Mass spectrometry-based, label-free quantitative proteomics of round spermatids in mice

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Received March 3, 2014; Accepted March 10, 2014

DOI: 10.3892/mmr.2014.2460

Abstract. Round haploid spermatids are formed at the completion of meiosis. These spermatids then undergo morphological and cytological changes during spermiogenesis. Although sperm proteomes have been extensively studied, relatively few studies have specifically investigated the proteome of round spermatids. We developed a label-free quantitative method in combination with 2D-nano-LC-ESI-MS/MS to investigate the proteome of round spermatids in mice. Analysis of the proteomic data identified 2,331 proteins in the round spermatids. Functional classification of the proteins based on Gene Ontology terms and enrichment analysis further revealed the following: 504 of the identified proteins are predicted to be involved in the generation of precursor metabolites and energy; 343 proteins in translation and protein targeting; 298 proteins in nucleotide and nucleic acid metabolism; 275 and 289 proteins in transport and cellular component organization, respectively. A number of the identified proteins were associated with cytoskeleton organization (183), protein degradation (116) and response to stimulus (115). KEGG pathway analysis identified 68 proteins that are annotated as components of the ribosomal pathway and 17 proteins were related to aminoacyl-tRNA biosynthesis. The round spermatids also contained 28 proteins involved in the proteasome pathway and 40 proteins in the lysosome pathway. A total of 60 proteins were annotated as parts of the spliceosome pathway, in which heterogeneous nuclear RNA is converted to mRNA. Approximately 94 proteins were identified as actin-binding proteins, involved in the regulation of the actin cytoskeleton. In conclusion, using a label-free shotgun proteomic approach, we identified numerous proteins associated with spermiogenesis in round spermatids.

Introduction

Mammalian spermatogenesis is a complex and highly ordered process, in which a diploid progenitor germ cell transforms to highly specialised spermatozoa. This process involves successive mitotic, meiotic and post-meiotic phases. Once meiosis is completed, haploid germ cells termed ‘round spermatids’ are produced; these spermatids subsequently undergo a series of differentiation steps collectively known as ‘spermiogenesis’. In spermiogenesis, round spermatids develop a distinct head, midpiece and tail region; round spermatids also undergo chromatin remodelling, develop an acrosome and become almost completely devoid of cytoplasm. These changes lead to the formation of slender, elongated, mature spermatids, which are released into the lumen of the seminiferous tubule during spermiation (1).

Round haploid spermatids initiate spermiogenesis; successful spermiogenesis is necessary for fertilization, and alterations of this process constitute an important cause of male infertility. This process requires a precise and well-coordinated system that regulates the constantly changing patterns of gene and protein expression (2). Therefore, the identification of proteins present in the spermatids can not only provide insights into the molecular basis of spermiogenesis, but also facilitate the identification of cell-specific targets for the diagnosis or induction of male infertility.

Numerous genes involved in spermatogenesis have been identified by differential display (3), serial analysis of gene expression (SAGE) (4) and microarray methods (5). Nevertheless, these methods do not provide pivotal information on the post-transcriptional control of gene expression, changes in protein expression levels and/or protein modifications. In this context, proteomics research has emerged and enhanced our knowledge on cell behavior at the system level, by revealing global patterns of protein content, modification and activity during development (6). Experiments have also been conducted to initiate differential protein expression profiling studies and/or systematic analyses of testicular proteomes in entire organs or isolated spermatogenic cells from various species. Several groups have focused on sperm proteomes, and identified numerous proteins that characterise sperm cells in
different mammals (7-11). Although proteomic analyses of the sperm and of different developmental stages of the tests have been performed in different mammalian species, the protein expression profiles of spermiogenesis, particularly of round spermatids, remain unclear.

Mass spectrometry (MS)-based proteomics technology is a powerful tool for large-scale protein identification and quantification (12). Previous proteomic studies have used techniques such as two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) and 1-D PAGE of the extracted protein mixture prior to liquid chromatography (LC)-MS/MS identification. Although these techniques require the reduction of sample complexity prior to LC-MS/MS analysis, proteins present in small amounts may not be detectable on the gel, thereby limiting the capacity of MS to identify a number of protein components. A number of quantitative proteomic methods have been developed, including stable isotope labeling and label-free methods (11). The latter is applicable in complex biological systems; in addition, this technique has a number of advantages, such as faster, cleaner and simpler results (13,14). Numerous researchers have employed label-free shotgun proteomic techniques (15-17).

Animal models are commonly used to study the molecular regulation of spermatogenesis. Numerous murine models have been established and applied to study the genes that are up- or downregulated in spermatogenesis. Biologically, meiosis and spermiogenesis are quite similar processes between humans and rodents. In the current study, label-free quantitative shotgun proteomics and mass spectrometry were combined to investigate the protein content of the round spermatids of mice, in order to provide new insights into the molecular regulation of spermiogenesis.

Materials and methods

Sample preparation. Round spermatids were isolated according to a previously described method (18) with slight modifications. In the first wave of mouse spermatogenesis, different spermatogenic cells were found at specific time points (days 6, 9, 14, 21, 35 and 60 postpartum). Based on the majority of germ cell types, male mice of different ages are commonly used to isolate differently developed stages of spermatogenic cell types. In this study, ten 35-day old male Balb/c mice were used to isolate round spermatids. The mice were anesthetized with CO2 and sacrificed by cervical dislocation. The testes were removed and decapsulated. The tubulous tissue was cut into small pieces and incubated in 5 ml of phosphate-buffered saline (PBS) containing 0.5 mg/ml of collagenase (Sigma-Aldrich, St. Louis, MO, USA) with continuous agitation at 33°C for 15 min. The dispersed seminiferous cords and cells were allowed to sediment for 5 min and the supernatant was decanted. The sample was then diluted in a solution of 50 mM NH4HCO3 (Sigma-Aldrich). The protein mixture was digested by incubating in grade-modified trypsin (Promega) at a 1:50 enzyme:protein ratio, at 37°C for 20 h. The tryptic peptide mixture was lyophilized and stored at -80°C until use.

Immunofluorescent detection. Cells were attached to poly-L-lysine coated microscopy coverslips and were fixed with 2% formaldehyde in microtubule-stabilizing buffer (50 mmol/l PIPES, 5 mmol/l EGTA and 5 mmol/l MgSO4) for 1 h. Coverslips were rinsed in PBS and permeabilized for 1 h in 1% Triton X-100 in PBS. Nonspecific antibody binding was prevented by incubation for 1 h in 10% normal goat serum. Microtubules were labeled with anti-α-tubulin monoclonal (Sigma-Aldrich). Primary antibodies were detected using FITC-conjugated rabbit anti-mouse immunoglobulin (Jackson ImmunoResearch Inc., West Grove, PA, USA). DNA was detected by labeling with DAPI. The coverslips were mounted in a drop of VectaShield mounting medium (Vector Laboratories Inc, Burlingame, CA, USA). Coverslips were examined using BX43 Epifluorescence microscope (Olympus Co.).

