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REVIEW

The "omics" of human male infertility: integrating big data in a systems biology approach

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Abstract Spermatogenesis is a complex process in which >2300 genes are temporally and spatially regulated to form a terminally differentiated sperm cell that must maintain the ability to contribute to a totipotent embryo which can successfully differentiate into a healthy individual. This process is dependent on fidelity of the genome, epigenome, transcriptome, and proteome of the spermatogonia, supporting cells, and the resulting sperm cell. Infertility and/or disease risk may increase in the offspring if abnormalities are present. This review highlights the recent advances in our understanding of these processes in light of the "omics revolution". We briefly review each of these areas, as well as highlight areas of future study and needs to advance further.

Keywords Male infertility · Genomics · Epigenomics · Proteomics · Methylation · Polymorphism

Introduction

Modern genetics, the combination of epigenetics with traditional genetics, is proving to be an important tool in understanding normal gene expression and disease. It is well established that noncoding changes to the genome, i.e., epigenetic modifications, facilitate regulation of gene expression, and that alterations to the epigenome are associated with many diseases. The genome and epigenome regulate the transcriptome and proteome, and together with phenotypic and environmental information provide an opportunity for a comprehensive systems biology approach to understanding normal and abnormal physiology.

Characterization of putative underlying genetic causes of male infertility have been hampered by the inherent complexity of spermatogenesis as well as the prohibitive costs for whole genome studies; however, this is changing rapidly (Fig. 1). The "omics revolution" has brought about ever more powerful and affordable means to interrogate the genome, epigenome, transcriptome, and the proteome, and is proving effective in identifying novel causes of male infertility (Fig. 2). As the 'omic' technologies and analyses continue to mature, characterization of the genetic, epigenetic, and proteomic architecture of male infertility will undoubtedly continue to accelerate.

In this manuscript, we review current understanding of the sperm genome, epigenome, and proteome. We also highlight some tools that have been successfully used for studying spermatozoa and the prospects for future advances in male infertility research.

Genetic complexity of spermatogenesis

For many years, the mainstay of genetic research of male infertility involved the targeted sequencing of individual genes in modestly sized cohorts of infertile men and fertile or normozoospermic controls. Based on testicular gene expression studies, it is estimated that >2300 genes are required

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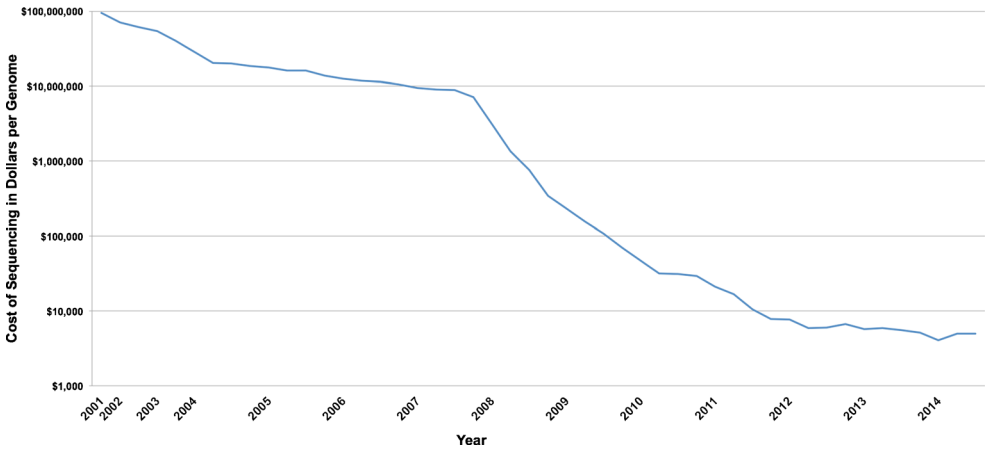


Fig. 1 Improved affordability of genome sequencing. Rapidly declining sequencing costs (normalized as cost per human genome) over the past 15 years (modified from http://www.genome.gov/sequencingcosts/)

for normal spermatogenesis (Schultz et al. 2003). As expected for a process as complex as spermatogenesis, these targeted approaches proved largely ineffective at identifying genomic variants responsible for infertility. Numerous papers have reported single nucleotide polymorphisms (SNPs) associated with male infertility, but most reported associations are based on a small number of samples, and are generally not replicated in additional cohorts (Carrell and Aston 2011). In spite of their limitations, these approaches have been successful in identifying a few common genomic variants that modulate

