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REVIEW

Proteomics and the genetics of sperm chromatin condensation

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Spermatogenesis involves extremely marked cellular, genetic and chromatin changes resulting in the generation of the highly specialized sperm cell. Proteomics allows the identification of the proteins that compose the spermatogenic cells and the study of their function. The recent developments in mass spectrometry (MS) have markedly increased the throughput to identify and to study the sperm proteins. Catalogs of thousands of testis and spermatozoan proteins in human and different model species are becoming available, setting up the basis for subsequent research, diagnostic applications and possibly the future development of specific treatments. The present review intends to summarize the key genetic and chromatin changes at the different stages of spermatogenesis and in the mature sperm cell and to comment on the presently available proteomic studies.
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INTRODUCTION

Spermatogenesis involves extremely marked cellular, genetic and chromatin changes resulting in the generation of the highly specialized sperm cell (Figure 1). Spermatogonial stem cells replicate and differentiate into primary spermatocytes that undergo genetic recombination to give rise to haploid round spermatids.[1–4] Round spermatids then undergo a differentiation process called spermiogenesis where marked cellular, epigenetic and chromatin remodeling takes place.[2,5–12] The nucleosomes are disassembled and the histones are removed and replaced by the high positively charged protamines forming tight toroidal complexes, organizing 85–95% of the human sperm DNA (Figure 1). At the cellular level, most of the cytoplasm is removed, and a large flagella and the acrosomal vesicle are assembled (Figure 1). Finally, the spermatozoon undergoes a maturation process through its transit in the epididymis where the chromatin is further compacted through the formation of disulfide bonds and zinc bridges among protamines, and the acquirement of different membrane and cellular functionalities.[13–15] Once in the female tract, the spermatozoon must be capacitated, a process involving many signaling changes and the attainment of hyperactivated motility.[16–19] Before the sperm cell penetrates the oocyte, the sperm–oocyte recognition and the acrosomal reaction must take place.[20] Finally, once in the oocyte, the male pronucleus must undergo another extremely marked chromatin remodeling process where the nucleoprotamine structure is disassembled and a new nucleosomal and chromatin structure is assembled (Figure 1). The accessibility of the spermatozoon has facilitated the study of its composition and mechanisms involved in its function and makes this cell particularly well suited for proteomic analysis.[21] In addition, dissecting the differentiation process of spermatogenesis through proteomic analysis provides important potential biomedical applications in regenerative medicine,[22,23] in the identification of the genetic basis of male infertility,[24–28] in understanding the origin of genetic and epigenetic mutation,[5,9,10,26,29–32] in reproductive toxicology[33] and in the development of potential contraceptive strategies.[34,35] Different studies have investigated the genetic and protein changes and the mechanisms involved in the different stages of spermatogenesis and function of spermatozoa. The present review intends to complement different recent reviews focusing on the proteomics of the mature sperm cell,[21,36–43] on testicular proteomics[44,45] or on the proteomic changes upon epididymal maturation and capacitation.[46] To reach this goal, the structure followed will be to describe the key genetic and chromatin changes at the different stages of spermatogenesis (Figure 1), with indication of the related proteomic studies being performed based on large-scale mass spectrometry (MS) identification of proteins.

TESTICULAR PROTEOMICS: SPERMATOGONIAL STEM CELLS, SPERMATOCYTES AND SPERMATIDS

One of the initial approaches applied to identify proteins present in the different stages of spermatogenesis exploited the changes in cellular abundance during testis development. Using this approach, the two-dimensional (2D) proteome profile changes during mouse testis development led to the identification using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) of 44 proteins with substantial changes in protein abundance during development.[47] Subsequent application of MALDI-TOF/TOF using a similar approach allowed identification of 257 different proteins that clustered into six different expression patterns.[48] A limitation of the analysis of the entire testis is the existence of mixed cellular population including the presence of somatic cells. Therefore, other approaches

