Epigenetics, spermatogenesis and male infertility

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ABSTRACT

Epigenetic modifications characterized by DNA methylation, histone modifications, and chromatin remodeling are important regulators in a number of biological processes, including spermatogenesis. Several genes in the testes are regulated through epigenetic mechanisms, indicating a direct influence of epigenetic mechanisms on the process of spermatogenesis. In the present article, we have provided a comprehensive review of the epigenetic processes in the testes, correlation of epigenetic aberrations with male infertility, impact of environmental factors on the epigenome and male fertility, and significance of epigenetic changes/aberrations in assisted reproduction. The literature review suggested a significant impact of epigenetic aberrations (epimutations) on spermatogenesis, and this could lead to male infertility. Epimutations (often hypermethylation) in several genes, namely MTHFR, PAX8, NFR3, SPN, HRAS, JPH3DDA, IGF2, H19, RASGRF1, CTL2, PLAG1, D1RAS3, MEST, KCNQ1, LIT1, and SNRPB, have been reported in association with poor semen parameters or male infertility. Environmental toxins/drugs may affect fertility via epigenetic modifications. For example, 5-aza-2'-deoxycytidine, an anticancer agent, causes a decrease in global DNA methylation that leads to altered sperm morphology, decreased sperm motility, decreased fertilization capacity, and decreased embryo survival. Similarly, Endocrine disruptors, such as methoxychlor (an estrogenic pesticide) and vinclozolin (an anti-androgenic fungicide) have been found by experiments on animals to affect epigenetic modifications that may cause spermatogenic defects in subsequent generations. Assisted reproduction procedures that have been considered rather safe, are now being implicated in inducing epigenetic changes that could affect fertility in subsequent generations. Techniques such as intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI) may increase the incidence of imprinting disorders and adversely affect embryonic development by using immature spermatozoa that may not have established proper imprints or global methylation. Epigenetic changes, in contrast to genetic aberrations, may be less deleterious because they are potentially reversible. Further research could identify certain drugs capable of reversing epigenetic changes.

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1. Introduction

Male infertility has proven to be a complex pathology—its underlying physiology and biochemistry leaves much to be understood. A number of men who are identified as infertile are given this diagnosis without an accompanying explanation of its cause. Many research projects have focused on exploring the genetic basis of male infertility, but thus far they have been able to explain not more than 15% of infertile cases [1]. Therefore, much remains to be understood, and we need to involve several other approaches to completely understand the etiology of male infertility. One such promising approach is epigenetics, which has the potential to explain the etiology of numerous disorders, at least partially.

The genome is defined as the total amount of information encoded by the nucleotide sequence of an organism’s DNA. The epigenome is comprised of the modifications made in gene expression by changing DNA and histone structure without changing the DNA sequence itself. Epigenetic processes include actions such as DNA methylation, posttranslational histone modifications, and chromatin remodeling. These changes can have short- or long-term effects and could be trans-generational [2]. DNA wrapped around histones forms a nucleosome, which is the basic subunit of chromatin. The manner in which a region of DNA is wrapped around histones determines whether or not a gene or a family of genes is available for transcription. Regions of DNA that are tightly compacted are called heterochromatin and are transcriptionally inactive; conversely, regions that are bound loosely to histones are called euchromatin and are transcriptionally active. The compactness of DNA in a particular region of chromatin is determined by epigenetics; therefore, epigenetic changes play a crucial role in determining which genes are expressed and when in specific cells. Although the genetic code is considered to be static, or the same in every cell for an organism’s entire life, the epigenetic code is dynamic and tissue-specific [3]. Therefore, the genetic code defines the permanent imprint of information determining the phenotype and characteristics, whereas the epigenetic code provides a dynamic imprint to fine tune the phenotype and characteristics according to environmental and other factors. It is thought that reversal of epigenetic changes can be induced; consequently, aberrant epigenetic modifications are being explored as therapy targets for a number of diseases [4]. Because the field of epigenetics is still rather young, much remains to be discovered about the processes and mechanisms involved in its regulation of gene expression. In this review we provide an overview of the role of epigenetic processes in spermatogenesis and male infertility.

2. Overview of epigenetic processes

Of the known epigenetic processes, the three most common and best characterized are DNA methylation, histone modification, and chromatin remodeling (Fig. 1). A brief summary of these processes is provided below.

2.1. DNA methylation

Methylation patterns of cytosine residues within CpG dinucleotides convey important epigenetic information about gene expression. These dinucleotides consist of cytosine bound to guanine by a phosphodiester bond. CpG dinucleotides can be found in clusters that have been termed CpG islands. The number of methylated cytosine in a particular CpG island may vary depending on several factors discussed later in this article. In 1987, Gardiner-Garden and Frommer formed the accepted definition of a CpG island as a 200-base pair length of DNA with a C + G content of 50% or greater and a CpG frequency of at least 0.6 of the statistically expected value [5]. This definition, however, was termed by Takai and Jones as arbitrary and in 2002 they developed a search algorithm to limit and more accurately establish the term. By their analysis, a more appropriate definition of a CpG island is a 500-base pair stretch of DNA with a C + G content of 55% or greater and a CpG frequency of at least 0.65 of the statistically expected value [6]. However, still there is no concrete definition of a CpG island and criteria used by different algorithms may vary. CpG islands have been found near promoters [7], indicating that they play a role in regulating gene expression. Indeed, hypermethylation of DNA in CpG islands is associated with the maintenance of gene suppression, while hypomethylation in these regions is associated with gene expression [8]. Hypermethylation is also found in centromeric and pericentric heterochromatin sites [9]. In vertebrates, CpG islands are often associated with “housekeeping” genes, which are required for general cell functions, as well as certain tissue-specific genes [5].

