 consequently of the focusing position (Garbis, et al., 2005).

The testis is an organ enriched in alternative splicing. In this study, we also identified different splicing forms of one gene product. For example, ACP1, named as low molecular weight protein tyrosine phosphatase, is an 18-kDa enzyme that is distantly related to other protein tyrosine phosphatases. The single gene of ACP1 is known to undergo an alternative splicing event 20 resulting in 3 isoforms (Tailor, et al., 1999, Wo, et al., 1992). In the present study, we have detected 2 spots representing ACP1—spots 1789 and 1927; the latter spot is more mobile during

electrophoresis. Tryptic fragments of the spots 1789 and 1927 matched specific sequences of isoform 1 and isoform 2, respectively; the 2 isoenzymes utilize different exons (exons 3 and 4) and are expected to possess distinct substrate specificities (Wo, McCormack, Shabanowitz, Hunt, Davis, Mitchell and Van Etten, 1992). Another example is hnRNPA2/B1; 6 spots on the 2D gel of
5 the human testis matched hnRNPA2/B1. In 5 of these spots, isoforms A2 and B1 could not be distinguished. However, in spot 1379, the specific peptide of isoform B1 was detected, thereby confirming the isoform. Similar results were obtained in the spots that matched lamin A/C. A total of 15 spots corresponding to lamin A/C were identified, and these spots were dispersed on the 2D gel at 2 different MW levels. Ten spots (spots 237, 238, 247, 252, 255–257, 259, 260, and 277)
10 with a similar MW of approximately 76 kDa and with pI ranging from 6.39 to 6.91 were identified. Analysis of these spots confirmed the presence of isoform A in a few spots; however, in certain spots, isoform A and isoform ADelta10 could not be distinguished. Isoform ADelta10 is an alternative splicing product of the lamin A/C gene cloned from lung adenocarcinoma cell line. This product differs from lamin A in the absence of a 5′ region corresponding to exon 10 that was
15 predicted to result in a shift in the MW of the protein from 74 kDa to 70 kDa and a shift in pI from 7.4 to approximately 8.6 (Machiels, et al., 1996). Considering the different MW and pI of the 2 isoforms, we estimated that the 10 spots that matched Lamin A/C corresponded to isoform A. For the other 5 spots (spot 392, 408, 419, 451, and 455), only peptides that were common between isoform A, isoform ADelta10, and isoform C were detected; however, their low MW of
20 approximately 65 kDa enabled us to designate them as isoform C of lamin A/C. Abundant alternate transcripts in the testis may expand the functions of the testes proteins and play an

essential role in spermatogenesis.

Protein heterogeneity could also be a consequence of PTMs. An SP_PIR_Keywords analysis of proteins with more than 1 spot indicated that 50 proteins could be phosphorylated, while 39 proteins could have acetylated forms. Reversible protein phosphorylation is one of the most common and important covalent PTMs and is involved in the regulation of almost all fundamental cellular activities such as gene transcription, cell-cycle progression, cell division and proliferation, energy storage, apoptosis, and is also important in spermatogenesis (Hubbard and Cohen, 1993, Kierszenbaum, 2006) Based on these knowledge, we used a Pro-Q diamond phosphoprotein gel stain to obtain the phosphoproteome of the human testis; among the identified spots, a total of 68 spots were stained, corresponding to 52 proteins. Among these proteins, the presence of lamin A/C is conspicuous. Nine of the 15 spots matching lamin A/C were stained with the Pro-Q Diamond stain. These spots with a MW of approximately 76 kDa were considered as isoform A of lamin A/C. Lamins are intermediate filament-type proteins that form a major component of the nuclear lamina, a filamentous meshwork associated with the inner nuclear membrane (Moir and Goldman, 1993, Nigg, 1992). In cells undergoing mitosis, cyclin-dependent kinases mediate the phosphorylation of lamins A, B, and C. This event follows the interphase phosphorylation of lamins by PKC (Buendia, et al., 2001). Lamin phosphorylation is a key event in the mitotic breakdown of the nuclear lamina (Lee, et al., 2000). Lamin A/C is an A-type lamin that has been detected in spermatocytes. *Lmna*$^{-/-}$ mice display impaired spermatogenesis, with a significant accumulation of spermatocytes I during the early stages of prophase I; however, pachytene spermatocytes are severely defective, particularly in the synaptic pairing of the sex chromosomes, leading to massive

apoptosis during the pachytene stage of meiosis I (Alsheimer, et al., 2004). These results revealed that the A-type lamin also plays an important role in meiosis. The multiple phosphorylated forms of lamin A detected in the human testis suggested that phosphorylation is an important mechanism regulating lamin A/C function in the human testis and may play a crucial role in meiosis. However,
5 further studies are required to verify this hypothesis.

