Acetylproteomic Analysis Reveals Functional Implications of Lysine Acetylation in Human Spermatozoa (sperm)*

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Male infertility is a medical condition that has been on the rise globally. Lysine acetylation of human sperm, an essential posttranslational modification involved in the etiology of sperm abnormality, is not fully understood. Therefore, we first generated a qualified pan-anti-acetyllysine monoclonal antibody to characterize the global lysine acetylation of uncapacitated normal human sperm with a proteomics approach. With high enrichment ratios that were up to 31%, 973 lysine-acetylated sites that matched to 456 human sperm proteins, including 671 novel lysine acetylation sites and 205 novel lysine-acetylated proteins were identified. These proteins exhibited conserved motifs XXXXYXXX, XXXKXXX, and XXXKXXXX, were annotated to function in multiple metabolic processes, and were localized predominantly in the mitochondrion and cytoplasmic fractions. Between the uncapacitated and capacitated sperm, different acetylation profiles in regard to functional proteins involved in sperm capacitation, sperm-egg recognition, sperm-egg plasma fusion, and fertilization were observed, indicating that acetylation of functional proteins may be required during sperm capacitation. Bioinformatics analysis revealed association of acetylated proteins with diseases and drugs. Novel acetylation of voltage-dependent anion channel proteins was also found. With clinical sperm samples, we observed different lysine acetyltransferases and lysine deacetylases expression between normal sperm and abnormal sperm of asthenospermia or necrospermia. Furthermore, with sperm samples impaired by epigallocatechin gallate to mimic asthenospermia, we observed that inhibition of sperm motility was partly through the blockade of voltage-dependent anion channel 2 Lys-74 acetylation combined with reduced ATP levels and mitochondrial membrane potential. Taken together, we obtained a qualified pan-anti-acetyllysine monoclonal antibody, analyzed the acetylproteome of uncapacitated human sperm, and revealed associations between functional protein acetylation and sperm functions. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.041384, 1009–1023, 2015.

Infertility rates are increasing in modern society. Approximately 9% of couples worldwide are infertile (1), and in at least one-third of the cases, the infertility is due to male factors (2). The underlying causes are complex, and factors such as lifestyle and nutrition, genetic and environmental factors, epidemic infections, and sexually transmitted diseases are known (3). Although 15% of infertility cases are due to genetic defects (4), the majority of other cases have unknown etiology. Treatment of infertile males with assisted reproductive technologies can facilitate fatherhood; however, this approach raises concerns for the genetic/epigenetic integrity of the children. This in turn requires greater understanding of the complex mechanisms of sperm formation and sperm functions.

Large scale “omics” studies hold great promise in terms of establishing cause and effect in human disease. For example, analysis of data from the human genome project has led to the identification of new genetic causes of male infertility (5), particularly those associated with specific defects in semen parameters, testicular pathologies (6), epididymal regulatory elements for genes coordinating epididymal function (7), and cell-free seminal DNA in human semen (8). However, a combination of alternative splicing and posttranslational modifications (PTMs)1 can yield an estimated one million different

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1 The abbreviations used are: PTM, posttranslational modification; KAT, lysine acetyltransferase; KDAC, lysine deacetylase; EGCG, epigallocatechin 3-gallate; KLH, keyhole limpet hemocyanin; VDAC, voltage-dependent anion channel; CsA, cyclosporin A; mAb, monoclonal anti-
protein species that may vary with time and location as well as according to physiologic, pathologic, and pharmacologic perturbations (9) and that hold an even greater promise at the protein level. In somatic cells, such diversity is required for the selected activation and inhibition of signaling pathways in a context-appropriate manner. PTMs regulate diverse processes, including enzyme activation or inactivation, DNA interaction, protein stability, changes in subcellular localization, and protein-protein interaction. Proteomic studies of human seminal plasma (10), sperm-specific proteins (11), subcellular proteins (12), interactomes (13), and clinical studies (14) have provided information on the proteins related to sperm function and dysfunction. However, proteomic studies of sperm protein PTMs have not been widely conducted.

Unlike somatic cells, mature sperm are almost transcriptionally and translationally silent except for minor translation of mRNAs by mitochondrial-type ribosomes (15), which requires that PTMs in sperm play more important roles in regulation by replacing the roles that could be burdened on transcriptional and translational machines in somatic cells. The importance of phosphorylation, an essential PTM in sperm differentiation, maturation, and fertilization, has been well established (16, 17). Acetylation as a broad and abundant PTM comparable with phosphorylation (18), however, has not been well analyzed in human sperm.

Acetylation is a prominent covalent modification among over 200 others (including phosphorylation, ubiquitylation, and glycosylation) found in mammalian cells (UNIMOD database). Dynamic addition or removal of acetyl groups to or from the ε-amino group of lysine residues (19) is catalyzed by lysine acetyltransferases (KATs) and deacetylases (KDACs), respectively. In somatic cells, nuclear histones and transcription regulators and a plethora of cytoplasmic proteins are subject to lysine acetylation (20, 21). In the cell cytoplasm, lysine acetylation plays an indispensable role in cellular metabolic regulation (21) because most mammalian metabolic enzymes are acetylated, and acetylation-mediated metabolic regulation is highly conserved during evolution (22). Moreover, modulation of protein acetylation has been considered an important anticancer strategy (23, 24). Although the roles of lysine acetylation in somatic cell are readily accepted, the roles in human sperm are less clear.

Protein acetylation can modulate proteasomal degradation of core histones (25) and axonemal microtubule construction (26, 27) consistent with findings that poor sperm motility and male infertility are associated with perturbed α-tubulin acetylation (27). Previous in vitro studies also showed that a histone acetylase inhibitor causes spermatid apoptosis, down-regulation of histone acetylation, and a cease in translation in advance (29). These previous observations suggest that protein acetylation might be correlated with the etiology of sperm abnormality and thus imply that protein acetylation (catalyzed by histone acetylases) may be essential for sperm functions. Besides, sperm histone acetylation activity was found to be positively correlated with the sperm DNA fragmentation index in normozoospermic men (30), which also indirectly supports the point above. However, there are few publications on global protein acetylation of human sperm. Although in most cases global identification of acetylation sites relies on polyclonal antibodies (21, 31, 32), there are many drawbacks, such as limited antibody amount and uncertainty of antibody quality due to batch variation. Therefore, monoclonal antibodies (mAbs) are a feasible choice to overcome the drawbacks of polyclonal antibodies. Because commercial pan-anti-lysine acetylation mAbs lack evidence for global application with a high enrichment ratio, we generated high quality anti-acetyl-lysine mAbs first and subsequently analyzed the acetylproteome of human sperm with an aim to uncover the human sperm acetylproteome and its implications for sperm functions.