2.2. Histone modifications

Posttranslational histone modifications are essential for proper cell function. The N-termini of histone tails contain amino acid residues that are affected by methylation, acetylation, phosphorylation, ubiquitylation and sumoylation. The sum of these modifications and the information they communicate is referred to as the histone code. Methylation is one of the most prevalent histone posttranslational modifications. It is monitored by histone methyltransferases [HMTases] and is generally associated with gene silencing. Methylation of H3K9, for example, is a classic indication of gene silencing and is commonly found in heterochromatin, as well as silenced promoters [10]. In some cases, however, methylation of arginine and lysine residues can lead to gene activation [11]. For example, methylation of histone H3K4 is implicated in gene expression [12]. More than one methyl group may be transferred to a single amino acid residue. The location and number of methyl groups in a region of DNA convey a specific epigenetic signal.

Histone acetylation is associated with increased levels of transcription and is modulated by both histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs activate gene
expression, while HDACs inhibit gene expression [13]. Acetylated lysines are specifically recognized by bromodomain-containing proteins and act to enhance chromatin remodeling [14]. Phosphorylation of histones occurs on serine residues and generally leads to gene activation [13]. However, chromosome condensation and gene silencing is seen when the histone variant H2AX is phosphorylated [15]. Covalent modification of histones by ubiquitylation of lysine residues can cause both gene expression and repression. For example, addition of ubiquitin to histone H2A is linked to gene silencing [16] whereas ubiquitylation of H2B is linked to gene activation [17]. Lysines may also undergo sumoylation, or the attachment of small ubiquitin-related modifier proteins (SUMOs) [18]. This not only serves to silence gene expression, but also inhibits other types of histone modifications like acetylation [18].

2.3. Chromatin remodeling

In contrast to the two previously described epigenetic processes, chromatin remodeling is not based on covalent interactions. Instead, ATP-dependent chromatin remodeling complexes use energy from hydrolyzing ATP to alter the location and/or structure of nucleosomes [19]. This changes which genes are available for transcription and can lead to either gene expression or silencing [19]. In yeast, the positions of nucleosomes can generally be predicted based on the primary DNA sequence; this is because nucleosomes must be situated in regions where the DNA can bend [20]. Chromatin remodeling complexes primarily act by sliding histones along the DNA, but they can also twist, spool and bulge the DNA [21]. Two of the common families of such complexes include the SWI/SNF family and ISWI family [22]. The SWI/SNF and ISWI families are ubiquitous, as well as act in a non-specific fashion on various loci [23]. Each family has a conserved catalytic ATPase subunit to carry out its function [22].

3. Regulation of epigenetic processes

An ever-expanding number of molecules have been implicated in the regulation of epigenetic processes. Some of these factors have been isolated and characterized, while others remain unknown and are only speculated to be present based on the action of other epigenetic regulators. A description of some known regulatory molecules is provided below.

3.1. DNA methylation

DNA methylation is regulated by DNA methyltransferases (DNMTs), which add methyl groups from S-adenosyl-methionine to the 5’ position of cytosine residues of CpG dinucleotides [8]. DNMT1 plays a role in maintaining already-established methylation patterns, whereas DNMT3A and DNMT3B act to make new methylation patterns [24–26]. Goll et al. [27] showed that DNMT2 methylates RNA in humans, but this remains unconfirmed in mice. Finally, DNMT3L has no enzymatic activity of its own [28] but is required for DNMT3A2 activity [29].

3.2. Histone modifications

Posttranslational covalent histone modifications are regulated by a number of different molecules. Methylation of histones is
modulated by histone methyltransferases which can add or remove methyl groups to or from arginine and lysine residues [30]. SuV39h1 trimethylates lysine residues on histone H3 to form pericentric heterochromatin [31] and establishes de novo gene silencing [32]. Another lysine methyltransferase, G5a, is responsible for mono- and dimethylation of H3K9 in euchromatin [33]. LSD1 and jmjC-domain-containing proteins are methyltransferases with lysine demethylase activity. Jhdn2a, also known as Jmjd1a, is known to interact with chromatin remodeling proteins to affect chromatin condensation [34]. Histone acetylation occurs on lysine residues and is regulated by HATs and HDACs. One family of HATs is the MYST family, which affects a variety of physiological developmental processes [35]. Within this family, MYST1 acetylates H3K16 to impact chromatin architecture [36]. SIRT1, on the other hand, is a deacetylase that removes acetyl groups from H1, H3 and H4 [37]. Histone phosphorylation of serine and threonine residues is mediated by MUTp kinase in hisones H3 and H2A [38]. Additionally, NHK-1 phosphorylates threonine residues on H2A [39] and MSK1 [40], MSK2 [40] and PKA phosphorylate serine residues on H3 [41]. The ubiquitin-conjugating enzyme HR6B plays a role in ubiquitylation of histone lysine residues to ultimately affect cross-talk frequency and formation of synaptonemal complexes during meiosis [16]. Sumoylation of lysine residues on histone H4 is dependent upon the E1 SUMO-activating enzyme (SAE1) and SAE2, in addition to UBC9. This modification may mediate gene suppression by recruiting both histone deacetylases and heterochromatin protein 1, supporting the suggestion that there is crosstalk between histone modifications [18].

