Sperm Chromatin

FERTILE GROUNDS FOR PROTEOMIC DISCOVERY OF CLINICAL TOOLS*

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Sperm are remarkably complex cells with a singularly important mission: to deliver paternal DNA and its associated factors to the oocyte to start a new life. The integrity of sperm DNA is a keystone of reproductive success, which includes fertilization and embryonic development. In addition, the significance in these processes of proteins that associate with sperm DNA is increasingly being appreciated. In this review, we highlight proteomic studies that have identified sperm chromatin proteins with fertility roles that have been validated by molecular studies in model organisms or correlations in the clinic. Up to 50% of male-factor infertility cases in the clinic have no known cause and therefore no direct treatment. In-depth study of the molecular basis of infertility has great potential to inform the development of sensitive diagnostic tools and effective therapies that will address this incongruity. Because sperm rely on testis-specific protein isoforms and post-translational modifications for their development and function, sperm-specific processes are ideal for proteomic explorations that can bridge the research lab and fertility clinic. ***Molecular & Cellular Proteomics 7:1876–1886, 2008.***

Sperm are intricate yet streamlined cells with the underlying purpose of producing a healthy baby. Sperm cells in every sexually reproducing animal are highly specialized delivery vehicles for the chromatin cargo within, which is composed of DNA and its associated proteins. It is clear that sperm chromatin is essential for sperm function and subsequent embryonic development because defects in sperm chromatin are linked to natural reproductive malfunctions like spontaneous abortion as well as assisted reproductive failure (1–3). These defects can include disrupted DNA integrity caused by genetic mutations, apoptotic DNA fragmentation, or exposure to environmental agents and free radicals (4). Furthermore, disruption of chromatin proteins contributes to decreased fertility (5). Despite this evidence, most clinical assays for sperm chromatin only detect gross defects in DNA integrity (4, 6). This, combined with the typical assessments of sperm production, including motility, morphology, and male hormone levels, still result in a striking 30–50% of male-factor infertility cases having no known cause (7). With 2.1 million couples in the United States facing infertility as of 2002, there is a pressing need for an expanded array of sensitive tests to improve diagnostic capabilities in the clinic (8).

The requirement for more research extends beyond diagnosis, however, because many of the basic cellular mechanisms that underlie male infertility remain unknown. Consequently, virtually no therapies exist that remedy the molecular causes of sperm dysfunction. Rather, prolonged or invasive assisted reproductive technology (ART)[1] procedures and intracytoplasmic sperm injection (ICSI) are used to bypass male infertility, with a modest 42% success rate (9). In addition, the safety of widespread ICSI use has recently been called into question. It is speculated that sperm from infertile patients contain cytologically subtle chromatin abnormalities that can affect the resulting embryo (10, 11). Thus it is important to define the epigenetic features of paternal chromatin that can affect future generations. Further knowledge about sperm chromatin protein function can lead to the development of novel therapies that target only sperm, reducing possibilities for side effects or unintended consequences on resulting offspring.

Any comprehensive understanding of sperm biology must include proteomic analysis. Sperm are particularly well suited to proteomic approaches. Compared with most other cell types, they are easily isolated from other tissues and fluids. Importantly, sperm development and function rely heavily on sperm-specific protein isoforms of somatic counterparts, as well as the post-translational modification of key sperm proteins (12, 13). For example, during spermatogenesis, histone proteins in developing sperm are replaced by testis-specific histone variants important for fertility (14). Also, because *de novo* transcription in post-meiotic sperm is largely silenced, the cell depends on post-translational modifications to implement subsequent stages of sperm formation, maturation, and activation (15). The development of clinical applications arising from the proteomic discovery of sperm proteins and sperm-specific protein isoforms is a budding field, with abundant opportunities for innovation. Of particular promise is the

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[1] The abbreviations used are: ART, assisted reproductive technology; ICSI, intracytoplasmic sperm injection; SNBP, sperm nuclear basic protein; IVF, *in vitro* fertilization; 2D-PAGE, two-dimensional poly-acrylamide gel electrophoresis; LC-MS^2, liquid chromatography-tandem mass spectrometry; GO, Gene Ontology; RNAi, RNA interference; MudPIT, Multidimensional Protein Identification Technology; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

identification of sperm-specific post-translational modifications that are functionally important during and after sperm development.

