Mapping of the Human Testicular Proteome and its Relationship With That of the Epididymis and Spermatozoa*

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The testis produces male gametes in the germinal epithelium through the development of spermatogonia and spermatocytes into spermatids and immature spermatozoa with the support of Sertoli cells. The flow of spermatozoa into the epididymis is aided by testicular secretions. In the epididymal lumen, spermatozoa and testicular secretions combine with epididymal secretions that promote sperm maturation and storage. We refer to the combined secretions in the epididymis as the sperm-milieu. With two-dimensional-PAGE matrix-assisted laser desorption ionization time-of-flight MS analysis of healthy testes from fertile accident victims, 725 unique proteins were identified from 1920 two-dimensional-gel spots, and a corresponding antibody library was established. This revealed the presence of 240 proteins in the sperm-milieu by Western blotting and the localization of 167 proteins in mature spermatozoa by ICC. These proteins, and those from the epididymal proteome (Li et al., 2010), form the proteomes of the sperm-milieu and the spermatozoa, comprising 525 and 319 proteins, respectively. Individual mapping of the 319 sperm-located proteins to various testicular cell types by immunohistochemistry suggested that 47% were intrinsic sperm proteins (from their presence in spermatids) and 23% were extrinsic sperm proteins, originating from the epididymis and acquired during maturation (from their absence from the germinal epithelium and presence in the epididymal tissue and sperm-milieu). Whereas 408 of 525 proteins in the sperm-milieu proteome were previously identified as abundant epididymal proteins, the remaining 22%, detected by the use of new testicular antibodies, were more likely to be minor proteins common to the testicular proteome, rather than proteins of testicular origin added to spermatozoa during maturation in the epididymis. The characterization of the sperm-milieu proteome and testicular mapping of the sperm-located proteins presented here provide the molecular basis for further studies on the production and maturation of spermatozoa. This could be the basis of development of diagnostic markers and therapeutic targets for infertility or targets for male contraception. Molecular & Cellular Proteomics 10.3 10.1074/mcp.M110.004630, 1–11, 2011.

Two contradictory global issues are of serious current concern. On the one hand, aging populations, largely caused by a fall in birth rates, aggravate social burdens in developed countries. On the other, overpopulation is straining the available resources in the developing world and threatening the sustainable environment of the planet, partly because of the lack of convenient methods of family planning. These problems, which lie on each side of the same reproductive coin, can be tackled by increasing knowledge of the biological regulation of fertility, in particular that of men, so that fertility can either be prevented or promoted, depending on the particular demographic situation.

The testis is the key organ for male reproduction, providing an irreplaceable biological function that enables procreation of the species by the production of the male gamete. An adult testis comprises unique cell types for the endocrine and exocrine activities responsible for complete and efficient spermatogenesis. The two major cell types for the endocrine function are Sertoli cells in the seminiferous tubules, secreting peptide hormones, and Leydig cells in the interstitium, producing androgens. For spermatogenesis, Sertoli cells also provide structural and functional support to the germ cells developing in the germinal epithelium, which begin as spermatogonia at the base of the tubules, form spermatocytes as they undergo meiosis, and give rise to haploid round spermatids, which differentiate into elongated spermatids ready to be released into the tubule lumen as immature spermatozoa (1). These testicular spermatozoa still have to undergo maturation during their passage through the epididymis before they become fully competent for natural fertilization as ejaculated spermatozoa (2). Immature spermatozoa released into the seminiferous tubular lumen pass into the epididymis in their native testicular fluid, which is gradually modified during passage through the convoluted epididymal tubule by the absorptive and secretory activities of the efferent duct and epididymal epithelia. Maturation of spermatozoa occurs while...
they are bathed in this unique sperm-milieu in the epididymal lumen (3).

In our recent study of the human epididymal tissue and fluid proteomes by two-dimensional-gel MALDI-TOF MS/MS (4), reverse transcription (RT)-PCR analysis indicated the expression of the genes of some luminal fluid proteins by the testis, raising the question of the possible contribution of the testis to the sperm-milieu for maturation. With the advance of cDNA microarrays, databases of gene expression in the different testicular cell types have been constructed and published online for rats and mice (http://mrg.genetics.washington.edu/). On the other hand, a global transcriptome of the normal adult human testis is still lacking, although differences among pathological cases have been reported, albeit without information on the individual patterns of cellular expression (5, 6). There are publications on whole testicular proteomes of pigs (7), mice (8–10) and rats (11). More recently a human testicular proteome (12, 13) and proteomic changes associated with contraceptives usage (14) have been published. However, the cellular localization of these proteins is largely unknown.