**EXPERIMENTAL PROCEDURES**

**Peptides and Proteins—**Peptide libraries with and without lysine acetylation (Table I) were synthesized by GL Biochem (Shanghai, China). A cysteine residue (Cys) was added to the peptide N terminus to facilitate conjugation with carrier proteins (BSA and KLH) (32). BSA was chemically acetylated to generate acetylated BSA (32). The concentration of acetylated BSA was determined using a BCA protein assay kit (Pierce) and further analyzed by SDS-PAGE.

**Generation of Pan-anti-acetyllysine Monoclonal Antibodies—**Reagents for immunization of mice, including complete Freund’s adjuvant, incomplete Freund’s adjuvant, polyethylene glycol 4000, and mouse monoclonal antibody isotype reagents, were purchased from Sigma-Aldrich. Cell culture medium DMEM was from Gibco. Hypoxanthine-aminopterin-thymidine and hypoxanthine-thymidine media were from Invitrogen. ECL kits for Western blotting were purchased from Amersham Biosciences. PVDF membrane was from Millipore. BALB/c mice (female; 6 weeks old) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All animal experiments were performed according to the laboratory animal care protocols approved by the Institutional Animal Care Committee of Shanghai Institute of Planned Parenthood Research. Immunization of mice and mAb production followed previous protocols (33).

Mice were immunized with acetylated BSA, and splenocyte fusion with myeloma cells (sp2/0) was mediated by polyethylene glycol 4000. Positive clones were evaluated by indirect ELISA with KLH-P1(Ac) protein conjugate antigen (Table I). After three to four successive limiting dilutions of hybridomas in Sigma-Aldrich hypoxanthine-aminopterin-thymidine medium, positive clones with continuous and stable antibody secretion were cultured and injected into mice to induce ascitic fluid (33). mAbs were then purified on a Protein G column and stored at 1 mg/ml at −20 °C. The subclass of mAbs was determined by indirect ELISA (acetylated BSA as antigens) with mouse monoclonal antibody isotyping reagents. mAbs were conjugated with activated Sepharose 4B.

**ELISA and Dot Blot Assays—**Indirect ELISA was used to characterize the specificity of mAbs. Microtiter plates were coated with 1 mg/liter antigen dilutions (BSA, acetylated BSA, BSA-P1 conjugate, BSA-P1(Ac), BSA-P2, and BSA-P2(Ac)). Primary antibodies derived from different clones were serially diluted, and the secondary anti-
BSA-P1 and BSA-P1(Ac) antigens were dialyzed in dialysis buffer (20 mM KCl, 1.5 mM MgCl2, 10 mM Tris-Cl (pH 7.4), 5 µM TSA, 0.5% Nonidet P-40) containing deacetylase inhibitors. After centrifuging at 4 °C for 10 min, supernatants were immunoprecipitated with tubulin antibody. Pan-acetyllysine mAb 1G5, tubulin (lysine acetylation, Lys(Ac)-40) antibody, and tubulin antibody were used to detect the immunoprecipitation products, respectively, by Western blotting. See supplemental Table 1 for antibody information.

The protein extract or immunoprecipitation products were separated by SDS-PAGE (4–20% acrylamide gel) and transferred to PVDF membranes (GE Healthcare). The membranes were incubated for 2 h at room temperature in TBS containing 5% nonfat milk powder and incubated with primary antibody. For anti-acetyllysine antibody incubation, mAb 1G5 and polyclonal anti-acetyllysine antibody (provided by Zhao et al. (21)) were diluted in TBS (containing 5% BSA) overnight at 4 °C with or without competitive agent-acetylated KLH. After washing four times in TBS-Tween 20, the membranes were incubated with secondary antibody goat anti-rabbit IgG-HRP antibody or goat anti-mouse IgG-HRP antibody (Sigma-Aldrich) for 2 h at room temperature. ECL reagents (GE Healthcare) and a Tanon™ image system (Tanon, Shanghai, China) were utilized to detect proteins on the blots.

Measurement of the Affinity between mAb and Lysine-acetylated Peptides—The affinity between mAb and peptides containing Lys(Ac) was measured by using FortBio’s Octet RED 96. Synthesized BSA-P1 and BSA-P1(Ac) antigens were dialyzed in dialysis buffer (20 mM sodium phosphate, pH 7.4) and biotinylated at room temperature for 30 min. The biotin-conjugated antigens were purified using a Zeba Spin desalting column (Fisher, catalogue number 89882) and diluted to 50 µg/ml. The sensors (super streptavidin) were pre-wet in dialysis buffer for 15 min prior to use and then loaded with biotinylated proteins for 15 min. Controls were sensors loaded with BSA-P1. mAb 1G5 was serially diluted (250, 125, 60, 30, and 15 ng) in a 96-well plate. The measurements were carried out automatically at room temperature.

Human Sperm Samples—The collection of semen was approved by Shanghai Institute of Planned Parenthood Research’s ethics committee and carried out according to criteria in the World Health Organization Manual (5th Edition) with the consent of donors at the Shanghai Sperm Bank. Briefly, semen samples obtained by masturbation after at least 3 days of abstinence were allowed to liquefy for at least 30 min before centrifugation in a 60% Percoll gradient to remove seminal plasma, immature germ cells, and non-sperm cells. The purified sperm were washed in PBS three times before subsequent proteomic analysis.