Functional analysis of sperm-specific factors following proteomic identification is crucial to our understanding of male reproduction. Direct experimental approaches can identify relevant variables and reduce experimental complexity. Such strategies are facilitated by the use of model organisms like sea urchins, fish, mice, worms, and flies. Model organisms often have simplified anatomical structures and streamlined developmental programs in comparison to humans. Their male germ cells can be obtained in large quantities in various developmental stages. Though some male reproductive proteins evolve rapidly (16), others involved in fundamental processes of sperm development like meiosis are conserved (17–19). As in humans, the chromatin of each of these animals is at its most compact in mature sperm. Ultimately, defining the molecular functions of conserved proteins can aid in identifying biologically relevant clinical markers of human infertility, as well as targets for infertility therapies.

One challenge currently facing proteomicists, basic biologists, and clinicians is how to make sense of the vast amount of data being generated through proteomic, genomic, functional, and clinical studies. Only through solving this challenge can the full potential of proteomics be translated into meaningful clinical options for male infertility. This review will highlight a selection of recent advances in our understanding of sperm chromatin biology that allow proteomic data to be interpreted within cellular or functional contexts. These studies include strategies for employing bioinformatics to generate testable hypotheses, increasing the specificity of proteomic analysis, analyzing function in model organisms, and correlating expression changes with detailed observations from the clinic. The proteomics of other sperm subcellular compartments, fluid components, or physiological reproductive structures are presented elsewhere (20, 21). By discussing sperm chromatin-based studies, we illustrate the contributions that chromatin studies can make toward understanding fertility, as well as the need to develop sophisticated molecular tools for the study of male-factor infertility. We also promote communication and integration between lab and clinic when designing and interpreting studies.

Sperm Are Ideal for Proteomic Analysis—Sperm are arguably the most specialized cells in the human body (Fig. 1*A*). As such, a remarkable set of complex events must unfold to ensure the proper development and function of each cell. These include a) configuring chromatin for efficient delivery to the egg and preserving the epigenetic information needed for subsequent zygotic development (22); b) using a flagellated force-generating tail structure for long-distance travel to the egg (23, 24); and c) unleashing a series of membrane-associated cellular changes for penetrating the substantial corona and zona pellucida layers surrounding the egg (25–27). The sperm cell develops these capacities at distinct time points before fertilization (Fig. 1*B*). Chromatin changes occur in the testis during meiosis (in which copies of the genome are partitioned into haploid spermatid cells) and spermiogenesis (in which spermatids elongate to form sperm with fully compacted chromatin). Sperm motility is gained in the epididymis upon exit from the testis, and sperm capacitation for penetrating the zona pellucida occurs within the female reproductive tract. These events are largely controlled by post-translational events, for transcription and translation greatly subside as DNA becomes tightly compacted and cytoplasm is jettisoned during spermiogenesis (13, 15). Progression of subsequent developmental stages is mediated by existing signaling molecules (26, 27). For example, the phosphatase protein PP1γ is involved in multiple aspects of sperm development, including spermiogenesis and acquisition of sperm motility (28–30). Much is yet unknown regarding the molecular basis of sperm biology, particularly how these drastic changes in cell morphology and function occur in the absence of new protein synthesis.

The wide array of changes in chromatin during sperm development provides an ideal platform for proteomic exploration of essential sperm-specific chromatin proteins. During meiosis, sperm chromosomes are segregated in a distinct fashion from that of oocyte chromosomes with unique timing and generated end products (31). After meiosis, sperm DNA experiences extreme chromosome compaction during spermiogenesis. This compaction is mediated by drastic changes at the most fundamental level of DNA packaging where a nucleosomal architecture shifts to a toroidal structure (32). Sperm nuclear basic proteins (SNBPs), which include variants of histone subunits, transition proteins, and protamine proteins, implement this change (33, 34). This transition occurs in a stepwise fashion, replacing somatic histones with testis-expressed histone variants, then transition proteins, and finally protamines (35). Deficits in SNBPs are known to cause male infertility (36–38). A growing body of work indicates that these chromatin proteins do not act exclusively to compact sperm DNA. Histone localization and post-translational modification of histones encode epigenetic information that may regulate transcription important for sperm development (22). They may also serve to mark the heterochromatic state of specific regions of the genome that may be important after fertilization, when somatic histones are incorporated back into paternal chromatin or during subsequent zygotic development (39). Thus achieving a deep understanding of the molecular basis of sperm chromatin composition and dynamics will impact multiple levels of fertility biology.