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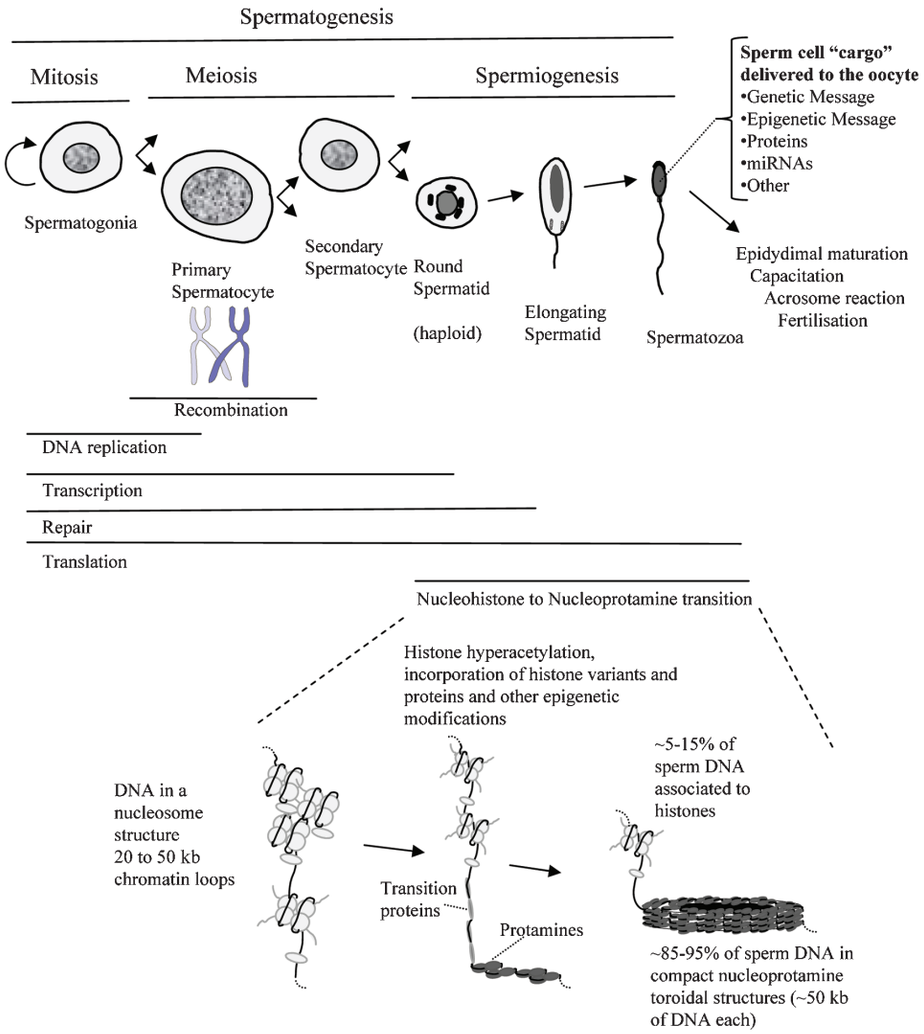

Figure 1 Cellular, genetic and chromatin changes at the different stages of spermatogenesis and sperm cell maturation. At the top of the figure, the different cellular stages of spermatogenesis are represented along with the genetic activities.[2,60] At the bottom of the image, a chromatin model of the mammalian nucleohistone to nucleoprotamine transition is shown.[2,5,11,21]

have been used such as the isolation of the different cellular components of the testis or the study of cultured germ cells.

One of the testicular cells studied using proteomics is the spermatogonial stem cell.[49–51] The interest in the study of the spermatogonial stem cells is multiple. Pathological perturbation of the stem cell is suspected as the origin of certain types of testicular cancers and male infertility, so that identification of the mechanisms involved would facilitate the development of preventive or treatment options.[52,53] In addition, the discovery of pluripotent stem cells within the testis raises important biomedical applications in regenerative medicine.[23] Important issues in stem cell research are the identification of key genes and proteins needed to maintain the pluripotent state or that can be used as markers for their identification.[49] A very elegant application of a comparative proteomics approach has been applied to demonstrate that after comparison of the proteomic profiles of cultured mouse multipotent adult germ line stem cells with embryonic stem cells, only 18 proteins were detected as differentially expressed out of a total of 409 proteins identified using 2D separation of the proteins followed by MS.[22] The interpretation of this result was that the proteomes of multipotent adult germ line stem cells were highly similar to those of embryonic stem cells.[22]