Although we are unaware of the origin or biological significance of most of the observed heterogeneities, similar results have been reported in other studies. HSPA5 matched 10 spots on the 2D gel of human testis. The MW of 2 spots was much lower than their theoretical value, and the peptides detected on these 2 spots confined to the N-terminal sequence of HSPA5. The
10 prediction of the MW based on the detected sequence was in accordance with the observed MW. A similar phenomenon was observed in human pancreatic tissue. HSPA5 was also identified in 3 gel spots with a MW lower than that of this protein; 2 spots had peptides confined to the N-terminal of the protein, and 1 spot showed peptides confined to the C-terminal of the protein. Thus, these spots were considered to correspond to the protein fragments of HSPA5 resulting from limited
15 proteolysis (Person, et al., 2006). According to our previous study, HSPA5 is closely associated with spermatogenesis (Huo, Zhu, Ma, Lin, Zhou and Sha, 2004), but the biological significance of its fragments in the human testis requires further investigation. Eight spots that were identified as the α-enolase protein migrated to the regions of similar MW and different pI and were not stained by the phosphoprotein stain. Similar distribution pattern was observed in the 2-DE of rat mesangial
20 cells, and the 4 spots identified as α-enolase were also not stained with the Pro-Q Diamond stain (Jiang, et al., 2005). This indicates that α-enolase underwent a certain form of posttranslational

processing or modification but not phosphorylation.

In this study, we identified 202 proteins which were also expressed in the human sperm (Baker, Reeves, Hetherington, Muller, Baur and Aitken, 2007, Martinez-Heredia, Estanyol, Ballesca and Oliva, 2006, Yoshii, Kuji, Komatsu, Iwahashi, Tanaka, Yoshida, Wada and Yoshimura, 2005), suggesting their roles in spermatogenesis. As 92 of the 202 proteins also exhibited heterogeneity in human testis, they maybe play roles by some specific functional forms. For example, HSPA2 (Heat shock 70 KDa protein 2), a testis-specific member of the HSP70 family, has unique functions in spermatogenic cells. Besides the important role in meiosis (Allen, et al., 1996, Dix, et al., 1996, Dix, et al., 1997, Zhu, et al., 1997), recently HSPA2 acquires a new function as a chaperone of spermatid-specific DNA packaging proteins, and hence could be considered as the first identified factor controlling the histone to TP transition (Govin, et al., 2006). The central roles of HSPA2 have also been established, as the expression level of this protein was related to sperm cellular maturity, DNA integrity, chromatin maturity, chromosomal aneuploidy frequency and sperm functions, including fertilizing potential (Cayli, et al., 2003). HspA2 ratios of <10% in the diminished sperm maturity range predict the failure to cause pregnancies by IVF (Ergur, et al., 2002). Further, the detected over-expression of the HSPA2 protein in asthenozoospermic samples could be related to the dysfunction of motility (Martinez-Heredia, et al., 2008). Although HSPA2 has multiple roles in spermatogenesis and sperm function, its heterogeneity in testis has not been reported. In this study, 7 spots on this 2D gel were identified to be HSPA2, with different MW and pI. One of them was stained with phosphorstaining. It indicated that HSPA2 may be regulated by phosphorylation modification.

Pathway Studio analysis of the human testis proteome showed that 27 proteins were annotated to be related to infertility, including HSPA2 with diverse heterogeneity, and GPX4. GPX4 is a glutathione peroxidase with 2 spots (spot 1884 and spot 1778) having different pIs and MWs in the proteome. It can catalyze the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides by reducing glutathione, and functions in the protection of cells against oxidative damage. Previous studies showed that PHGPx may be important in many aspects such as structural stability of sperm chromatin (Conrad, et al., 2005), related to oligoasthenozoospermia (Diaconu, et al., 2006) and linked to male fertility (Foresta, et al., 2002). This proteome contains an extensive repertoire of proteins important for testis function, and their dysfunctions may be related to certain diseases.