Cell Lysis, Digestion, Peptide Enrichment, and MS Analysis—Purified sperm cells (~0.3 g) were resuspended in 2 ml of homogenization buffer (10 mM KCl, 1.5 mM MgCl2, 10 mM Tris-Cl (pH 7.4), 5 µM TSA, 0.5% Triton X-100). Sperm cells were successively freeze-thawed, sonicated, and then homogenized with a Fluko homogenizer. The process was repeated three times to allow complete cell lysis. Lysates were centrifuged at 10,000 × g at 4 °C for 30 min. DTT (5 mM final concentration) was added, and the lysate was incubated at room temperature for 10 min. Freshly prepared iodoacetamide was then added to the protein solution to a final concentration of 15 mM; a 30-min incubation at room temperature in the dark followed. The reaction was stopped by adding cysteine to a final concentration of 15 mM at room temperature for 30 min. Chilled acetone was added to a final concentration of 85% (v/v), and the solution was stored at −20 °C for 1 h to precipitate proteins. The precipitated materials were isolated by centrifuging at 800 × g for 4 °C for 5 min. Protein pellets were vacuum-dried for 30 min, dissolved in 5 ml of 50 mM NH4HCO3 (pH 8.0), and sonicated to increase solubility. Five milligrams of protein was digested with sequencing grade trypsin at a 1:50 ratio (trypsin:protein) at 37 °C overnight. Additional trypsin was added at a 1:100 ratio (trypsin:protein) to allow complete digestion at 37 °C for 3 h. The solution was heated at 99 °C for 5 min to inactivate trypsin and centrifuged at 12,000 × g for 10 min. The supernatant was vacuum-dried and redissolved in 1 ml of deionized water. The vacuum drying and redissolving steps were repeated four times to remove NH4HCO3 completely. The pellet was resuspended in binding buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) and incubated with 200 µl of Sepharose 4B beads conjugated with pan-anti-acetyllysine antibody at 4 °C with gentle agitation for 6 h. Beads were washed with PBS three times and rinsed with deionized water. Enriched peptides were eluted (0.1% TFA buffer containing 5% acetonitrile), vacuum-dried, separated by nanoflow LC, and analyzed by Q Exactive MS (Thermo Fisher Scientific) via a nanoelectrospray ion source (Thermo Fisher Scientific) (34). Mass spectra were acquired in a data-dependent mode with an automatic switch between a full scan and the “top 20” mode scan. The target value for the full-scan MS spectra was 3,000,000 with a maximum injection time of 30 ms and a resolution of 70,000 at m/z 200. Resolution for MS/MS spectra was set to 17,500 at m/z 200, and the ion target value for MS/MS was set to 500,000 with a maximum injection time of 120 ms. Normalized collision energy was 30%, and the stepped width was 20%. Repeat sequencing of peptides was kept to a minimum by dynamic exclusion of the sequenced peptides for 25 s. Raw data were analyzed using MaxQuant software (version 1.3.0.5) with standard settings against the UniProt human sequence database (released June 2013; 88,381 entries). All searches were carried out with precursor mass tolerance of 7 ppm and fragment mass tolerance of 20. Six trypsin missed cleavages were allowed. Raw data were analyzed with fixed modification carbamidomethyl (Cys) and variable modifications oxidation (Met) and acetyl (protein N terminus). Peptide identifications were accepted (false discovery rate <1%) and subsequently assembled by MaxQuant into proteins (false discovery rate <1%). Acetylation sites were taken from the acetylation sites.txt files (MaxQuant score >32, localization probability >0.75, and score difference >5).

Protein-Protein Interaction Network and Pathway Enrichment—The updated databases we used were ConsensusPathDB, PINA, HiPPIE, and dbPTM. The software R was used for statistical computing, and Perl scripts were used for the other processing. The protein-protein networks were created with Cytoscape (version 3.2.0). To build the protein-protein interaction network, we selected the candidates with the following standards. Our acetylated proteins as a seed data set were used to search the protein-protein interactions among the PINA database with the experiment proof information from the HiPPIE database. And the proteins directly interacting with the seed candidates were selected as the interaction data set among the PINA and HiPPIE databases, whereas the interactions among the seed data set and the selected interaction data set were used to build the protein-
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confirmed that the mAb 1G5 could recognize peptides of the P1(Ac) library rather than those of unmodified lysine, propionyllysine, butyryllysine, or crotonyllysine libraries (Fig. 1B). The minimal P1(Ac) peptide amount recognized by 1G5 was at least 100 pg/dot (Fig. 1B); thus this mAb was proved suitable for Western blot assays. The selectivity of both polyclonal and monoclonal (1G5) antibodies in Western blotting was further examined by addition of either competing protein-acetylated KLH or KLH during the incubation of primary antibody with the membranes. It was observed that competing acetylated KLH but not KLH inhibited the recognition of primary antibodies in the Western blotting assays (Fig. 1C), indicating that the lysine acetylation specificity of both antibodies is similar. The mAb 1G5 was further applied in the analysis of specific proteins in immunoprecipitation products by Western blotting. It was reported that α-tubulin underwent lysine acetylation in most eukaryotes (35), and TSA treatment increased tubulin acetylation at Lys-40 (36). With the α-tubulin antibody precipitation products of GC-1 cells, mAb 1G5 detected increased lysine acetylation of α-tubulin in the samples receiving TSA treatment when compared with the control, and this was further confirmed by a site-specific tubulin acetylation (Lys(Ac)-40) antibody (Fig. 1D). In addition to its specificity, mAb 1G5 must exhibit high affinity for Lys(Ac) peptides to make it suitable for global acetylome analysis. Therefore, peptide libraries P1 and P1(Ac) (Table I) covalently conjugated to BSA via an N-terminal cysteine residue were immobilized to measure the affinity of mAb 1G5 by ForteBio’s Octet RED 96. Subsequent measurements revealed that mAb 1G5 bound to acetylated lysine with a $K_d$ of 2.85 nM (Fig. 1E), underscoring its suitability for acetylproteomic analysis. All together, these results validated that mAb 1G5, as a pan-reactive anti-acetyl-lysine mAb with specific recognition and good accessibility to antigen peptides, was suitable for lysine acetylation analysis.