Automated 2D-nano-LC-ESI-MS/MS peptide analysis. The extracted peptides were desalted using 1.3 ml C18 solid-phase extraction column (Sep-Pak® Cartridge; Waters Corp., Milford, MA, USA). The peptides were dried using a vacuum centrifuge and were resuspended in loading buffer containing 5 mM ammonium formate (NH4FA) and 5% acetonitrile at pH 3.0. Next, peptides were separated and analyzed by 2D strong cation-exchange (SCX)/reversed-phase (RP) nano-scale LC/MS. The experiments were performed on a Nano Aquity UPLC system (Waters Corp.) connected to an LTQ Orbitrap XL mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with an online nano-electrospray ion source (Bruker, Auburn, CA, USA). A total of 106 cells were bottom-loaded in a cell separator apparatus with a 12.5 cm diameter (TH-300A; Shanghai Huxi Analysis Instrument Factory Co., Ltd., Shanghai, China) and then incubated in a 2-4% BSA linear gradient in RPMI-1640 medium (Gibco, Grand Island, NY, USA). After 3 h of velocity sedimentation at unit gravity, the cell fractions (10 ml/fraction) were collected from the bottom of the separator apparatus at a rate of 5 ml/min. The cell types, in terms of diameter and morphological characteristics, as well as the purity of each fraction, were assessed under a light microscope (BX43; Olympus Co., Tokyo, Japan). Only fractions with the expected cell type were pooled together. The average purity of round spermatids was 95%.
A 180 µm x 2.4 cm SCX column (Waters Corp.), which was packed with 5 µm of polysulfoethyl aspartamide (PolyLC Inc., Columbia, MD, USA) was used for the first dimension. To recover hydrophobic peptides retained on the SCX column after a conventional salt step gradient, an RP step gradient from 5 to 50% acetonitrile (ACN) was applied to the SCX column. A 15-µl plug was performed at each step of the gradient. The SCX column was cleaned once using 1 M NH₄FA. The plugs were then loaded onto the SCX column with loading buffer, at a flow rate of 15 µl/min for 6 min. A peptide sample (15 µl) was loaded onto the SCX column prior to injection of the gradient plugs. The eluted peptides were then captured using a trap column (Waters Corp.), and salts were diverted to waste. The trap column (2 cm x 180 µm) was packed with a 1.7 µm桥接ethyl hybrid (BEH) C18 particle (Waters Corp.) and then used for protein separation at the second dimension.

The peptides on the RP analytical column were eluted with a three-step linear gradient, balancing with the 95% A buffer 10 min, then starting from 5 to 40% B in 40 min (A, water with 0.1% formic acid; B, ACN with 0.1% formic acid) and increased up to 80% B in 3 min. Afterwards, this solution was reduced to 5% B for 2 min. The column was left to re-equilibrate for 15 min. The column flow rate was maintained at 500 nl/min and the column temperature was maintained at 35°C. Eluted peptides were ionized at 1.9 kV and the ions were analyzed by an LTQ Orbitrap XL Mass spectrometer (Thermo Fisher Scientific Inc., Marietta, OH, USA).

The LTQ Orbitrap XL mass spectrometer was operated in a data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra with two microscans (300-1800 m/z) were acquired in the Orbitrap with a mass resolution of 60,000 at 400 m/z. Ten sequential LTQ-MS/MS scans were then conducted. Dynamic exclusion was used with two repeat counts, 10 sec repeat duration and 90 sec exclusion duration. For MS/MS, precursor ions were activated using a 35% normalized collision energy at the default activation q-value of 0.25.

Peptide sequencing data analysis. The acquired MS/MS spectra were searched against the IPI mouse.v3.68 fasta-formatted protein database using the SEQUEST v.28 (revision 12) software (Thermo Electron Corp.). To reduce identification of false positives, we appended to the database its decoy version containing the reverse sequences. The search parameters were the following: partial trypsin (KR) cleavage with two missed cleavages; the variable modification was oxidation (M); peptide mass tolerance, 50 ppm; and fragment ion tolerance, 1 Da. The open-source Trans Proteomic Pipeline software (revision 4.0; Institute of Systems Biology, Seattle, WA, USA) was then used to identify proteins based on the corresponding peptide sequences and a ≥95% confidence threshold. The peptides results were filtered by Peptide Prophet (19) with a p-value >0.95 and a Protein Prophet (20) probability of 0.95 was used for the protein identification results.

Bioinformatic analyses. The predicted cellular localization of the proteins identified in the round spermatids was retrieved based on the information available at the Gene Ontology (GO) project website (http://www.geneontology.org/). Functional classification of the proteins was based on biological process and molecular function GO terms. Assignment of the proteins to signaling pathways was based on information available at the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/pathway.html) and the BioCarta (http://www.biocarta.com/genes/index.asp) databases. Enrichment analysis for these categorizations was performed with tools available at DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/); DAVID is a web-based application that enables visualization, discovery and analysis of molecular interactions and associations with disease for a given list of genes or proteins.

Results

Identification of proteins in round spermatids by shotgun proteomics. Following isolation of murine testicular cells by a gradient method, the purity of the sorted round spermatids was assessed by immunofluorescent staining using the anti-α-tubulin antibody. α-tubulin is the main component of manchette, which is a spermatid-specific microtubular structure. The purity of the sorted round spermatids was >95%, as assessed by counting 500 sorted cells under the microscope (Fig. 1).

We employed a label-free shotgun proteomic technique to identify proteins, gain insights into the protein expression profile of round spermatids, and investigate the relevant molecular mechanisms. The reproducibility of the method was evaluated, with a reliability coefficient of 95% estimated from independent
Table I. Enriched biological processes in the proteome of round spermatids based on Gene Ontology (GO) terms.

