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Epigenetics in Sperm, Epigenetic Diagnostics, and Transgenerational Inheritance

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INTRODUCTION

Sperm are highly specialized cells transmitting the paternal genome to the oocyte and thus, to the next generation. For many years, it was believed that sperm was only contributing its DNA and nothing else. A growing body of evidence now supports the importance and relevance of the sperm epigenome for male fertility and offspring health. Sperm DNA methylation, sperm histones alterations, and non-coding RNA are all involved and can contribute to observable epigenetic alterations inherited by subsequent generations (1–4). Moreover, sperm has been shown to be particularly sensitive to different environmental exposures which can subsequently affect embryonic development and offspring (4). Several studies have observed that such environmental insults cause changes in the sperm epigenome which can be passed to subsequent generations transgenerationally (4–11). Recent data suggest that the different sperm epigenetic mutations (DNA methylation, ncRNA, histone modifications, and retention) offer predictive diagnostic tools in the assessment of male infertility and potential impacts on subsequent generations' disease etiology (8, 12–18). This chapter will focus on the different epigenetic factors, epigenetic diagnostics, concept of transgenerational inheritance, and the potential use of epigenetics in the context of male infertility.

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EPIGENETIC MODIFICATIONS

Individuals typically respond to their environment through changes in gene expression and these changes are generally mediated by epigenetic processes. Additionally, the mechanisms which generate individual cell types allow one cell type to differentiate and develop into another cell type are primarily mediated by epigenetic mechanisms. Epigenetics is defined as the "molecular factors/processes around the DNA that regulate genome activity independent of DNA sequence, and that are mitotically stable" (19). Several distinct epigenetic factors or processes act to control genome activity in a cell and regulate gene expression. These include DNA methylation, histone modification/retention, chromatin remodeling, non-coding RNAs (ncRNAs), and RNA methylation (Figure 7.1).

DNA Methylation

DNA methylation was the first epimutation to be characterized. DNA methylation involves a small (methyl) chemical group that is enzymatically attached to DNA through DNA methyltransferase (DNMT), primarily at the cytosine base when adjacent to guanine in most species (20, 21) and the product is 5-methylcytosine (5mC) (Figure 7.1). In mammals, the addition of a methyl group to a CpG site results in the alteration (often inhibition) of a transcription factor binding to DNA, while the removal of a methyl group to a CpG site facilitates the recruiting of proteins involved in gene expression (22). Other chemical modifications of cytosine bases in DNA have since been described, but are infrequent and function not elucidated. The ten-eleven translocation (TET) family of enzymes can successively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), which is the precursor to DNA methylation erasure (23). In broad terms, the presence of 5mC often represses DNA transcription, while 5hmC is permissive to transcription (24, 25). The functions of the other epigenetic modifications to cytosine are under investigation. N(6)-methyladenine is an epigenetic modification to the adenine base of DNA that was once thought to only be present in prokaryotic organisms, but has now been described in mammalian embryonic stem cells associated with RNA methylation (26).

Histone Modification and Retention

The histone proteins that DNA is wrapped around create the nucleosome and can be chemically modified to alter gene expression (Figure 7.1). Histone proteins are often subject to post-translational modifications

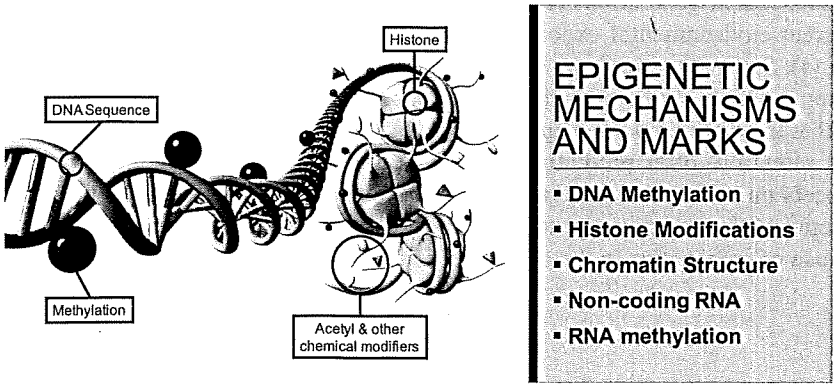

FIGURE 7.1 Epigenetic mechanisms and marks. Modified from (98).

which form a complex molecular mechanism that subsequently results in regulation of gene expression and downstream biological functions (27). Numerous different histone post-translational modifications interact and generate combinatorial patterns to influence gene expression. Among the known histone modifications are lysine acetylation, lysine and arginine methylation, arginine citrullination, lysine ubiquitination, lysine sumoylation, ADP-ribosylation, proline isomerization, and serine/threonine/tyrosine phosphorylation (28).

In many species, including humans, the sperm histones are removed from DNA following meiosis during spermiogenesis. The DNA is condensed with protamines resulting in highly compact nucleoprotamine complexes (29). In human sperm, roughly 5%–15% of the histones are retained in nucleosomes while the remainder is condensed in nucleoprotamine complexes (30, 31). Environmental exposures transgenerationally can modify the histone retention sites in sperm and dramatically increase the number of retention sites (32). The differential distribution of genes in protamine-associated versus histone-associated regions in the sperm yields crucial epigenetic information which is delivered to the oocyte and zygote (33, 34).

Chromatin Structure

The coiling, looping, and general structure of DNA, termed chromatin structure, is also an epigenetic factor (35) (Figure 7.1). The three-dimensional structure of DNA can make certain regions of the genome accessible to transcriptional machinery, or bring enhancer regions near gene promoters to affect gene expression. Many of the major events in the developmental progression of an organism, notably gametogenesis and fertilization, involve dramatic remodeling events of the germ cell chromatin (36). Chromatin modifications serve as crucial epigenetic modifications in development and reproduction. The condensation of DNA with protamines and removal of histones transcriptionally silences the sperm to allow effective delivery of the sperm DNA to the oocyte.