Before using this mAb in human sperm in global acetylproteomic analysis, we further compared the present mAb 1G5 with a previously reported polyclonal antibody (21) using sperm samples (from the caput, corpus, and cauda regions of epididymis; testis; and vas deferens). The mAb 1G5 revealed similar lysine acetylation patterns when compared with patterns stained by polyclonal antibody in the one-dimensional and two-dimensional Western blots (supplemental Fig. 1). Note that monoclonal antibody 1G5 stained the same membrane that was probed by the polyclonal antibody first and regenerated. Because of the use of different antibodies, there is minor difference in the patterns. These data support that mAb 1G5 has potential use for acetylproteomic analysis.

Acetylproteome Analysis of Human Sperm—Because the importance of protein acetylation in many somatic cells is readily accepted, a global survey of protein acetylation in sperm with mAb would represent a significant addition to the existing knowledge in this area. We therefore enriched acetylated peptides of normal human sperm using mAb 1G5 and

protein interaction network. The protein-protein interaction networks were created with Cytoscape (version 3.2.0). The pathway enrichment was statistically evaluated using the hypergeometric distribution and false discovery rate-adjusted p value.

Sperm Motility, Mitochondrial Membrane Potential, and ATP Measurement—Normal human sperm were purified with 60% Percoll (GE Healthcare, catalogue number 17-0891-02) and washed twice with human tubal fluid (Quinn’s Advantage fertilization medium, In-Vitro Fertilization Inc., catalogue number SPN-00549). Purified sperm were incubated in human tubal fluid with 10% Quinn’s Advantage SPS serum protein substitute (In-Vitro Fertilization Inc., catalogue number SPN-00234) and then analyzed. For sperm motility, computer-assisted sperm analysis was used to monitor and record sperm parameters. For concomitant sperm mitochondrial membrane potential analysis, cyclosporin A (CsA; Sigma, catalogue number 30024) or control buffer was added to the sperm suspension 15 min before EGCG treatment. After incubation with EGCG for 20 min, sperm were incubated with JC-1 (Sigma, catalogue number T4069) for 10 min in the dark and washed three times with PBS. Sperm mitochondrial membrane potential was assessed by flow cytometry (FACS Calibur). For ATP measurement—
Fig. 1. Specificity of monoclonal antibodies for N-ε-acetyllysine. Reactivity of 1G5, 6A11, 6G11, 7C1, 8B2, and 10B6 antibodies with bound BSA, acetylated BSA, and BSA-peptide conjugates (Table I) was examined by ELISA (A). Various concentrations of the synthesized random peptide libraries carrying lysine and modified lysine were dotted on a nitrocellulose membrane and detected with mAb 1G5 (B). C, Western blot analysis of the human sperm using anti-acetyllysine antibodies. Lanes 1 and 2 were detected by mAb 1G5. Lanes 3 and 4 were detected by polyclonal anti-acetyllysine antibody. Both antibodies were diluted 1:10,000. 100 μg/ml acetylated KLH was added in lanes 2 and 4, whereas the same concentration of KLH was added in lanes 1 and 3 during incubation of the primary antibodies with the PVDF membranes. α-Tubulin was detected as a loading control. D, GC-1 cells with and without TSA treatment (control) were lysed and immunoprecipitated (IP) with α-tubulin antibody, and then the immunoprecipitation products were analyzed by Western blotting using mAb 1G5, tubulin(Lys(Ac)-40) antibody, and α-tubulin antibody, respectively. E, the real time association and dissociation of mAb 1G5 with immobilized antigen peptides recorded by ForteBio’s Octet RED 96 (see “Experimental Procedures”). pr, propionyllysine; bu, butyryllysine; cr, crotonyllysine.
submitted samples for LC-MS/MS analysis. Single peptide and peptide mass fingerprinting identifications, annotated spectra, and annotated mass labeled spectra for all modified peptides were accessible at BiooTools. We identified a total of 973 Lys(Ac) sites that matched to 456 proteins (supplemental Table 2). The percentage of acetylated peptides over total peptides ranged from 12–31%, which was higher than the previous enrichment ratio of polyclonal antibodies (10–20%; see Ref. 32).

We used Motif-X to determine whether the enriched Lys(Ac) peptides could be clustered based on a particular amino acid motif. The search returned three major potential motifs: XXXKYXXX, XXXKFXXX, and XXXKHXXX. As shown in Fig. 2A, the three motifs were enriched from 188, 177, and 167 protein members, respectively. Each of the conserved amino acids at the +1 position in the motifs has different types of side chains in terms of polarity, charge (pH 7.4), and hydrophathy index (34, 35). The motif XXXKYXXX as the most highly enriched motif was consistent with a previously reported motif enriched with polyclonal antibody (31). These data suggested that mAb 1G5 had no essential difference with a previous home-made polyclonal antibody (31) in terms of the major motifs of the enriched peptides.

Brief analysis using DAVID v6.7 (the Database for Annotation, Visualization and Integrated Discovery) revealed that the identified proteins were involved in multiple metabolic processes and especially in the generation of precursor metabolites and energy (Fig. 2B). The majority of the enriched proteins were annotated in the mitochondria and cytoplasm (Fig. 2C). In the activities annotated, catalytic activity ranked first and was followed by oxidoreductase activity (Fig. 2D). KEGG analysis revealed a marked association of Lys(Ac) proteins with the citric acid (tricarboxylic acid) cycle (Fig. 2E) consistent with the previous results observed in liver (21). Furthermore, a comprehensive pathway enrichment in multiple databases revealed that the five top associated pathways were metabolism, disease, the citric acid (tricarboxylic acid) cycle and respiratory electron transport, Huntington disease, and Parkinson disease (supplemental Table 3). In addition, the protein location in different parts of sperm was 191 in mitochondria, 108 in membrane, 79 in acrosome, 13 in nucleus, 12 in flagellum, and five in microtubule (Fig. 2F).

