Semen Analysis: Assaying Sperm Epigenetics

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Abstract

The mammalian sperm contains a very specialized and singular epigenetic landscape that is essential for biology and fertility. DNA methylation, spermatozoal RNAs and nuclear protein composition are marks thought to be remnants of epigenetic transitions occurring during spermatogenesis. This unique nature of the sperm epigenome has led some labs to begin to study the links between sperm epigenetic marks and various forms of infertility. This article will focus on the different techniques used in the context of sperm epigenetics and their potential diagnostic utilization in male infertility and pregnancy outcomes.

Key Points

- Sperm epigenetics provides biomarkers for disease susceptibility.
- Sperm epigenetics provides biomarker for male infertility.
- Sperm epigenetics provides biomarker for exposure toxicity.
- Sperm epigenetics provides biomarker epigenetic transgenerational inheritance and generational impacts.

Introduction

The mammalian sperm contains a very specialized and singular epigenetic landscape that is essential for biology and fertility. DNA methylation, spermatozoal RNAs and nuclear protein composition are marks thought to be remnants of epigenetic transitions occurring during spermatogenesis even though some of them can influence fertilization and embryogenesis (Hammoud *et al.*, 2009). The main difference between sperm and somatic cells is the replacement of 90%–95% of histone proteins with protamines, thus allowing a tighter DNA compaction with the formation of toroid structures necessary for sperm motility and protection of DNA from oxidation and fragmentation (Jenkins and Carrell, 2011; Oliva, 2006; Caroppo and Skinner, 2024; Nilsson *et al.*, 2022; Ben Maamar *et al.*, 2021).

Epigenetics in normal cell function is well described and has been implicated in many diseases. Yet, its use in the field of fertility is only beginning to be explored even though it is still not well understood. The use of epigenetic signatures as potential markers for

Table 1 Experimental approaches for profiling genome-wide epigenetic marks in sperm

Technique	*Analysis*	*Advantages*	*Disadvantages*
CHARM	Methylation	• Analysis of CpG sites genome-wide regardless of proximity to genes or CpG islands	• Moderate resolution • Limited regions near enzymes recognition sites
Affinity enrichment-based methods	Methylation	• Detection of DMRs within high or low CpG density regions • Can discriminate 5mC from 5hmC • No mutation introduced • More sensitive than MeDIP in regions with higher CpG density	• Low resolution • Biased toward hypermethylated regions
MeDIP	Methylation	• No mutation introduced • Specific to 5mC/5hmC depending on the antibody • More sensitive in regions with low CpG density	• Biased toward hypermethylated regions • No identification of single 5mC sites • No prediction of absolute methylation level
Bisulfite conversion-based methods	Methylation	• Evaluate methylation state of almost every CpG sites	• High cost • Bisulfite treatment can degrade DNA • No discrimination between 5mC and 5hmC
Single-cell methylome	Methylation	• Highly sensitive • Detect target CpG sites at high coverage with low number of sequence reads	• Bisulfite treatment can degrade DNA • No discrimination between 5mC and 5hmC • Poor coverage for imprinting loci
Pyrosequencing analysis	Methylation	• Measurements of individual CpG dinucleotides	• Not a genome-wide approach
ChIP	Histones	• Very efficient immunoprecipitation	• Not applicable to non-histone proteins • Risk of protein rearrangement during chromatin preparation and precipitation • May have selective digestion of particular chromatin domains during preparation
RNA extraction	ncRNAs	• Pure preparations of RNA	• Depending on the method used, small RNAs cannot be recovered

diseases related to fertility or as predictors in assisted reproductive techniques (ART) is currently being developed (Skinner, 2024). Because of the unique nature of the sperm epigenome and how the patterns found in mature sperm can possibly perturb spermatogenesis, researchers believe that these marks could provide predictive insight on infertility and pregnancy outcomes.

Several techniques have been developed to study these different epigenetic marks (**Table 1**). Each of them can yield tremendous amounts of data about the patterns associated with different forms of fertility phenotypes. Furthermore, these techniques could potentially be useful as diagnostic tools in the foreseeable future and provide so much more data than the current tests used clinically to diagnose male fertility (Skinner, 2024).