In the comparison between human testes of fertile and azoospermic patients, all the differentially expressed proteins were identified in our proteome. And in the study of human sperm, 7 of 10 differentially expressed proteins between sperm of normozoospermic donors and asthenozoospermic patients were identified in our testis proteome. Thus, many of the proteins involved in male infertility such as azoospermia and asthenozoospermia were identified in our human testis proteome. This proteome could provide a reference for future proteomics study of male fertility using 2-DE. And the technology of 2-DE followed by mass spectrometry identification can be a promising technology to study the mechanisms of defects of testicular function.

In conclusion, the first reference 2-DE maps of the human testis stained with silver or Pro-Q Diamond dye specific for phosphoproteins have been established in this study. The different splicing and phosphorylated forms observed in this study will provide a useful resource enabling

the understanding of the regulatory mechanism underlying the functions of the human testis proteins. Other posttranslational processing and modification mechanisms indicated by protein heterogeneity and shifts in protein location are of biological significance and need to be studied in further detail to facilitate better understanding. And such a 2-DE database of the human testis will be valuable for the comparative proteomics analysis of normal and pathological human testis and can enable the identification of the functional forms of specific proteins related to spermatogenesis and steroidogenesis.

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Table 1. Analysis of the proteins with at least 2 spots identified in the human testis 2-D proteome according to SP_PIR_Keywords analysis

Term	Entrez Gene ID*	Count
Alternative initiation	2023; 2271; 2879; 2934; 25824	5
Acetylation	52; 216; 231; 301; 302; 759; 873; 1072; 2271; 2539; 2782; 3043; 3187; 3925; 3945; 3956; 3958; 4670; 5230; 5315; 5478; 5573; 6624; 6876; 6950; 7001; 7169; 7415; 7416; 7532; 9961; 10487; 10606; 10694; 10963; 23576; 51056; 51181; 3039/3040	39
Phosphorylation	52; 132; 226; 301; 302; 1072; 1809; 1938; 2023; 2288; 2539; 2597; 2664; 2934; 3181; 3187; 3190; 3308; 3312; 3315; 3320; 3329; 3615; 3925; 3945; 3958; 4000; 4735; 5052; 5093; 5230; 5315; 5515; 6624; 7178; 7414; 7415; 7416; 7430; 7431; 7532; 8880; 9184; 9588; 9948; 9961; 10376; 10694; 10963; 84823	50

*The analysis is based on unique Entrez Gene IDs of the identified proteins in human testis proteome.

5

Figure legends

Figure 1. HE staining of normal human testis used

Morphologies of normal human testes used were stained using hematoxylin/eosin staining. The histologically normal human testis used for 2-DE analysis was shown, and the spermatogenesis proceeds in an orderly manner.

Figure 2. Reference 2-DE map of human testis.

The silver-stained reference 2-DE map of human testis proteins. The identified spots are indicated with gray asterisks.

Figure 3. Identification information of HSPA5 protein.

A). The sequence of the human HSPA5 protein with peptides identified by MALDI-TOF MS in spots 218, 1535, and 1569. Bold format, peptides visualized in spot 218; solid underline, peptides detected in spot 1535; italic format, peptides observed in spot 1569. B). MALDI spectra of HSPA5 spots 218, 1535, and 1569. The matched peaks were labeled with m/z values and the “start” and “end” positions of the peptides in the HSPA5 sequence.

Figure 4. Phosphoproteome of the human testis.

The phosphorylated forms of proteins stained with the Pro-Q Diamond dye are visualized as green fluorescence.

Figure 5. Enlarged region of the human testis 2D gel.

A pseudocolor was used in panel A. Red signal represents silver staining; green signal indicates the spots stained with Pro-Q Diamond phosphostain; a yellow color represents an overlap of these 2 signals. The corresponding region of the silver-stained gel is shown in panel B; the spots