Because different cell types are functionally distinct, a protein distribution map in this context is crucial for establishing the cellular basis for the highly diversified functions of the testis. We present in this paper the result of a comprehensive undertaking to map the proteome of human adult testes. Obtained from accident victims of proven fertility, with emphasis on proteins that are also found in mature spermatozoa. We also define a common subset of proteins found in epididymal fluid and mature spermatozoa in an attempt to shed light on the proteome in the sperm-milieu for maturation. This was done by continuing and completing the screening of nonabundant proteins present in this sperm-milieu by Western blotting. The relationship among these proteins is largely unknown.

EXPERIMENTAL PROCEDURES

**Experimental Design**—The experimental protocol was to extract proteins from adult testes from four accident victims of proven fertility for separation by two-dimensional-PAGE and analysis by their MALDI-MS spectra. Antibodies were raised against the identified nonstructural proteins and their identities confirmed by Western blotting. These antibodies were used to detect expression of any such proteins in functional ejaculated spermatozoa by immunofluorescence cytochemistry, and to study in which testicular cell types they were localized by immunohistochemistry. To explore the possibility of any contribution of testicular proteins to the microenvironment for sperm maturation in the epididymis, and to extend the proteomic identification of this epididymal fluid initiated in our previous study (4), the newly raised antibodies were used for further screening of nonabundant proteins present in this sperm-milieu by Western blotting. Proteomic and gene ontological analysis were performed on the three proteomes, namely those of the testis, mature spermatozoa and the sperm-milieu for maturation. The relationship among these proteomes and that of the epididymal proteome previously published was analyzed, and the protein subsets common to the published partial proteomes of human epididymal fluid and ejaculated spermatozoa were highlighted.

**Adult Human Testes and Ejaculated Spermatozoa**—Testes from four accident victims aged 27–32 y were obtained from YuHuangDing Hospital with permission from the Ethics Committee of the hospital. These men had records documenting a history of fatherhood and no disease of the reproductive system, and informed consent from family members for the donation of organs for medical research. Both testes were immediately removed after death and preserved after cleaning of fat, connective tissues, and blood. Tissue aliquots from each testis were deep-frozen at −70 °C for later protein extraction, snap-frozen for RT-PCR and fixed in Bouin’s fixative for histological processing. Isolation and preparation of sperm-free epididymal luminal fluid was as previously described (4).

Permission was obtained from the YuHuangDing Hospital Ethics Committee to use ejaculates from young men for research purposes. Ejaculated spermatozoa were obtained from the semen of 20 healthy young men (23–30 years old) giving informed consent for the use of their spermatozoa for research. Ejaculates were produced by masturbation at the Hospital after 3–7 days of sexual abstinence. Only normozoospermic samples, according to the World Health Organization criteria (15), were used for the study.

**Testicular Protein Extraction**—Stored tissue aliquots of equal weights from the four donors were pooled before processing. Mincing tissue was frozen in liquid nitrogen and ground to powder, taken up 1:6 (w/v) in lysis solution (BioRad: 7 M urea, 2 M thiourea, 4% (w/v) 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid and 65 mm dithiothreitol) at 4 °C, sonicated for 5 min, shaken for 1 h and centrifuged at 20,000 × g for 1 h. Proteins in the supernatant were precipitated by mixing it with 4 volumes ice-cold acetone and allowing it to stand at −20 °C for 1 h. After being pelleted at 12,000 × g for 1 h, washed with 90% (v/v) acetone and dried, the proteins were taken up in 2 ml lysis solution and stored at −80 °C. Proteins in epididymal fluid were extracted as previously described (4).

**Gel Electrophoresis, Mass Spectrometric Analyses and Protein Identification**—Two-dimensional gel electrophoresis was performed as previously described (16). Before the second dimensional electrophoresis, nonlinear pH 3–10 IPG strips (18 cm) used for one-dimensional isoelectric focusing were equilibrated in two steps: reduction with dithiothreitol and carboxymethylation with iodoacetamide. The equilibrated strips were run on 12% (w/v) SDS-PAGE at 25 mA per gel and stained with Coomassie brilliant blue R-350 (Amersham Biosciences, Buckinghamshire, England). Gels were made in triplicate to confirm the spot topography and were scanned with a Z2000 scanner (Founder; Beijing, China) and gel images were processed with an Imagemaster (GE Healthcare, Piscataway, NJ). To detect low abundance proteins in the fluid fraction, one-dimensional Western blots were probed with each of the antibodies raised. Other proteins may not have been identified because of even lower abundance, limited by the sensitivity of the WB method or imperfect MS data searching software to identify them.