As shown in Fig. 3A, a total of 205 novel lysine-acetylated proteins and 671 novel lysine acetylation sites were identified when compared with previously published data of human sperm (31); 251 acetylated proteins were reported in both the present and previous studies. About 276 of the previously reported acetylated proteins (31) were not identified in the present study. It was observed that 187 of 251 consensus proteins had at least one consensus lysine acetylation site, whereas the other consensus proteins we identified had novel acetylation sites when compared with the previous report (31). See supplemental Table 4 for the detailed information on the acetylation sites. To further evaluate the novelty of the acetylated proteins we identified, comparison with the experimentally verified human protein lysine acetylation in dbPTM 3.0 (37) was conducted, revealing that 206 proteins we identified were never retrieved in the background of lysine acetylation of human proteins, whereas 250 proteins were already recorded in the database (Fig. 3B and supplemental Table 5). It should be noted that 75 of the 250 proteins had no consensus lysine acetylation sites when compared with the known acetylated sites in dbPTM 3.0, indicating that novel acetylation sites were identified (supplemental Table 6) in these known acetylated proteins. Therefore, both the consensus and novel acetylation sites of each protein were combined and listed in detail (supplemental Table 7).

| Peptides  | Peptide sequences |
|-----------|-------------------|
| P1        | CXXXXXKXXXXXXX    |
| P1(ac)    | CXXXXXK(ac)XXXXXX |
| P2        | DENLIRKHTGSGIL    |
| P2(ac)    | DENLIRK(ac)HTGSGIL|
| P(pr)     | CXXXXXK(pr)XXXXXX |
| P(bu)     | CXXXXXK(bu)XXXXXX |
| P(cr)     | CXXXXXK(cr)XXXXXX |
| BSA-P1    | BSA conjugated with P1 |
| BSA-P1(ac)| BSA conjugated with P1(ac) |
| KLH-P1(ac)| KLH conjugated with P1(ac) |
| BSA-P2    | BSA conjugated with P2 |
| BSA-P2(ac)| BSA conjugated with P2(ac) |

Notes: X is any amino acid. C represents cysteine. K is lysine. K(ac) represents acetyllysine in the motifs. K(pr) is propionyllysine. K(bu) is butyryllysine. K(cr) is crotonyllysine.

### Table I

| Synthesized peptides, peptide libraries and peptide-BSA/KLH conjugates with different types of lysine modification |
|-------------------------------------------------------------|
| Peptides | Peptide sequences |
|-----------|-------------------|
| P1        | CXXXXXKXXXXXXX    |
| P1(ac)    | CXXXXXK(ac)XXXXXX |
| P2        | DENLIRKHTGSGIL    |
| P2(ac)    | DENLIRK(ac)HTGSGIL|
| P(pr)     | CXXXXXK(pr)XXXXXX |
| P(bu)     | CXXXXXK(bu)XXXXXX |
| P(cr)     | CXXXXXK(cr)XXXXXX |
| BSA-P1    | BSA conjugated with P1 |
| BSA-P1(ac)| BSA conjugated with P1(ac) |
| KLH-P1(ac)| KLH conjugated with P1(ac) |
| BSA-P2    | BSA conjugated with P2 |
| BSA-P2(ac)| BSA conjugated with P2(ac) |
If the key proteins of sperm functions were taken into account, different profiles of lysine-acetylated proteins were observed in the capacitated sperm (31) and uncapacitated sperm (Table II). All the acetyl proteins related to sperm motility highlighted by the previous study (31) except DPCD were identified in both studies (Table II), indicating that a minor difference between capacitated and uncapacitated human sperm in terms of protein types might exist, although capacitated sperm were apparently different from uncapacitated sperm in motility. However, because DPCD appeared to be a novel candidate for primary ciliary dyskinesia (38) and Dpcd/Pol(−/−) mice had deficient ciliary motility (39), DPCD acetylation might be a key event in the process of sperm capacitation and ciliary motility that is worth further investigation in the future. Besides, of the four acetyl proteins that might be involved in fusion of sperm-egg plasma (31), only ROPN1B was identified in the present study (Table II), which implied that there are dramatic changes after capacitation in terms of lysine acetylation of these proteins. Of the proteins tagged with sperm capacitation, PRKACA, a catalytic subunit of cAMP-dependent protein kinase, was the only one that was not identified in the present study (Table II). It is known that mouse sperm lacking the sperm-specific PRKACA fail to respond to adenosine and catecholamine agonists that stimulate sperm flagellar beat frequency (40). Therefore, identification of PRKACA acetylation in capacitated sperm instead of uncapacitated sperm implied that PRKACA acetylation might be correlated with stimulated sperm kinetic activity in capacitation. During the sperm capacitation process, the increased number of acetylated proteins involved in fusion of sperm-egg plasma (31), only ROPN1B was identified in the present study (Table II), which implied that there are dramatic changes after capacitation in terms of lysine acetylation of these proteins. Of the proteins tagged with sperm capacitation, PRKACA, a catalytic subunit of cAMP-dependent protein kinase, was the only one that was not identified in the present study (Table II). It is known that mouse sperm lacking the sperm-specific PRKACA fail to respond to adenosine and catecholamine agonists that stimulate sperm flagellar beat frequency (40). Therefore, identification of PRKACA acetylation in capacitated sperm instead of uncapacitated sperm implied that PRKACA acetylation might be correlated with stimulated sperm kinetic activity in capacitation. During the sperm capacitation process, the increased number of acetylated proteins involved in fertilization
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![Diagram](image)

**Fig. 3. The numbers of novel acetylated sites and proteins.** A, comparison of the protein lysine acetylation identified in the present study (red circle) with that of a previous publication (31) (blue circle). Green numbers, shared proteins or Lys(Ac) sites in both studies. Red numbers, novel proteins or sites. B, determination of novel proteins and lysine acetylation sites by searching the experimentally verified human proteins in dbPTM 3.0. The blue pie represents the number of novel proteins we identified. The green pie, known lysine acetylated proteins. ***indicated that 75 of the 250 known acetylated proteins had virtually novel lysine acetylation sites that had never been described in the database dbPTM 3.0.

and sperm-egg recognition (Table II) implied that acetylation during capacitation might also be required for the subsequent sperm-egg recognition and sperm fertilization.