| GO id            | Description                                                                 | Count | %     | Q<0.01     |
|------------------|-------------------------------------------------------------------------------|-------|-------|------------|
| GO:0055114       | Oxidation reduction                                                           | 234   | 10.3356890460 | 4.40E-58  |
| GO:0008104       | Protein localization                                                           | 198   | 8.7455830389  | 1.24E-50  |
| GO:0045184       | Establishment of protein localization                                          | 180   | 7.9505300353  | 4.11E-33  |
| GO:0015031       | Protein transport                                                              | 179   | 7.9063604240  | 9.98E-31  |
| GO:0006091       | Generation of precursor metabolites and energy                                  | 142   | 6.2720848057  | 2.54E-24  |
| GO:0046907       | Intracellular transport                                                        | 135   | 5.9628975265  | 2.68E-24  |
| GO:0006412       | Translation                                                                   | 126   | 5.5653710247  | 2.77E-24  |
| GO:0042592       | Homeostatic process                                                           | 114   | 5.0353556890  | 5.88E-24  |
| GO:0016192       | Vesicle-mediated transport                                                     | 105   | 4.6378091873  | 1.32E-23  |
| GO:0007155       | Cell adhesion                                                                 | 100   | 4.4169611307  | 1.30E-21  |
| GO:0022610       | Biological adhesion                                                           | 100   | 4.4169611307  | 2.13E-21  |
| GO:0034613       | Cellular protein localization                                                  | 97    | 4.284452968   | 7.58E-20  |
| GO:0070727       | Cellular macromolecule localization                                           | 97    | 4.284452968   | 5.06E-19  |
| GO:0006886       | Intracellular protein transport                                                | 93    | 4.1077738516  | 1.53E-18  |
| GO:0006396       | RNA processing                                                                | 90    | 3.9752650177  | 2.20E-18  |
| GO:0044271       | Nitrogen compound biosynthetic process                                         | 88    | 3.8869257951  | 2.54E-18  |
| GO:0016071       | mRNA metabolic process                                                         | 84    | 3.7102473498  | 5.99E-18  |
| GO:0055085       | Transmembrane transport                                                        | 83    | 3.6660777385  | 1.56E-17  |
| GO:0019725       | Cellular homeostasis                                                           | 81    | 3.5777385159  | 5.15E-17  |
| GO:0043933       | Macromolecular complex subunit organization                                    | 78    | 3.445229682   | 5.78E-17  |
| GO:0006397       | mRNA processing                                                               | 77    | 3.4010600707  | 7.10E-16  |
| GO:0065003       | Macromolecular complex assembly                                                | 76    | 3.3568904594  | 1.18E-15  |
| GO:0051186       | Cofactor metabolic process                                                     | 73    | 3.2243816254  | 1.27E-15  |
| GO:0007010       | Cytoskeleton organization                                                      | 71    | 3.1360424028  | 5.24E-15  |
| GO:0022900       | Electron transport chain                                                       | 67    | 2.9593693576  | 8.39E-15  |
| GO:0008380       | RNA splicing                                                                  | 67    | 2.9593693576  | 1.13E-14  |
| GO:0005996       | Monosaccharide metabolic process                                               | 64    | 2.8268551237  | 1.89E-14  |
| GO:0006732       | Coenzyme metabolic process                                                     | 60    | 2.6501766784  | 1.89E-14  |
| GO:0019318       | Hexose metabolic process                                                       | 60    | 2.6501766784  | 1.97E-14  |
| GO:0006163       | Purine nucleotide metabolic process                                            | 59    | 2.6060070671  | 1.97E-14  |
| GO:0034654       | Nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process       | 59    | 2.6060070671  | 2.69E-14  |
| GO:0034404       | Nucleobase, nucleoside and nucleotide biosynthetic process                    | 59    | 2.6060070671  | 3.59E-14  |
| GO:0034621       | Cellular macromolecular complex subunit organization                           | 59    | 2.6060070671  | 5.07E-14  |
| GO:0009165       | Nucleotide biosynthetic process                                                | 58    | 2.5618374558  | 5.92E-14  |
| GO:0016044       | Membrane organization                                                          | 58    | 2.5618374558  | 9.05E-14  |
| GO:0048878       | Chemical homeostasis                                                           | 58    | 2.5618374558  | 1.13E-13  |
| GO:0006631       | Fatty acid metabolic process                                                   | 57    | 2.5176678445  | 1.46E-13  |
| GO:0034622       | Cellular macromolecular complex assembly                                        | 57    | 2.5176678445  | 1.48E-13  |
| GO:0009259       | Ribonucleotide metabolic process                                               | 56    | 2.4734982332  | 1.53E-13  |
| GO:0009611       | Response to wounding                                                           | 56    | 2.4734982332  | 1.89E-13  |
| GO:0009150       | Purine ribonucleotide metabolic process                                        | 55    | 2.4293286219  | 4.22E-13  |
| GO:0032989       | Cellular component morphogenesis                                                | 55    | 2.4293286219  | 4.40E-13  |
| GO:0006006       | Glucose metabolic process                                                       | 54    | 2.3851590106  | 5.52E-13  |
| GO:0030030       | Cell projection organization                                                    | 54    | 2.3851590106  | 6.21E-13  |
| GO:0015980       | Energy derivation by oxidation of organic compounds                             | 53    | 2.3409893993  | 7.03E-13  |
| GO:0006164       | Purine nucleotide biosynthetic process                                         | 53    | 2.3409893993  | 1.41E-12  |
| GO:0007264       | Small GTPase mediated signal transduction                                      | 53    | 2.3409893993  | 1.62E-12  |
Table I. Continued.