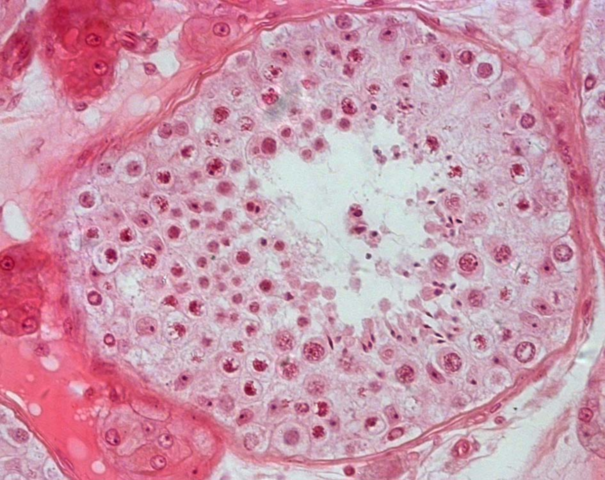
identified as lamin A/C protein are indicated with arrowheads, and the red arrowheads indicate that the spots were stained by the Pro-Q Diamond phosphostain.

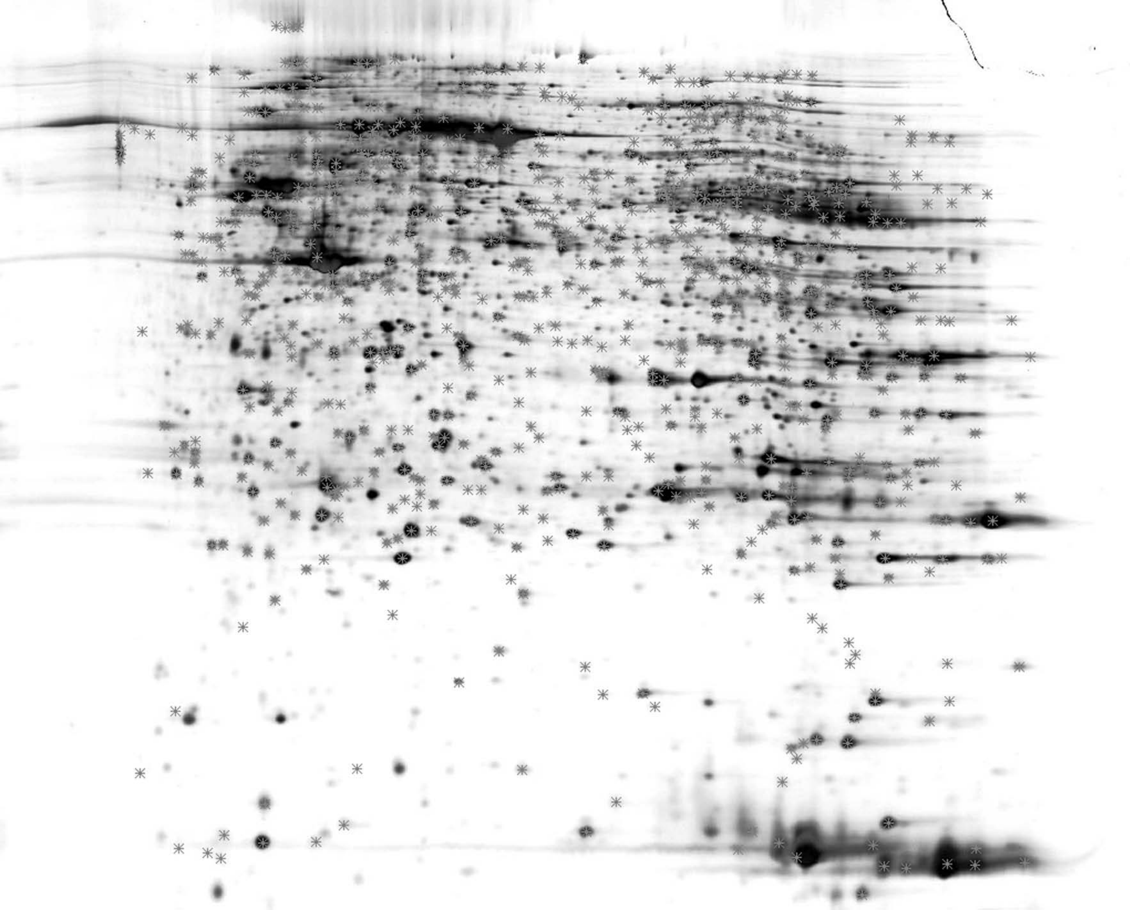
Figure 6. The involvement of proteins from the human testis proteome in diseases

The involvement of proteins from the human testis proteome in diseases was analyzed using the

5 Pathway Studio software. Proteins are shown as ovals; regulated diseases, by squares. Regulation relationships are indicated by arrows and documented by literature citations.