Lessons Learned from Whole Sperm Proteomics—Any single component of a system must be understood within the context of the whole. To date, most proteomic studies on human sperm have concentrated on determining the protein content of the whole sperm cell. Estimates of sperm proteome size have been made based on two-dimensional poly-acryl-

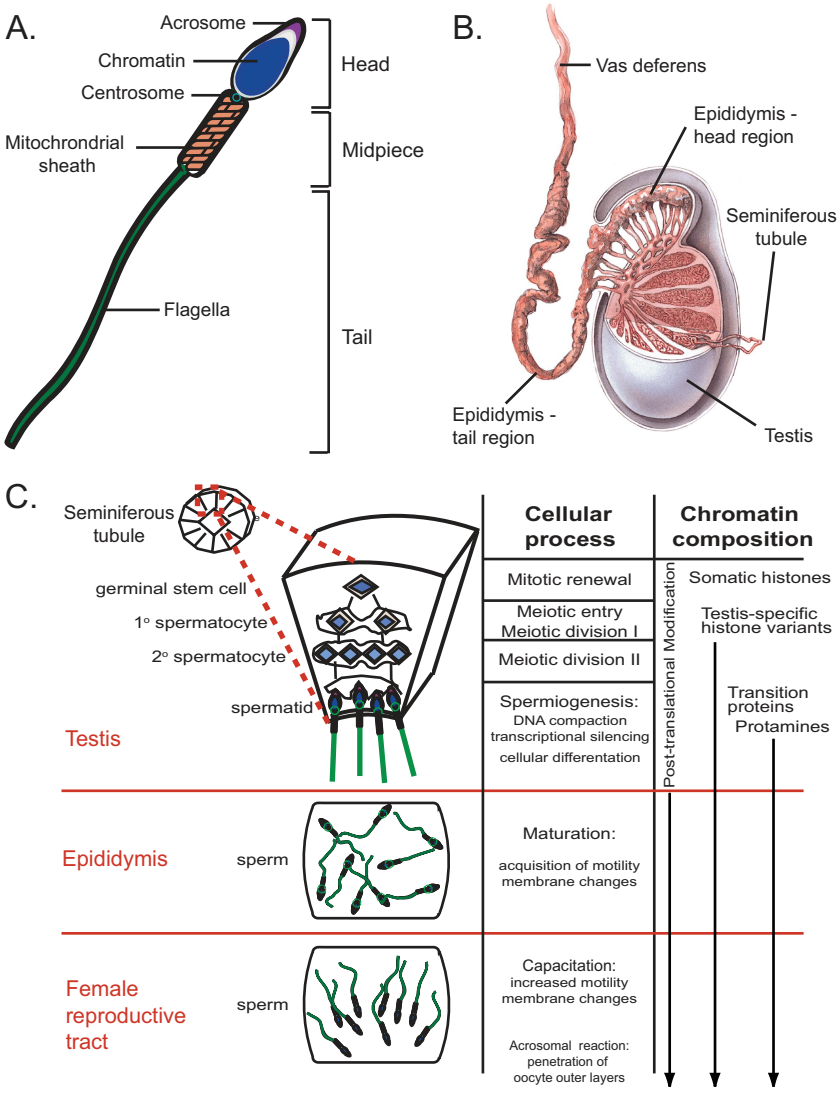

FIG. 1. **Human sperm and sperm development.** *A*, schematic of a human sperm cell, which is composed of three regions. The head region contains the highly compacted sperm DNA and associated proteins (*blue*) surrounded by the cellular membrane (*black*) that in the head region have receptors for recognizing oocyte factors. The acrosome (*purple*) houses digestive enzymes used for penetrating the oocyte outer layers. The mid-piece region consists of the paternally contributed centrosome (*green circle*) and mitochondria (*tan*) used for energy generation. The flagellar tail (*green*) provides motility. *B*, diagram of testis, epididymis, and vas deferens. Sperm develop within the seminiferous tubules that are coiled within the testis. Sperm transit to the epididymis where they further mature and are then stored in the vas deferens. Contraction of the vas deferens propels sperm through the male reproductive tract during ejaculation. Image from LifeART (and/or) MediClip® (2008) Wolters Kluwer Health, Inc.- Lippincott Williams & Wilkins. All rights reserved. *C*, diagram of human sperm formation and associated processes. A cross-section of a seminiferous tubule found within the testis is illustrated. Germinal stem cells renew through repeated rounds of mitosis then shift to meiosis to form primary and secondary spermatocytes. After meiosis, haploid cells undergo spermiogenesis, where DNA is tightly compacted, and transcription is largely silenced. Cells also differentiate morphologically to form flagella. In the epididymis, sperm mature to become motile, and cellular membranes are prepared for fertilization. After transfer to the female reproductive tract, sperm cellular membranes undergo further changes, and sperm become highly motile, a process known as capacitation. Upon binding with the oocyte, acrosomes release enzymes to penetrate oocyte outer layers to allow sperm-egg membrane fusion for fertilization.