DNA Methylation

DNA methylation is one of the most studied epigenetic modifications. A methyl group will be added on the fifth carbon of cytosine (C) forming 5-methylcytosine (5mC) by catalysis of DNA methyltransferases (Dnmts) (Smith and Meissner, 2013). DNA methylation occurs mainly in CpG dinucleotides (CpGs) even though it can also be found, less frequently, in non-CpG context (Kim *et al.*, 2014). DNA methylation has been associated with a large number of cellular processes such as X chromosome inactivation, embryonic development, genomic imprinting, transcriptional repression, alteration of chromatin structure, and transposon inactivation (Wilson *et al.*, 2012). These methyl marks are heritable but more interestingly certain methylation patterns have epigenetic transgenerational inheritance effects (Li et al., 2015b; Nilsson et al., 2022). DNA methylation is very different depending on the organism studied. In human embryonic stem cells for instance, DNA methylation occurs in up to 95% of CpGs with the remaining unmethylated CpG residues enriched in high density CpG islands (CGI) located at gene promoters (Chen *et al.*, 2011; Ben Maamar *et al.*, 2023). On the contrary, in invertebrates like *Drosophila*, methylation levels are low (Lyko *et al.*, 2000).

These important findings on DNA methylation would not have been possible without the development and advancement of experimental and computational profiling approaches.

Sperm DNA Extraction

Classic high quality DNA extractions developed for mammalian somatic cells are ineffective for sperm. In fact, unlike somatic cells, sperm DNA is highly compacted by the replacement of histones with sperm-specific proteins called protamines. These protamines and the disulfide bridges formed within and between protamines inhibit the extraction of sperm DNA by standard techniques (Griffin, 2013).

A wide variety of methods have been developed to extract DNA. In 1988, Bahnak *et al*., described a guanidinium protocol for the isolation of mammalian spermatozoa (Bahnak *et al*., 1988). This protocol was later modified by Hossain *et al*. replacing the lengthy ultracentrifugation steps with the use of isopropanol and proteinase K for digestion (Hossain *et al*., 1997). Commercial companies have also based their kits on this guanidine thiocyanate method for the isolation of RNA, DNA, and protein. But, these kits utilize toxic chemicals such as chloroform and phenol. This was counterbalanced by the development of column purification of the DNA following lysis.

In round spermatids, somatic histones are replaced by testis-specific transition proteins during spermiogenesis. To have a highly condensed chromatin, transition proteins are later replaced by protamines in the elongated spermatid which will then be phosphorylated (Brewer *et al*., 2002; Dadoune, 1995). Once the protamines bind to the DNA, most of the phosphate groups are removed and cysteine residues become oxidized and form disulfide bridges that link the protamines together. During sperm DNA extraction, a supplemental dissociation of nucleoproteins from nucleic acids is required. Dithiothreitol (DTT) and 2-mercaptoethanol enable to cleave disulfide bonds and allow proteins to unfold completely. DTT is known to be more effective, less toxic, quicker and the odor is more tolerated than 2-mercaptoethanol. Sodium dodecyl sulfate (SDS) is routinely used in DNA extraction methods. Sodium lauroyl sarcosinate (Sarkosyl), like SDS is also used to denature proteins and disrupt biological membranes. However, Sarkosyl, on the opposite of SDS, has an excellent solubility in chaotropic solutions. Extraction buffers used contain EDTA which removes metal ions potentially present in the seminal fluid, or on the cell surface and will thus reduce contamination in the final DNA extract. The addition of isopropanol facilitates DNA precipitation. An incubation at room temperature with sodium citrate and alcohol will concentrate any remaining chaotropic salts into the solution while the DNA will stay precipitated. In fact, sodium ions in the presence of alcohol neutralize the negatively charged phosphate groups on the DNA backbone facilitating its precipitation.

The first studies on DNA methylation determined the methylation status of genes of interest and quantified the total amount of 5mC. Thanks to microarray hybridization technology, studies on DNA methylation were able to scale up to the genome-wide-level. Nowadays, next-generation sequencing platforms are able to construct genomic maps of DNA methylation at single-base resolution. Enzyme digestion, affinity enrichment and bisulfite conversion are currently used for that purpose. The most recent development in the epigenomic profiling is a single-cell methylome, and the use of third-generation sequencing in detecting DNA methylation in real time.

Restriction Enzyme-Based Methods

These techniques use methylation-sensitive restriction enzymes (MREs) such as *BstUI*, *HpaII*, *NotI*, and *SmaI* which cleave only unmethylated target sequences leaving then the methylated DNA intact. This MRE digestion coupled with sequencing technologies can predict genome-wide DNA methylation levels (Maunakea *et al*., 2010). During the sequencing step, the DNA fragments from this cleavage are size-selected and sequenced. The results reveal the locations of the unmethylated CpG sites within the recognition sites of the enzyme utilized (Li *et al*., 2015a).

In conclusion, MRE-Seq estimates the relative DNA methylation levels with a low coverage of the genome.