A different approach to characterize the proteome of the spermatogonial stem cell exploited the peculiarities of the testis developmental biology in the dogfish *Scyliorhinus canicula*.[54] These authors isolated, under stereomicroscope and dissection, the testicular germinative zone, highly enriched in spermatogonial stem cells, and used 2D and MALDI-TOF/TOF to identify 16 proteins and to also demonstrate the feasibility of this model to study the stem cell niche.[54] Still, a different set of studies has used testicular cell sorting to obtain enriched cellular fractions with which proteomic analysis is performed. This approach was applied to separate spermatogonia from 9-day-old rats followed by protein 2D analysis and identification of the proteins using MALDI-TOF.[55] More recently, the same group applied a similar procedure on immature and mature rat testis combined with sedimentation at unit gravity or elutriation to obtain highly enriched fractions of spermatogonia, spermatocytes and early spermatids.[56] Subsequently, 2D difference in gel electrophoresis allowed identification of the relative abundance of 1274 proteins of which 265 differed

significantly in the three groups of cell types. MALDI-TOF/TOF was then used to identify 123 non-redundant proteins clustering into the clades of mitotic, meiotic and post-meiotic cell types.[56] It is also important to consider the close relationship between the Sertoli cell and the spermatogenic cells. Recently, the effect of the loss of Dicer in the Sertoli cell, required for microRNA biogenesis, on the testicular proteome, has been studied.[57]

Once the diploid spermatogonium is committed, it divides mitotically to produce two diploid intermediate cells called primary spermatocytes. Each primary spermatocyte then duplicates its DNA and subsequently undergoes meiosis I to produce two haploid secondary spermatocytes (Figure 1). Very importantly, this stage involves genetic recombination of homologous chromosomes to increase the genetic variability of the gamete. Many of the genetic causes of male infertility stem from meiotic anomalies. For instance, an important proportion of cases of male infertility are due to meiotic arrest.[25,26,28] Also, many chromosomal structural anomalies result in incorrect pairing at meiosis and in the generation of chromosomally unbalanced gametes responsible for embryo lethality or severe anomalies in the offspring.[58] Well-known causes and risk factors of male infertility such as the presence of Y-chromosome microdeletions also result in a variety of phenotypes which may include Sertoli cell-only syndrome, spermatogenic arrest and hypospermatogenesis resulting in azoospermia or oligospermia.[24,27,59] Thus, proteomics, through the indicated above strategies, allows the identification of the proteins involved in the meiotic stage of spermatogenesis with the potential to contribute to the identification of the involved pathogenic mechanisms associated to male infertility in these cases.[47,48,56]

After the completion of the meiosis, the haploid round spermatids are generated (Figure 1). Haploid round spermatids are still transcriptionally active.[60] However, another aspect of the biology of the spermatogenesis that deserves consideration is that each cell division from a spermatogonium to a spermatid is incomplete. The cells remain connected to one another by bridges of cytoplasm to allow synchronous development. It has been proposed that these cellular bridges allow the exchange of proteins and gene products so that, even though the round spermatids are genetically haploid, they may express proteins as if they were diploid.[60] The haploid spermatids have been the focus of different proteomic studies. Fluorescence-activated cell sorting sorting has been applied to isolate haploid mouse spermatids followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify 2116 proteins, 299 of which were testis specific and 155 were novel.[61] Interestingly, the analysis of the chromosomal distribution of the haploid identified genes showed an underrepresentation of the X chromosome, interpreted as owing to meiotic X-chromosome inactivation, and overrepresentation of chromosome 11 upon expansion of the gene families.[61]