amide gel electrophoresis (2D-PAGE), which creates a visual reference map of the sperm proteome by separating proteins according to isoelectric point and mass to ideally resolve protein isoforms (40). Using this technique, the number of sperm proteins has been estimated at 1000–2000 (41, 42). Subsequently, the resolution of 3872 separate protein spots was achieved via multiple 2D-PAGE, each corresponding to a restricted pH range for separation in the first dimension (43). Shotgun proteomic approaches have also contributed substantially to sperm proteome characterization (44, 45). Human sperm proteins were identified after proteolytic digestion and high-resolution liquid chromatography to separate peptides before tandem mass spectral identification (LC-MS^2). To decrease sample complexity, sperm proteins were divided into

Triton X-100 soluble or insoluble fractions, then further separated via one-dimensional SDS-PAGE before protease treatment and LC-MS^2. Repeated rounds of LC-MS^2 identified peptides corresponding to 1760 and 1056 proteins, respectively; though the identified protein list is only available for the latter (44, 45).

Such proteomic technologies are powerful and have yielded remarkably large lists of proteins. However, each still has its challenges, which include the detection of low abundance proteins (46). For 2D-PAGE, the excision and mass spectrometric characterization of spots to assign protein identity can be laborious. For example, Li *et al.* (43) verified the protein identities of 16 of 3872 spots, whereas Martinez-Heredia *et al.* (41) identified 131 of 1000 spots, providing just a glimpse of the proteins and their functions (47). While shotgun proteomic techniques can give a more thorough picture, the proteolytic digestion of whole proteins into individual peptides *en masse* may obfuscate detection of multiple isoforms or combinations of post-translational modifications of any one protein. The difference in the number of protein species detected via 2D-PAGE (3872) and the number of protein identities resolved by shotgun proteomics (1056) may attest to differences in technology. For example, the smaller number of proteins detected by shotgun proteomics may reflect that there are fewer proteins and protein isoforms in sperm because mature sperm have eliminated almost all cytoplasm. The larger number of protein spots detected by 2D-PAGE may suggest that there are more post-translationally modified versions of the proteins present. The development of new or combined proteomic approaches leading to more comprehensive identification of the sperm proteome places a full appreciation of the complexity of post-translational regulation within our grasp.

The elucidation of the sperm proteome provides a rich source of raw material for bioinformatic investigation. The use of Gene Ontology (GO) classifications to generate putative functional or subcellular localization groups provides a general snapshot of the proteome (48). In addition to GO analysis, more sophisticated classification methods can be employed to reveal meaningful patterns. For example, analysis of the sperm proteome of *Drosophila melanogaster* has generated new hypotheses about germline gene regulation and chromatin organization (49). First, shotgun mass spectrometric analysis leading to the identification of 381 proteins in *Drosophila* sperm was performed. By analyzing the chromosomal distribution of corresponding genes, Dorus *et al.* (49) determined that sperm proteins are underrepresented on the X chromosome. This result is consistent with the finding from DNA microarray analysis that male-expressed genes are also underrepresented on X (50). Sub-chromosomal clustering analysis also shows that individual chromosomes have regions with high densities of sperm-expressed genes, suggesting that the genome itself is organized into clusters of similarly expressed genes. This analysis led to the hypothesis that cessation of transcription during spermatogenesis occurs in a hierarchical fashion, with sperm-specific genes being silenced last (49). We can speculate further that SNBPs may also be located in sub-chromosomal clusters whose expression is regulated by chromatin-based mechanisms. In this way, existing large data sets can be mined for testable hypotheses using various bioinformatic strategies, contributing greatly to the potential of proteomics to address specific cellular processes.

Are studies of whole sperm the best application of proteomic technology to yield information about subcellular compartments like the nucleus? How are proteins categorized within GO groups actually connected in functional pathways? Although whole sperm analysis has increased our knowledge of the composition of a sperm cell, we still do not understand how most of these proteins contribute to specific aspects of fertility. Ultimately, to understand the significance of proteomic data, functional studies must be performed. The following section describes strategies that increase our knowledge of mechanisms important for fertility by combining directed proteomic approaches for identification of sperm-specific proteins with functional studies in model organisms.