The protein-protein interaction analysis revealed a complex network of the proteins we identified. Only interactions with experimental evidence are shown (Fig. 4). Because the proteins were significantly enriched in energy generation, we have highlighted the ATP synthase network (Fig. 5A) by showing significant enrichment in the pathways (a) respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins (REACT_6305 reactome) and (b) formation of ATP by chemiosmotic coupling (REACT_6759 reactome). Voltage-dependent anion-selective channel 2 (VDAC2), a novel and important membrane channel protein in sperm functions, was annotated to be enriched in two pathways (Fig. 5A): (a) Huntington disease, Homo sapiens (human) path:hsa05016 KEGG and (b) Parkinson disease, H. sapiens (human) path:hsa05012 KEGG. Moreover, the networks of highlighted functional sperm proteins in Table II showed significant interaction of AKAP3 and AKAP4 in multiple sperm functions (Fig. 5B).

DrugBank data have been used to annotate human sperm proteome and facilitate in silico drug target discovery by revealing that mitochondrial and ciliary proteins are the major drug targets (41). In a complementary approach, we therefore used WebGestalt software (42, 43) to search for associations between known drugs or disease states using our sperm protein acetylation data set. This analysis revealed that the major Lys(Ac) proteins in human sperm showed association with adenosine triphosphate, adenosine, and NADH; these molecules are involved in energy production (supplemental Table 8). Proteins associated with flunisolide and hydroxocobalamin (enrichment ratio of 41.67 with 0.0002 adjusted \(p\) value) were also enriched. Surprisingly, proteins associated with antibiotics, vitamins, and nerve muscle-regulating drugs were also enriched. These antibiotics include netilmicin, ciprofloxacin, gentamicin, cerulenin, and cephalosporin analogues cefaclor and cefotaxime. Ciprofloxacin, a drug known to be cytotoxic to sperm, can induce apoptosis through the mitochondrial pathway (44). Cephalexin analogues, such as cefpodoxime(45) and ceftriaxone (46), are also known chemicals that impair sperm functions. Two \(\gamma\)-aminobutyric acid-related drugs, baclofen and gabapentin, also had associations with Lys(Ac)-modified proteins. Baclofen, a selective \(\gamma\)-aminobutyric acid type B receptor agonist, was found to significantly increase the percentage of active spermatozoa and lead to sperm hyperactivation (47). Gabapentin, a \(\gamma\)-aminobutyric acid analogue that was originally developed to treat epilepsy and to relieve neuropathic pain, was found to inhibit sperm motility and to be cytotoxic to sexual organs (48). Regarding the correlation with specific diseases, our enriched sperm protein acetylome was associated with mitochondrial diseases, human immunodeficiency virus, stress, shock, and metabolic diseases among others (supplemental Table 9). However, the most significant association was with very-long-chain acyl-coenzyme A dehydrogenase deficiency. Altogether, although the direct interactions between drugs and acetylated proteins need further evidence, our analysis indicated that sperm lysine acetylation might be associated with diverse sperm functions, and the key proteins with acetylation provide novel drug targets for potential clinical use.

**Involvement of KDACs and KATs in Sperm Functions**—Because commercially available antibodies that recognize site-specific lysine acetylation are rare at present, it is almost impossible to correlate lysine acetylation changes with sperm phenotypes by quantifying the fraction changes of novel acetylated proteins to their unmodified forms by Western blotting. In addition, sperm are specialized cells that do not proliferate, which hampers proper labeling in vitro and subsequent quantification by MS. Because protein acetylation was involved in many sperm functions and impaired enzymes might be a pathogenic hint for the unbalanced protein acetylation and deacetylation in abnormal sperm, we selected asthenospermia samples to see whether KDACs and KATs were
involved. Unlike monogenic diseases, asthenospermia is not caused by defects in one single protein. The disease has a complex etiology as many proteins involved in cytoskeletal structure, microtubule movement, and cell movement are associated with asthenospermia (49). We hypothesized that a more global perturbation of acetylation might contribute to the multifactorial etiology of asthenospermia. We first examined the expression of KATs and KDACs by Western blotting in the samples.

Comparison of the enrichment of lysine-acetylated functional sperm proteins in the previous (31) and present study. “1” indicates that the protein was identified, whereas “0” represents not identified.

| Sperm functions      | Gene symbol | Capacitated sperm | Uncapacitated sperm |
|----------------------|-------------|------------------|---------------------|
| Sperm motility       | AKAP4       | 1                | 1                   |
|                      | ODF2        | 1                | 1                   |
|                      | TEKT4       | 1                | 1                   |
|                      | GAPDHs      | 1                | 1                   |
|                      | TEKT3       | 1                | 1                   |
|                      | LDHC        | 1                | 1                   |
|                      | GPX4        | 1                | 1                   |
|                      | TEKT2       | 1                | 1                   |
|                      | DPCD        | 1                | 0                   |
| Fusion of sperm-egg  | ROPN1B      | 1                | 1                   |
| plasma               | PIDIA3      | 1                | 0                   |
|                      | CRISP1      | 1                | 0                   |
|                      | ADAM32      | 1                | 0                   |
| Sperm capacitation   | DLD         | 1                | 1                   |
|                      | CABYR       | 1                | 1                   |
|                      | AKAP3       | 1                | 1                   |
|                      | AKAP4       | 1                | 1                   |
|                      | PRKACA      | 1                | 0                   |
| Fertilization        | ELSOBP1     | 1                | 0                   |
|                      | PLCZ1       | 1                | 0                   |
|                      | KLHL10      | 1                | 0                   |
|                      | SPACA3      | 1                | 0                   |
|                      | SPA17       | 1                | 0                   |
|                      | CRISP1      | 1                | 0                   |
|                      | ROPN1B      | 1                | 1                   |
|                      | AKAP1       | 1                | 1                   |
|                      | AKAP4       | 1                | 1                   |
|                      | ZPB3        | 1                | 0                   |
|                      | ATP8B3      | 1                | 1                   |
|                      | ACR         | 1                | 1                   |
|                      | ACRV1       | 1                | 0                   |
|                      | PARK7       | 1                | 0                   |
|                      | SPACA3      | 1                | 0                   |
|                      | ACE         | 1                | 1                   |
|                      | ATP8B3      | 1                | 1                   |
|                      | ZPB3        | 1                | 1                   |

a Protein lysine acetylation identified in a previous publication (31).

b Protein lysine acetylation identified in the present study.

normal sperm. However, acetyltransferases KAT5 and KAT2A were down-regulated in the asthenospermia and necrospermia samples, whereas KAT1 was up-regulated at the protein level (Fig. 6A). At present, it is technically impossible to tell the specific enzymatic activity of a KAT or KDAC from a pool. So we measured neither the specific nor the total enzymatic activities of KATs or KDACs in the samples.