3.3. Chromatin remodeling

Chromatin remodeling is regulated by ATP-dependent chromatin remodeling complexes. The restructuring of chromatin architecture can serve to both activate and suppress gene expression based on which sequences are exposed. Nucleosome remodeling ATPases restrict the free rotation of DNA to generate negative superhelical torsion in a linear stretch of DNA to create a cruciform [42]. The two possible mechanisms for this action are tracking [43,44] and wrench action [43,45]. Each of these models is based on the anchoring of a currently unknown molecule, called BLOCK, to the DNA while it simultaneously slides along the helix to force the DNA to rotate about its axis [43]. The mechanism by which this torsion causes chromatin remodeling is still unknown [43].

4. Epigenetics in the testes and spermatogenesis

The epigenetic processes described above not only occur in the testes and male germ cells, but also create distinct epigenetic patterns from other tissues. The epigenetic marks and processes specific to the testes and spermatogenesis are detailed below.

4.1. DNA methylation

Genome-wide analysis has shown that the testes have a highly unique pattern of DNA methylation. Indeed, compared to somatic tissues, testicular DNA has eight times the hypomethylated loci [46]. Most of these loci are non-repetitive, non-CpG island sequences and are generally away from 5’ regions of genes [46]. Hypomethylation was usually not found in gene regulatory regions; therefore correlation between gene expression patterns and hypomethylation of those sequences was neither expected nor found [46]. Hypomethylation of non-CpG island loci does, however, correlate with the GC content in that chromosomal region [46]. Interestingly, the opposite pattern is seen in somatic tissues [46]. Marchal et al. [47] found that chromosomes in Sertoli cells had low levels of methylation in euchromatin and high levels of methylation in juxtapositional regions.

Testicular germ cells also have distinct methylation patterns. These genome-wide patterns are established prior to meiosis, primarily by the type A spermatogonial stage but also in spermatagonia and spermatocytes in early prophase I, through de novo methylation and demethylation [48]. Regions that are methylated de novo are usually non-repetitive [48]. Additionally, demethylation does not take place in spermatogenic cells that do not replicate their DNA [48]. DNA methylation occurs in a sequence-specific manner and is almost always completed by termination of the pachytene spermatocyte stage [48]. The main targets of methylation are non-CpG island sequences in both distinct loci and repetitive sequences, but CpG islands can also be methylated [48]. These regions can be within known genes or between genes [48]. The methylation status of certain genes may vary as the germ cell passes through the various stages of spermatogenesis and may or may not correspond to the gene’s expression pattern [49]. Prenatal germ cell euchromatin is hypermethylated, but global passive demethylation occurs postnatally [47]. Conversely, juxtapositional heterochromatin is hypomethylated before birth and after birth becomes increasingly methylated [47]. Juxtapositional regions in spermatogonia are known to be hypomethylated relative to somatic tissues [47]. It is suggested that acquired genome-wide DNA methylation does not control gene expression specific to spermatogenic cell types, but rather plays a role in germ-cell-specific organization of chromatin [48].

4.2. Genetic imprinting

Sex-specific epigenetic patterns on discrete loci are referred to as genetic imprints. Genetic imprinting is dependent upon the parent-of-origin and causes a gene to be expressed mono-allelically. A majority of imprinted loci are methylated at CpG islands, which become differentially methylated regions [50]. All of these differentially methylated regions include short indirect repeats and many are further arranged in larger unit repeats [51]. The structure of these tandem repeats may indeed be the signal for imprinted loci [51]. Modulation of expression of these genes is executed by imprint control regions between the parental chromosomes, which are differentially methylated [52]. Since imprinted genes generally are found in clusters, it is believed that regulation occurs at the chromosomal level instead of the gene level [52].

Paternally imprinted genes are found in the male germ line and are primarily silenced by DNA methylation. Only a small number of genes have been found to have paternal imprinting, including the IGF2/H19, Rasgrf1 and Gtl2 loci [53]. The best-characterized of these is the IGF2/H19 locus, which displays reciprocal parental IGF2 and maternal H19 gene expression [8]. Insulator proteins, such as CTCF, are known to bind to differentially methylated regions to block interaction between the promoter and enhancer as a means to regulate gene transcription. Whereas CTCF is found in both somatic and germ cells, the insulator BORIS is specific to male germ cells [54,55]. It has been proposed that BORIS interacts with both demethylases to remove imprints and methylases to establish de novo DNA methylation patterns [54,55]. It has also been suggested that that paternal-specific de novo methylation is regulated by the combination of BORIS activation and CTCF suppression [54].