In a different approach, the proteomic analysis in testis biopsies of testosterone-treated men allowed the identification of proteins potentially related to the induced testis regression.[62] As an experimental model, the effect of hyperthermia on mouse spermatogenesis has also been studied.[63] The same group also investigated the whole human testis, identifying 462 unique proteins.[64,65] Evidence for protein heterogeneity was concluded upon the identification of 180 different proteins in more than one protein spot. Also, phosphoprotein staining allowed identification of 52 phosphorylated proteins.[65] Proteins related to altered fertility in fish have also been identified using a whole-testis proteomic approach.[66]

The proteomic studies during spermiogenesis are highly relevant in the identification of all the proteins involved and the mechanisms of the nucleohistone-to-nucleoprotamine transition, which probably represents the most marked chromatin change that cells may undergo (Figure 1). One of the initial chromatin changes in the nucleohistone-to-nucleoprotamine transition is the incorporation of histone variants.[67–72] Another important early event is histone hyperacetylation that occurs during spermiogenesis just before the nucleosome disassembly.[73–78] It was postulated that histone hyperacetylation and rapid turnover of acetyl groups could rapidly and reversibly expose binding sites in chromatin for subsequent binding of chromosomal proteins.[74] More recently, it was also shown *in vitro* that histone hyperacetylation facilitated nucleosome disassembly and histone displacement by protamines.[79,80] Also, hyperacetylated nucleosomes were shown to appear in a more relaxed structure upon binding to electron microscopy grids.[80,81] It has been shown that the testis-specific bromodomain-containing protein binds to hyperacetylated histone-4, triggering a reorganization of the chromatin.[3,10,82,83] Impaired histone-4 hyperacetylation has been detected in infertile patients.[84,85]

Once the nucleosomes are disassembled, transition proteins are incorporated.[2,86] Transition proteins are then finally replaced by protamines to form a highly compact nucleoprotamine complex (Figure 1).[2,3,6,7,10,86–90] It is known that protamines are phosphorylated before binding to DNA and that a substantial dephosphorylation takes place concomitant to nucleoprotamine maturation.[2,91–93] The dynamics of binding of the protamines to DNA has also been studied.[94–96] After binding to DNA, the formation of interdisulfide bonds between protamines further stabilizes the nucleoprotamine complex.[15,97] Different models for the structure of nucleoprotamine have been proposed.[98–104] These chromatin changes during spermiogenesis take place in the context of a marked metamorphosis of the sperm cell and shaping of the head and associated structures such as the perinuclear theca and manchette.[8,105] However, despite substantial amount of information available, the identification of the molecular mechanisms governing the nucleohistone-to-nucleoprotamine transition and all the sperm cell changes still requires substantial effort. The available catalogs that are becoming available as derived from the proteomic projects represent a very important first step in the complete identification of the proteins and mechanisms involved.

EPIDIDYMAL MATURATION

Testicular spermatozoa are haploid, have completed the nucleohistone-to-nucleoprotamine transition and have acquired most phenotypic features but they are not yet functional (Figure 1). Motility and the ability to bind to the oocyte are lacking and the sperm chromatin still needs to further complete maturation through the formation of disulfide bonds and zinc bridges in the nucleoprotamine (Figure 1). All these functionalities are acquired during the transit of the sperm cell through the epididymis.[13–15,106,107] Proteomics has also been applied to the study of the sperm cell during epididymal maturation. 2D difference in gel electrophoresis has been used to isolate and to identify 60 protein spots, significantly modified as sperm traverse the epididymis.[46] In one of the proteins, the change was found to represent a serine phosphorylation.[46] The proteomic changes in mammalian spermatozoa during epididymal maturation have also been the subject of review.[13]

Epididymal secretions may also determine many aspects of the physiology of the spermatozoa. There is evidence that some of the proteins present in the fully mature ejaculated sperm cell may have been acquired during the epididymal transit.[108] Therefore, several proteomic initiatives have also focused their efforts on the identification of the proteins present in the fluid secretions, which surround the

sperm cell during its passage through the male genital tract. The human and stallion epididymal secretome and the human seminal plasma proteome have been reported.[109–113] Also, the proteomic component of epididymosomes and accessory gland fluid has been characterized in several species.[108,114–123]