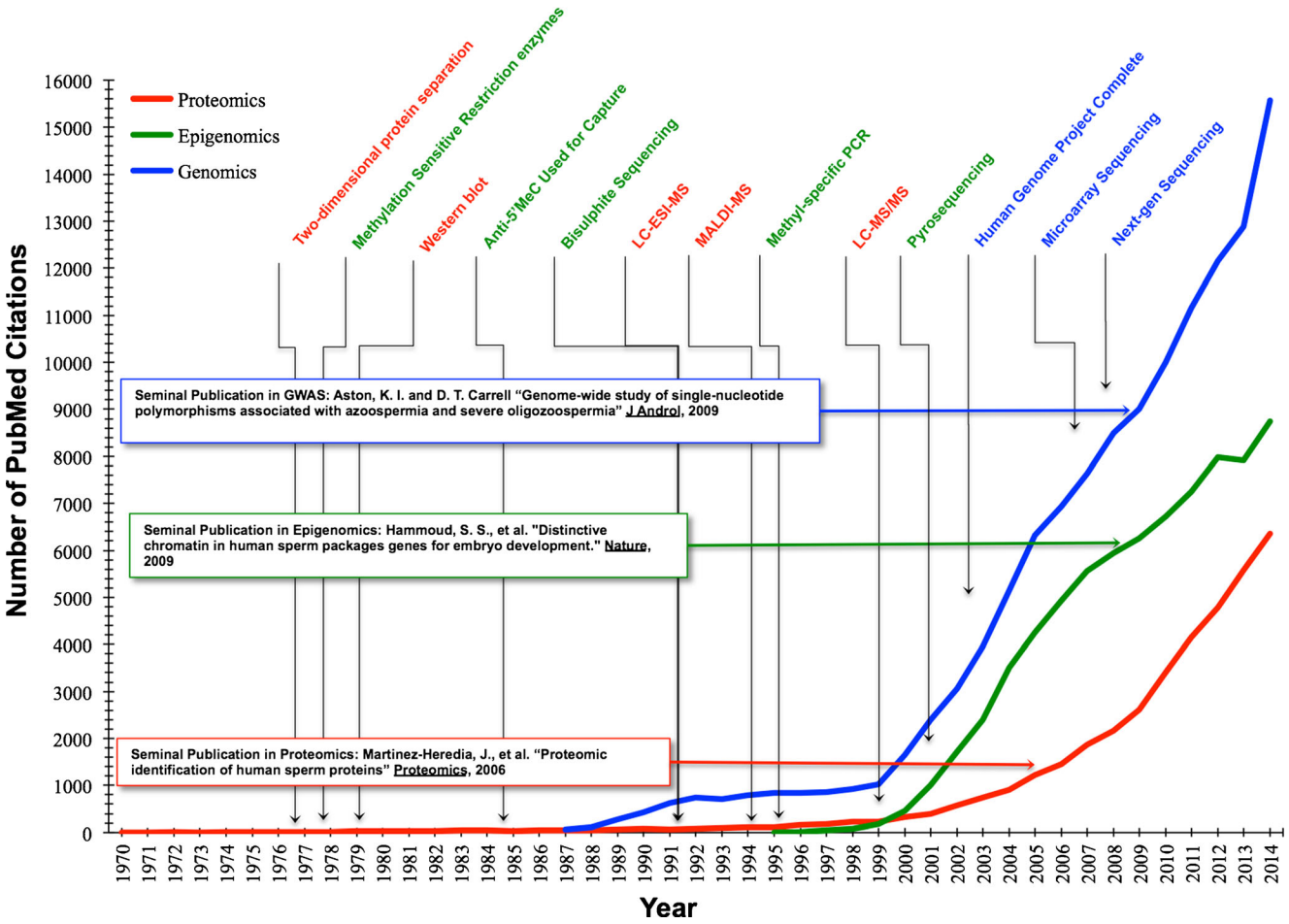


Fig. 2 The rise of "omics" publications. Annual publication volume for papers related to proteomics, epigenetics, and genomics based on the number of PubMed citations per year. The rapid increase in research focused on these topics is largely related to advancements in molecular and analytical tools as highlighted on the timeline. Also listed are pioneering manuscripts that applied these tools in the context of spermatogenesis and male infertility

spermatogenesis (e.g., polymorphisms in MTHFR, FSHB, and GSTM1, and gr/gr deletions) and provided additional insight into the genomic architecture of spermatogenesis defects (Song et al. 2013; Tuttelmann et al. 2007, 2012; Visser et al. 2009; Wei et al. 2012). However, targeted sequencing studies strongly indicate that whole genome analysis will be necessary to more fully characterize the genetic basis of male infertility.

Tools for genomic studies: Benefits and Liabilities

Genomic analytical tools can be broadly classified into two main categories: array- and sequencing-based approaches (Aston and Conrad 2013). These approaches have been used extensively for the discovery of genetic variants involved in numerous complex diseases. The ability to interrogate the whole genome at various levels of resolution enables the localization of genetic associations without specific a priori knowledge of genomic regions involved in the disease or trait. Use of these technologies has accelerated our understanding of the genetic basis for many disease states (Solinas-Toldo et al. 1997).

Microarray-based approaches are designed to evaluate a subset of the genome, with resolution and coverage depending on the array design and the number of probes on the array. Microarray-based approaches have been used extensively in the study of complex diseases and have proven modestly effective for identifying genomic features associated with various diseases. Their primary limitations are that they typically interrogate only a small fraction of the genome, and they are usually designed to identify variants that occur at relatively high frequencies (>1 %) in the population. Based on the importance of rare variants in complex disease, microarray manufacturers such as Affymetrix and Illumina have begun to include rare, non-synonymous coding variant content on some of their SNP arrays.

Sanger-based DNA sequencing, developed in the 1970s, formed the basis for the Human Genome Project. The 90 % complete first draft sequence of the euchromatic portion of the human genome, published in 2001 (Lander et al. 2001), required more than 12 1/2 years at a cost of approximately US$2.7 billion. Today, a human genome can be sequenced in a single day for well under $10,000 (Fig. 1).

Next-generation-sequencing-based approaches overcome many of the limitations of microarrays, but the improved resolution and increase data generated using sequencing technologies results in increased analysis complexity and much higher costs. In addition, the value of whole genome data is limited by the fact that a significant portion of the genome has unknown function. This significantly limits interpretability of associations identified in non-annotated regions of the genome. Strategies to enrich DNA for specific regions of interest (most commonly coding regions of the genome) are effective in significantly reducing sequencing costs and simplifying data analysis and interpretation, but these approaches are not well suited to identifying important genetic variants with regulatory functions outside of coding regions.

As genomic technologies continue to advance and their associated costs decrease, they are becoming increasingly accessible for the study of complex diseases including male infertility. Researchers have begun to apply these powerful genomic tools to the study of spermatogenesis and male infertility. These approaches have yielded early successes, but, given the genetic complexity of spermatogenesis and the heterogeneity of male infertility phenotypes, a comprehensive characterization of the genetic basis of male infertility will continue to be an active area of research for many years to come.

Application of genomic tools in the study of male infertility

Early microarray-based gene expression studies of testis and sperm gave insight into the complexity of spermatogenesis by identifying numerous testis expressed and testis-specific genes in human and animal models (Ostermeier et al. 2002). The human genome project elucidated the localization of testis-expressed genes, and several important papers have highlighted the enrichment of genes important in spermatogenesis within the sex chromosomes. These studies were critical for annotating genes involved in spermatogenesis as well as putting into context the findings of studies aimed at identifying genetic variants associated with male infertility.

As costs have declined, whole genome studies have begun to emerge in the field of male infertility. In 2007, researchers performed SNP array analysis on three globozoospermic brothers from a consanguineous family and identified a 17-Mb region common to all three men (Dam et al. 2007). The causal mutation was identified in the gene SPATA16 within this homozygous region. More recently, two other groups utilized SNP arrays to evaluate cohorts of globozoospermic men, and both studies identified a 200-kb homozygous deletion that included the gene DPY19L2 in many of the men with complete globozoospermia (Harbuz et al. 2011; Koscinski et al. 2011).

In 2009, Aston et al. performed the first genome-wide association study (GWAS) of spermatogenesis impairment (Aston and Carrell 2009). A few common variants were identified that were more frequent in men with spermatogenesis impairment compared with controls; however, significance was marginal after multiple comparison correction. In a subsequent study, a larger number of idiopathic infertile men were targeted, and 172 SNPs were evaluated based on the initial GWAS data as well as other published data. The marginal

associations for the majority of SNPs detected in the initial study could not be replicated. However, a few new SNPs showed moderately significant associations in the follow-up study (Aston et al. 2010).

Since these early studies, the body of genomic data for infertile men has expanded rapidly. GWAS have been performed to identify polymorphisms associated with testicular dysgenesis syndrome (Dalgaard et al. 2012), family size and birth interval in the Hutterite population (Kosova et al. 2012), as well as more recent, larger studies to investigate the genetic basis for severe spermatogenesis impairment (Hu et al. 2012; Zhao et al. 2012). In the majority of these studies, few small-effect common variants were identified. Several groups have attempted to replicate the identified associations in other infertile populations, but in most cases the associations do not replicate (Chihara et al. 2015; Sato et al. 2013; Tu et al. 2015; Zou et al. 2014). However, a very large study in Han Chinese men replicated three nonobstructive azoospermia (NOA) susceptibility loci, strongly implicating a small number of SNPs, with modest affect on the NOA phenotype in that population. In a smaller replication study, three of the SNPs identified in the Hutterite study were also identified in Japanese men (Sato et al. 2015). Cumulatively, these studies indicate that common genomic variants do not contribute appreciably to male infertility and that future studies should evaluate rare variants on a genome-wide scale.

Unlike the standard array-based SNP analysis, which typically targets relatively common genetic polymorphisms, the same array data can be used to identify extremely rare or novel copy number variants (CNVs), since the analysis simply depends on identifying differences in signal intensity across the genome. The role of CNVs in male infertility is becoming increasingly apparent based on recent array-based SNP and CGH studies in which duplications, deletions, and, in some cases, rearrangements of the genome can be inferred.