Directing Proteomics Toward Functional Analysis—Knowledge of male-factor infertility can be greatly expanded by in-depth analysis of each stage of sperm cell development in model organisms such as *Caenorhabditis elegans*, *Drosophila*, and mouse (18, 51). Because many essential elements of sperm development are evolutionarily conserved, the use of model organisms allows a reductionist approach to the syndrome of male infertility in the context of hypothesis-based experiments with controlled variables. In these organisms, sperm develop rapidly in tissues analogous to the human testis and are accessible to a variety of experimental approaches at each stage in the development (52–55). For example, cytological assays to view cells during all stages of sperm development are well established. Also, biochemical protocols for the stage-specific isolation of developing sperm are commonly employed, as are subcellular fractionation techniques to enrich for certain classes of proteins. Each of these organisms has extensively annotated genomes accessible through on-line databases like WormBase, FlyBase, and the Mouse Genome Informatics website (56–58). Loss-of-function analyses of specific proteins using genetic mutations or RNA interference (RNAi) are also straightforward to conduct.

A traditional challenge encountered using model organisms is the discovery of candidate proteins to be analyzed for specific functions. Today, modern shotgun proteomic technology and sensitive methods of detection can create the opposite dilemma. Proteomic lists on the order of thousands of proteins present an overwhelming number of candidates for functional analysis. Several strategies can focus the scope of proteomic identification upon a process of interest. One is

to employ subcellular fractionation techniques that focus on the organelles or fractions of interest (59). An additional method is the application of abundance criteria from mass spectrometry to prioritize likely resident proteins of subcellular organelles or regions (60). The utility of abundance criteria in prioritizing factors that make up various subcellular compartments of embryonic stem cells (cytoplasm, nucleoplasm, and chromatin/membrane) was validated by the finding that abundant proteins in each compartment corresponded to expected functional clusters based on GO (61). The refinement of the target protein list can be accomplished through subtractive or comparative analysis against a different cell type or developmental stage, thereby enriching for relevant proteins and eliminating general factors (62, 63).

One example of the use of these criteria is a study that prioritized the identification of sperm-enriched chromatin proteins with the goal of finding evolutionarily conserved fertility factors (17). Each of these three strategies was employed to generate a focused list that was subsequently used in functional studies. First, subcellular biochemical fractionation was used to purify sperm chromatin proteins, which reduced overall proteomic complexity. Large-scale protein identification was carried out using Multidimensional Protein Identification Technology (MudPIT), which employs a tandem set of liquid chromatographic steps (strong cation exchange and reverse phase) to separate proteolytic peptides before MS^2 identification (64). The peptides identified corresponded to 1099 predicted *C. elegans* proteins (65). Second, reproducibility from multiple repetitions of MudPIT analysis was used to generate a prioritized list of 502 proteins, which represented 88% of the relative mass of all sperm chromatin proteins identified and were thus the most abundant proteins in the samples. Third, this group was subtracted against a list of all oocyte chromatin proteins generated in parallel, reducing the number of sperm chromatin-associated fertility candidates to 132. By taking this step, a large number of shared housekeeping and DNA proteins as well as canonical histones and shared meiotic proteins were eliminated. Thus, members of this list were likely to have sperm-specific chromatin localization and function.

The rationale for this experimental design was supported by functional analysis of these candidate proteins in worms (17). First, sperm chromatin localization was demonstrated for 11 candidates using immunolocalization. Subsequent functional analysis using RNAi and assays for fertility showed that 38% percent of these 132 proteins are important for germ cell formation or embryonic viability. The actual percentage is likely to be higher because functional redundancy between co-expressed protein isoforms may obscure detection of a fertility role for any single protein, and because genes required for spermatogenesis are often resistant to RNAi in *C. elegans*. Significantly, 59% percent of the 132 proteins have human homologs, whereas 37% of genes with mouse homologs that had been characterized showed male infertility in genetic knock-out strains. Through the application of directed proteomic approaches combined with functional and bioinformatic analysis, a focused and testable group of prime candidates was identified for future studies in *C. elegans* or other organisms.

Exploring Mechanism through Proteomics and Functional Studies—Focused analyses of proteins that are expressed exclusively in sperm can further advance our knowledge of the molecular pathways required for chromatin function. For example, new SNBPs can be identified through proteomic analysis of biochemical fractions of sperm chromatin and characterized for roles in organizing chromatin. During spermatogenesis, a massive amount of epigenetic information is potentially being "wiped clean" by the histone replacement process that must be re-established in the zygote (22, 39). Although protamines ultimately displace the majority of somatic histones during spermiogenesis, 15% of histones in humans are retained, some at transcriptionally active sites (66, 67). This suggests that some epigenetic information is preserved in sperm. Therefore, elucidating not only the composition but also the spatio-temporal patterns of sperm nuclear basic proteins within chromatin has implications for understanding germline gene regulation and embryonic contributions of sperm chromatin.