PROTEOMICS OF MATURE SPERMATOZOA AND THE SPERM CELL GENOME, EPIGENOME AND PROTEOME DELIVERED TO THE OOCYTE

The most extensive report on the identification of mature human sperm proteins used LC-MS/MS to identify 1056 gene products.[124] This catalog is complementary to additional catalogs obtained using the 2D approaches.[21,125–127] In other mammals, spermatogenic proteomic profiles are also available.[21,128–131] In invertebrates, MS identification of sperm proteins has been applied to the fruit fly.[36,132,133] and the worm *Caenorhabditis elegans*[134] among several others.[21] One of the aspects amenable to study by proteomic analysis is the identification of the evolution and intensified selection of some sperm proteins.[131,135–137]

Knowledge of the sperm cell proteome is also relevant to understand the different functions of the sperm cell. It is well known that the most essential function of the sperm cell is to deliver an intact paternal genomic DNA sequence to the oocyte, and that alterations in DNA integrity are a cause of male infertility, failed assisted reproduction and pregnancy loss.[127,138–140] More recently, it has been recognized that, in addition to the DNA sequence, the existence of imprints determined by the DNA methylation status is also important for a proper embryonic development, and that infertile patients have an aberrant DNA methylation at specific loci.[29–32] The analysis of the proteins identified in the different mature sperm proteomic projects has more recently identified proteins that may have a role in fertilization. For example, transcription factors, DNA-binding proteins and proteins involved in chromatin metabolism have been identified in cells that are transcriptionally inactive and that have most of its DNA tightly packaged with protamine.[5,21,124] It will be interesting to determine whether these nuclear proteins are marking some regions of the male genome and may have an epigenetic function.[5] An alternative explanation for the presence of some of these proteins is that they could represent leftovers from spermiogenesis, although in this case, the identification of these proteins could be useful as they could represent a 'window' to the later stages of spermatogenesis with potential clinical implications. Recent reports indicate the presence of a complex chromatin organization of the genes in sperm, with an appreciable fraction containing both nucleohistone and nucleoprotamine domains that is suggested to be of potential relevance for embryo development.[141–143] In addition to the sperm chromatin proteins, it will also be interesting to consider the possibility of additional sperm proteins having a potential role in fertilization.[21,37,144]

PROTEOMIC ALTERATIONS OF EJACULATED SPERM CELLS IN INFERTILE PATIENTS

The presence of proteomic anomalies in infertile patients has been assessed by comparing the proteome of abnormal sperm samples from infertile patients with the proteome of control normozoospermic samples from fertile donors. One of the initial reports that demonstrated the potential of 2D proteomics in the study of sperm defects reported the proteomic mapping of a patient who experienced a failure in *in vitro* fertilization, where 20 different proteins were identified as compared with controls.[145] Subsequently, different protein differences associated with asthenozoospermia have also been identified.[146–148] Following a similar approach, the abundance of the proteins present in the sperm cells from 47 sperm samples from infertile patients and from 10 semen donors were analyzed in our laboratory by 2D polyacrylamide gel electrophoresis.[127] In each of the 2D maps, the intensity of 101 spots previously identified by MALDI-MS analysis was measured. In addition, other parameters related to male infertility such as the protamine content and DNA integrity were also determined in each independent sample. Several interesting proteins such as transcription factors, prohibitin, heat shock and proteasome proteins were identified and linked to altered DNA integrity and abnormal protamination.[127] Proteomics has also been applied to the analysis of round-headed spermatozoa by 2D fluorescence difference in gel electrophoresis, resulting in the identification of 35 protein spots (out of 61 identified) exhibiting significant changes in expression (9 proteins upregulated and 26 proteins downregulated) between normal and round-headed spermatozoa.[149] It will be interesting to extend the analysis of patients through differential proteomics to incorporate the use of more robust methods based on non-isotopic labeling of the proteins and LC-MS/MS identification of the proteins.[19]