4.3. Epigenetic reprogramming

Epigenetic reprogramming is the process by which most, but not all, genomic methylation patterns are erased and reestablished
in a sex-specific fashion. This takes place immediately after fertilization and in primordial germ cells as a way to reestablish totipotency [56]. The reprogramming that occurs following fertilization during preimplantation development removes most of the epigenetic marks that were established during spermatogenesis [56]. Prior to reprogramming, primordial germ cells are methylated in a similar manner to somatic tissues; however, between 10.5 and 12.5 days post-coitum, before these cells migrate to the gonadal ridge, most DNA methylation patterns are erased [56]. This allows for sex-specific de novo methylation to occur both during embryonic development and spermatogenesis [56]. The removal and subsequent re-establishment of DNA methylation patterns may be a mechanism to prevent aberrant methylation from being transmitted from parent to offspring [57].

### 4.4. Nuclear protein transitioning

Male germ cells package their chromatin into a smaller volume than that of a somatic cell and therefore require specific epigenetic modifications to meet this size requirement. Such a stringent restriction on volume is placed on spermatozoa in order to ensure that the transmission of paternal DNA to the oocyte is as efficient and safe as possible [58]. This volume compaction is attained by exchanging histones for protamines, highly basic nuclear proteins unique to spermatozoa that act to tightly compact DNA by neutralizing its negative charge [59]. In this process, histones are first replaced by transition proteins (TPPs) and transition proteins are then replaced by protamines. Approximately 15% of the histone complement remains after protamines are incorporated [60], much of which lies in pericentric regions [61]. It has been proposed that the nucleohistone–nucleoprotamine structure conveys additional epigenetic information, such as classification of chromosomal regions based on their functional roles [62].

Before histones can be removed from haploid spermatids, histone variants must first be incorporated [61] and histone H4 must be hyperacetylated [63]. It is thought that this hyperacetylation decreases affinity for the DNA, which creates loose nucleosomal architecture that facilitates easy removal of histones as well as addition of TNPs and protamines [63]. Hyperacetylation of histone H4 is also accompanied by pericentric heterochromatin reprogramming [61]. Testis-specific histone variants that are incorporated include variants such as H1T [64], H1LS1 [65], H2A.X [66], TH2B [67], H3.3A [68] and H3.3B [68]. In *Homo sapiens*, four TNPs (TNP1–4) have been identified and described to be more basic than histones yet less basic than protamines [69]. Immediately following these two critical steps, protamines replace most histones to facilitate chromatin condensation. There are two classes of protamines found in humans—Prm1 and Prm2. Prm1 protamines can be posttranslationally modified by phosphorylation, dephosphorylation [70] and disulfide bond formation [71]. Prm2 protamines are biosynthesized through posttranslational cleavage from a larger precursor molecule during spermiogenesis [72]. Both of these protamines are required for chromatin condensation. The mechanism of action of these molecules has yet to be elucidated due to conflicting data; a study by Balhorn suggested that protamines bind to the minor groove of DNA, whereas a study by Fita et al. proposed that protamines actually bind to the major groove of DNA [73]. Moreover, a study by Raukas and Mikelsaar suggested that protamines can really be found in channels between DNA molecules [74]. A clear DNA–protamine structure has yet to be described.

#### 4.5. Histone variants

Even though spermatogenesis is characterized by the exchange of histones for protamines, histones still play a critical role in the development and maintenance of spermatozoa. Indeed, mature sperm chromatin have been shown to retain some level of each of the four core histones—H2A, H2B, H3 and H4 [66]. Variants of these core histones have been identified in mature spermatozoa and have subsequently been named H2A1L, H2A2L and H2B1L [61]. It is possible that H2A1L, H2A2L and H2B1L take part in reprogramming pericentric heterochromatic regions during spermatogenesis [61]. A male gonad-specific histone H2B, named TH2B, has been found in a sub-population of mature human sperm [75]. Data suggests that TH2B may supply a platform for the changing chromosome architecture associated with the addition of new histone variants [61]. Moreover, H1T, a testes-specific linker histone variant, is known to be exchanged for somatic H1 linker histones during spermatogenesis [76]. It is possible that H1T is in turn replaced by a different linker histone, H1LS1, which may aid in chromatin remodeling in condensing spermatids [65,76]. Finally, the variant H1T2 is expected to play a significant role in post-meiotic chromatin remodeling [77,78], although this has not yet been confirmed. Despite their low frequency in comparison to nucleoprotamines, nucleohistones are still important players in male gametogenesis. The genes/loci important for epigenetic modifications are listed in Table 1.

### 5. Aberrant epigenetic regulation, male infertility and embryonic development

It is crucial that proper regulation of epigenetic processes is maintained throughout spermatogenesis to not only ensure proper sperm function, but also proper embryonic development. It has been found that the sperm epigenetic environment plays a role in establishing epigenetic marks in the embryo [79], thus aberrant epigenetic regulation in spermatogenesis has a profound effect on both male fertility and embryonic development.