10

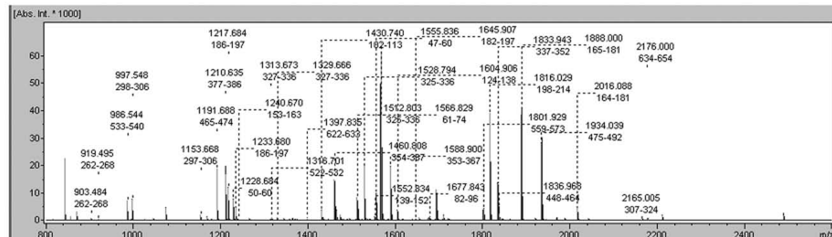


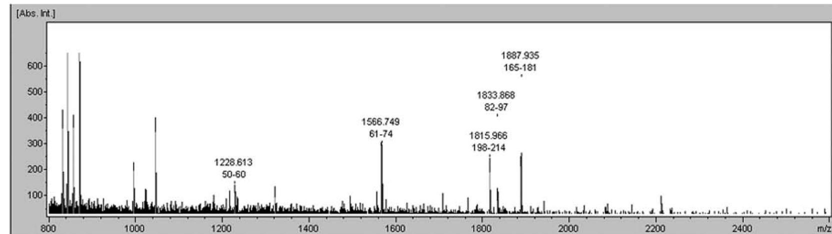


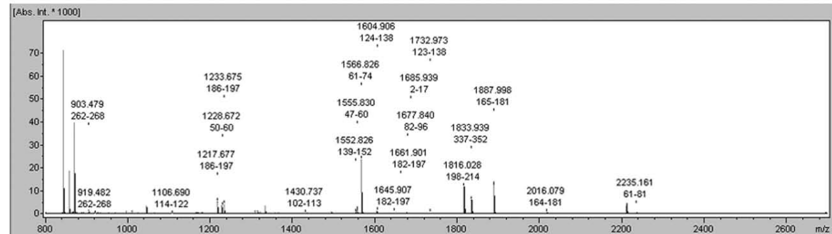
(A)

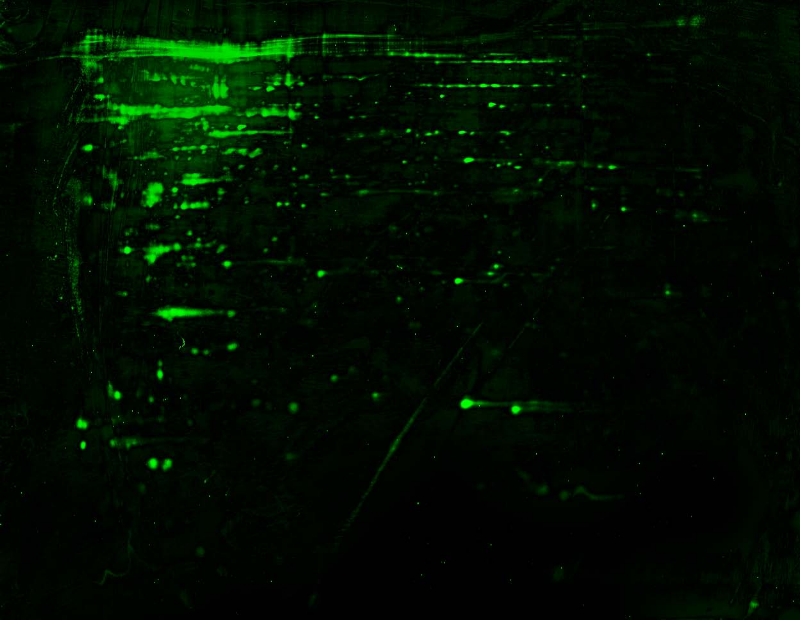
1 MMKFTVVAAA LLLLGAVRAE EEDKKEDVGT VVGIDLGTTY SCVGVFKNGR
51 VEIIANDQGN RITPSYVAFT PEGERLIGDA AKNQLTSNPE NTVFDAKRLI
101 GRTWNDPSVQ QDIKFLPFKV VEKKTKPYIQ VDIGGGQTKT FAPEEISAMV
151 LTKMKETAEA YLGKKVTHAV VTVPAYFNDA QRQATKDAGT IAGLNVMRII
201 NEPTAAAIAY GLDKKEGEKN ILVFDLGGGT FDVSLLTIDN GVFEVVATNG
251 DTHLGGEDFD QRVMEHFIKL YKKKTGKDVR KDNRAVQKLR REVEKAKRAL
301 SSQHQARIEI ESFFEGEDFS ETLTRAKFEE LNMDLFRSTM KPVQKVLEDS
351 DLKKSDIDEI VLVGGSTRIP KIQQLVKEFF NGKEPSRGIN PDEAVAYGAA
401 VQAGVLSGDQ DTGDLVLLDV CPLTLGIETV GGVMTKLIPR NTVVPTKKSQ
451 IFSTASDNQP TVTIKVYEGE RPLTKDNHLL GTFDLTGIPP APRGVPQIEV
501 TFEIDVNGIL RVTAEDKGTG NKNKITITND QNRLTPEEIE RMVNDAEKFA
551 EEDKKLKERI DTRNELESYA YSLKNQIGDK EKLGGKLSSE DKETMEKAVE
601 EKIEWLESHQ DADIEDFKAK KKELEEIVQP IISKLYGSGG PPPTGEEDTS
651 EKDEL