CAPACITATION

Capacitation is the activation process that leads to hyperactivated motility facilitating the sperm–oocyte interaction, binding and preparation for the acrosome reaction to penetrate the zona pellucida.[16–18,20,150] The fact that most of the male genome is heavily condensed by protamines and transcription is completely blocked in the nucleoprotamine domains, together with the loss of most of the sperm cytoplasm, has classically favored the hypothesis that any protein changes concomitant to sperm capacitation had to be because of post-translational modification (Figure 1). However, some evidence suggests that the mature sperm cell is capable of translation of nuclear mRNA by mitochondrial-type ribosomes.[151] Further support for this hypothesis of translation by mitochondrial-type ribosomes during sperm capacitation has been provided using a proteomic approach.[17] In this study, differential proteomics was applied to identify 44 proteins with lower expression in *D*-chloramphenicol (a specific inhibitor of mitochondrial translation)-treated sperm cells in comparison with capacitated sperm. In addition, evidence was provided that 26 of 44 of these proteins were involved in critical processes for the sperm–egg interaction.[17]

However, in addition to the potential of *de novo* protein syntheses, the most widely accepted hypothesis is that most of the protein changes concomitant to capacitation are likely to be because of the post-translational modification of existing proteins. One of the most common post-translational modifications is phosphorylation. This modification was studied in capacitated human sperm resulting in the mapping of 60 sites of phosphorylation.[152] The protein profiles of capacitated versus ejaculated human sperm have also been studied after 2D separation of the proteins.[18] This study resulted in the identification of the 25 most abundant spots in ejaculated sperm, the 23 most abundant spots in capacitated sperm and the identification of proteins with substantial variation between uncapacitated and capacitated sperm.[18] The role of the nitric oxide as an inducer of capacitation has also been studied to identify 240 *S*-nitrosylated human sperm proteins.[153] A focused approach applied to the detergent-resistant membranes in capacitated sperm allowed the identification of 100 proteins, many of which were implicated in sperm–oocyte interaction.[154]

Isotopic labeling has also been applied to analyze the capacitation-associated changes in 42 different phosphopeptides.[155] A radically

different and robust approach has been recently applied to identify the proteomic changes associated with sperm capacitation through the combined use of immobilized pH gradient-strip prefractionation followed by LC-MS/MS analysis.[19] Using this approach, label-free quantitative analysis of proteomic changes associated with capacitation identified 71 peptides corresponding to 52 proteins changing during capacitation many of which had not been previously implicated in this process.[150]

CONCLUSION

Proteomics applied to sperm cell research has so far led to the generation of catalogs of thousands of proteins present in the testis and in the mature sperm cell. This information is already being applied to the identification of the molecular mechanisms involved in spermatogonial stem cell physiology, meiotic recombination and in chromatin condensation, function and evolution of the sperm cell. Proteomics is also applied to identify the post-translational protein modifications occurring during epididymal maturation and capacitation, and to identify the complete proteomic complement of the sperm cell chromatin delivered to the oocyte. Furthermore, it is also being applied to the identification of the proteins involved in male fertility and infertility leading to the identification potential infertility markers and additional biomedical applications. However, there are still different issues and challenges that must be considered. Methodologically, it will be necessary for laboratories to keep up with the constant improvement in throughput of the MS equipment. The potential development of efficient spermatogenic *in vitro* culture systems, allowing synchronous differentiation of relatively pure cellular stages, would be a major accomplishment to potentiate the proteomic study of the sperm cell differentiation mechanisms. The alternative of separating and sorting testicular cells for proteomic analysis must take into account the cellular purity of the resulting cellular fractions. Even considering only the mature sperm cell, apparently a single cell type, it turns out to be a complex heterogeneous mixture of different quality sperm cells with substantial biochemical, functional and morphological differences. This fact together with the variation present in independent individuals and the physiological changes that the spermatozoa undergo upon ejaculation generates an enormous potential for variation, which must be taken into account in proteomic studies. Ultimately, the proteomic information will be very valuable in the context of the genetic, genomic, transcriptomic and metabolomic information. Altogether, we are now at an exciting and challenging momentum in proteomic sperm cell research with lots of work still to be done.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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