| GO id.          | Description                                           | Count | %         | Q<0.01     |
|-----------------|-------------------------------------------------------|-------|-----------|------------|
| GO:0030029      | Actin filament-based process                          | 52    | 2.2968197880 | 1.65E-12   |
| GO:0009260      | Ribonucleotide biosynthetic process                   | 51    | 2.2526501767 | 2.84E-12   |
| GO:0006457      | Protein folding                                       | 51    | 2.2526501767 | 4.68E-12   |
| GO:0008610      | Lipid biosynthetic process                            | 51    | 2.2526501767 | 5.69E-12   |
| GO:0050801      | Ion homeostasis                                       | 51    | 2.2526501767 | 5.93E-12   |
| GO:0009152      | Purine ribonucleotide biosynthetic process            | 50    | 2.2084805654 | 6.56E-12   |
| GO:0030036      | Actin cytoskeleton organization                       | 50    | 2.2084805654 | 7.59E-12   |
| GO:0009141      | Nucleoside triphosphate metabolic process            | 49    | 2.1643109541 | 8.27E-12   |
| GO:0009144      | Purine nucleoside triphosphate metabolic process      | 48    | 2.1201413428 | 8.27E-12   |
| GO:0009205      | Purine ribonucleoside triphosphate metabolic process | 47    | 2.0759717314 | 9.26E-12   |
| GO:0009199      | Ribonucleoside triphosphate metabolic process         | 47    | 2.0759717314 | 1.50E-11   |
| GO:0006461      | Protein complex assembly                               | 47    | 2.0759717314 | 1.50E-11   |
| GO:0070271      | Protein complex biogenesis                            | 47    | 2.0759717314 | 1.50E-11   |
| GO:0006873      | Cellular ion homeostasis                              | 47    | 2.0759717314 | 2.68E-11   |
| GO:0055082      | Cellular chemical homeostasis                         | 47    | 2.0759717314 | 4.11E-11   |
| GO:0032268      | Regulation of cellular protein metabolic process      | 46    | 2.0318021201 | 2.46E-10   |
| GO:0008283      | Cell proliferation                                    | 45    | 1.9876325088 | 2.89E-10   |
| GO:0009145      | Purine nucleoside triphosphate biosynthetic process   | 44    | 1.9434628975 | 4.24E-10   |
| GO:0009142      | Nucleoside triphosphate biosynthetic process          | 44    | 1.9434628975 | 9.73E-10   |
| GO:0009206      | Purine ribonucleoside triphosphate biosynthetic process| 43    | 1.8992932862 | 1.48E-09   |
| GO:0009201      | Ribonucleoside triphosphate biosynthetic process      | 43    | 1.8992932862 | 1.48E-09   |
| GO:0045333      | Cellular respiration                                  | 42    | 1.8551236749 | 1.53E-09   |
| GO:0046034      | ATP metabolic process                                 | 42    | 1.8551236749 | 2.38E-09   |
| GO:0033043      | Regulation of organelle organization                  | 42    | 1.8551236749 | 2.96E-09   |
| GO:0006605      | Protein targeting                                     | 41    | 1.8109540636 | 5.08E-09   |
| GO:0032535      | Regulation of cellular component size                 | 41    | 1.8109540636 | 8.13E-09   |
| GO:0006119      | Oxidative phosphorylation                            | 39    | 1.7226148410 | 9.12E-09   |
| GO:0043062      | Extracellular structure organization                   | 39    | 1.7226148410 | 1.14E-08   |
| GO:0016052      | Carbohydrate catabolic process                        | 38    | 1.6784452297 | 1.16E-08   |
| GO:0006754      | ATP biosynthetic process                              | 38    | 1.6784452297 | 1.21E-08   |
| GO:0006575      | Cellular amino acid derivative metabolic process      | 38    | 1.6784452297 | 1.63E-08   |
| GO:0019226      | Transmission of nerve impulse                         | 38    | 1.6784452297 | 3.36E-08   |
| GO:0007017      | Microtubule-based process                             | 37    | 1.6342756184 | 3.37E-08   |
| GO:0009719      | Response to endogenous stimulus                       | 36    | 1.5901060071 | 4.09E-08   |
| GO:0051493      | Regulation of cytoskeleton organization               | 35    | 1.545936958 | 6.21E-08   |
| GO:0046164      | Alcohol catabolic process                             | 34    | 1.5017667845 | 8.50E-08   |
| GO:0016053      | Organic acid biosynthetic process                     | 34    | 1.5017667845 | 1.21E-07   |
| GO:0046394      | Carboxylic acid biosynthetic process                  | 34    | 1.5017667845 | 1.21E-07   |
| GO:0010324      | Membrane invagination                                 | 34    | 1.5017667845 | 1.42E-07   |
| GO:0006897      | Endocytosis                                           | 34    | 1.5017667845 | 1.81E-07   |
| GO:0009725      | Response to hormone stimulus                          | 33    | 1.457591731 | 1.85E-07   |
| GO:0007517      | Muscle organ development                              | 33    | 1.457591731 | 3.23E-07   |
| GO:0055080      | Cation homeostasis                                    | 33    | 1.457591731 | 4.24E-07   |
| GO:0044275      | Cellular carbohydrate catabolic process               | 32    | 1.4134275618 | 7.66E-07   |
| GO:0008202      | Steroid metabolic process                             | 32    | 1.4134275618 | 1.18E-06   |
| GO:0007268      | Synaptic transmission                                 | 32    | 1.4134275618 | 1.27E-06   |
| GO:0046395      | Carboxylic acid catabolic process                     | 30    | 1.3250883392 | 1.31E-06   |
| GO:0016054      | Organic acid catabolic process                        | 30    | 1.3250883392 | 1.31E-06   |
| GO:0044087      | Regulation of cellular component biogenesis           | 30    | 1.3250883392 | 1.49E-06   |
| GO:0006979      | Response to oxidative stress                          | 29    | 1.2809187279 | 1.49E-06   |
| GO:0033365      | Protein localization in organelle                     | 29    | 1.2809187279 | 1.50E-06   |
| GO:0030198      | Extracellular matrix organization                     | 29    | 1.2809187279 | 1.50E-06   |
| GO id.          | Description                                           | Count | %         | Q<0.01    |
|----------------|-------------------------------------------------------|-------|-----------|-----------|
| GO:0043623     | Cellular protein complex assembly                      | 29    | 1.2809187279 | 1.50E-06 |
| GO:0055066     | Di-, trivalent inorganic cation homeostasis           | 29    | 1.2809187279 | 1.65E-06 |
| GO:0015992     | Proton transport                                      | 28    | 1.2367491166 | 2.14E-06 |
| GO:0019320     | Hexose catabolic process                              | 28    | 1.2367491166 | 2.24E-06 |
| GO:0006007     | Glucose catabolic process                             | 28    | 1.2367491166 | 2.36E-06 |
| GO:0006818     | Hydrogen transport                                    | 28    | 1.2367491166 | 2.42E-06 |
| GO:0046365     | Monosaccharide catabolic process                      | 28    | 1.2367491166 | 3.04E-06 |
| GO:0032956     | Regulation of actin cytoskeleton organization          | 28    | 1.2367491166 | 3.40E-06 |
| GO:0032970     | Regulation of actin filament-based process            | 28    | 1.2367491166 | 3.88E-06 |
| GO:0045454     | Cell redox homeostasis                                | 28    | 1.2367491166 | 4.20E-06 |
| GO:0006913     | Nucleocytoplasmic transport                           | 28    | 1.2367491166 | 4.55E-06 |
| GO:0051169     | Nuclear transport                                     | 28    | 1.2367491166 | 6.02E-06 |
| GO:0051130     | Positive regulation of cellular component organization| 28    | 1.2367491166 | 6.58E-06 |
| GO:0016042     | Lipid catabolic process                               | 28    | 1.2367491166 | 7.04E-06 |
| GO:0010608     | Post-transcriptional regulation of gene expression     | 28    | 1.2367491166 | 8.39E-06 |
| GO:0030003     | Cellular cation homeostasis                           | 28    | 1.2367491166 | 9.47E-06 |
| GO:0015674     | Di-, trivalent inorganic cation transport             | 28    | 1.2367491166 | 1.03E-05 |
| GO:0010035     | Response to inorganic substance                       | 27    | 1.1925795053 | 1.42E-05 |
| GO:0006790     | Sulfur metabolic process                              | 27    | 1.1925795053 | 1.43E-05 |
| GO:0006333     | Chromatin assembly or disassembly                      | 26    | 1.1484098940 | 2.23E-05 |
| GO:0015986     | ATP synthesis coupled proton transport                 | 26    | 1.1484098940 | 2.39E-05 |
| GO:0015985     | Energy coupled proton transport, down                  | 26    | 1.1484098940 | 2.64E-05 |
| GO:0034220     | Ion transmembrane transport                           | 25    | 1.1484098940 | 2.89E-05 |
| GO:0008064     | Regulation of actin polymerization or depolymerization| 25    | 1.1484098940 | 4.22E-05 |
| GO:0030832     | Regulation of actin filament length                    | 26    | 1.1484098940 | 4.27E-05 |
| GO:0043254     | Regulation of protein complex assembly                 | 26    | 1.1484098940 | 5.34E-05 |
| GO:0051129     | Negative regulation of cellular component organization| 26    | 1.1484098940 | 5.34E-05 |
| GO:0042692     | Muscle cell differentiation                            | 26    | 1.1484098940 | 5.49E-05 |
| GO:0060537     | Muscle tissue development                              | 26    | 1.1484098940 | 6.85E-05 |