(B)



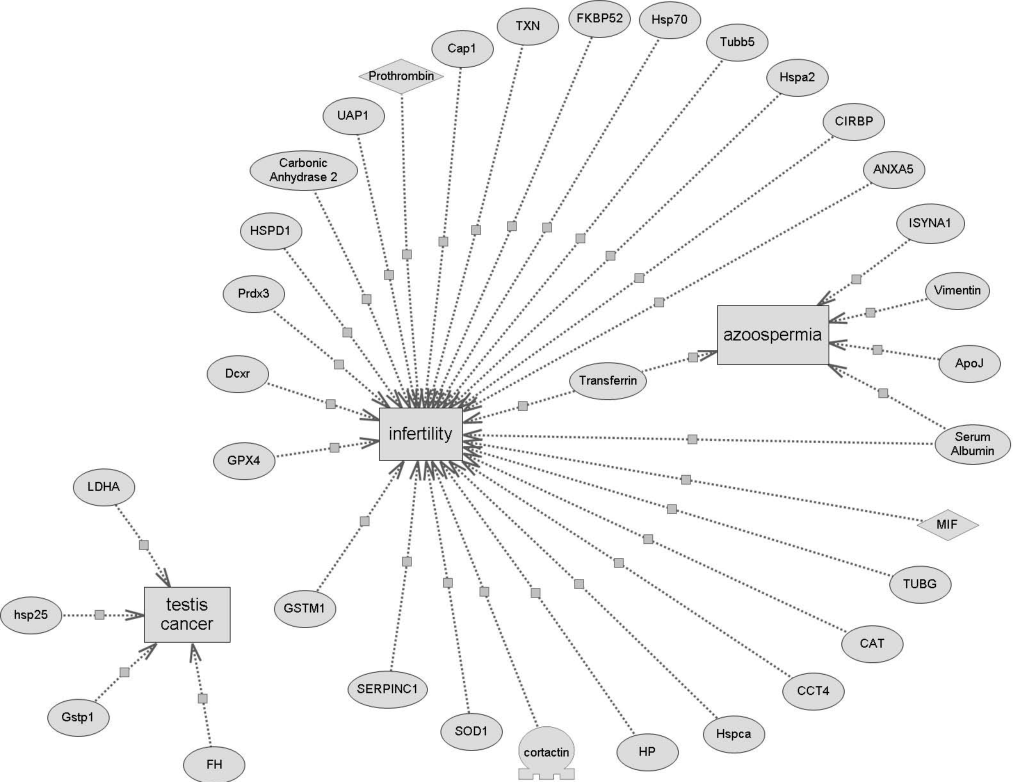






(A)

(B)



infertility
azoospermia
testis cancer
TXN
FKBP52
Hsp70
Cap1
Tubb5
Prothrombin
Hspa2
UAP1
CIRBP
Carbonic Anhydrase 2
ANXA5
HSPD1
ISYNA1
Prdx3
Vimentin
ApoJ
Dcxr
Transferrin
GPX4
Serum Albumin
LDHA
MIF
hsp25
GSTM1
TUBG
CAT
Gstp1
SERPINC1
CCT4
FH
SOD1
cortactin
HP
Hspca