#### 5.1. DNA methylation

Improper DNA methylation of various genes has been implicated in abnormal semen parameters, as well as several instances of male factor infertility. This aberrant methylation can occur globally or be limited to one specific locus. A study by

**Table 1** List of the genes/proteins important for epigenetic modifications.

<table>
<thead>
<tr>
<th>Genes/proteins</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR</td>
<td>Maintains the pool of methyl donors</td>
<td>[7]</td>
</tr>
<tr>
<td>DNMT1, DNMT3A, DNMT3B</td>
<td>DNA methylation</td>
<td>[24-26]</td>
</tr>
<tr>
<td>DNMT3L</td>
<td>Required for DNMT3A2 activity</td>
<td>[29]</td>
</tr>
<tr>
<td>SWI/SNF, ISWI</td>
<td>Chromatin remodeling</td>
<td>[22,23]</td>
</tr>
<tr>
<td>Jhd1n2a</td>
<td>Chromatin remodeling</td>
<td>[34]</td>
</tr>
<tr>
<td>Swi90H</td>
<td>Histone methylation</td>
<td>[31,32]</td>
</tr>
<tr>
<td>G9a</td>
<td>Histone methylation</td>
<td>[33]</td>
</tr>
<tr>
<td>LSD1-domain proteins, JmJC-domain proteins</td>
<td>Histone demethylation</td>
<td>[34]</td>
</tr>
<tr>
<td>HATs</td>
<td>Histone acetylation</td>
<td>[35]</td>
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<td>MYST</td>
<td>Histone acetylation</td>
<td>[36]</td>
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<tr>
<td>HDAC5</td>
<td>Histone deacetylation</td>
<td>[35]</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Histone deacetylation</td>
<td>[37]</td>
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<tr>
<td>MUTp</td>
<td>Histone phosphorylation</td>
<td>[38]</td>
</tr>
<tr>
<td>NIK-1</td>
<td>Histone phosphorylation</td>
<td>[39]</td>
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<tr>
<td>MSI1, MSI2</td>
<td>Histone phosphorylation</td>
<td>[40]</td>
</tr>
<tr>
<td>PKA</td>
<td>Histone phosphorylation</td>
<td>[41]</td>
</tr>
<tr>
<td>HHR8</td>
<td>Histone ubiquitilation</td>
<td>[16]</td>
</tr>
<tr>
<td>E1 SUMO-activating enzyme 1, E1 SUMO-activating</td>
<td>Histone sumoylation</td>
<td>[18]</td>
</tr>
<tr>
<td>enzyme 2, UBC9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTCF</td>
<td>Interacts with differential</td>
<td>[54,55]</td>
</tr>
<tr>
<td></td>
<td>methylated regions</td>
<td></td>
</tr>
<tr>
<td>BORIS</td>
<td>Interacts with demethylases</td>
<td>[54,55]</td>
</tr>
</tbody>
</table>
Houshdaran et al. demonstrated that poor sperm concentration, motility and morphology were associated with broad DNA hypermethylation across a number of loci [80]. Four of these sequences — PAX8, NTF3, SPN and HRAS — were single copy sequences unique to non-imprinted genes [80]. Moreover, the repetitive element Satellite 2 was also found to be hypermethylated [80]. The authors proposed that hypermethylation of these loci results from the improper erasure of already established methylation marks rather than aberrant de novo methylation following epigenetic reprogramming [80]. The data from this study suggest that methylation defects present outside of implanted loci may be a key factor in some cases of infertility. Recent research has identified a critical role for the JHMD2A (Jumonji C-terminal containing histone demethylase 2A) histone demethylase in male infertility, obesity [81] and spermatogenesis [34]. Studies using knock-out mice models identified a critical role for JHMD2A in the regulation and expression of protease 1 and transition nuclear protein 1, both of which are critical for DNA condensation during chromosomal packaging in sperm [34]. The lack of proper DNA packaging in sperm has been associated with infertility in mice [82,58]. It is possible that aberrations of a number of other epigenetic proteins regulating the activities of the proteins involved in DNA compaction could cause infertility.

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate pathway that catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate [83]. The enzyme maintains bioavailability of methionine so it can be converted to S-adenosylmethionine, a methyl donor for a variety of substrates including DNA [83]. To better characterize the role of MTHFR in spermatogenesis and male fertility, Khazamipour et al. compared the methylation status of the MTHFR gene promoter in the testes of men with non-obstructive azoospermia to men with obstructive azoospermia without defects in spermatogenesis. It was found that 53% of men with non-obstructive azoospermia had hypermethylation of the MTHFR promoter, whereas none of the men with obstructive azoospermia exhibited hypermethylation of this region [84]. These statistically significant data indicate that MTHFR hypermethylation is a specific epigenetic aberration and may strongly contribute to certain cases of male infertility. Interestingly, a study by Kelly et al. demonstrated that administration of betaine during pregnancy, nursing and post-weighing can indeed improve testicular histology and fertility [85]. Wu et al., in a very recent study concluded that hypermethylation of MTHFR gene promoter in sperm was associated with idiopathic male infertility [86]. The authors demonstrated that the number of patients with hypermethylation was three times to that of control individuals [86].