Involvement of VDAC2(Lys(Ac)-74) in the Impaired Sperm Motility by EGCG—Apart from the over-representation of metabolic enzymes, we observed that membrane proteins and channel proteins (e.g. VDACs 2 and 3) were novel acetylated proteins in human sperm. In mammals, VDACs are pore-forming proteins mainly located in the outer mitochondrial membrane (50); they have already been identified as key determinants of male fertility (51). In addition, human VDAC2 has been found in the acrosomal membrane or plasma membrane of sperm where it mediates Ca\(^{2+}\) transmembrane transport (52). We thus hypothesized that lysine acetylation of VDAC2 might also be associated with sperm motility. Previously, we observed that EGCG treatment of human and mouse sperm in vitro significantly reduced sperm motility and progressive motility in a dose-dependent manner (Fig. 6, B and C), which may be associated with reduced ATP levels (Fig. 6D). We observed that VDAC2(Lys(Ac)-74) was reduced upon treatment with EGCG (Fig. 6E). Therefore, with this model, we evaluated the association of VDAC2 lysine acetylation with sperm membrane potential by flow cytometry (Fig. 7, A–E). Treatment of sperm with EGCG significantly reduced sperm mitochondrial membrane potential (Fig. 7, F and G; p < 0.01, Student’s t test). However, pretreatment with CsA (an inhibitor of VDAC2) protected sperm against EGCG-dependent membrane potential loss (Fig. 7, B–G) and concomitantly protected against loss of sperm motility and progressive motility (Fig. 7, H and I). This observation suggests that VDAC2(Lys(Ac)-74) rather than VDAC2 (Fig. 6E) may be involved in the impairment of sperm motility induced by EGCG.

The incomplete rescue of sperm function by CsA might be due to the involvement of other pore-forming channels. However, the lysine acetylation of other VDAC proteins was not examined due to a lack of acetyllysine-specific VDAC antibody.

DISCUSSION

Prior to this report, detection of protein acetylation has been challenging due to the particular physical and biochemical properties of the acetyl group (32). A combination of subcellular fractionation and antibody affinity purification has enhanced the enrichment of acetylated proteins, particularly because this approach reduces interference from high abundance acetylated histone peptides (32, 53). However, most research to date has been based on the use of polyclonal antibodies, which have low affinity, low selectivity, low enrichment efficiency, and high batch-to-batch variation. The novel, specific, and high affinity anti-acetyllysine mAb we generated here makes it possible to circumvent many of these issues.
and has facilitated the successful global survey of human sperm protein lysine acetylation. When enrichment efficiency was compared, the percentage of acetylated peptides over total peptides ranged from ~12 to 31%, which was higher than that of polyclonal antibodies (10–20%; see Ref. 32). If the number of acetylated proteins was taken into account, the number of proteins we identified was relatively high. The number of acetylated proteins isolated from cellular lysates in previous studies ranges from 191 in the prokaryote Salmonella enterica (54) to more than 1000 in human liver (21); the spectrum of acetylated proteins is very different depending on tissue sources. The number of acetylated proteins we identified was comparable with that obtained with polyclonal antibodies in a previous publication (31). However, in the present study, we used uncapacitated sperm rather than capacitated sperm, which may account for the difference in the protein numbers, protein types, and acetylation sites. Moreover, because of the verified mAb tool, we identified 205 novel lysine-acetylated proteins of human sperm and 671 novel acetylation sites. The successful application of this mAb for acetylproteome analysis proved it to be a very powerful tool for lysine acetylation analysis.

Similar to data obtained in previous publications, most of the proteins we identified have roles in metabolic pathways. Almost all key glycolytic enzymes were found to be lysine-acetylated, which is consistent with a previous observation in human liver samples (22). Similarly, almost every enzyme in the tricarboxylic acid cycle was acetylated. By contrast with the liver study, gluconeogenesis enzymes (such as fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase) were not identified in sperm. These data support the notion that sperm have specific acetylome profiles and that glycolysis might be modulated through protein acetylation in sperm.

In addition to the extensive acetylation of metabolic enzymes, we identified nine tubulin isoforms (supplemental Table 10) and five histone and histone variants with multiple acetylation sites (supplemental Table 11). Lysine acetylation of sperm tubulins is essential for sperm motility, and a recent publication showed an association between acetylated α-tubulin isoforms (TUBA3C and TUBA4A) and asthenospermia (27). We also identified site-specific acetylation of these α-tubulin isoforms in the current study (supplemental Table 10). These data may provide insights into the mechanisms by which α-tubulin isoforms are acetylated and how this impacts the regulation of sperm motility. The site-specific lysine acetylation of histones is known to regulate genomic histone reten-

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Fig. 4. Network graph of the protein-protein interactions. Green color represents proteins identified in the present acetylproteome study. Gray color indicates proteins that were not identified in the present acetylproteome study. Only interactions with experimental evidence are shown. The graph was created by Cytoscape (version 3.2.0).
tion (55), transfer of epigenetic information to the oocyte (56), and vertical transfer of epigenetic markers (57). Although acetylation of H4K8, H3K9, H4K12, and H4K16 has been detected in human sperm, there have been no global studies of histone (or other protein) lysine acetylation sites in mature human sperm. The novel histone acetylation sites we identified (supplemental Table 11) reveal novel facets to histone regulation in human sperm.