Comprehensive High-Throughput Arrays for Relative Methylation (CHARM)

This method uses the enzyme McrBC digesting methylated DNA to fractionate it. McrBC cleaves half of the methylated DNA and all the methylated CpG islands (CGI). This enables to relatively size-select unmethylated DNA and subsequently utilizes hybridization arrays. Thus, CHARM is able to detect differentially methylated regions (DMRs) at CGI shores (sequences up to 2 kb away from CGI) which are not detectable with methods such as methylated DNA immunoprecipitation (MeDIP).

Affinity Enrichment-Based Methods

As the MeDIP, these affinity enrichment-based methods use antibodies specific for 5mC or methyl-CpG-binding domain (MBD) proteins to enrich methylated DNA regions. This MBD protein-based approach relies on the capacity of MDB proteins to bind specifically to methylated DNA sequences followed by a microarray (MBD-chip) or sequencing (MBDCap-seq/MethylCap-seq) (Brinkman *et al*., 2010), methylated DNA capture by affinity purification technologies. This technique is biased to high density CpG sites (Beck *et al*., 2022).

Methylated DNA Immunoprecipitation (MeDIPs)

Using an anti-methylcytosine antibody, MeDIP is able to immunoprecipate DNA with methylated CpG sites. These DNA fractions enriched by MeDIP can be evaluated using tiling arrays (MeDIP-chip) or high-throughput sequencing (MeDIP-seq). This technique usually yields a resolution of 100–300 bp. MeDIP-seq generates the relative enrichment of methylated DNA across the genome instead of predicting the absolute DNA methylation level. Because of the low amount of DNA needed (as low as 1 ng), this method can be used to profile DNA methylation in minute DNA samples, rare cell types and microdissected tissues (Taiwo *et al.*, 2012; Zhao *et al.*, 2014). However, in MeDIP, CpG-rich fragments are more likely to be enriched than CpG-poor ones even if they are fully methylated, a computational correction to normalize CpG content across a wide range of CpG densities is always required (Down *et al.*, 2008). The MeDIP analysis is biased to lower density CpG regions so can identify greater than 95% of the epigenome, compared to the other procedures (Beck *et al.*, 2022).

Bisulfite Conversion-Based Methods

This technique is based on the deamination of unmethylated C to uracil (U) residues on the genomic DNA, whereas methylated C residues remain unaffected. The U eventually converts to thymine (T) in a subsequent polymerase chain reaction (PCR). The methods based on this bisulfite conversion provide single-base resolution. When investigating specific DNA sequences or studying genome-wide methylation via a methylation array, whole-genome bisulfite sequencing and reduced-representation bisulfite sequencing are commonly used. This procedure is biased to higher density CpG regions due to bioinformatics issues so typically assesses around 30–40% of the epigenome (Beck *et al.*, 2022).

Single-Cell Methylome

Generally, most genome-wide DNA methylation profiling techniques need bulk cell populations as starting materials. It is also impossible to assess the methylation heterogeneity among individual cells. To overcome this issue, single-cell bisulfite-based techniques have been developed. To reduce DNA loss and provide information on approximately 1 million CpG sites within an individual mouse or human cell, steps of *Msp*I digestion have been integrated to the bisulfite conversion. These techniques are ideal in studies with limited cell amounts and heterogeneous cell populations. They are especially practical for sperm cells, oocytes, primordial germ cells, and embryonic stem cells (Guo *et al.*, 2015; Schwartzman and Tanay, 2015; Kelsey *et al.*, 2017).

Pyrosequencing Analysis

Pyrosequencing provides an analysis of the methylation pattern at a specific site of 25–50 bp. It allows measurement of the methylation percentage of individual CpG dinucleotides, a certain flexibility in sequencing primer position to analyze any CpG sites. This technique is only used for specific sites and it is not possible to use it as a genome-wide approach as many regions of the genome cannot be analyzed with this protocol.

Third-Generation Sequencing

The epigenetics field is evolving rapidly and with it the technologies associated. Emerging third-generation sequencing such as single-molecule real-time sequencing or nanopore sequencing are perfect examples. The first technique enables to detect directly modifications by monitoring the activity of DNA polymerase during the incorporation of different nucleotides labeled with fluorescence into complementary DNA strands. In the second technique, single-strands of DNA are pulled by a phage DNA polymerase through a bacterial pore in single-nucleotide steps and the ion current through the pore is recorded (Laszlo *et al.*, 2013).