5.2. Genomic imprinting

Appropriate establishment of genomic imprints is critical to the maintenance of fertility. Indeed, both paternal and maternal imprinting defects have been identified in several groups of men experiencing male factor infertility. Substantial work has been directed towards confirming the presence of these imprinting abnormalities. A study by Houshdaran et al. demonstrated that poor semen parameters were linked with increased DNA methylation at several differentially methylated loci, more than likely due to a defect in methylation erasure during epigenetic reprogramming [80]. These loci included PLG1, a maternally imprinted gene, DIRAS3 and MEST [80]. An additional study by Kobayashi et al. examined seven imprinted genes, including the paternally imprinted Gtl2 and H19 loci, in 97 infertile men. They found that 14.4% of the patients studied exhibited abnormal paternal imprinting and 20.6% exhibited abnormal maternal imprinting [87]. Donors with normal sperm count and motility were methylated at the H19 locus, whereas one patient with moderate oligospermia and three patients with severe oligospermia showed no methylation of the locus [87]. Similarly, healthy donors displayed methylation at the Gtl2 locus while two patients with moderate oligospermia and four with severe oligospermia displayed no methylation [87]. Further sequencing of both loci concurrently showed that one patient with severe oligospermia exhibited a defect in both the H19 and Gtl2 loci [87]. Moreover, five of the ten patients with severe oligospermia and three of the eight patients with moderate oligospermia displayed aberrant methylation of maternally imprinted loci and differentially methylated regions [87].

Poplinski et al. compared the methylation status of the H19/IGF2 imprinting control region 1 (ICR1) and MEST loci in 33 healthy donors and 148 men with idiopathic infertility. Normozoospermic men displayed high methylation at the H19/IGF2 ICR1 locus and low methylation at the MEST locus, whereas low methylation of the H19/IGF2 ICR1 locus and high methylation of the MEST locus was found to be associated with low sperm concentration [88]. The H19/IGF2 ICR1 locus of men with idiopathic infertility was 89.6% methylated with less than 40 million sperm in comparison to 95.9% methylated in normal donors [88]. As methylation of this locus increased, sperm count increased linearly [88]. Moreover, decreased methylation of H19/IGF2 ICR1 locus directly correlated with decreased sperm motility [88]. Data from two different studies by Marques et al. [89,90] also indicated that some oligozoospermic patients and secretory azoospermic patients with hypospermatogenesis exhibit loss of methylation at H19 [89,90]. Similarly, in 2010 Boissonnas et al. found that many patients with teratozoospermia and oligoasthenoteratozoospermia exhibited hypomethylation at variable CpG islands at the H19 locus [91]. Hypermethylation at MEST was more strongly linked with poor sperm quality than hypomethylation at H19/IGF2 ICR1. This hypermethylation of MEST was observed in samples from infertile men with less than 40% sperm motility and less than 5% normal sperm morphology [88]. Men with idiopathic infertility exhibited MEST methylation of 9.6% in comparison to 4.3% in controls [88]. Furthermore, increased methylation of MEST linearly correlated with decreased sperm motility [88]. These data are supported by the findings of a study by Marques et al. [92].

5.3. Nuclear protein transitioning

The exchange of protamines for histones is a crucial step in the process of spermatogenesis, causing the DNA to be tightly wrapped for efficient transmission of nuclear material to the oocyte upon fertilization. It is known that the Prm1 to Prm2 ratio (P1/P2 ratio) is strictly maintained and regulated. Indeed, deviation from the standard ratio of 0.8–1.2 has been shown to lead to infertility [93]. A change in either direction of this ratio adversely affects semen quality and DNA integrity [93]. Patients with abnormally depressed or elevated P1/P2 ratios are characterized by poor sperm concentration, motility and morphology as well as decreased fertilization capabilities [93]. Studies suggest that the most common cause of infertility by aberrant protamine exchange is an increase in the P1/P2 ratio caused by a decrease in Prm2 levels, although improper regulation of Prm1 levels has also been implicated in some cases of male infertility [93]. Furthermore, a study by de Yebra et al. showed that men with a higher P1/P2 ratio were also more likely to have lower total protamine levels and higher intermediate protein levels [94]. Moreover, this same study additionally demonstrated that some infertile men completely lack Prm2 in their sperm nuclei [94]. There have been no cases reported of fertile men with severely altered P1/P2 ratios; indeed, it appears to be a characteristic limited exclusively to certain infertile males [95]. Interestingly, a link between abnormal protamine incorpo-
ration and aberrant genomic imprinting has recently been discovered. Hammond et al. found that infertile males with abnormal protamines exhibited statistically significant hypermethylation at the imprinted loci KCNQ1, LIT1 and SNRPN [96]. Moreover, this study also demonstrated that these patients showed hypomethylation of the H19 locus [96].

As discussed previously, hyperacetylation of histone H4 is required in the transition from histones to protamines. This step decreases the affinity of the interaction between the sperm histones and DNA to allow the exchange for transition proteins to occur. A study by Sonnack et al. showed that men exhibiting qualitative and/or quantitative infertility have significantly decreased levels of histone H4 acetylation associated with impaired spermatogenesis [63]. In the seminiferous tubules of men with round spermatid maturation arrest, only approximately 60% of spermatids were immunopositive for this hyperacetylation and many were multinucleated [63]. Moreover, infertile men with qualitatively normal spermatogenesis exhibited approximately 90% immunopositive spermatids and infertile men with qualitatively abnormal spermatogenesis exhibited approximately 75% immunopositive spermatids [63]. This contrasts significantly with the almost all hyperacetylated round spermatids in fertile men [63]. Interestingly, spermatocytes in the seminiferous tubules of men with round spermatid maturation arrest exhibited an additional signal, indicating that early hyperacetylation of histone H4 may lead to premature nuclear protein transitioning and subsequent infertility [63].