To examine the stage-specific composition and organization of sperm chromatin undergoing condensation, a recent proteomic study utilized MS^2 identification of SDS-PAGE-separated proteins from acid-extracted nuclei in later stages of spermatogenesis in mice (68). Acid-soluble fractions are expected to contain fundamental proteins mediating sperm chromosome condensation, which are highly basic. Five new histone variants with exclusive expression during late spermiogenesis were identified through proteomic analysis. Cytological analysis showed that in condensing spermatids, two of these histone variants, H2AL1 and H2AL2, are incorporated specifically into heterochromatin regions around centromeres, known as pericentromeres. The appearance of H2AL1 and H2AL2 coincides with the removal of post-translational modifications marking heterochromatin, such as histone H3 lysine 9 trimethylation and heterochromatin protein 1 (HP1) association to chromatin (69). H2AL1 and H2AL2 are also retained after protamine incorporation (68). Thus, sperm-specific histone variants may serve as potential epigenetic regulators for the re-establishment of heterchromatin at specific sites after fertilization. As such, the absence or reduced levels of these sperm-specific proteins may correlate with incompletely formed or defective sperm chromatin.

Although there are no direct human counterparts for these two histone variants, testis-specific human histone variants have been characterized and have potential clinical applications (14, 70). Because other types of epigenetic marks occur in many cell types, such as histone modifications, sperm-specific histone variants are ideally suited for further development as infertility biomarkers that reveal defects in

chromatin packaging or sperm development that may have significant consequences on reproductive outcome or resulting offspring.

Functional Inquiry with an Eye on the Clinic—Fertility research is at an important juncture, for technological innovations are allowing the convergence of large-scale proteomic studies and basic biological studies with clinical outcomes. Such connections are the major aim of biomedical research. The previous examples illustrate that proteomics can be harnessed to shed light on the composition of sperm chromatin through strategies that prioritize targets and reduce complexity, whereas biological function can be addressed by model organism research. However, the practical potential of these studies will not be realized if their results are not considered in the context of human infertility. This disconnect represents one of the fundamental challenges facing all fields involved. One story involving several groups, discussed below, is an example that makes the link between mechanistic characterization of a sperm chromatin component in mouse and predictors of reproductive outcome in humans.

In the mouse proteomic study described in the previous section, acid-soluble sperm chromatin fractions were shown to include previously unidentified stage-specific markers of pericentromeric heterochromatin (68). Using the same biochemical approach, another class of less basic proteins was identified to contain chaperone proteins, including HSPA2, a testis-specific member of the HSP70 chaperone family (71). Two separate roles in chromatin dynamics have been found for HSPA2 during sperm formation (71–73). During sperm meiosis, HSPA2 functions in desynapsis at the synaptonemal complex (74, 75). Mice lacking HSPA2 are male infertile, with meiotic arrest and apoptotic phenotypes (76, 77). A second role for HSPA2 was suggested by the observation that HSPA2 is present in both meiotic and post-meiotic sperm (71, 73, 77). HSPA2 becomes acid-soluble only after meiosis, during transition protein incorporation (71). Co-immunoprecipitation studies demonstrated that HSPA2 and transition proteins form acid-soluble complexes in elongating spermatids. Thus, HSPA2 is the first identified transition protein chaperone (71).

With such critical roles in spermatogenesis, the evolutionarily conserved chaperone HSPA2 is a prime candidate for fertility assessment in humans. In fact, two separate studies demonstrated that the ratio of HSPA2 levels to those of creatine kinase-B, another identified biochemical marker of sperm maturity, is predictive of *in vitro* fertilization outcome (72, 78, 79). Interestingly, levels of HSPA2, as well as 16 other proteins, were also found to be aberrant in a proteomic comparison of sperm from 10 normal donors and 20 patients that suffer from low motility of sperm (asthenozoospermia) (80). HSPA2 has been hypothesized to function in several post-meiotic sperm processes including cytoplasmic extrusion and plasma membrane remodeling, which may affect sperm morphology or cellular interactions (81). Additional data correlates HSPA2 expression with the formation of hyaluronic acid binding sites on the plasma membrane (82). In fact, ICSI performed with sperm selected for their affinity for hyaluronic acid resulted in 4- to 6-fold reduced incidence of chromosomal abnormalities (82–84). HSPA2 is therefore one example of a biologically relevant marker of reproductive potential that can be found integrating directed proteomics, functional characterization, and correlations with clinical observations. The body of work on HSPA2 demonstrates how clinical tools like biomarkers and effective protocols are validated from a full understanding of the biological significance of sperm or testis-specific proteins.