Unexpectedly, ribosomal cytoplasmic proteins (60 S) RPL10A and RPL3, which have been isolated from human sperm nuclei (58), were also identified in the acetylproteome. This suggests that cytoplasmic translation in sperm may be regulated by lysine acetylation. Because of the successful enrichment of lysine-acetylated peptides, we were able to identify novel VDACs, one of which was recently characterized in both bovine and human sperm (50, 52). Modification of channel proteins by acetylation is virtually unexplored territory except for a recent report in which incretin regulates β-cell function via the acetylation of multiple Kvα and Kvβ subunits (59). We did not fully explore the mechanistic impact of lysine acetylation on VDAC2 function here. However, our data showing that CsA (a known VDAC modulator) changes sperm mitochondrial membrane potential and VDAC acetylation status suggest that acetylation and mitochondrial function are closely correlated in sperm. We suggest that improved extraction of membrane proteins combined with acetyllysine peptide enrichment will be a powerful and informative approach to be exploited in the future.

Comparison of the acetylation of key functional proteins involved in sperm functions (Table II) suggested that post-ejaculation sperm might require dynamic acetylation to support the subsequent sperm capacitation, sperm-egg recognition, fertilization, and fusion of sperm-egg plasma just like the occurrence of tyrosine phosphorylation in the sperm head.

Fig. 5. Representative protein-protein interaction networks of key pathways and proteins involved in sperm functions. A, annotation of the acetylated proteins by protein-protein network and pathway enrichment. The two significantly enriched pathways in the ATP synthase network were: (a) respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins (REACT_6305 reactome) and (b) formation of ATP by chemiosmotic coupling (REACT_6759 reactome). The two significantly enriched pathways of the VDAC2 network were (a) Huntington disease, H. sapiens (human) path:hsa05016 KEGG and (b) Parkinson disease, H. sapiens (human) path:hsa05012 KEGG. Green color represents pathway a enrichment, and yellow color represents pathway b. Red color indicates enrichments in both pathways a and b. Gray color indicates the interactive proteins that were not enriched in the designated pathways. B, protein-protein interaction networks of the highlighted functional human sperm proteins in Table II. Green color represents proteins identified in the present acetylproteome analysis, whereas gray color indicates proteins that were not identified.
during capacitation (60, 61) to enable sperm to display capa-
citamtion, to undergo acrosome reactions, and to penetrate
zona-free oocytes. Therefore, acetylation profiles of different
types of human sperm can lay a solid basis for the discovery
of novel biomarkers if site-specific antibodies could be devel-
oped to verify the different modifications of key functional
proteins in future.

To our knowledge, this is the first report to identify the
global lysine acetylation profile of human sperm using mAb
and to describe the potential correlations between lysine
acetylation and sperm functions. In the future, the lysine
acetylproteome might be a source of novel biomarkers of
sperm abnormalities that can be exploited for both diagnostic
and therapeutic purposes. Currently, clinicians rely primarily

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**Fig. 6.** Lysine acetyltransferases, deacetylases, and VDAC2(Lys-74) acetylation are correlated with sperm motility. A, expression of lysine acetyltransferases and deacetylases in normal and abnormal human sperm samples. Human sperm samples (five semen samples for each group) were pooled and examined by Western blotting. GAPDH, β-actin, and α-tubulin were detected, respectively, in cases where sperm samples of asthenospermia (AS) and necrospermia (NS) had abnormal GAPDH, actin, or tubulin. B and C, the impairment effects of EGCG on human and mouse sperm motility. Purified human and mouse sperm were incubated with EGCG (0, 35, or 175 μg/ml) for 0, 30, 90, or 180 min and analyzed with computer-assisted sperm analysis. Sperm motility was defined by the percentage of spermatozoa with rapid and slow motility (“grade A,” sperm with progressive motility; “grade B,” sperm with non-linear motility; “grade C,” sperm with non-progressive motility). D, relative ATP concentrations of sperm with and without 35 μg/ml EGCG treatment. CN, control; NTC, non-treatment control. E, inhibition of human sperm VDAC2(Lys-74) acetylation by EGCG. Lysates of human sperm treated with EGCG for different periods of time were analyzed by Western blotting for acetyl-VDAC2(Lys-74), α-tubulin, VDAC2, and acetyl-α-tubulin(Lys-40). Error bars, S.D.
on semen analysis, which is limited to count, motility, and morphology, to predict male reproductive potential. Proteomics has great potential for expanding the repertoire of clinical diagnostic tests of sperm in the infertility field. For example, reports of the entire human sperm proteome have increased our understanding of human sperm function (41, 62), and comprehensive comparative studies between normal and abnormal spermatozoa have led to the discovery of novel peptides and metabolites that may be correlated with male infertility. With the novel antibodies we report here, clinicians can go a step further to examine whether impaired sperm function is due to altered PTMs. We also identified novel lysine-acetylated proteins that might be targeted by antibiotics, vitamins, and other drugs. This information may

Fig. 7. Effects of EGCG and CsA on sperm mitochondrial membrane potential and sperm motility. Sperm pretreated with various concentrations (0, 7.5, and 15 μM) of CsA were incubated with 35 μg/ml EGCG and stained with JC-1 before flow cytometry analysis. Gates R2, R3, and R4 represent sperm with high, medium, and low mitochondrial membrane potential, respectively. A–E, representative flow cytometry assays of three independent experiments. The percentage of events calculated in gates R2 and R2/R3 is shown in F and G. The percentage of rapid motility (grade A, A%) and the total motility (grades (A + B + C), (A+B+C)% analyzed by computer-assisted sperm analysis is shown in H and I. CN, control. Error bars, S.D. *, p < 0.05; **, p < 0.01, n = 3, paired Student’s t test.
be useful in both retrospective and prospective studies to look for correlations among sperm abnormalities, infertility, and treatment. Our findings also have provided a candidate list for sperm impairment factors and pathways that are worth further study. Along with recent findings in the fields of genetics, proteomics, and metabolomics (28), our contribution has added knowledge and provided tools for PTM proteomics research to discover theranostic biomarkers.

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