| GO:0006511     | Ubiquitin-dependent protein catabolic process          | 26    | 1.1484098940 | 7.99E-05 |
| GO:0006518     | Peptide metabolic process                             | 25    | 1.1042402827 | 8.11E-05 |
| GO:0032271     | Regulation of protein polymerization                   | 25    | 1.1042402827 | 8.43E-05 |
| GO:0014706     | Striated muscle tissue development                     | 25    | 1.1042402827 | 1.02E-04 |
| GO:0051050     | Positive regulation of transport                       | 25    | 1.1042402827 | 1.25E-04 |
| GO:0030005     | Cellular di-, tri-valent inorganic cation homeostasis  | 25    | 1.1042402827 | 1.79E-04 |
| GO:0006323     | DNA packaging                                          | 24    | 1.0600706714 | 1.90E-04 |
| GO:0042060     | Wound healing                                          | 24    | 1.0600706714 | 1.91E-04 |
| GO:0006084     | Acetyl-CoA metabolic process                           | 23    | 1.0159010601 | 2.00E-04 |
| GO:0006096     | Glycolysis                                             | 23    | 1.0159010601 | 2.24E-04 |
| GO:0030833     | Regulation of actin filament polymerization            | 23    | 1.0159010601 | 2.66E-04 |
| GO:0006399     | tRNA metabolic process                                 | 23    | 1.0159010601 | 2.72E-04 |
| GO:0051187     | Cofactor catabolic process                             | 22    | 0.9717314487 | 2.96E-04 |
| GO:0043244     | Regulation of protein complex disassembly              | 22    | 0.9717314487 | 3.73E-04 |
| GO:0031589     | Cell-substrate adhesion                               | 22    | 0.9717314487 | 4.24E-04 |
| GO:0051146     | Striated muscle cell differentiation                   | 22    | 0.9717314487 | 4.24E-04 |
| GO:0051188     | Cofactor biosynthetic process                         | 22    | 0.9717314487 | 4.87E-04 |
| GO:0007018     | Microtubule-based movement                             | 22    | 0.9717314487 | 4.87E-04 |
| GO:0022904     | Respiratory electron transport chain                   | 21    | 0.9275618374 | 4.87E-04 |
| GO:0009109     | Coenzyme catabolic process                            | 21    | 0.9275618374 | 5.98E-04 |
| GO id.          | Description                                                                 | Count | %          | Q<0.01   |
|----------------|------------------------------------------------------------------------------|-------|------------|----------|
| GO:0015931     | Nucleobase, nucleoside, nucleotide and nucleic acid transport                | 21    | 0.9275618374 | 6.05E-04 |
| GO:0031497     | Chromatin assembly                                                          | 21    | 0.9275618374 | 6.14E-04 |
| GO:0065004     | Protein-DNA complex assembly                                                | 21    | 0.9275618374 | 6.22E-04 |
| GO:0017038     | Protein import                                                              | 21    | 0.9275618374 | 6.40E-04 |
| GO:0050878     | Regulation of body fluid levels                                             | 21    | 0.9275618374 | 6.65E-04 |
| GO:0005976     | Polysaccharide metabolic process                                            | 21    | 0.9275618374 | 6.65E-04 |
| GO:0009060     | Aerobic respiration                                                          | 20    | 0.883922261  | 6.65E-04 |
| GO:0006418     | tRNA aminoacylation for protein translation                                  | 20    | 0.883922261  | 8.53E-04 |
| GO:0043039     | tRNA aminoacylation                                                          | 20    | 0.883922261  | 8.84E-04 |
| GO:0043038     | Amino acid activation                                                        | 20    | 0.883922261  | 9.32E-04 |
| GO:0007160     | Cell-matrix adhesion                                                        | 20    | 0.883922261  | 9.32E-04 |
| GO:0007015     | Actin filament organization                                                  | 20    | 0.883922261  | 1.02E-03 |
| GO:00061069    | Negative regulation of organelle organization                               | 20    | 0.883922261  | 1.13E-03 |
| GO:0050657     | Nucleic acid transport                                                       | 20    | 0.883922261  | 1.13E-03 |
| GO:0051236     | Establishment of RNA localization                                            | 20    | 0.883922261  | 1.22E-03 |
| GO:0050658     | RNA transport                                                                | 20    | 0.883922261  | 1.23E-03 |
| GO:0006403     | RNA localization                                                             | 20    | 0.883922261  | 1.32E-03 |
| GO:0006334     | Nucleosome assembly                                                           | 20    | 0.883922261  | 1.42E-03 |
| GO:0034728     | Nucleosome organization                                                      | 20    | 0.883922261  | 1.45E-03 |
| GO:0006099     | Tricarboxylic acid cycle                                                     | 19    | 0.839222614  | 1.46E-03 |
| GO:0046356     | Acetyl-CoA catabolic process                                                 | 19    | 0.839222614  | 1.47E-03 |
| GO:0090964     | Glutamine family amino acid metabolic process                                | 19    | 0.839222614  | 1.47E-03 |
| GO:0051494     | Negative regulation of cytoskeleton organization                             | 19    | 0.839222614  | 1.71E-03 |
| GO:0006200     | Secondary metabolic process                                                   | 19    | 0.839222614  | 1.84E-03 |
| GO:0030031     | Cell projection assembly                                                     | 19    | 0.839222614  | 2.06E-03 |
| GO:0007599     | Hemostasis                                                                   | 19    | 0.839222614  | 2.06E-03 |
| GO:0016125     | Sterol metabolic process                                                      | 19    | 0.839222614  | 2.06E-03 |
| GO:0006417     | Regulation of translation                                                    | 19    | 0.839222614  | 2.06E-03 |
| GO:0043242     | Negative regulation of protein complex disassembly                           | 18    | 0.7950530035 | 2.06E-03 |
| GO:0006900     | Oxygen and reactive oxygen species metabolic process                         | 18    | 0.7950530035 | 2.16E-03 |
| GO:0048193     | Golgi vesicle transport                                                       | 18    | 0.7950530035 | 2.26E-03 |
| GO:0051028     | mRNA transport                                                                | 18    | 0.7950530035 | 2.33E-03 |
| GO:0008203     | Cholesterol metabolic process                                                | 18    | 0.7950530035 | 2.34E-03 |
| GO:0007596     | Blood coagulation                                                            | 18    | 0.7950530035 | 2.44E-03 |
| GO:0050817     | Coagulation                                                                  | 18    | 0.7950530035 | 2.73E-03 |
| GO:002526      | Acute inflammatory response                                                   | 18    | 0.7950530035 | 2.78E-03 |
| GO:0042943     | Response to drug                                                             | 18    | 0.7950530035 | 2.84E-03 |
| GO:0006749     | Glutathione metabolic process                                                | 17    | 0.7508833922 | 2.86E-03 |
| GO:0030834     | Regulation of actin filament depolymerization                                | 17    | 0.7508833922 | 2.86E-03 |
| GO:0044242     | Cellular lipid catabolic process                                              | 17    | 0.7508833922 | 3.04E-03 |
| GO:0051170     | Nuclear import                                                               | 17    | 0.7508833922 | 3.19E-03 |
| GO:0055001     | Muscle cell development                                                      | 17    | 0.7508833922 | 3.26E-03 |
| GO:009310      | Amine catabolic process                                                      | 17    | 0.7508833922 | 3.28E-03 |
| GO:0009309     | Amine biosynthetic process                                                   | 17    | 0.7508833922 | 3.34E-03 |
| GO:0051248     | Negative regulation of protein metabolic process                             | 17    | 0.7508833922 | 3.34E-03 |
| GO:0006633     | Fatty acid biosynthetic process                                              | 17    | 0.7508833922 | 3.34E-03 |
| GO:0060267     | Regulation of vesicle-mediated transport                                      | 17    | 0.7508833922 | 3.77E-03 |
| GO:0009791     | Post-embryonic development                                                   | 17    | 0.7508833922 | 3.99E-03 |
experiments. We found that the peptide spectral intensity is higher than the spectral counts in the quantification of proteomic analysis. The average peptide spectral intensity was used as a standard for the relative quantification of proteins. A total of 2,331 proteins were identified by using the sequenced peptides as queries in searches against the IPI mouse database. Repeating the search against the related decoy database with the same parameters yielded a low (1%) false discovery rate (FDR) at the peptide level, indicating that our approach has high specificity.