Protein spots excised from two-dimensional gels were destained with 25 mm NH4HCO3/50% (v/v) acetonitrile and dried, followed by in-gel digestion with 0.01 μg trypsin in 0.01 ml 25 mm NH4HCO3 for 12 h at 37 °C. Digestion buffer was renewed to a new 1.5 ml clear microtube (Axygen Scientific, Union City, CA), 50 μl of 1% trifluoroacetic acid (v/v) in 50% (v/v) acetonitrile was added to the gel plugs, which were sonicated for 30 min. This extract was removed and combined with the digestion buffer and freeze-dried (Labconco, Kansas City, MO) (17). Peptides were then resuspended in 15 μl of 0.5% (v/v) trifluoroacetic acid in Milli-Q water. Peptides were analyzed by a Voyager DE-STR biospectrometry work station (Applied Biosystems/ MDS SCIEX, Foster City, CA) or a 4800 matrix-assisted laser desorption ionization time-of-flight (MALDI TOF)/TOF Analyzer (Applied Bio-
The MALDI-TOF mass spectrometer was operated in the delayed extraction/reflector mode with an acceleration voltage of 20 kV, a grid voltage setting of 72% and a 120 ns delay. Positively charged ions were analyzed in reflection mode. External calibration was performed with the ProteomMass peptide and protein MALDI/MS calibration kit (Sigma, St. Louis, MO, USA). Each mass spectrum represents the sum of 150–200 laser shots collected from ≥30 different positions within each spot. Mass spectra were processed by Data explorer 4.0 (Applied Biosystems/MDS SCIEX, Foster City, CA). The parameters used were m/z range 800–4000, resolution >10,000, S/N threshold >10.0 and internal calibration by trypsin autodigestion peptides (trypsin_[108–115] MH+ 842.509; trypsin_[58–77], MH+ 2211.104). Masses frequently detected that arose from the matrix, trypsin or known contaminants (e.g. keratins) were not analyzed. MALDI tandem mass spectrometry was performed on a TOF/TOF system (4800 Proteomics Analyzer, Applied Biosystems). The 4800 calibration mixtures (Applied Biosystems) were used to calibrate the spectrum to a mass tolerance within 150 ppm. For MS mode, peptide mass maps were acquired in positive reflection mode, and 800–4000 m/z mass range was used with 1000 laser shots per spectrum. A maximum of five precursors per spot with minimum signal/noise ratio of 50 were selected for tandem MS (MS/MS) analysis. Two kilovolts energy was used for collision-induced dissociation, and 2000 acquisitions were accumulated for each MS/MS spectrum. All of the automatic data analysis and database searching were fulfilled by the GPS Explorer™ software (version 3.6, Applied Biosystems) running mascot search algorithm (v2.1, Matrix Science, London, UK) for protein identification.

Data Mining and Bioinformatic Analysis—Database searches for MS was performed with Mascot (http://www.matrixscience.com/, MatrixScience Ltd., UK) against the SWISS-PROT protein database (Swissprot Release 55.0; 356,194 sequences; 127,836,513 residues) for Homo sapiens. MS/MS was performed with GPS Explorer v.3.6. (Applied Biosystems/MDS SCIEX) programs that incorporate the Mascot (v.2.1.) search algorithm (Matrix Science Inc. Boston, MA) against the NCBI nh database (NCBI n 20080210; 5,947,209 sequences; 2,045,123,248 residues) for Homo sapiens. The algorithm was set to use trypsin as the enzyme, allowing for one missed cleavage site and assuming carbamidomethyl as a fixed modification of cysteine and oxidized methionine as a variable modification. For PMF data, peptide mass tolerance was set to ±0.3 Da. For MS/MS database searches, mass tolerance of precursor ions and fragment ions was set to 150 ppm and ±0.4 Da. Protein hits were considered identified if the Mascot score was greater than 60 and matched at least four peptides for peptide mass fingerprinting and 37 for MS/MS analysis (significance level, p < 0.05). If more than one protein was identified in a spot, the single protein member with the highest score (top rank) was chosen from the multiprotein family.

Proteins were distinguished functionally by a step-by-step classification and each protein was placed in only one category. The proteins were first scored according to their function reported in the literature and the KEGG database (Release S2.0); proteins that could not be defined were sought in the PIR database (Release 15.9). Proteins were also scanned for protein domains by InterPro database (Release 23.0) and those with no annotation and supporting information were categorized as “Unclassified.”

Generation of Antibodies for Mapping the Testicular Proteome—Attempts were made to raise antibodies in rabbits to all identified nonstructural testicular proteins in collaboration with Shandong Drug Target Research Company Ltd (Yantai, Shandong Province, China). Synthetic peptides designed for 2–3 epitopes for each protein were generated and used for this purpose, and the antibodies produced were affinity purified to achieve an effective dilution of 1:10,000 when tested for specificity with Western blotting of testis extracts.