CNV analyses in infertile men have identified a large number of patient-specific or patient-enriched CNVs that may contribute to spermatogenesis impairment (Eggers et al. 2015; Fruhmesser et al. 2013; Krausz et al. 2012; Lopes et al. 2013; Stouffs et al. 2012; Tuttelmann et al. 2011). The clinical relevance of most of these CNVs remains to be determined through subsequent replication and functional studies. In addition, several studies have observed that, in general, rare CNVs occur significantly more frequently in infertile men across the genome, with more profound differences observed in the sex chromosomes (Krausz et al. 2012; Lopes et al. 2013), and that the number of CNVs per genome is inversely correlated with sperm count (Tuttelmann et al. 2011). The GWAS and CNV analysis findings suggest that the underlying genetic etiology of male infertility is driven predominantly by numerous rare genetic events. Hence, the application of genomic tools that can identify these rare variants genome-wide will improve our power to detect meaningful genetic events related to male infertility.

Whole exome sequencing has been used to identify causal variants in a number of complex diseases, and data are also beginning to emerge from infertile men. Current exome studies target diseases for which male infertility is a secondary phenotype. For example, several groups have performed exome sequencing on individuals and families with primary ciliary dyskinesia (PCD), a disease characterized by chronic respiratory symptoms as well as sperm motility impairments resulting in infertility. These studies have successfully identified mutations in multiple genes resulting in PCD (Moore et al. 2013; Olbrich et al. 2012; Onoufriadis et al. 2014; Sui et al. 2015; Zariwala et al. 2013). Additionally, exome sequencing studies of a family with early onset pulmonary fibrosis (Alder et al. 2015) and cerebello-oculo-renal syndrome (Alazami et al. 2014) successfully identified mutations to explain those diseases as well as their associated infertility phenotypes. A recent study performed exome sequencing for two consanguineous families with multiple cases of idiopathic azoospermia (Ayhan et al. 2014), and another sequenced the exomes of two families which displayed both primary amenorrhea in females and azoospermia in males (Tenenbaum-Rakover et al. 2015). Both studies successfully identified homozygous mutations that are likely causal of the infertility in those families.

Future directions in genomic research

The successful application of whole genome tools to better characterize the etiology of male infertility has demonstrated their power and, in general, stimulated the field. Consanguineous family-based studies have largely proven successful because the genetic architecture lends to straightforward analysis. Studies of this nature will continue to be valuable for the discovery of novel genes and variants involved in male infertility. However, male infertility is a common disease and, in most cases, the underlying genetic cause will not be explained by consanguineous family relationships. Carefully designed population-based whole genome studies will be required to identify the majority of variants responsible for male infertility. The greatest challenge will be developing analytic tools effective at accurately prioritizing and calling genomic variants based on one or very few occurrences in a population. The accumulation of genome sequence data from carefully phenotyped infertile men in the coming years will invariably shed new light on the complexities of male infertility, and will hopefully pave the way for improved diagnostic and treatment strategies.

The sperm epigenome

Broadly defined, epigenetics is the study of factors in a cell, other than DNA coding changes that alter gene expression. The epigenome is primarily responsible for the differentiation of cell types. The epigenome of a cell consists primarily of three major factors: DNA methylation, histone modifications, and microRNAs and other small RNAs. DNA methylation and histone modifications influence gene transcription directly by blocking or facilitating binding of transcription factors to the genome, while microRNAs primarily affect gene expression via their interaction and modification of transcripts.

The sperm epigenome is of critical importance in understanding spermatogenesis, as well as understanding the role of the sperm genome after fertilization. Sperm are terminally differentiated cells that combine with another terminally differentiated cell, the oocyte, to develop the totipotent embryo. The sperm epigenome provides a means to better understand the biology of development, as well as several translational realms, including stem cell-based therapies. Second, the sperm epigenome is likely important for establishing a functional epigenome in the developing embryo, and it appears that some sperm epigenetic defects may contribute to a greater risk of embryo growth arrest and/or risk to the offspring. Better understanding of the contribution of the sperm epigenome in the establishment of the embryo epigenome may improve patient care by (1) using epigenetically normal sperm to produce higher quality embryos, and (2) selecting embryos for transfer after preimplantation epigenetic screening (PES), and identification of transmissible risk factors by the gametes to the offspring.

The normal sperm epigenome

Unique chromatin and histone modifications in sperm

Sperm chromatin undergoes a unique and critical remodeling during the last stage of spermatogenesis, called spermiogenesis, which includes a two-step removal of 90–95 % of the histones and their replacement with two protamines, protamine 1 (P1) and protamine 2 (P2), which are small molecules that are negatively charged due to high arginine content (Balhorn 2007; Carrell et al. 2007; Ward and Coffey 1991) (Fig. 3). The initial step is a replacement of the majority of the histones with two transition proteins (T1 and T2), which are subsequently replaced with an equal ratio of P1 and P2 (Brewer et al. 2002). The temporal expression of the protamines is delayed due to the cessation of transcription during late spermiogenesis. Abnormal expression of the protamines is rare in men of normal fertility, but relatively common (10–20 %) in patients with male infertility (Aoki et al. 2006b). Protamination levels are heterogeneous within an ejaculate, but abnormalities resulting in generalized poor protamination are usually associated with decreased sperm quality, including an increased risk of epigenetic defects (Aoki et al. 2006a).

The retention of 5–10 % of the histones during protamination introduces an intriguing question in regards to the possible biological role of sperm histone retention (Carrell et al. 2007). One would not expect that the evolutionarily important process of spermiogenesis would have a "leaky" process of protamination in fertile men unless the retained histones played a potentially important function. A hypothesis for the role of histone retention is that the histone epigenetic marks (histone tail modifications) may play a role in normal sperm function, perhaps in post-fertilization paternal genome regulation.

Hammoud et al. (2009) reported the first genome-wide analysis of the human sperm epigenome and noted an important pattern in histone retention. They found that retained histones were consistently located in three regions of the genome: genes related to development and embryogenesis, imprinted genes, and microRNAs. This observation was supported by subsequent studies that noted evidence for histone retention in the promoter regions of important genes (Arpanahi et al. 2009; Erkek et al. 2013). Importantly, it was shown by Hammoud et al. that the promoter regions are bivalently marked with both activating (H3K4me3) and silencing (H3K27Me3) histone modifications, a pattern first observed in stem cells. This bivalent marking is thought to confer a 'poised' sperm genome (in conjunction with DNA methylation patterns discussed below), possibly facilitating activation of the paternal genome during embryogenesis. Two recent papers failed to identify the nucleosome enrichment patterns reported in previous studies. In contrast to the patterns originally reported by Hammoud et al., evaluation of histone localization in mouse, bovine, and human sperm indicated that the majority of retained histones reside in gene-poor regions of the genome (Carone et al. 2014; Samans et al. 2014). The discrepant conclusions across studies reflect the complexity of these types of studies and indicate that additional studies are necessary to fully characterize patterns of histone retention and modifications in mature sperm and the developmental roles sperm epigenetics play in the developing embryo.