Enriched pathways and functional categories. Among the 2,331 identified proteins, 2,287 were found to correspond to unique genes. To characterize these proteins, we initially categorized them based on biological process terms of GO and conducted an enrichment analysis. The most significant categories are shown in Table I. These processes include the generation of precursor metabolites and energy (504), translation and protein targeting (343), nucleotide and nucleic acid metabolism (298), transport (275) and cellular component organization (289). Some of the identified proteins were associated with cytoskeleton organization (183), protein degradation (116) or response to stimulus (115). Approximately 164 proteins with unknown functions were also identified in the proteome of round spermatids. The full classification of the unknown-function proteins with regards to the biological processes they are associated with is demonstrated in a pie chart in Fig. 2.

Furthermore, the predicted molecular function and subcellular localization of the identified proteins was retrieved from GO and enrichment analysis was performed with DAVID tools. A total of 1,818 identified proteins were classified into 9 groups according to their molecular function: binding (866); catalytic activity (400); structural molecule activity (155); motor activity (150); translation regulator activity (35); anti-oxidant activity (19); and enzyme inhibitor activity (43). The full classification of 1,818 proteins is shown in a pie chart in Fig. 3.

Fig. 4 shows the classification of the proteins identified in this study according to their predicted subcellular localization. If an individual protein was predicted to localize in more than one cellular compartment, all localizations were counted non-exclusively. The largest proportion of the identified proteins was associated with the mitochondrion (486), followed by the following cell parts/organelles: cytoplasm (327); cytoskeleton (227); endoplasmic reticulum (260); nucleus (194); Golgi apparatus (151); membrane (148); and lysosome (45).

| GO id.             | Description                                      | Count | %     | Q<0.01    |
|-------------------|--------------------------------------------------|-------|-------|-----------|
| GO:0018130        | Heterocycle biosynthetic process                 | 16    | 0.7067137809 | 4.28E-03 |
| GO:0055002        | Striated muscle cell development                 | 16    | 0.7067137809 | 4.35E-03 |
| GO:0034504        | Protein localization in nucleus                  | 16    | 0.7067137809 | 4.53E-03 |
| GO:0032269        | Negative regulation of cellular protein metabolic process | 16    | 0.7067137809 | 5.12E-03 |
| GO:0002449        | Lymphocyte mediated immunity                     | 16    | 0.7067137809 | 5.13E-03 |
| GO:0030835        | Negative regulation of actin filament depolymerization | 15    | 0.6625441696 | 5.13E-03 |
| GO:0032272        | Negative regulation of protein polymerization    | 15    | 0.6625441696 | 5.25E-03 |
| GO:0031333        | Negative regulation of protein complex assembly  | 15    | 0.6625441696 | 5.46E-03 |
| GO:0000302        | Response to reactive oxygen species              | 15    | 0.6625441696 | 5.56E-03 |
| GO:0051258        | Protein polymerization                           | 15    | 0.6625441696 | 5.56E-03 |
| GO:0009063        | Cellular amino acid catabolic process            | 15    | 0.6625441696 | 5.56E-03 |
| GO:0006606        | Protein import into nucleus                      | 15    | 0.6625441696 | 6.69E-03 |
| GO:0070482        | Response to oxygen levels                        | 15    | 0.6625441696 | 6.69E-03 |
| GO:0006694        | Steroid biosynthetic process                     | 15    | 0.6625441696 | 6.69E-03 |
| GO:0042773        | ATP synthesis coupled electron transport          | 14    | 0.6183745583 | 6.74E-03 |
| GO:0051693        | Actin filament capping                           | 14    | 0.6183745583 | 7.11E-03 |
| GO:0042743        | Hydrogen peroxide metabolic process              | 14    | 0.6183745583 | 7.27E-03 |
| GO:0034599        | Cellular response to oxidative stress            | 14    | 0.6183745583 | 7.31E-03 |
| GO:0042542        | Response to hydrogen peroxide                    | 14    | 0.6183745583 | 8.12E-03 |
| GO:0030837        | Negative regulation of actin filament polymerization | 14    | 0.6183745583 | 8.13E-03 |
| GO:0034330        | Cell junction organization                       | 14    | 0.6183745583 | 8.55E-03 |
| GO:0006413        | Translational initiation                         | 14    | 0.6183745583 | 8.62E-03 |
| GO:0008652        | Cellular amino acid biosynthetic process         | 14    | 0.6183745583 | 8.74E-03 |
| GO:0006997        | Nucleus organization                             | 14    | 0.6183745583 | 9.32E-03 |

Q, value calculated from p-value by Benjamini-Hochberg-Yekutieli multiple testing correction with Fisher discriminant analysis.

Table I. Continued.
To investigate the pathways governing the behavior of round spermatids, we further classified the proteins based on KEGG pathway terms. As expected, an important proportion of the identified proteins (370) were involved in metabolic pathways. Among these proteins, 81 were involved in the oxidative phosphorylation pathway (Fig. 5A) that supports spermatid maturation, and 34 were related to the fatty acid metabolism pathway. This pathway provides the necessary energy for spermatid maturation. In addition, 27 proteins were bound to the citric acid (TCA) cycle (Fig. 5B) and 92 proteins were involved in sugar metabolism pathways, such as glycolysis, gluconeogenesis, pyruvate metabolism, starch and sucrose metabolism and the pentose phosphate pathway (data not shown).

In addition to the proteins involved in metabolism, a large group of proteins essential for translation were identified in round spermatids. A total of 68 proteins were annotated as parts of the ribosomal pathway, and 17 proteins as related to aminoacyl-tRNA biosynthesis. Numerous proteins were also involved in protein degradation. We found that the round spermatid proteome contained 28 proteins in the proteasome pathway and 40 proteins in the lysosome pathway (Fig. 6A and B). Pathway annotation of the haploid proteome by the Pathway Studio software (http://www.elsevier.com/online-tools/pathway-studio/pathway-studio-web) revealed that 60 proteins are components of the spliceosome pathway, in which heterogeneous nuclear RNA (hnRNA) is converted to mRNA (Fig. 7).

Numerous actin and actin-binding proteins participate in the formation of sperm. LC-MS/MS analysis performed in this study identified ~94 actin-binding proteins, involved in the regulation of the actin cytoskeleton KEGG pathway in round spermatids of mice (Fig. 8A and B).

A total of 25 proteins involved in gap junctions, 44 proteins in tight junctions and 26 proteins in adherens junctions were also detected. Seven proteins involved in the nucleocytoplasmic transport pathway (Fig. 9) and nine proteins related to the caspase cascade of the apoptotic signaling pathway were also identified. Full results from the pathway analysis are shown in Table II.

Discussion

The proteome of a cell or an organelle provides information regarding the ensemble of proteins expressed in that particular cell or organelle, and the modification of proteins under specific physiological conditions and time points (21). Label-free proteomics is a rapidly growing MS-based quantitative proteomic workflow, since it does not require chemical labeling; quantification is thus unaffected by labelling efficiencies (22). In order to fully characterise spermiogenesis, and in particular the biological characteristics of round spermatids, we obtained, using a label-free proteomic approach, the full proteome of 2,331 proteins of round spermatids of mice; among these proteins, 2,287 mapped to unique genes.

Spermatogenesis is a complex and highly ordered process, which begins with the differentiation of spermatogonial stem cells and ends with the formation of mature sperm. In haploid germ cell differentiation (or spermiogenesis), round spermatids undergo marked morphological changes. The nucleus
Figure 5. Pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database. Proteins involved in the (A) oxidative phosphorylation pathway (n=81) and (B) the citrate (TCA) cycle (n=27). Red stars denote the detected proteins.
Figure 6. Pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database. Proteins involved in the (A) proteasome pathway (n=28) and (B) the lysosome pathway (n=40). Stars denote the detected proteins.
becomes more compact, the mitochondria are rearranged, the flagellum develops and an acrosome is formed (23). In the present study, β-1-globin, β-2-globin and histone H4 were found to be expressed in round spermatids (data not shown). These proteins are constituents of the chromatin structure and participate in gene regulation (24).