All together, these third-generation sequencing techniques will allow researchers to make more discoveries on the different epigenetic modifications and could reveal novel functions of these epigenetic marks in gene expression. However, before their application is extended, the throughput and accuracy must be substantially improved before applying them to studies involving complex genomes (Yong *et al.*, 2016; van Dijk *et al.*, 2023). A limitation is only single molecules are sequenced at a time, so large numbers of molecules need to be assessed to obtain an assessment of the methylation sites of a CpG site, compared to other methods that assess millions of cells to assess the methylation of the CpG site.

Histone Modifications

Histones order DNA into structural units called nucleosomes. They are the major protein component of chromatin and are subject to several different post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation.

The positive charge of the lysine residue on histone tails usually leads to a tight association with negatively charged DNA. Histone acetyltransferases (HATs) are enzymes initiating the formation of euchromatin from heterochromatin. HATs catalyze the transfer of an acetyl group (CH_3CO) to a conserved lysine on the histone tail. Acetylation of histones typically leads to relaxed chromatin and is associated with activation of transcription. In contrast, histone deacetylases (HDACs) catalyze the removal of acetyl groups from histones, consequently leading to more tightly packaged chromatin. The recruitment of HATs and HDACs is tightly regulated by DNA binding proteins located near epigenetic target regions. The methylation of specific amino acids on histones can lead to the formation of heterochromatin with consequent repression of transcription.

The interaction between histones and DNA is most often studied using a technique known as chromatin immunoprecipitation (ChIP). This method requires the use of highly specific antibodies directed against DNA-binding proteins and can be followed by a number of nucleic acid analysis techniques, such as PCR, qPCR, sequencing, and microarray hybridization. ChIP determines whether certain histones are associated with specific genomic regions and also identifies regions of the genome associated with specific histone modifications—methylation and acetylation being the most studied ones.

Chromatin Immunoprecipitation (ChIP)

To map histone modifications, chromatin is normally isolated under either fixed or native conditions. Sperm is treated with a reducing agent in order to reduce and prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues. The sperm is subsequently resuspended in a lysis buffer containing some detergents, whose concentrations are crucial to an efficient chromatin shearing. This shearing can be proceeded by an enzymatic digestion (usually a micrococcal nuclease) or by a sonication method. The genomic DNA that is not protected by histones will be divided in fragments around 150–250 bp (150 bp being the size of one nucleosome). After that histones will be detected in the soluble chromatin fraction while protamines are spun down. This chromatin can be directly used in ChIP to map histone variants, modifications or retention in sperm. To do the immunoprecipitation, an incubation with an antibody against histones or histone modifications is used. Then the antibody–chromatin complexes will be captured using beads and the bound chromatin is eluted. The immunoprecipitated DNA is obtained by a phenol/chloroform extraction. The DNA associated with histones and histone modifications can be characterized by candidate specific quantitative PCR (qPCR) approaches or genome-wide high-throughput sequencing. However, even if qPCR is a valid technique, because the isolated DNA is around 150 bp in size, designing one or both primers outside these small fragments can lead to mistaken conclusions (Hisano *et al.*, 2013).

Non-Coding RNA

A very large number of transcripts do not appear to encode for proteins. These transcript species are known as noncoding RNAs (ncRNAs). For a long time, they have been considered as "junk" in the genome, but emerging evidence has highlighted that ncRNAs can have a wide variety of functions. Complex eukaryotes such as fungi, plants, and animals have > 50%, a number rising to ~ 98.5% noncoding DNA in humans (Amaral *et al.*, 2008). Non-coding RNAs can be classified according to length: the small ncRNAs (sncRNA) with a length < 200 bp; anything longer than that is referred to as long ncRNA (lncRNA). Examples of short ncRNAs include rRNA, microRNA (miRNA), siRNA, and Piwi-interacting RNA (piRNA). Both of these ncRNAs have been shown to be implicated in spermatogenesis (Bettegowda and Wilkinson, 2010; Chuma and Nakano, 2012; Lee *et al.*, 2012; Yadav and Kotaja, 2014).

Small non-coding RNAs are generally identified by RT-PCR analysis, in situ hybridization, or small RNA sequencing studies. Besides, the recent development of microarray technologies has permitted the global analysis of spermatozoal microRNAs (miRNAs) allowing different research teams to determine different expression profiles between fertile and infertile men (Hamatani, 2011; Kotaja, 2014). Large non-coding RNAs are also predominant in sperm and can be analyzed in a standard RNA sequencing analysis (Tosar *et al.*, 2024).