Western Blotting (WB) and RT-PCR—These were performed with conventional techniques as already described (4). Western blotting involved incubation of polyvinylidene fluoride membrane blots from one-dimensional SDS-PAGE gels with primary antibodies and horseradish peroxidase-conjugated secondary antibody, each for 1 h at room temperature, and visualization of protein bands by the DAB kit (ZhongShan Biotechnology, Beijing, China). For RT-PCR, total RNA from human tissues was extracted with Trizol (Tianwei Corporation, Beijing, China) following the manufacturer’s recommendations. cDNAs were synthesized according to instructions provided with the AMV reverse transcriptase (Promega, Madison, WI). Forward and reverse primers for the genes of testis proteins were designed from Primer Premier 5.0 Software (PREMIER Biosoft International, Palo Alto, CA) on the basis of sequences published in GenBank and are listed in supplemental Table S1. PCR was performed with a PE9700 device (Applied Biosystems) and PCR products were separated on 1% (w/v) agarose gel and visualized by staining with ethidium bromide. Human β-actin expression was used as internal positive control and deionized water was used in place of cDNA as negative control. To control for any DNA contamination of the RNA extracts and cDNA samples, most primer-pairs were designed to span gene introns so that any contaminating DNA would be revealed by the larger size of the PCR product than the expected sequence of the mRNA-derived cDNA.

Immunohistochemistry (IHC) of Testicular Tissue Sections—After fixation in Bouin’s solution for 8–12 h, testicular tissue blocks were processed for paraffin embedding by conventional methods. Tissue sections of 4 μm thickness were dewaxed and treated in a microwave oven for 20 min for antigen retrieval. Sections were incubated with 3% (v/v) H2O2 to inhibit endogenous peroxidases before being incubated for 1 h with 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline at room temperature to block nonspecific binding with antibodies. Sections were then incubated overnight at 4 °C with primary antibody (diluted 1:50 in blocking solution). After several washes with TBS, sections were incubated for 1 h at 37 °C with horseradish peroxidase-conjugated anti-rabbit IgG (ZhongShan Biotechnology) at a final dilution of 1:200 in blocking solution. Peroxidase activity at binding sites was revealed by a DAB kit (ZhongShan Biotechnology). Sections were counterstained with hematoxylin, dehydrated and mounted for bright-field microscopy (DM LB2, Leica, Nussloch, Germany). Normal rabbit IgG was used instead of the primary antibody as a negative control.

Immunocytochemistry (ICC) of Ejaculated Spermatozoa—Freshly produced ejaculates were allowed to liquefy for 30 min at 37 °C before the gentle addition of 1.5 ml phosphate-buffered saline (PBS) to top to allow spermatozoa to swim up for 1 h. Motile spermatozoa were collected and washed twice in PBS. The sperm pellet was resuspended to a concentration of 1 × 107/ml, placed on 1% (w/v) gelatin-coated slides, air-dried, and fixed with ice-cold methanol for 10 min. Slides were blocked for 1 h at room temperature with 3% (v/v) BSA in PBS and incubated at 37 °C for 30 min with primary antibodies (diluted 1:50 in PBS containing 3% BSA (PBS-BSA)). After three washes with PBS, the corresponding secondary antibody was applied (fluorescein isothiocyanate-labeled anti-rabbit IgG, 1:200 in PBS-BSA). Samples were subsequently washed in PBS and deionized water. Sperm heads were visualized by staining the nuclei with propidium iodide (0.01 mg/ml, Invitrogen, Carlsbad, CA). After the staining, all sections were mounted in 80% (v/v) glycerol and examined with a confocal laser scanning microscope (LSM-510 META; Carl Zeiss, Jena, Germany). Normal rabbit IgG was used instead of the primary antibody as a negative control.

1 The abbreviations used are: BSA, bovine serum albumin; cDNA, complementary DNA; ICC, Immunocytochemistry; IHC, Immunohistochemistry; PBS, phosphate buffered saline.
**RESULTS**

**Section I: The Testicular Proteome**—This comprises all proteins identified from the normal testes used for the present work.

**Gel Electrophoresis and MALDI-TOF/MS-MS**—Gel electrophoretic analysis of proteins from a total of four human testicular samples, which were pooled for protein extraction and separated on two-dimensional gels, revealed 1920 spots that were consistently detected in three gels (see Fig. 1). From the spots analyzed by MALDI-TOF/MS, of the 725 total testicular proteins identified (supplementary Table S2), 228 had been found and categorized in the epididymal proteome (4) so that 497 were found for the first time here, designated as “new testicular” proteins below. Fifty nine percent of the total 725 proteins were represented by a single spot and the rest by two or up to more than 10 spots revealing the same unique proteins, presumably representing spliced variants or isoforms (supplementary Fig. S1). Of the antibodies raised to 405 new testicular (571 total) nonstructural proteins, 379 new testicular (544 total) were suitable for use in sperm ICC and Western blotting. Fig. 2 shows that 112 new testicular (167 total) antibodies detected proteins on washed ejaculated spermatozoa (“sperm-located proteins”) and that 267 new testicular (377 total) gave no signal. Of the sperm-located proteins 49 new testicular (100 total) were detectable in epididymal fluid by WB (supplementary Fig. S2) and 63 new testicular (67 total) were not; of the nonsperm-located proteins, 68 new testicular (140 total) were found in epididymal fluid and 199 new testicular

![Fig. 1. Separation and identification of human epididymal proteins by two-dimensional-PAGE and MALDI-MS.](image-url)
There was thus a total of 240 (100/1100140), including 116 (49/67) new, testicular proteins in the epididymal fluid (sperm-milieu) identified in the present study. Classification of Biological Function of the Testicular Proteome—Proteomic analysis of the functions of these proteins is given in Fig. 3. Of the total number of proteins (725) (Fig. 3A), the majority (32%) was related to general metabolism and the next prevalent (19%) were structural proteins; the smallest group consisted of those related to immune defense.