Other interesting histone modification patterns within the sperm have emerged. First, testes-specific histone 2B (TH2B) is retained in lesser amounts than H3K4 and H3K27, but is preferentially located in the promoter regions of genes involved in spermatogenesis, specifically ion channel genes (Hammoud et al. 2009). TH2B has also been implicated as a possible regulator of the transition to protamines (Montellier et al. 2013). Retained histone H2AZ is located in pericentric heterochromatin regions, which had previously been reported in immunohistochemical staining studies (Hammoud et al. 2009; Montellier et al. 2013; Rangasamy et al. 2003). Interestingly, regions of the genome associated with

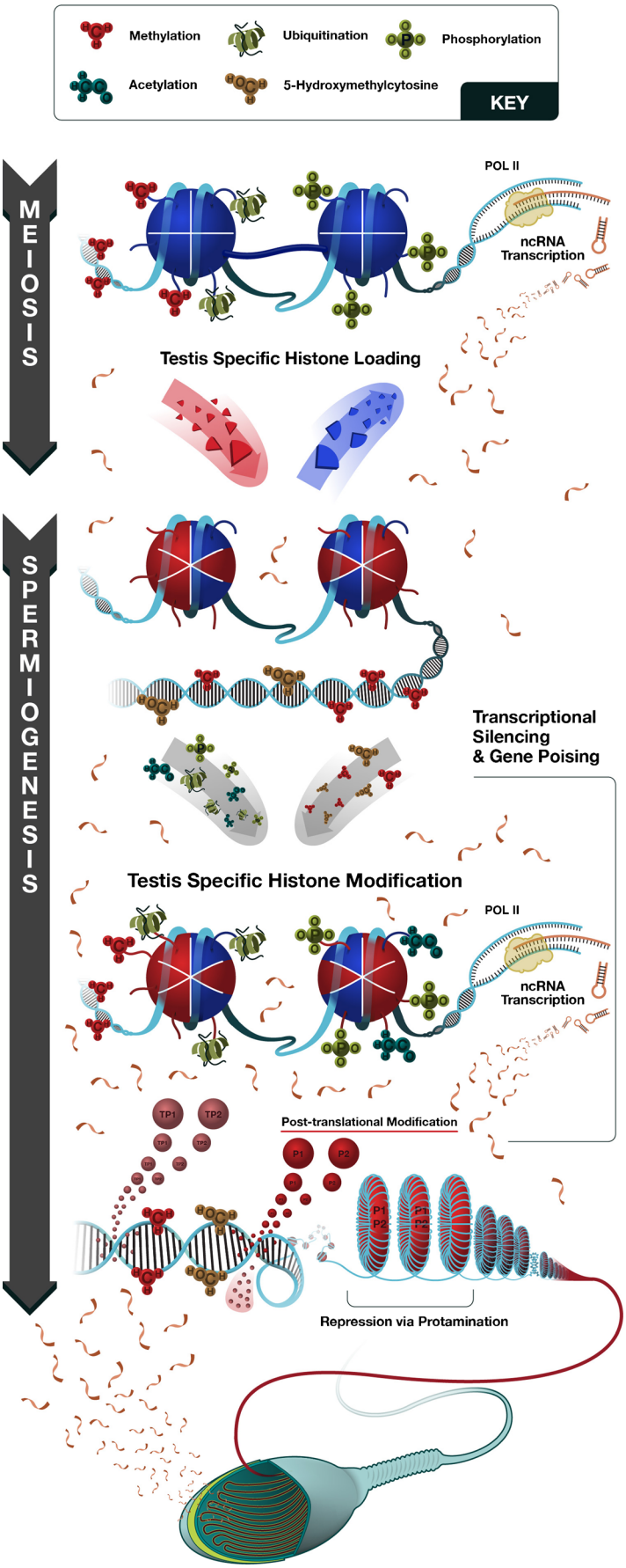


Fig. 3 Epigenetics and spermatogenesis. Illustration of epigenetic modifications that throughout the process of spermatogenesis, including the production of germ-cell specific non-coding RNAs (*ncRNA*), the integration of testis-specific histones following meiosis, epigenetic modifications to DNA and histones during the early stages of spermiogenesis, and, finally, the replacement of the majority of histones by transition proteins (TP) and subsequent protamine (P1, P2) integration during the final stages of spermiogenesis

H3K4me3, but not bivalently marked with H3K27me3, are also associated with spermatogenesis genes. These observations indicate that histone retention may not only indicate future function, as seen in the marks of developmental genes, but may also provide a partial historical record of spermatogenesis gene expression.

Sperm DNA Methylation

DNA methylation at the 5-carbon position of cytosines, largely at cytosine-phosphate-guanine dinucleotides (CpGs), is a potent inhibitor of transcription (Jaenisch and Bird 2003). The most potent inhibitor of transcription is methylation of CpG "islands", which are CpGs found in the promoter regions and which act by allowing or inhibiting the binding of DNA-binding proteins to the promoter regions. DNA methylation is facilitated and maintained by a family of DNA methyltransferases (DNMTs) (Sanli and Feil 2015). The interaction of gene sequence, histone modifications, and DNA methylation is complex and not well understood.

The life cycle of sperm DNA methylation is complex and intriguing. Briefly, following fertilization, the paternal genome undergoes active demethylation of most of the genome (Jenkins and Carrell 2012). This has caused some to question the importance of sperm DNA methylation in embryogenesis and transmission of epigenetic marks to the offspring. While many questions still remain, it is important to note that clearly some sites retain methylation marks throughout the programmatic methylation resets, including imprinted genes, retrotransposons, and some non-imprinted genes (Borgel et al. 2010; Gkountela and Clark 2014; Smallwood et al. 2011; Smith et al. 2014). Furthermore, data clearly indicate that transgenerational inheritance of epigenetic marks occurs, including methylation effects (Wei et al. 2015).

The normal sperm epigenome has been described in detail (Hammoud et al. 2009, 2014; Molaro et al. 2011) and contains important features. First, similar to the stem cell epigenome, the promoter regions of both bivalently and monovalently marked developmental genes in sperm are hypomethylated compared to somatic cells (Molaro et al. 2011). These observations further support the hypothesis of a poised sperm epigenome, transcriptionally silenced, but 'poised' to facilitate rapid initiation of transcription, an observation confirmed in the zebrafish (Wu et al. 2011). It is important to note that a recent study by Hammoud et al. (2014) has shown that the sperm epigenome is largely set and maintained in adult germline stem cells, rather than being modified during spermiogenesis. This includes not only the bivalent poising pattern of developmental genes but also unique epigenetic marks for genes previously expressed in spermatogenesis, including an atypical pattern of active transcription in some genes, such as piRNAs (piwi-interacting RNAs) with low CG content, that have bivalent histone modifications but DNA methylation, mostly 5-hydroxymethylation (5hmC) (Hammoud et al. 2014).

RNA In Sperm

Transcription ceases in the round spermatid stage of spermatogenesis and RNA transcripts are either retained in ribonucleoproteins (RNPs), for future use in the developing sperm or embryo, or they are degraded. Krawetz and others first reported the retention of RNAs, including both coding and non-coding transcripts, in mature sperm (Ostermeier et al. 2005). This observation has since been confirmed in multiple laboratories, and the types of RNAs identified now include virtually all classes of small micro RNAs (mi), small interfering (si), small nucleolar (sno), and piwi-interacting (pi), as well as long noncoding RNAs (lnRNAs). Sequencing of the sperm transcriptome has greatly expanded the number of RNAs identified that are expressed in a stage-specific manner and for which there is good homology between mice and humans, and between fertile men (Hammoud et al. 2014).