5.4. Chromosome structure

The global structure of chromatin is known to affect gene expression by modulating which regions are available to be accessed by transcription factors and other transcriptional proteins and which are not. This feature of epigenetic regulation becomes particularly important when considering normally expressed genes that are crucial for proper spermatogenesis and subsequent oocyte fertilization. It has now been established that an increase in total heterochromatic variants is strongly linked to some cases of male factor infertility [97]. Indeed, it has been demonstrated that there is an increase in the frequency of chromosomal variants, from 32.55% to 58.68%, in infertile men compared to controls [97]. The large polymorphic variation 9hq, located in centromeric heterochromatin on chromosome 9, was shown to increase in frequency from 4.25% in controls to 14.69% in men with severe infertility. It is thought that this rise in heterochromatic regions, not only on chromosome 9 but amongst many chromosomes, may down-regulate normally active genes [97]. Indeed, polymorphic variations on the Y chromosome have been implicated in male infertility based on this reasoning [97]. All the major genes/loci known to be epigenetically different in some infertile individuals are listed in Table 2.

6. Environmental factors and epigenetics

Epigenetic regulation is known to change based on environmental stimuli. As illustrated throughout this review, epigenetic markers are very tightly monitored throughout spermatogenesis and disruption of proper epigenetic regulation can lead to sub- and infertility. Indeed, certain environmental factors have been found to cause aberrant epigenetic regulation that adversely affects semen parameters. A study by Tunc and Tremellen demonstrated that the production of seminal reactive oxygen species is positively correlated with DNA fragmentation and negatively correlated with DNA methylation [98]. Moreover, a negative correlation was seen between DNA methylation and DNA fragmentation [98]. Infertile men were found to have greater DNA fragmentation and higher reactive oxygen species levels than fertile men [98]. These results indicate that DNA damage caused by oxidative stress may facilitate aberrant global DNA methylation. Interestingly, subjects treated with antioxidants for three months saw a decrease in DNA damage and reactive oxygen species levels, as well as an increase in proper global DNA hypomethylation [98]. Another study by Oakes et al. showed that 5-aza-2′-deoxycytidine, an anticancer agent, causes a decrease in global DNA methylation which leads to altered sperm morphology, decreased sperm motility, decreased fertilization capacity and decreased embryo survival [99]. 5-Aza-2′-deoxycytidine prevents de novo DNA methylation in male germ cells, thus affecting genes that normally become methylated during spermatogenesis [99].

Endocrine disruptors, such as methoxychlor and vinclozolin, have been implicated by several studies in the disruption of spermatogenesis and development of male infertility. Moreover, the deleterious effects of these agents have also been observed in males of subsequent generations. Both of these results, however, remain controversial. A study by Anway et al. [2] suggested that transient maternal exposure to methoxychlor [an estrogenic pesticide] and vinclozolin [an anti-androgenic fungicide] during pregnancy led to decreased sperm count and viability, increased spermatogenic cell apoptosis, abnormal seminiferous tubule morphology and complete infertility in male offspring. The authors treated the animal models with methoxychlor and vinclozolin which are estrogenic and anti-androgenic endocrine disruptors. The gestating mothers were exposed to these compounds during the stage of epigenetic reprogramming, and the next progeny was further bred to get F1, F2, F3 and F4 generations. Analysis of the cellular apoptosis demonstrated a greater than two fold increases in spermatogenic cell apoptosis for the F1–F4 generations.

By conducting further experiments the authors proved that the transmission of the epigenetic changes was taking place through male germ cell line. The authors explained the very high number of animals (>90%) with spermatogenic defects on the basis of epigenetic changes. The authors also indicate that these effects were conserved in some members of the F1 through F4 generations [2]. Furthermore, Stouder and Paolini-Giacobino demonstrated

<table>
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<th>Genes/proteins</th>
<th>Aberration and male infertility</th>
<th>Reference</th>
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<tr>
<td>MTHFR</td>
<td>DNA hypermethylation results in poor semen quality and infertility</td>
<td>[83,84]</td>
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<tr>
<td>PAX9, NTF3, SFN, HRAS</td>
<td>DNA hypermethylation associates with poor sperm concentration, motility and morphology</td>
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<tr>
<td>JM20A</td>
<td>Knockout results in loose packaging of DNA and may cause infertility</td>
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<tr>
<td>IGFL, H19</td>
<td>Low methylation associates with low sperm concentration</td>
<td>[88]</td>
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<td>RASGRF1</td>
<td>Hypermethylation at the imprinted locus associates with poor semen parameters</td>
<td>[53]</td>
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<td>GTL2</td>
<td>Hypermethylation at the imprinted locus associates with poor semen parameters</td>
<td>[87]</td>
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<tr>
<td>PLAG1, D1RAS3, MEST</td>
<td>Hypermethylation at the imprinted loci associates with poor semen parameters</td>
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<tr>
<td>KCNQ1, LIT1, SNRPN</td>
<td>Hypermethylation at the imprinted loci associates with poor semen parameters</td>
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that exposure to vinclozolin decreased the percentage of CpG methylation in the paternally imprinted genes H19 and Git2, as well as increased the percentage of CpG methylation in the maternally imprinted genes Peg1, Sertpn and Peg3 [100]. This phenotype was passed on to subsequent generations; however, the authors did observe a gradual, near-complete recovery of normal phenotype by the F3 generation [100]. Unlike these two studies, Inawaka et al. determined that exposure to vinclozolin and other anti-androgenic compounds does not affect spermatogenesis, DNA methylation or fertility of F1 males [101]. A consensus on these conflicting data has yet to be reached at this time.