Molecular Weights, Isoelectric Points of the Testicular Proteins and Chromosomal Locations of Their Genes—An overview of the distribution of the pi, molecular weights, and chromosomal location of the testicular proteins found in testicular tissue, luminal fluid and on spermatozoa is given in Table I. The molecular weights of the human testicular and sperm-milieu proteins were generally 10–100 kDa with most between 20 and 50 kDa. The percentages of testicular proteins, testicular sperm-milieu proteins and sperm-located proteins in this range were 50%, 52%, and 50%. The isoelectric points of the human testicular proteins ranged from 4.5 to 9. In the range from 5.5 to 7.0, the proportion of testicular proteins, testicular sperm-milieu proteins and sperm-located proteins were, respectively, 45%, 37%, and 46%. The chromosomal location of the testicular genes transcribing the tissue, sperm-milieu and sperm-located proteins was the autosomes or the X-, but not on the Y-, sex chromosome.

Location of Testicular Proteins in Spermatozoa—By ICC analysis of the testicular proteins, a combination of 13 sperm locations was observed (Table II), which are shown in Fig. 4. These included the acrosome (Fig. 4A), the equatorial region (Fig. 4B), the postacrosomal region (Fig. 4C), the cytoplasmic droplet at the neck (Fig. 4D), the midpiece only (Fig. 4E), the principal piece only (Fig. 4F), the end-piece (Fig. 4G), the head, midpiece, and tail (Fig. 4H), the annulus only (Fig. 4I), the acrosome and the neck (Fig. 4J), the equatorial region and principal piece (Fig. 4K), the postacrosomal region and annulus (Fig. 4L), the midpiece and proximal principal piece (Fig. 4M). Table II indicates the total number of spermatozoa found at each of these locations.

Analysis in silico of all the proteins (Fig. 3A) and the 167 sperm-located proteins (Fig. 3B) indicated that their functions were loosely associated with the location on the spermatozoa: those on the acrosome were mainly signal transduction proteins, molecular chaperone, and proteases whereas the equatorial proteins had no function as molecular chaperones (supplementary Table S3). Neck proteins were mostly related to metabolism and tail proteins were mainly metabolism, structure-related, and signal transduction pathway proteins.
Nomenclature of Testicular Proteins—On the basis of the results of MALDI-TOF, MS/MS, WBs of testicular tissue and the sperm-milieu (epididymal luminal fluid), and ICC location of the proteins in spermatozoa, the testicular proteins were given a unified nomenclature. This was based on that designed for epididymal proteins (4) and comprised (a) tissue proteins, (b) sperm-milieu (epididymal luminal fluid) proteins, and (c) sperm-located proteins (Fig. 5). As further illustrated in the flow chart (Fig. 2), if the results of testicular WB were positive and WB of epididymal luminal fluid and sperm ICC were negative, i.e. the proteins were found in testicular tissue only, the proteins were termed Human Testis protein-LiTissue (HTL-T-n; where n is a different number for each protein in this category); (b) if the result of WB of both testicular tissue and luminal fluid was positive but sperm ICC negative, i.e. testicular proteins were also found in epididymal luminal fluid but not in spermatozoa, they were termed Human Testis protein-Li Sperm-milieu (HTL-S-n); (c) if the result of testicular IHC and WB of tissue and epididymal fluid were positive and spermatozoa reacted to antibodies raised against testicular proteins, i.e. testicular proteins were also found in epididymal fluid and in spermatozoa, the sperm-milieu sperm-located proteins were termed Human Testis protein-Li Sperm-milieu sperm-located (HTL-S-nx; where x indicates the location of the protein in different sperm compartment; see Fig. 4). If WB of epididymal luminal fluid was negative, sperm-located proteins were termed Human Testis protein-LiTissue sperm-related (HTL-T-nx).