Characterizing the sperm transcriptome has broad and intriguing implications. First, the transcriptome may yield markers of spermatogenesis, and hence be more accurate than current laboratory assays to detect specific defects in sperm function pathways, such as protamination, motility, and fertilization (Jodar et al. 2013; Luk et al. 2014). Second, it is possible that the sperm provides key miRNAs and other non-coding RNAs that may have a biological function in the embryo. For example, the miRNA most abundant in the sperm of fertile men was reported to be miRNA34c, a microRNA that has previously been reported to be involved in embryo cleavage (Liu et al. 2012). Given the inherent function of miRNAs in gene regulation, this line of study is intriguing and important. Lastly, miRNAs have been shown in multiple studies to provide another pathway of transgenerational inheritance either directly, or by regulation of DNA methylation profiles (Dunn et al. 2011; Watanabe et al. 2011; Wei et al. 2015). Although the sperm transcriptome has been less studied than sperm methylation, this area of epigenetic programming offers another area of potential clinical importance.

The Sperm Epigenome of Infertile Men

Early studies of abnormal epigenetic marks in the sperm of infertile men were largely precipitated by a few reports of a small, but significantly increased, risk of imprinting disorders in the offspring of men undergoing intracytoplasmic sperm injection (ICSI) due to severe oligozoospermia (Odom and Segars 2010; Owen and Segars 2009). Although absolute risk of imprinting disorders remained low, the increased relative risk lead to the study of the methylation status of imprinted genes, and the studies generally showed an altered

methylation of some imprinted genes in sperm from oligozoospermic compared to normozoospermic males (Jaenisch and Bird 2003; Rangasamy et al. 2003; Sanli and Feil 2015). Further studies showed that sperm DNA methylation was often altered in other patient phenotypes; including abnormal protamination and dysmorphic embryos resulting from IVF (Aston and Carrell 2012; Carrell and Hammoud 2010; Nanassy and Carrell 2011). The rates of imprinted gene defect transmission to offspring are much lower than the observed rate of abnormalities reported in the sperm population, which suggests heterogeneity of sperm methylation defects and the selective process of ICSI itself (the most morphologically normal sperm are injected), as well as possible corrective mechanisms during embryogenesis.

Methylation defects have also been reported in non-imprinted genes in the sperm of infertile men. Aston et al. (2012) reported that, in a genomewide analysis of >28,000 methylation sites, about 10 % of patients with abnormal protamination or a history of poor embryogenesis during IVF therapy had severely abnormal, chaotic methylation profiles with aberrant methylation in about 6 % of non-imprinted loci and 43 % of imprinted loci. Montjean et al. (2015) have recently shown that global sperm DNA methylation is negatively associated with semen analysis parameters, as well as chromatin packaging and DNA damage. Interestingly, abnormal sperm DNA methylation is reportedly linked to unexplained infertility, highlighting the potential use of this area to better characterize infertility (Hotaling and Carrell 2014; Klaver and Gromoll 2014; Urdinguio et al. 2015).

Several reports have reported abnormal protamination in the sperm of infertile men, but studies of abnormal histone modifications are few, due to the increased difficulty and cost of such analyses. Hammoud et al. (2011) studied genomewide histone distribution in 7 men with known aberrant protamination or increased embryo dysmorphogenesis and reported a gross lack of enrichment of histones at developmental gene promoters in 5/7 of the patients, and subtle defects of histone modifications of specific loci, as well as subtle methylation abnormalities. Another study has demonstrated abnormal shifts in the location of H3K9ac-modified histones in the sperm of some infertile men (Steilmann et al. 2011). Similarly, Vieweg et al. (2015) have reported H4K12ac distribution is altered in infertile men. These studies may highlight the role of improper histone modifications in affecting chromatin compaction, which then leads to subsequent sperm function alterations.

It is likely that a general relationship exists between abnormal histone modifications and methylation profiles. The use of epigenetic analysis as a marker of fertility and embryogenesis capability is a rapidly expanding area of applied research.

The Transient Sperm Epigenome: Paternal Aging

One of the most intriguing aspects of the sperm epigenome is its possible relationship mediating environmental influences, including biological factors such as aging, disease, obesity, and fertility. Reports are emerging of environmental influences, e.g., diet alterations, causing not only epigenetic modifications in the exposed animal but also transgenerational epigenetic inheritance through successive generations (Martos et al. 2015; O'Doherty and McGettigan 2014; Szyf 2015). While further studies are needed to characterize the effects of environmental influences on sperm epigenomes, one factor that has received attention recently is the role of paternal aging on the sperm epigenome and the offspring.

The role of advanced paternal age as a risk factor to offspring is an important area of interest in sperm epigenetics. It is well documented that there is a continuing rise in the age of fathers in most regions of the world (Sartorius and Nieschlag 2010). Concomitantly, there are increasing reports of a link between advanced paternal age and an increased risk of neuropsychiatric disorders, such as autism, bipolar syndrome, and schizophrenia (Frans et al. 2008; Hare and Moran 1979; Miller et al. 2011a, b; Sandin et al. 2015). Epigenetic alterations as a possible contributor to this increased risk was recently reported by Jenkins et al. (2015) who evaluated 2 semen samples from fertile, normozoospermic men collected 9–19 years apart. They observed consistent methylation changes in 147 distinct regions of the genome. The data did not show a "critical point" of change, rather a gradual and consistent change, primarily in the form of hypomethylated regions. Importantly, when the hypomethylated regions were evaluated for associated gene/diseases two significant disease associations were found, schizophrenia and bipolar syndrome. In a separate confirmation study (Jenkins et al. 2015), using men from the general population, including some men with reduced sperm quality, an even stronger association was observed (a 2.3-fold increase). These data demonstrate the potential of epigenetic analysis for providing benefit to patients in the form of risk assessment as a part of informed consent

The Sperm Proteome

Proteomics is defined as the systematic analysis of all the proteins in a tissue or cell and aims to assess the expression levels of all proteins of one functional state of a biological system (Domon and Aebersold 2006). One of the first pioneering studies of the protein content of the spermatozoa was done by Friedrich Miescher in 1874 (Miescher 1874), in which he identified a basic (positively charged) component from the sperm of the salmon that he called "protamin", which was coupled to an acidic component that he called "nuclein". Later, the "nuclein" was to become known as DNA whereas the "protamin" was identified as the protein known as

protamine (Dahm 2005). Protamines are the most abundant sperm nuclear proteins in many species and were one of the initial proteins identified in the sperm cell (Balhorn 2007; Carrell 2012; Felix 1960; Oliva and Dixon 1991). In the past, the study of the proteins was based mainly on their electrophoretic or chromatographic characterization followed by the determination of the amino-acid sequence using Edman protein sequencing methods (Gilboa et al. 1973; McKay et al. 1986; Mohri 1968) or the use of antibody-based protein detection methods (de Yebra et al. 1998) (Fig. 4). However, using these approaches, less than 100 sperm proteins were identified and studied in the sperm cell until the 1990s. Recently, advances in mass spectrometry have allowed the identification and quantification of hundreds to thousands of proteins per experiment (Amaral et al. 2014a; Codina et al. 2015; Oliva et al. 2008) (Fig. 4). Mass spectrometry is based on the extremely accurate detection of the mass of peptides generated from the proteins extracted from cells or tissues (Domon and Aebersold 2006). Following extraction of the proteins from the cells, reduction of the complexity of the extract is accomplished using two alternative approaches (Oliva et al. 2009): (1) electrophoretic separation of proteins followed by protein digestion into peptides, and (2) initial digestion of proteins to generate peptides and peptide separation (Codina et al. 2015; de Mateo et al. 2013). Peptides are then finally identified using mass spectrometry (Fig. 4).