Dissecting the Complexity of Human Male Infertility—Another exciting avenue that exploits the power of proteomics is the large-scale identification of biomarkers that correlate with clinical reproductive success or failure. For example, comparison of proteomes of human sperm samples from fertile and infertile individuals may identify many candidate proteins with altered expression (80, 85, 86). However, prioritizing these proteins for biomarker development is not a straightforward task. The sperm proteome of an individual may vary from sample to sample taken on different days (86). Likewise, fertility of individuals classed in either fertile or infertile categories can also span a large range (87). Thus it may be difficult to ascertain which proteins consistently differ in expression across studies that use different sample sizes and methodologies.

Similar to cell biological studies of the sperm proteome, clinical studies using proteomics can also benefit from reductionist approaches that focus on defined variables. Because infertility can be a complex syndrome with multiple causes, one such strategy is to first classify individuals based on known molecular markers of specific fertility defects then to identify other proteins that also show altered expression. These newly identified candidate proteins can then be further investigated for roles in the same processes mediated by the original molecular marker. Because changes in chromatin integrity and key sperm chromatin proteins such as protamines are already known to correlate with infertility, protamines represent an ideal starting point for such explorations (88, 89).

One such study by de Mateo *et al.* (47) applied proteomic analysis to find proteins showing altered expression in correlation with sperm DNA damage and protamine content. An altered ratio of the two protamine proteins, protamine 1 (P1) and protamine 2 (P2), correlates with male infertility (90–93). Sperm samples were obtained from 47 infertile patients and 10 sperm donors, all of those who presented with normal numbers of sperm (normozoospermia). Each sample was assessed for DNA fragmentation using terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) and extracted for protamines to determine the ratio of protamine proteins. First, the authors observed a range of differences in DNA fragmentation and P1/P2 ratios among all 57 samples. Each sample was then subjected to 2D-PAGE, resolving

~1000 spots. MS^2 analysis identified 33 new sperm proteins in addition to the 98 proteins the authors had found in previous studies (41). Of these, 101 were selected for comparison among the 57 sperm samples (47). Over half of the 101 proteins showed correlated differences with another protein among the 57 samples. These results could represent substantial individual variation between donors. Alternatively, they may represent functional associations. In support of the latter hypothesis, 8 proteins were found to vary in correlation with differences in TUNEL results and 7 with altered P1/P2 ratios.

This preliminary study indicates that proteomic analysis is able to detect a small number of protein expression differences within the sperm proteome and has identified two groups of proteins showing expression levels that co-vary with sperm chromatin assessments. Both TUNEL and P1/P2 ratios are known to correlate with fertility (4, 88); although it is not known whether the proteins found in this study do so. Further examination of larger numbers of sperm profiles will clarify which cohort differences are because of individual variation and which differences consistently correlate with these assays of sperm chromatin state. Such proteins can be further validated for their utility as biomarkers and functionally characterized. In summary, the strategic extension of correlative studies to these and other measures of infertility will yield relevant biomarkers that are useful in the clinic.

DISCUSSION

Though the application of proteomics to understanding fertility is still developing, our understanding of sperm development, function, and dysfunction has grown considerably through its use. To achieve continued progress and concrete results for couples battling infertility, the arenas of proteomics, basic biology, and the clinic must become integrated partners. Each discipline brings unique tools and perspectives to the table. Proteomics provides rapid and in-depth discovery of fertility factors and proteins with altered expression in infertile patients. Detailed molecular studies facilitated by model organisms can provide insight about the function or dysfunction of such proteins in sperm. Finally, clinical studies that correlate differences in protein expression with fertility measures can be used to translate molecular data into widespread patient benefit. The studies highlighted in this review focus on sperm chromatin to illustrate that the synergy of technically divergent fields increases knowledge of fundamental processes in sperm and facilitates development of new clinical tools.

The interdisciplinary sharing of information is integral for the incorporation of proteomics with basic biology and clinical research. Bioinformatic tools that are widely available to researchers and proteomicists need to be comprehensively developed and used in fertility studies. This includes the effective organization, management, and dissemination of standardized and detailed fertility data via accessible web-based portals, so that meaningful correlations can be ascertained and verified (94–96). In addition to making proteomic data available, proteomicists and basic biologists should describe their protocols and workflow thoroughly, promoting reproducibility of results. Likewise, it is necessary for clinical scientists to include the parameters and cut-off for fertility assessments with detailed characterizations of patients and clinical samples to aid in cross-study comparisons and meta-analyses. Because in today's world no one researcher can wear all hats, opportunities abound for productive and meaningful collaborations for scientific discovery as well as the translation of these discoveries into clinical application.