**Summary of the sites of sperm-located proteins in the testicular and epididymal proteomes. Of the 15 location patterns reported in the previous study (4) on the epididymal proteome, two patterns (11 and 12, shaded) were not found for the testicular proteins identified in the present work.**

![Table I](image)

**Table I**

| Characteristic of testicular proteome (including distribution of pI, MW, chromosome) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MW (kDa) | <10 | 10–20 | 20–30 | 30–40 | 40–50 | 50–60 | 60–70 | 70–80 | 80–90 | 90–100 |
| Testicular proteins (%) | 2 | 7 | 18 | 19 | 16 | 13 | 7 | 6 | 3 | 3 | 6 |
| Testicular sperm-milieu proteins (%) | 0 | 6 | 17 | 21 | 18 | 12 | 6 | 8 | 2 | 4 | 9 |
| Sperm-located testicular proteins (%) | 0 | 4 | 15 | 15 | 13 | 8 | 8 | 4 | 5 | 10 |
| pI | <4.5 | <5.0 | <5.5 | <6.0 | <6.5 | <7.0 | <7.5 | <8.0 | <8.5 | <9.0 | >9.0 |
| Testicular proteins (%) | 1 | 8 | 15 | 18 | 16 | 10 | 3 | 5 | 8 | 9 | 8 |
| Testicular sperm-milieu proteins (%) | 0 | 10 | 13 | 16 | 16 | 9 | 8 | 5 | 7 | 5 | 10 |
| Sperm-located testicular proteins (%) | 0 | 9 | 13 | 13 | 14 | 4 | 7 | 6 | 6 | 9 | 13 |
| Chromosome no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X | Y |
| Testicular proteins (%) | 11 | 7 | 6 | 5 | 3 | 5 | 5 | 3 | 6 | 4 | 7 | 4 | 2 | 3 | 3 | 4 | 6 | 1 | 7 | 2 | 1 | 3 | 3 | 0.1 |
| Testicular sperm-milieu proteins (%) | 6 | 8 | 4 | 4 | 3 | 6 | 6 | 5 | 3 | 2 | 8 | 4 | 1 | 3 | 3 | 6 | 6 | 1 | 3 | 3 | 2 | 5 | 8 | 0 |
| Sperm-located testicular proteins (%) | 10 | 8 | 7 | 9 | 4 | 6 | 4 | 4 | 7 | 4 | 7 | 3 | 1 | 2 | 2 | 3 | 5 | 1 | 3 | 2 | 2 | 4 | 3 | 0 |

**Table II**

| Protein localisation | Total protein number | In testicular proteome only | In epididymal proteome only | In both |
|---|---|---|---|---|
| Pattern No. | Sperm domains | | | |
| 1 | Acrosome | 82 | 31 | 38 | 13 |
| 2 | Equatorial | 32 | 13 | 16 | 3 |
| 3 | Post-acrosomal | 33 | 5 | 16 | 12 |
| 4 | Neck | 64 | 27 | 28 | 9 |
| 5 | Midpiece | 18 | 7 | 6 | 5 |
| 6 | Principal piece | 34 | 12 | 18 | 4 |
| 7 | End-piece | 8 | 3 | 4 | 1 |
| 8 | Whole sperm | 3 | 1 | 2 | 0 |
| 9 | Acrosome+neck | 4 | 1 | 3 | 0 |
| 10 | Acrosome+midpiece | 2 | 0 | 1 | 1 |
| 11 | Acrosome+principal piece | 2 | 0 | 2 | 0 |
| 12 | Equatorial+principal piece | 1 | 0 | 1 | 0 |
| 13 | Post-acrosomal+annulus | 1 | 0 | 0 | 1 |
| 14 | Neck+annulus | 1 | 0 | 0 | 1 |
| 15 | Midpiece+principal piece | 34 | 12 | 17 | 5 |

**Section II: The Sperm Proteome**—This comprises proteins originally identified in the testicular or epididymal proteome.

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and later found to be present in ejaculated spermatozoa by using the antibodies raised (Fig. 5). A list of the identities of all these 319 sperm-located proteins found in the testicular and epididymal proteomes is given in supplementary Table S5. Of these, 55 (17%) were found in both organs, whereas 152 (48%) were found only in the epididymis and 112 (35%) only in the testis. Around one-third of these sperm proteins were detected on part of the tail, and 90 were detected in the
acrosome, most exclusively so (Table II). Comparison of the epididymal with testicular proteins in this set of sperm-located proteins showed that a higher proportion of proteins had a principal piece location and a lower proportion had an acrosomal location in the former. In all, 77 of the 319 proteins in the present sperm proteome were found among the 1053 proteins identified in the proteome of the human spermatozoon (20) (see Venn diagram in supplementary Fig. S3A for the size of the common subsets). Among the proteins identified by our work, the 55 common to the testis and epididymis represent 33% of the testicular and 55% of the epididymal proteome; the 51 proteins common to the testicular and sperm proteomes represent 31% of the testicular but only 5% of the sperm proteome and the 50 proteins common to the epididymal and sperm proteomes were 24% of the epididymal and 5% of the sperm proteome.

In all, 223 of the 319 sperm-located epididymal (174) and testicular (49) proteins were also present in the sperm-milieu (those with nomenclature -S-nx in Fig. 5 and individually marked in supplementary Table S5). This includes 59 of the 1053 in the published human sperm proteome as shown in supplementary Fig. S3B.