Energy metabolism is a key process for the development of round spermatids. Round spermatids require ATP, most probably to sustain morphological changes, as well as active protein degradation and synthesis. In round spermatids, lactate and pyruvate are the preferred substrates for the generation of energy; the use of glucose is limited (25). In our study, 504 proteins were identified as involved in the generation of precursor metabolites and energy (Table I). The TCA cycle is the main energy resource of round spermatids, although glycolytic and pentose phosphate pathways also contribute to energy production in the spermatids (26). Citrate synthase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase are expressed in round spermatids (Table I and Fig. 5B). L-lactate dehydrogenase, pyruvate kinase and pyruvate dehydrogenase, which are involved in the glycolytic pathway, are also expressed in round spermatids. Pyruvate kinase is fully activated in round spermatids when glucose is metabolized by the glycolytic pathway (27). A total of 81 proteins were identified as involved in the oxidative phosphorylation pathway in round spermatids (Fig. 5A); these proteins may be involved in the formation and in reactions occurring in the acrosome, which require energy provided by oxidative phosphorylation (25).

At the stage of development of round spermatids, numerous proteins and organelles are degraded; the ubiquitin-proteasome and the lysosome pathways are important, particularly in facilitating the formation of condensed sperms. In the present study, 28 proteins were found as involved in the proteasome pathway and 40 proteins in the lysosome pathway (Fig. 6A and B). Post-translational protein modification by ubiquitination is a signal for lysosomal or proteasomal proteolysis. UBA6 is an E1-activating enzyme, which can activate ubiquitin and FAT10 (28,29). UBA6 uses a specific E2 enzyme, namely, Use1, which cooperates with E3 enzymes to ubiquitylate a unique subset of protein substrates (30). CUL4 is an E3 ubiquitin ligase; in the absence of a functional CUL4 gene, a decreased number of spermatozoa, reduced sperm motility and defective acrosome formation are observed (31). The ubiquitination of proteins can be regulated and reversed by deubiquitinating enzymes.
Ubiquitin C-terminal hydrolases (UCHs) are responsible for the removal of polyubiquitin chains during substrate priming for proteasomal proteolysis. UCHL1 and UCHL3, which were identified in round spermatids in our study, are involved in sperm acrosomal formation and function; these enzymes are known to be important for fertilization (32,33).

Transcription during spermatogenesis begins in almost-round spermatids; these transcripts are then translated during spermatid elongation and acrosome formation (34,35). In our study, 60 proteins were annotated as parts of the spliceosome pathway, in which hnRNA is converted to mRNA and translated to proteins (Fig. 7). Following protein synthesis, some proteins are translocated between the nuclear and cytoplasmic compartments to allow the essential cellular responses to extracellular and intracellular signals. In our study, seven proteins, such as SRP19 and SRP72, were identified as involved in protein transport and regulation of signal transduction (36,37).

Acrosome formation and spermatid nuclear shaping are two major processes of spermiogenesis. Actin and actin-binding proteins are implicated in various aspects, including acrosome formation and nuclear shaping of the spermatids during spermiogenesis. Actin is also involved in germ cell movement, protein transport and nuclear modifications. Numerous actin-binding proteins are found in actin-rich sites, and these...
Table II. Pathway analysis in the round spermatid proteome using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the BioCarta Pathway databases.

| Source       | Term                                      | Count | %     | P          | Q<sup>a</sup> |
|--------------|-------------------------------------------|-------|-------|------------|---------------|
| KEGG         | Ribosome                                  | 68    | 3.0   | 6.5E-32    | 1.2E-29       |
| KEGG         | Oxidative phosphorylation                 | 81    | 3.6   | 3.2E-28    | 2.9E-26       |
| KEGG         | Parkinson’s disease                       | 73    | 3.2   | 6.9E-21    | 4.2E-19       |
| KEGG         | Alzheimer's disease                       | 85    | 3.8   | 2.1E-18    | 9.6E-17       |
| KEGG         | Valine, leucine and isoleucine degradation| 36    | 1.6   | 1.9E-17    | 7.0E-16       |
| KEGG         | Huntington's disease                      | 83    | 3.7   | 4.8E-17    | 1.5E-15       |
| KEGG         | Fatty acid metabolism                     | 34    | 1.5   | 1.1E-15    | 2.9E-14       |
| KEGG         | Citrate (TCA) cycle                       | 27    | 1.2   | 4.2E-15    | 9.8E-14       |
| KEGG         | Spliceosome                               | 60    | 2.7   | 6.8E-14    | 1.4E-12       |
| KEGG         | Glycolysis/Gluconeogenesis                | 38    | 1.7   | 2.3E-11    | 4.3E-10       |
| KEGG         | Propanoate metabolism                     | 23    | 1.0   | 7.5E-11    | 1.3E-9        |
| KEGG         | Glutathione metabolism                    | 30    | 1.3   | 1.6E-9     | 2.5E-8        |
| KEGG         | Proteasome                                | 28    | 1.2   | 2.4E-9     | 3.5E-8        |
| KEGG         | Pyruvate metabolism                       | 25    | 1.1   | 1.1E-8     | 1.5E-7        |
| KEGG         | Focal adhesion                            | 66    | 2.9   | 5.7E-7     | 7.0E-6        |
| KEGG         | Butanoate metabolism                      | 21    | 0.9   | 1.1E-6     | 1.3E-5        |
| KEGG         | ECM-receptor interaction                   | 35    | 1.5   | 1.3E-6     | 1.4E-5        |
| KEGG         | Drug metabolism                           | 31    | 1.4   | 9.6E-6     | 9.9E-5        |
| KEGG         | PPAR signaling pathway                    | 32    | 1.4   | 1.1E-5     | 1.1E-4        |
| KEGG         | Arginine/proline metabolism               | 24    | 1.1   | 2.2E-5     | 2.1E-4        |
| KEGG         | Tight junction                            | 44    | 1.9   | 1.0E-4     | 9.2E-4        |
| KEGG         | Lysosome                                  | 40    | 1.8   | 1.1E-4     | 9.2E-4        |
| KEGG         | Metabolism of xenobiotics by P450 cytochrome | 26 | 1.1 | 1.5E-4 | 1.2E-3        |
| KEGG         | β-alanine metabolism                      | 13    | 0.6   | 1.5E-4     | 1.2E-3        |
| KEGG         | Tryptophan metabolism                     | 18    | 0.8   | 3.5E-4     | 2.6E-3        |
| KEGG         | Alanine, aspartate and glutamate metabolism | 15 | 0.7 | 3.7E-4 | 2.6E-3        |
| KEGG         | Protein export                            | 7     | 0.3   | 7.7E-4     | 5.3E-3        |
| KEGG         | Fatty acid elongation in mitochondria      | 7     | 0.3   | 7.7E-4     | 5.3E-3        |
| KEGG         | Starch and sucrose metabolism             | 16    | 0.7   | 1.0E-3     | 6.6E-3        |
| KEGG         | Pentose phosphate pathway                 | 13    | 0.6   | 1.1E-3     | 6.9E-3        |
| KEGG         | Leukocyte transendothelial migration      | 37    | 1.6   | 1.1E-3     | 6.8E-3        |
| KEGG         | Cardiac muscle contraction                | 27    | 1.2   | 1.1E-3     | 6.7E-3        |
| KEGG         | Valine, leucine and isoleucine biosynthesis | 8     | 0.4   | 1.2E-3     | 6.8E-3        |
| KEGG         | Porphyrin and chlorophyll metabolism      | 14    | 0.6   | 1.4E-3     | 7.9E-3        |
| KEGG         | Adherens junction                         | 26    | 1.1   | 1.7E-3     | 9.3E-3        |
| KEGG         | Aminoacyl-tRNA biosynthesis               | 17    | 0.8   | 2.1E-3     | 1.1E-2        |
| KEGG         | Galactose metabolism                      | 12    | 0.5   | 5.8E-3     | 2.9E-2        |
| KEGG         | Regulation of actin cytoskeleton          | 56    | 2.5   | 6.2E-3     | 3.0E-2        |
| KEGG         | Pentose and glucuronate interconversions  | 9     | 0.4   | 6.5E-3     | 3.1E-2        |
| KEGG         | Arrhythmogenic right ventricular cardiomyopathy | 24 | 1.1 | 6.9E-3 | 3.2E-2        |
| KEGG         | Amino sugar and nucleotide sugar metabolism | 16 | 0.7 | 9.6E-3 | 4.4E-2        |
| KEGG         | Limonene and pinene degradation           | 8     | 0.4   | 1.2E-2     | 5.1E-2        |
| KEGG         | Ascorbate and aldarate metabolism         | 8     | 0.4   | 1.2E-2     | 5.1E-2        |
proteins bind to actin filaments and modulate their corresponding properties and functions. Myosin, an actin-dependent molecular motor, is involved in a number of important functions in spermiogenesis, such as acrosome biogenesis, vesicle transport, gene transcription and nuclear shaping (38,39). In the current study, ~94 proteins were predicted to be involved in the regulation of the actin cytoskeleton in the round spermatids of mice (Fig. 8).