The human sperm proteome

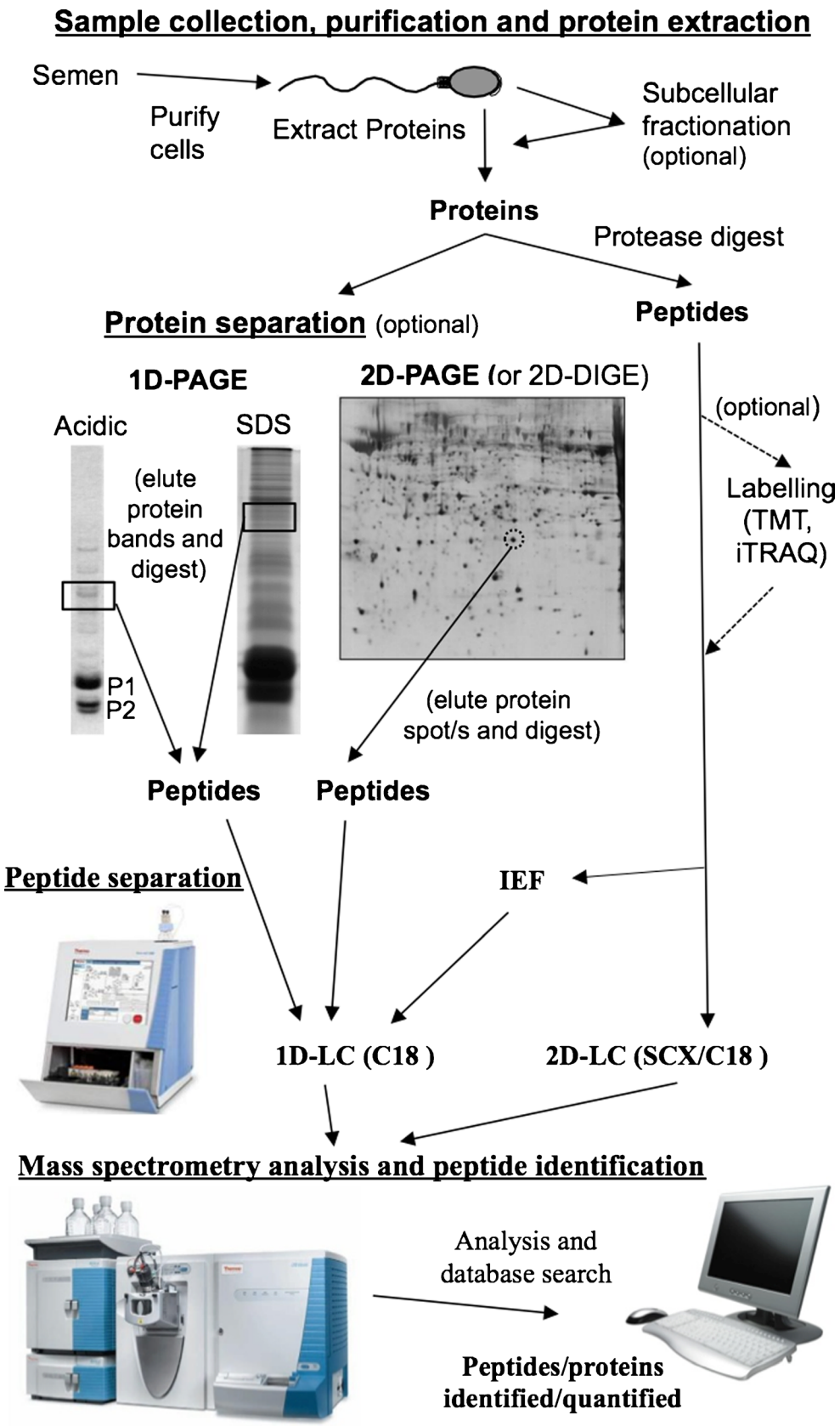
Several groups have investigated the proteins that constitute the normal human sperm proteome (defined as the proteins present in normozoospermic ejaculated spermatozoa) (Amaral et al. 2013; Baker et al. 2007, 2013; Mateo et al. 2009; Gu et al. 2011; Wang et al. 2013). A recent comprehensive compilation of all human sperm proteomic studies led to the generation of a list of 6198 different proteins identified in the normal mature ejaculated human sperm cell (Amaral et al. 2014a). The comparison of these proteins to the hypothetical proteins involved in known functional pathways in the sperm cell indicated that an estimated 78 % of the sperm proteins have already been identified. Using this rationale, it was concluded that the complete human sperm proteome includes at least 7500 different proteins (Amaral et al. 2014a). Data mining of the sperm proteome confirmed many expected functional pathways, including metabolism, apoptosis, and membrane trafficking (Amaral et al. 2014a; Paiva et al. 2015). But it has also identified many unexpected proteins such as those involved in RNA metabolism, translational regulation, ribosomes, peroxisomes and a large number of proteins with a potential epigenetic function (Amaral et al. 2014a; Castillo et al. 2014a).

Nuclear proteome of sperm

Sperm chromatin in mammals and in many additional vertebrates is largely formed by a highly compacted complex of DNA coupled to protamines (Aoki and Carrell 2003; Balhorn 2007; Oliva 2006; Oliva and Dixon 1991). In addition to histones and protamines, the recent application of mass spectrometry (MS) to study the whole sperm cell or the nuclear compartment revealed that the mammalian sperm contains many additional chromatin-associated proteins with potential regulatory roles during development (Amaral et al. 2014a; Baker et al. 2007, 2013.; de Mateo et al. 2011a; Kichine et al. 2013; Wang et al. 2013). A systematic compilation of the human sperm nuclear and chromatin-associated proteome demonstrated the presence of as many as 581 different proteins (Castillo et al. 2014a). This large number of chromatin-associated proteins was unexpected, since it was previously believed that nuclear proteins consisted almost entirely of protamines and a minor fraction of retained histones.

Furthermore, a recent detailed genomic and proteomic dissection of the human sperm chromatin followed by a high-throughput identification of the proteins and high-throughput sequencing of the associated DNA identified that there are two distinct sets of sperm proteins differing in chromatin affinity (Castillo et al. 2014a). Histone variants, transcription factors, chromatin-associated and modifying proteins involved in regulatory roles, such as the histone lysine demethylase PHF8, were identified as weakly attached to the sperm DNA, whereas proteins with structural roles were identified in the condensed fraction. Of relevance, the presence of this substantial number of chromatin-associated proteins, in addition to protamines and or histones, has also been detected in additional mammals (Castillo et al. 2014a). Thus, the proteomic structure of the sperm chromatin is much more complex than previously thought (Castillo et al. 2014a).