Section III: Cellular Localization in the Testis by IHC of all 319 Proteins Comprising the Present Sperm Proteome—Fig. 6 shows examples of the nine cellular locations within the human testis of the 319 proteins detected in spermatozoa by antibodies raised against testicular or epididymal proteins. The seminiferous tubules contain the fluid-secreting Sertoli cells and the developing germ cells that they support (forming the germinal epithelium); the tubules are bounded by the structural peritubular (contractile myoid) elements and the steroid-secreting Leydig cells that lie within the intertubular space. Proteins were found that were specific to one cell type (only Sertoli cells (Figs. 6D, 6E), spermatids (Figs. 6B, 6G),
peritubular myoid cells (Fig. 6H) or Leydig cells (Fig. 6J); two cell types (spermatocytes and spermatids (Fig. 6F), myoid and Leydig cells (Fig. 6I)) and germ cells plus somatic cells (spermatogonia, spermatocytes, spermatids, and Sertoli cells (Fig. 6C)). A total of 245 proteins was found in the germinal epithelium, with most of these also localized in the interstitial cells, whereas the remaining 23% (74) were found in Leydig cells or peritubular cells (supplementary Table S6). Several of these proteins had been previously identified in the epididymal proteome and also identified as sperm-milieu proteins, bearing the nomenclature HEL-S-nx (sperm-located sperm-milieu (4)). As expected for sperm-located proteins, the majority of those localized in the germinal epithelium (151 of 245) was found in round or elongated spermatids, although only 33 proteins were exclusively so located (Table III). These spermatic-exclusive proteins include those that are well known (e.g. lactate dehydrogenase C and protein kinase A anchor proteins 3 and 4) and those that have not been reported (e.g. prohibitin, F-actin-capping protein subunit α-2 and Ribonuclease-like protein 13 precursor).

On the other hand, Sertoli cells contained the smallest number of such proteins, with only three (cathepsin D, 1-acylglycerol-3-phosphate Oacyltransferase 9 and inositol-3-phosphate synthase 1) being absent from all germ cells. Four proteins found to be exclusively expressed by spermatocytes were Profilin-1, HEAT repeat-containing protein 4 (HEATR4), acyl-coenzyme A thioesterase 1 (ACOT1), and F-box/WD repeat-containing protein 1A (BTG). Only one protein, Kelch-like protein 15 (KLHL15) was exclusively found in primary spermatocytes.

Section IV Proteome of the Sperm-milieu—This comprises proteins originally identified in the testicular or epididymal proteome and later found to be present in the epididymal fluid, which is the milieu for sperm maturation (Fig. 5). The identities of all these 525 proteins found in the epididymal fluid, combined from those in this study (of testicular origin) and the previous study of the epididymal proteome (4), are given in supplementary Table S7. Those common to both organs, bearing the HEL- nomenclature (see Section I, nomenclature), are highlighted in purple supplementary Table S7.

Because epididymosomes are the vehicles for transfer of some proteins from the epididymal epithelium to spermatozoa in the tubule lumen (21), it is pertinent to compare the 146 proteins identified in the human epididymosomes (22) with those sperm-located testicular (100) and epididymal (174) proteins also found in the sperm-milieu (supplementary Fig. S3C). The 51 proteins common to the testis and epididymis represent 51% of the testicular and 29% of the epididymal proteins; the 15 proteins common to the testicular and epididymosomal proteomes represent 13% of the testicular and 19% of the epididymosomal proteome and the 21 proteins common to the epididymal and epididymosomal proteomes were 12% of the epididymal and 14% of the epididymosomal proteome.

Gene ontological analysis of all the 525 sperm-milieu proteins is given in supplementary Fig. S4A. Little difference in the distribution of functions was observed between the two subsets common to the epididymal or the testicular proteomes, except for a slightly higher proportion responsible for general metabolism and a lower proportion for signal transduction in the latter subset. Such differences were more marked when only those proteins that were found in both sperm-milieu and spermatozoa were considered (supplementary Fig. S4B).

**DISCUSSION**

The diagnostic assessment of testicular function by routine histological analysis has recently been supplemented by evaluation of the transcriptome. This includes, for example, a protein (profilin IV) involved in a specific spermatogenic process (acrosomal biogenesis) (23), groups of genes selectively expressed premeiotically, postmeiotically, and at terminal differentiation in tissue from a collective of patients exhibiting defined testicular pathology (24), and global expression of genes whose expression is associated with certain spermatogenic damage (25). The localization of a few genes within specific cell types has been supported by in situ hybridization (23) and RNA analysis of laser-captured cells (26).