The full complexity of the sperm proteome will be revealed using rapidly advancing proteomic technologies applied to different subcellular or biochemical fractions of sperm cells. Even with the application of directed strategies to enrich for typical DNA-associated factors, the studies discussed yielded unexpected proteins that function in male fertility. For example, proteomic analysis of acid-solubilized sperm chromatin revealed not only sperm nuclear basic structural proteins, but also a set of chaperone proteins that included HSPA2 (71). Likewise, of the 132 proteins identified in sperm chromatin proteomics in *C. elegans*, the majority of these proteins were actually in the categories of RNA binding, signaling, and unknown proteins (17). Such findings underscore a significant benefit of large-scale proteomic identification as a nonbiased approach to find a broad range of functionally significant candidate proteins for further functional analysis.

The dissection of sperm function and dysfunction will benefit greatly from proteomic analysis because sperm rely heavily on post-translational modifications for their development and function. It will therefore be necessary to define specific protein isoforms as well as combinations of post-translational modifications, for each may have unique regulatory functions at different stages in sperm development. Unique sets of sperm-specific "signatures" of post-translational modifications may regulate chromatin structure and gene expression from spermatogenesis through zygotic development (70). Sperm are therefore an ideal testing ground for new strategies that combine rapid protein identification with ways to easily distinguish relevant combinations of post-translational modifications on individual proteins (97–102). Such studies will have a significant impact on our understanding of the epigenetic information stored within the paternal chromatin contribution that may have long lasting effects on future generations.

The complexity of protein species in sperm is only one of the challenges facing scientists interested in converting proteomic knowledge into clinical applications. Infertility is a multifactorial syndrome, and any one case of infertility likely is caused by multiple genetic and environmental insults. Therefore approaches that define how individual factors act in specific fertility processes allow the dissection of infertility from the bottom up. We have highlighted examples in *C. el-*

egans and mouse that have effectively used proteomics to molecularly define crucial aspects of sperm chromatin (17, 68, 71); though studies in other organisms, including agricultural models, have made significant contributions as well (103–105).

Although lack of knowledge about the molecular function of a given protein does not preclude its use as a biomarker, such characterization enriches and informs the development of clinical tools and therapies. Characterization of specific sperm proteins are valuable from a practical standpoint to decide which candidates are the most compelling for further investment of time and resources. A temporal and mechanistic understanding of how a protein plays into a particular developmental or biochemical pathway would allow more refined characterization of sperm dysfunction. For example, inappropriate expression of stage-specific developmental markers in a sperm sample could prompt a diagnosis of aberrant sperm development and may influence decisions about that couple's treatment options. Moreover, molecular characterization of clinical targets can denote those that have the potential to lead to treatments that improve patient fertility outcomes.

How will proteins identified by proteomics be useful in the clinic? One important consideration is that a given clinical biomarker assay should have sufficient sensitivity to identify affected individuals correctly and yet show adequate specificity to avoid false detection of unaffected persons (106). Another consideration for a useful biomarker is that expression changes occur prevalently within a population of infertile patients (107, 108). Some candidate proteins may not meet all criteria. For example, certain biologically relevant markers may be highly sensitive and specific, detecting a defect in only a small subset of the population. Because the etiology of male infertility is complex, the most efficient assays may be those that combine such biomarkers into panels, improving the probability of detecting dysfunction for each patient using one clinical test (109, 110). The continued identification of sperm-specific proteins is essential for the development of a broad range of highly specific markers that can detect infertility arising from numerous possible events.

Finally, clinical tests that indicate sperm dysfunction help clinicians and patients make informed decisions (111–113). 50% of miscarriages have unexplained causes (114). Paternal genomic abnormalities, like sperm DNA damage, are correlated with fertilization success and may also be indicators of post-fertilization outcome, like spontaneous abortion after natural or *in vitro* fertilization (IVF) (115–117). Tests for the quality of sperm chromatin can be used to inform patients of the statistical likelihood of potential reproductive outcomes, like spontaneous abortion, before they make the decision to proceed with a particular course of ART. Developing reliable and well validated tests will arm both clinician and patient with the necessary information to proceed with the most appropriate treatment options.

Cures for infertility may no longer be just a far off dream. Given the modern revolution in systems-wide approaches like proteomics and genomics, our prospects for understanding this complex syndrome are greatly increased. In addition to proteomic studies of chromatin, proteomic analyses have been performed on seminal fluid and various sperm organelles, including the flagella to define motility and sperm membranes to understand sperm-egg interactions (118–120). Thus there is tremendous opportunity to make important findings and contributions to understand sperm biology, to define causes of infertility, and to bring hope to infertile couples with safe and effective treatment.

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