There are 3 potential explanations for this apparently complex proteomic structure in the sperm chromatin. First, these additional sperm chromatin proteins may constitute additional layers of sperm epigenetic information and be involved in early embryo development processes, such as sperm chromatin remodeling, and the transcriptional regulation of histone-bound paternal genes after fertilization. Under this hypothesis, these proteins would have a similar epigenetic function to the one recently proposed for the differential distribution of histones in the sperm chromatin (Carrell 2012; Carrell et al. 2008; Castillo et al. 2014a; Gannon et al. 2014). Second, this complex protein structure would be leftovers of an incomplete spermiogenesis. Under this hypothesis, the additional proteins may simply be the result of the sequence of events taking place during spermatogenesis with no function at all in the embryo. It would fit with the fact that the normal human ejaculate has a higher proportion of aberrant sperm cells (the lower reference limit for normal forms is only 4 %) (Cooper et al. 2010) and also


Sample collection, purification and protein extraction
Semen
Purify cells
Extract Proteins
Subcellular fractionation (optional)
Proteins
Protease digest
Protein separation (optional)
Peptides
1D-PAGE
2D-PAGE (or 2D-DIGE)
Acidic
SDS
(elute protein bands and digest)
P1
P2
(optional)
Labelling (TMT, iTRAQ)
(elute protein spot/s and digest)
Peptides
Peptides
Peptide separation
IEF
1D-LC (C18)
2D-LC (SCX/C18)
Mass spectrometry analysis and peptide identification
Analysis and database search
Peptides/proteins identified/quantified

◀ **Fig. 4** Proteomic methodologies. The first step in a proteomic study involves sample collection, purification and protein extraction (*top*). Extracted proteins can then be separated by protein electrophoresis systems or directly digested into peptides. In the protein separation option, the separated protein bands or protein spots must be eluted from the gel and digested into peptides. Peptide mixtures can then be separated using different alternative options and finally analyzed by mass spectrometry (*bottom*) resulting in the accurate identification of the peptides and corresponding proteins

has a higher proportion of the sperm chromatin organized in the form of nucleosomes (about 8–15 %) (Castillo et al. 2015; Gatewood et al. 1987), as compared to mice where the fitness of the sperm cells is much higher and also contain a much lower proportion of the chromatin organized in the form of nucleosomes (about 2 %) (Balhorn et al. 1977). The third potential explanation is that some of the proteins present in the normal sperm cell are delivered to the oocyte upon fertilization and may have different roles in the embryo unrelated to the chromatin structure. Known examples of sperm proteins crucial for embryo development are the centrosomal proteins since they are paternally inherited (Chatzimeletiou et al. 2008). Thus, it is possible that some of the additional proteins constituting the sperm proteome that are transmitted to the oocyte upon fertilization may also have roles in the zygote (Amaral et al. 2014a; Castillo et al. 2014b).

In addition to DNA and proteins, RNAs are also present in the sperm nucleus (Jodar et al. 2012; Jodar and Oliva 2014; Li et al. 2008). Of interest, a role for microRNA-34c has been shown to be required for the first cleavage division in mouse (Liu et al. 2012). Intriguingly, mature human sperm delivers to the oocyte many additional, potentially regulatory RNAs (Pantano et al. 2015) and RNA-associated proteins (Azpiazu et al. 2014; de Mateo et al. 2011b).

Some clues about the relative contribution of the above 3 hypotheses (not mutually exclusive) may also come from different model species. Zebrafish is a species that lacks protamines and instead uses increased amounts of histone H1 and depletion of histone decondensing modifications to accomplish sperm chromatin condensation. In this species, it has been reported that there is an apparent historical record of genes activated during spermatogenesis, and that genes important for embryo development are packaged in blocks of multivalent chromatin (Wu et al. 2011). *Drosophila melanogaster* contains protamines and Mst77F protein, similar to H1-like linker protein HILS1 in mammals (Rathke et al. 2014). In *Drosophila*, the existence of a paternal effect essential for zygote viability has been demonstrated (Loppin et al. 2005). Also, the centromere-specific histone H3 variant Cid can be detected in mature fly sperm; similar to the persistence of histones at mammalian centromeres, and lack of Cid precludes centromeres to integrate into the gonomeric spindle of the first mitosis (Raychaudhuri et al. 2012). Thus, even in these species with a radically different sperm chromatin structure, and very phylogenetically distant from mammals, there is a differential organization of the genes in sperm.

Differential proteomic studies on patients

Before the application of mass spectrometry, the study of the potential changes in the abundance of the sperm proteins in patients as compared to controls was largely based either on their electrophoretic separation and detection with dyes, with western analysis, or through their immunological detection and quantification. Some of the most widely studied human sperm proteins have been the two protamines P1 and P2, where the normal P1/P2 ratio is roughly 1 and variations above or below 1 have been shown to be present in many infertile patients (Aoki et al. 2005; Balhorn et al. 1988; Belokopytova et al. 1993; Carrell et al. 2007; Chevaillier et al. 1987; de Mateo et al. 2009; de Yebra et al. 1998; Mengual et al. 2003; Torregrosa et al. 2006). In addition, several groups have studied the protamine ratio in infertile patients undergoing IVF or ICSI treatments, detecting a correlation between the presence of an altered protamine content and the sperm penetration score, fertilization rates and the pregnancy rates (Aoki et al. 2005, 2006b; Carrell and Liu 2001; Castillo et al. 2011; de Mateo et al. 2009; Khara et al. 1997; Simon et al. 2011).

In addition to the selective study of specific proteins, mass spectrometry has been recently applied using shotgun (non-directed) approaches to identify proteins that are present in an altered abundance in sperm cells from infertile patients. Several methodological variants have been applied towards this objective. Initial approaches were based on the separation of the proteins using two-dimensional gel electrophoresis followed by the detection and quantification of the protein spots and finally excision of the differential protein spots and identification using mass spectrometry (Fig. 4) (Baker et al. 2005; de Mateo et al. 2007; Martinez-Heredia et al. 2006, 2008; Pixton et al. 2004). But more recently, these protein-based approaches are being replaced by higher-throughput peptide-based approaches (Amaral et al. 2014b; Baker et al. 2007, 2013; de Mateo et al. 2011a; Kichine et al. 2013; Wang et al. 2013). In this case, the extracted proteins are digested en masse at the beginning with a protease (usually trypsin) and then the peptides are separated through two-dimensional liquid chromatography or other systems, identified using tandem mass spectrometry (2D-LC-MS/MS) and quantified (Fig. 4) (Castillo et al. 2014a; Codina et al. 2015; de Mateo et al. 2013). There are basically two variants for peptide quantification: those based on peptide labeling using isobaric tags (either in vivo or in vitro) and those based on label-free quantification, and each variant has its own strengths and limitations (Amaral et al. 2014b; Codina et al. 2015). All these approaches have been applied to the study of the sperm cell from patients as compared to controls.