Numerous studies have focused on the proteomic analysis of spermatogenesis. Nevertheless, current knowledge on the proteome of round spermatids is limited, and the detailed protein patterns of round spermatids remain unknown. Thus, large-scale proteomic approaches such as the one employed in the present study, can provide a rich resource in the study of spermiogenesis, and enrich our knowledge on the biological functions of round spermatids.

Table II. Continued.

| Source | Term                                           | Count | %   | P     | Q*    |
|--------|------------------------------------------------|-------|-----|-------|-------|
| KEGG   | Phenylalanine metabolism                       | 10    | 0.4 | 1.2E-2| 5.1E-2|
| KEGG   | Long-term potentiation                         | 22    | 1.0 | 1.2E-2| 5.2E-2|
| KEGG   | Fe γ R-mediated phagocytosis                   | 28    | 1.2 | 1.7E-2| 6.8E-2|
| KEGG   | Glyoxylate and dicarboxylate metabolism        | 8     | 0.4 | 1.7E-2| 6.9E-2|
| KEGG   | Tyrosine metabolism                            | 14    | 0.6 | 1.9E-2| 7.3E-2|
| KEGG   | Gap junction                                   | 25    | 1.1 | 2.0E-2| 7.5E-2|
| KEGG   | Synthesis and degradation of ketone bodies     | 6     | 0.3 | 2.3E-2| 8.7E-2|
| KEGG   | Lysine degradation                             | 14    | 0.6 | 2.8E-2| 1.0E-1|
| KEGG   | N-glycan biosynthesis                          | 15    | 0.7 | 3.3E-2| 1.2E-1|
| KEGG   | Prion diseases                                 | 12    | 0.5 | 4.5E-2| 1.5E-1|
| KEGG   | Long-term depression                           | 20    | 0.9 | 6.0E-2| 2.0E-1|
| KEGG   | Fructose and mannose metabolism                | 12    | 0.5 | 6.5E-2| 2.1E-1|
| KEGG   | Oocyte meiosis                                 | 29    | 1.1 | 6.7E-2| 2.1E-1|
| KEGG   | Renin-angiotensin system                       | 7     | 0.3 | 9.7E-2| 2.9E-1|
| BioCarta| Shuttle for transfer of acetyl groups from     | 8     | 0.4 | 7.9E-5| 1.7E-2|
| BioCarta| mitochondria to the cytosol                  |       |     |       |       |
| BioCarta| uCalpain and friends in cell spread            | 8     | 0.4 | 3.7E-3| 3.3E-1|
| BioCarta| ERAD pathway                                   | 9     | 0.4 | 6.6E-3| 3.8E-1|
| BioCarta| AKAP95 role in mitosis and chromosome dynamics| 6     | 0.3 | 1.9E-2| 6.4E-1|
| BioCarta| Agrin in postsynaptic differentiation          | 11    | 0.5 | 2.2E-2| 6.1E-1|
| BioCarta| Cycling of Ran in nucleocytoplasmic transport  | 4     | 0.2 | 2.7E-2| 6.2E-1|
| BioCarta| Protein kinase A at the centrosome             | 7     | 0.3 | 2.9E-2| 6.0E-1|
| BioCarta| Caspase cascade in apoptosis                   | 9     | 0.4 | 4.1E-2| 6.8E-1|
| BioCarta| Endocytotic role of NDK, phosphins and dynamin | 5     | 0.2 | 5.5E-2| 7.4E-1|
| BioCarta| Role of β-arrestins in the activation and      | 7     | 0.3 | 6.0E-2| 7.3E-1|
| BioCarta| targeting of MAP kinases                      |       |     |       |       |
| BioCarta| How progesterone initiates the oocyte          | 8     | 0.4 | 8.5E-2| 8.2E-1|
| BioCarta| maturation                                     |       |     |       |       |
| BioCarta| Role of Ran in mitotic spindle regulation      | 5     | 0.2 | 8.5E-2| 7.9E-1|
| BioCarta| ChREBP regulation by carbohydrates and cAMP    | 5     | 0.2 | 8.5E-2| 7.9E-1|
| BioCarta| CFTR and b2AR pathway                          | 5     | 0.2 | 8.5E-2| 7.9E-1|
| BioCarta| Rho-selective guanine exchange factor          | 4     | 0.2 | 9.8E-2| 8.2E-1|

Q*, value calculated from p-value by Benjamini-Hochberg-Yekutieli multiple testing correction. ECM, extracellular matrix; PPAR, peroxisome proliferator-activated receptors; ERAD, endoplasmic-reticulum-associated degradation; AKAP, A-kinase anchoring protein; NDK, nucleoside diphosphate kinase; ChREBP, carbohydrate-responsive element-binding protein; CFTR, cystic fibrosis transmembrane conductance regulator; b2AR, β 2 adrenergic receptor.
In conclusion, this study is the first, to the best of our knowledge, to conduct a proteomic analysis of round spermatozids. Round spermatozids are formed in a specific phase of spermatogenesis. We performed label-free quantification analysis and identified 2,287 unique proteins, which are involved in energy metabolism, transcription, protein synthesis and degradation, and nucleocytoplasmic transport. These biological processes facilitate the morphological changes to which round spermatozids are subjected. The proteome analysis performed in the current study provided a comprehensive characterization of the protein expression profiles of round spermatozids. Therefore, the present study is expected to enhance our understanding of the molecular basis of spermatogenesis.

Acknowledgements

This study was supported by the Scientific Research Foundation for Returned Scholars of Shanxi Province (2011-043, 2010-677).

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