The sperm contains reduced cytoplasm, low amounts of full-length RNAs along with biologically degraded RNAs, and highly protamine-condensed DNA. Due to this complex nature, the isolation of RNA from sperm is unique compared to somatic cells.

To isolate total RNA, the sperm membrane and nucleoprotamine complex have to be completely dissolved. Variations of two methods have historically been used to prepare RNA from natural sources (e.g., tissue samples, whole organisms, cell cultures, bodily fluids): chemical extraction and immobilization on glass, often referred to as solid-phase extraction. Chemical extraction methods typically use highly concentrated chaotropic salts in conjunction with acidic phenol or phenol-chloroform solutions to inactivate RNases and purify RNA from other biomolecules. These methods provide very pure preparations of RNA. But, the RNA must typically be desalted and concentrated with an alcohol precipitation step. This alcohol precipitation does not quantitatively recover small nucleic acid molecules, making it ill-suited for the preparation of very small RNAs.

The second method, solid-phase extraction, is based on high salt or salt and alcohol to decrease the affinity of RNA for water and increase its affinity for the solid support used. However, this solid-phase purification of RNA does not effectively recover small RNAs.

Some companies have developed isolation procedures combining the advantages of organic extraction and solid-phase extraction while managing to recover small RNAs. The use of ethanol immobilizes the large RNAs through a glass-fiber filter and the small RNAs are collected in the filtrate. For non-coding RNAs (ncRNAs), an enrichment is usually necessary. Candidate-specific RT-PCR, microarrays or genome-wide high throughput sequencing analysis can be used to characterize the nature of these ncRNA.

Epigenetic Human Sperm Analysis

Clinical Potential Use

Epigenetics has been well studied in normal cell function and has also been implicated in many diseases (Momparler, 2003; Skinner, 2024). Its use in fertility is however just starting to be explored. The multiple factors involved in epigenetics such as the variables involved, the poor description of normality as each cell type has unique signatures with some degree of variation and the type of epigenetic mark being assessed; make it challenging and hard to understand. With our current understanding of the epigenome, we cannot predict the potential biological impact of a change in DNA methylation (Jenkins *et al.*, 2017). The emergence of extremely high resolution techniques enabling for instance a single cell resolution of complete transcriptomes, provides remarkable insight into epigenetics at the single cell resolution which can offer tremendous possibilities in both research and diagnostic development. Despite these limitations, many epigenetic signatures in sperm have been tightly associated with a number of sperm abnormalities and/or clinical outcomes such as protamine levels in sperm from fertile and infertile human males (Jenkins and Carrell, 2011; Caroppo and Skinner, 2024). Using epigenetic signatures as potential markers for various fertility related diseases and/or as predictors for success in ART is becoming more widely explored (Aston *et al.*, 2015; Jenkins *et al.*, 2016; Jodar *et al.*, 2015; Caroppo and Skinner, 2024). The high resolution techniques enable to screen large portions of the epigenome and create a huge amount of data available which can help understand the relationship between the sperm epigenome and various fertility phenotypes (Hrdlickova *et al.*, 2017; Caroppo and Skinner, 2024). 684 RNA "elements" have been identified as being required to facilitate the best chances at successful IVF outcomes and the absence of even one of these elements has been shown to decrease the success rate in various fertility treatments (Jodar *et al.*, 2015). Another study identified genetic markers which could be useful as a complement for classical semen analysis in order to identify sperm donors with less favorable in utero insemination reproductive outcome despite having normal semen parameters (Bonache *et al.*, 2012). DNA methylation techniques have progressed greatly these last years and they are commonly used in the assessment of sperm DNA methylation patterns. For instance, Aston *et al.* identified in the mature sperm some methylation patterns which could predict the likelihood for a man to go through IVF or if a less invasive procedure might be effective (Aston *et al.*, 2015).

Conclusion

It is well known that the current available diagnostic tools to establish a man's reproductive potential are lacking (WHO, 2010). Many researchers have highlighted the need to innovate and improve approaches with better predictive power for male infertility diagnosis purpose. Assessing the DNA methylation seems to be the easiest option as it remains remarkably stable throughout spermatogenesis (Hammoud *et al.*, 2014) whereas RNA transcripts and histone retention/modifications change dramatically. Yet, discriminating one epigenetic mark rather than another is less likely to offer a better understanding in the etiologies of commonly seen sperm disorders. An integration of all epigenetics is required to understand the biology and allow effective biomarkers (Caroppo and Skinner, 2024; Beck, *et al.*, 2021).These techniques, even though not presently applicable clinically, will be developed as predictive tools to assess an individual's ability to procreate (Caroppo and Skinner, 2024).

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