One of the first studies combined two-dimensional gel electrophoresis with in-gel digestion and MS peptide identification to study the sperm proteome of one patient with failed IVF, due to male factor as compared to the sperm proteomes from fertile donors, and resulted in the identification of 4 differential proteins (Pixton et al. 2004). In a similar study, the sensitivity was increased using fluorescent detection of the proteins (2D-DIGE), 3 IVF patients were compared to 3 controls and 12 differential proteins were identified (Frapsauce et al. 2009). Of relevance, two of the differential proteins identified were related to gamete interaction (Frapsauce et al. 2014).

Normozoospermic sperm used for artificial insemination with different reproductive success have been investigated (Azpiazu et al. 2014; Xu et al. 2012; Zhu et al. 2013). In a recent study, Tandem Mass Tag™ (TMT; Life Technologies) labeling of peptides and LC-MS/MS protein identification resulted in the detection of 31 proteins present at lower abundance and 35 at higher abundance in the no pregnancy group in comparison to sperm that resulted in pregnancies (Azpiazu et al. 2014). These differential proteins were involved in chromatin assembly and lipoprotein metabolism, suggesting that alterations in their levels may result in epigenetic errors during spermatogenesis, which could ultimately prevent embryonic development. Of interest, one of the differential proteins detected turned out to be a protamine 1 protein kinase (SRSF protein kinase 1). Proper protamine phosphorylation is needed for correct protamine deposition into chromatin (Azpiazu et al. 2014; Castillo et al. 2014a, 2015; Chatzimeletiou et al. 2008; Oliva and Dixon 1991).

An important group of sperm differential proteome studies focused on patients with altered semen analysis. The most commonly studied sperm phenotype has been asthenozoospermia, mainly because it is easy to extract a large amount of proteins from individual ejaculates. The initial studies were based on two-dimensional gel electrophoresis and all resulted in the identification of several proteins detected at an altered abundance in asthenozoospermic patients as compared to normozoospermic fertile donors (Martinez-Heredia et al. 2008; Siva et al. 2010; Zhao et al. 2007). Using higher-throughput TMT isobaric peptide labeling followed by LC-MS/MS protein identification and quantification, two independent sperm proteome comparisons of sperm differing in motility have been recently performed: the comparison of asthenozoospermic to normozoospermic sperm and the comparison of swim-up migrated to non-migrated normozoospermic sperm (Amaral et al. 2014b). Of relevance, similar proteomic alterations affecting proteins involved in sperm motility and its regulation were detected with both approaches. Similarly, analysis of obesity-associated asthenozoopsermic sperm identified several differential proteins (Kriegel et al. 2009; Liu et al. 2014; Paasch et al. 2011). Other studies have focused on different post-translational modifications (PTMs) of proteins such as N-glycosylation or phosphorylation on asthenozoospermic patients and have concluded that they may play an important role in sperm motility (Chan et al. 2009; Parte et al. 2012; Shen et al. 2013).

Future perspectives of proteomics in basic and translational research

Very few studies have so far studied the proteome of severely anomalous sperm cells such as those present in globozoospermic, severe oligoasthenozoospermic, or oligoasthenoteratozoospermic sperm, possibly due to technical issues related to the limiting amount of proteins that can be recovered (Liao et al. 2009; Thacker et al. 2011). But this is an area of great interest because these types of severely pathological samples are routinely being used in assisted reproduction. As the sensitivity of mass spectrometry improves in the near future, it can be expected that it will be possible to individually analyze patients with different diagnoses.

The intra- and inter-individual variations of the human sperm head proteome have also been studied, revealing a significant inter-individual variation in the protein profiles (Kichine et al. 2013). This variation may be partly responsible for the fact that some proteins have been reported in independent differential proteomic studies described above with differing results. Another reason for variation may be the presence of PTMs, with different protein variants detected in different studies. Thus, this is an area that will require further consideration in the near future.

Sperm proteomic studies have so far identified around 6000 proteins of the over 7500 that it is estimated make up the sperm cell. Differential proteomics has resulted in the identification of a substantial number of proteins present in an altered abundance or state in the sperm cells from infertile patients, but more research needs to be done before this knowledge can be applied in the clinic. Of particular interest is the definition of diagnostic and prognostic biomarkers in ART. An important limitation of ART is that it is effective in only ~25–40 % of all treatment cycles, and no markers are available at present to predict whether a treatment will be effective. The challenge now remains to develop the detected deregulated proteins into clinically relevant biomarkers. Toward this end, further research is needed to identify the mechanisms and pathways implicated in the regulation of spermatogenesis and differentiation and function of the sperm cell in animal models and in cell culture systems. The challenge will be to fully understand the function of the different proteins making up the sperm cell, and to uncover the relationship between the sperm proteome, transcriptome, chromatin structure, epigenome, metabolome and genome in health and disease from a systems biology approach.

Case–control studies should now also be feasible to determine which of the proteins so far detected as deregulated have

a predictive value. Once these proteins are established, the technical application to the clinic may be accomplished through specific antibody-based protein detection arrays or thought of as targeted rather than shotgun-based mass spectrometry approaches such as Select Reaction Monitoring (SRM) and Parallel Ion Monitoring (PIM).

Conclusions

This review of the "omics" of human sperm has briefly reviewed the sperm genome, epigenome, transcriptome, and proteome. It is evident that each of these areas of study is complex and employs techniques that derive large datasets requiring technical expertise to analyze and unravel significant biological insights. Indeed, one of the major necessities of a successful study in these areas is the collaboration of molecular biologists, geneticists, statistical experts and computational biologists.

A true systems biology approach of studying spermatogenesis requires integration of the data from each of these fields to gain deeper insights into disease mechanisms. This review has demonstrated a few such overlapping areas of significant insight gained from the genomic, epigenomic, and proteomic studies. However, generally, the integration of data into a systems biology approach is in its infancy in the field of male infertility. Progress is dependent on the improved accessibility and functionality of public, interactive databases, as well as the training of the next generation of researchers specializing in this type of data analysis.

In the years since Jacobsen et al. (2000) first published a relationship between decreased semen quality and an increased risk of cancer, several studies have identified mortalities and morbidities associated with reduced sperm quality (Eisenberg et al. 2015; Groos et al. 2006; Hotaling and Walsh 2009; Jensen et al. 2009; Walsh et al. 2010). It is clear that genomic and epigenomic abnormalities contributing to male infertility may be rare and subtle, and that increased "burden", rather than a few variants with high penetrance, may be contributory to a significant portion of male infertility (Aston and Carrell 2012; Lopes et al. 2013). This observation is consistent with the "testicular dysgenesis syndrome" hypothesis and highlights the need for study of genetic and epigenetic variants that may affect multiple pathways and diverse phenotypes (Skakkebaek et al. 2001). In this regard, one recent addition to the field of data analysis is the web server "DiseaseConnect"(http://disease-connect.org). The database uses large-scale omics data to identify common genes and pathways to help identify related pathologies (Tarin et al. 2015). This type of integrative analysis is important in further understanding the overall pathology, risks, and treatments of infertile males.

Improvements in technology and the progressive lowering of costs to perform large-scale omic studies has lead to important advancements in biological knowledge, including an improved understanding of spermatogenesis, sperm function, and post-fertilization events. It is likely that future studies will uncover low frequency polymorphisms and epigenetic variations that result in abnormal protein expression resulting in infertility or risk to the offspring.

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