7. Epigenetics in assisted reproductive technologies

As described previously, the DNA of spermatozoa is differentially methylated at several maternal and paternal imprinting regions, as well as exhibits unique global methylation patterns. Reprogramming of the epigenome and imprinted loci during gametogenesis and peri-implantation stages is very crucial for maintaining proper pattern of inheritance, particularly at imprinted loci [102]. There is a concern that assisted reproductive technologies such as intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI) may increase the incidence of imprinting disorders and adversely affect embryonic development by using immature spermatozoa that may not have proper imprints or global methylation established. Deregulation of imprinted regions has been associated with the onset of Angelman syndrome in the cases undergoing intracytoplasmic sperm injection [103,104]. Deregulation of Igf2 imprinted loci has been previously associated with malformed offspring in mice, characterized by retardation [105] and Beckwith-Wiedemann syndrome [106]. However, long term studies on imprinting disorders in assisted reproductive techniques have denied an association between the two [107,108]. The evidence describing ART procedures as increasing the frequency of imprinting disorders such as Prader-Willi Syndrome, Beckwith-Wiedemann Syndrome and Angelman Syndrome has remained contradictory. Doornbos et al., Manning et al. and others contend that use of ART does not increase the risk of imprinting disorders [109,110], while Manipalvira et al., Shiota et al. and others conclude that it indeed does [111,112].

Large longitudinal studies must be conducted to examine this connection more thoroughly. Evidence has been provided, however, that ROSI is linked to abnormal zygotic epigenetic regulation. Indeed, a study by Kishigami et al. demonstrated that injection with round spermatids versus mature spermatozoan results in distinguishable methylation patterns of the paternal zygotic genome [79]. Data from this study show that zygotic genomes derived from round spermatids are remethylated after initial demethylation before completion of the first mitosis [79]. This inability to prevent global DNA remethylation does indeed lead to abnormal genome-wide DNA methylation in the paternal zygotic genome [79]. Moreover, unlike spermatozoa, round spermatids exhibit H3K9 trimethylation, which is preserved through the first mitosis of the zygote [79]. The authors believe these differences in epigenetic patterns may account for the lower success rates of ROSI [79].

8. Conclusion and future directions

The epigenetic processes of DNA methylation, post-translational histone modifications, and chromatin remodeling are conserved across cell types and serve an important role in modulating gene expression. Hypermethylation of DNA is associated with gene silencing, and hypomethylation is associated with gene expression. Covalent histone modifications act to inhibit and/or enhance gene expression, and chromatin remodeling exposes or hides regions of DNA for transcription. It is known that DNA methylation patterns are highly specific in both the testes and spermatozoa, and they change throughout the course of spermatogenesis. Processes such as genomic imprinting and epigenetic reprogramming occur in the genesis of both male and female germ cells, whereas nuclear protein transitioning and incorporation of histone variants are unique to male gamete development. These specialized modifications serve to maximize efficiency of sperm DNA delivery to the oocyte, as well as provide important information to the zygote to ensure proper gene expression and growth. Aberrant modulation of these regulatory processes can have a profound, deleterious impact on semen parameters, fertility, and embryonic development. We have compiled a list of the genes/loci that are extremely important for epigenetic modifications and are good candidates for screening in infertile individuals (Table 1). Oxidative stress and chemotherapies are known to adversely affect fertility, whereas the effect of endocrine disruptors has yet to be conclusively defined. Similarly, the implications, if any, of assisted-reproductive technologies on proper transfer of epigenetic information are still relatively unknown. Epigenetics has proven to be a useful tool in addressing questions about idiopathic male infertility previously unanswerable by traditional genetics. Indeed, epigenetic regulation plays a crucial role in proper spermatogenesis and maintenance of fertility. In order to gain a greater understanding of the complexity of male fertility and infertility, further exploration into the mechanisms of these processes must be pursued.

Genetic changes are known to contribute to a number of disorders to a little or large extent; however, similar knowledge about the contribution of epigenetics is being unraveled. The understanding and investigation of the role of epigenetics has rather just begun, and we must uncover the role of epigenetic modifications in more disorders than we know today. This could explain the etiology of the disorders in several cases that could otherwise not be explained by genetic changes. Some of the already known epigenetic aberrations related to male infertility are listed in Table 2. The understanding of epigenetics has an additional advantage in that epigenetic changes can be modified/reversed with the help of certain drugs. Therefore, epigenetics-related disorders could be cured if we completely understand the process of epigenetic modification. On the other hand, it is not possible to treat genetic changes, and diagnoses of genetic changes, thus, do not help cure the disease. It is evident that understanding of epigenetic processes and their association with infertility would help us not only understand etiological factors but also treat male infertility. Exploration of both genetic and epigenetic changes could go hand in hand, and this could help us explain the interaction between the two factors, if any.

Conflict of interest statement

All the authors declare to have no actual or potential conflict of interest.

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