Although gene expression reflects the presence and absence or transcriptional activities of certain cell types, of more interest is whether these genes are translated. Other techniques have been employed for this, such as IHC to locate proteins in particular cell types (27, 28). More recent studies have applied proteomic techniques to the human testis that have indicated differences in proteins present in the testes of normozoospermic men and those with nonobstructive
azoospermia (12), increases in apoptotic pathways when
spermatogenesis is halted by hormonal contraception (14)
and the large heterogeneity of proteins in fertile men (13).

Despite being the primary sex organ, the testis delivers
functionally incompetent spermatozoa that only develop their
fertilizing potential after transit through the attached epididymis (3). This organ secretes proteins and glycoproteins, some
of which bind to, or insert into, the sperm membrane, thereby
influencing its fluidity, enzyme activity, and signal transduc-
tion molecular components and thus the physiology of the
spermatozoon. Knowledge of these proteins would aid under-
standing of human male fertility and infertility.

The present proteomic database comprising 725 proteins is
larger than, and contains 97% of, those reported by Guo et al.
(13). The proteome of the human epididymis has recently
been revealed by our laboratory, with emphasis on the sub-
sets of proteins detectable in the luminal fluid and in mature
spermatozoa (4). Because the flow of this fluid originates from
the testis, a testicular protein contribution to this fluid is
possible, either exclusively or in addition to the contribution of
epididymal secretions, and transcripts of the majority (93%)
of epididymal genes were found in the testis (4). Identification
of the testicular proteome confirmed that many (228, 31%)
proteins were common to the epididymal proteome. The dual
origin of these luminal proteins in both the testis and epididy-
mys is not unknown; examples of sperm-coating proteins in
rodents are SPAM1 (29), CRISP (30), and SGP-2 (31).

The generation of an antibody library against these pro-
teins, excluding structural proteins, enabled the detection of
proteins hitherto unknown in epididymal fluid, to add to the
epididymal luminal proteins previously reported (4). To be a
testicular exocrine secretion that could become a sperm-
located protein during maturation in the epididymis, testicular
proteins must fulfill the criteria of being expressed by Sertoli
cells (to be secreted), but not by spermatids (not intrinsic sper
proteins) or epididymal epithelial cells (not from ep-
ididymal secretions). Of the three germinal epithelial pro-
teins found exclusively in Sertoli cells, cathepsin D and
1-acylglycerol-3-phosphate O-acyltransferase 9 failed this
candidacy, because they were detected in the epithelium of
efferent ducts and principal cells of the corpus and cauda
epididymidis. The other candidate was inositol-3-phos-
phate synthase 1 (ISYNA1), which was only expressed in
basal cells in the epididymis and very weakly in ciliated cells
in the efferent ducts (data not shown). Stronger evidence for
such a candidacy, such as secretion by Sertoli cells and
absence from isolated human testicular spermatozoa, is
lacking.

ISYNA1 is involved in myo-inositol synthesis by catalyzing
the conversion of glucose-6-phosphate to myo-inositol-1-
phosphate, and this is dephosphorylated by inositol 1-phos-
phate phosphatase, which was also found in the human tes-
ticular proteome (HTL-T-94), but it was neither secreted nor
present on human spermatozoa. The enzymatically active
ISYNA1 has long been known to be present in the rat testis
(32, 33) and ovine testicular, but not ejaculated, spermatozoa,
which can convert glucose to myo-inositol in vitro (34). In mice
the ISYNA1 gene is transcribed by isolated primary spermatocytes and round spermatids (35). The present finding on the
absence of this protein from human spermatozoa warrants in-
vestigation as a species difference.

Of all the sperm-located proteins, IHC on testicular sections
indicated that 74 out of 319 were absent from the germinal
epithelium. As these were all identified in our previous study
as epididymal secretory sperm-located proteins (4), they were
probably acquired by the spermatozoa during passage
through the epididymis. Human epididymal secretions are
most likely to be responsible for the extrinsic sperm proteins
because of the testicular proteins detected in epididymal fluid,
60% were already found in the epididymis proteome as abun-
dant proteins appearing on two-dimensional gels (4). The
remaining 40% may not have originated from the testis, but
would be just common to both testis and epididymis. The
resorption of most testicular fluid, along with its proteins, by
the efferent ducts (36) makes it unlikely that testicular fluid
proteins would reach epididymal fluid. This has been illus-
trated in the rat where, although the Sertoli cell product SGP2
binds to late spermatids, it dissociates from spermatozoa in
the rete testis and efferent ducts (whose epithelia remove the
protein from the lumen) and it is the epididymal SGP2, se-
creted in the distal initial segment, that is found on epididymal
spermatozoa (31).

The present findings, which extend the proteome of the
sperm-milieu and identified a subset of the sperm proteome,
could serve as a molecular basis for the development of
biomarkers of normal or abnormal sperm maturation. To-
gether with the mapping of the testicular and epididymal
proteomes, clinical advances could be made in analyzing the
causes of infertility stemming from testicular dysfunction or
posttesticular mal-development of sperm function.

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