Non-Coding RNA (ncRNA)

Non-coding RNA molecules can act as epigenetic factors (37) (Figure 7.1). These are small RNA molecules that do not code for a protein, but rather function as RNA to regulate gene expression. The non-coding RNA molecules that act as epigenetic factors have secondary structure to facilitate DNA and protein interactions, but are not DNA sequence-dependent, so the majority do not depend on having a nucleotide sequence that is complimentary to a specific DNA or RNA region in order to function. Long non-coding RNAs (lncRNAs) (38) and small non-coding RNAs (sncRNAs) are the two major types. The sncRNA have many sub-families such as transfer RNA-derived small RNAs (tsRNAs) (39), which are examples of ncRNA classes that are present in sperm and can act as epigenetic factors that affect subsequent generations (39, 40).

RNA Methylation

Chemical modifications of RNAs can have dynamic regulatory roles similar to the epigenetic modifications of DNA and histone proteins (41) (Figure 7.1). The most prevalent known mammalian RNA modification is N6-methyladenosine (m6A), a reversible methylation of the messenger RNA (mRNA) (42). The methylation of RNA alters the structure of the RNA to change function and protein or DNA association. Methylation of numerous RNA species results in a diversity of functions on RNA including biophysical, biochemical, and metabolic stabilization of RNA and further crucial functional processes (43).

EPIGENETIC ALTERATIONS (EPIMUTATIONS) AND MALE INFERTILITY

Male infertility has increased at an alarming rate over the past 40 years and this crisis demands new approaches for prevention, diagnosis, and treatment (44). It is estimated as many as 1 in 20 men currently face reduced fertility (45). A myriad of environmental conditions ranging from increased exposure to toxicants to increased rates of obesity may explain this increase in male infertility (46). The molecular mechanisms driving this increase in male infertility likely include epigenetic alterations (47).

DNA Methylation

Idiopathic male infertility is associated with a reduction in the quality or quantity of sperm. An association between disrupted DNA methylation and abnormal human sperm was first described by Navarro-Costa (48). This group proposed a correlation between male gametogenic defects and incorrect epigenetics alterations (epimutations) in male germline genes. DNA isolated from poor quality sperm collected from an infertility clinic exhibits abnormal methylation at numerous sequences, implicating improper programming of the male germline (49). Poplinski (50) compared the DNA methylation of sperm among men exhibiting idiopathic infertility against that of control men. This research group found aberrant methylation patterns among the men with idiopathic infertility and suggest that these aberrant patterns may be passed onto children conceived through assistive-reproductive technologies (ART). Differential DNA methylation regions were found to be associated with male patients who are responsive to follicle-stimulating hormone (FSH) use, a therapeutic treatment for idiopathic infertility (51). Changes in the methylation of histones in sperm are suggested to have cumulative detrimental effects on fertility among men (52). Recent efforts have developed diagnostic tools that will identify aberrant DNA methylation and will provide a diagnostic tool in a clinical setting (18, 51).

Histone Modification and Retention

When the histones are replaced by protamines during spermatogenesis a strict ratio of protamine-1 (P1) to protamine-2 (P2) is regularly maintained. This ratio is strictly regulated and highly conserved among mammalian species (53). Aoki (54) found an association with a reduction in the P1/P2 ratio among a population of infertile men, indicating abnormal concentrations of either type of protamine are strongly associated with male infertility. In normal spermatogenesis, not all histones are replaced with protamines. There is a programmed pattern to which histones are retained in normal spermatogenesis. A population of infertile men exhibited random or abnormal histone retention as well as elevated levels of histone retention among their sperm (52, 55).

Acetylation of histones is a normal part of spermatogenesis, when histones are exchanged with protamines. Among infertile men, impaired spermatogenesis is associated with a decrease of histone acetylation in spermatids resulting in incorrect histone-to-protamine exchange (56). Aberrant histone acetylation of the promoters of developmentally important genes was found among subfertile men and suggested to adversely affect the transfer of epigenetic information to the oocyte (57).

Chromatin Modification

The structure of chromatin is known to affect gene expression and can be crucial for proper spermatogenesis. An increase in heterochromatic variations is strongly associated with male infertility (58). Any

perturbation to the normal chromatin remodeling involved in precursor sperm cells has negative consequences on spermatogenesis and eventually on male fertility (59).

Non-Coding RNA (ncRNA)

Sperm RNAs represent a portion of epigenetic information that is delivered to the oocyte from the paternal germ cells. Sperm RNA elements (SRE) from males presenting idiopathic infertility and found around 30% presented an incomplete set of the required SREs (60, 61). Short non-coding RNAs play an essential role in spermatogenesis and have recently been implicated in male fertility disorders (reviewed in [62]). Long non-coding RNAs (lncRNAs) are shown to play a role in sperm motility and potentially fertility (63). Zhang (64) identified lncRNAs which are crucial for human spermatogenesis and sperm function and the resulting causes of male infertility.

RNA Methylation

Human sperm fertility has been associated with altered levels of N6-methyladenine (m6A) in human sperm (65). These authors suggest that increased levels of m6A may be a risk factor for decreased sperm motility. A depletion of spermatagonial stem cells (SSC) is also associated with a reduction of m6A resulting in abnormal spermatogenesis (66).

EPIGENETIC TRANSGENERATIONAL INHERITANCE

The environment has been shown to be one of the most critical factors to impact the biology of an organism. An exposure to one or multiple environmental factors (e.g., nutrition, toxicants, stress) can trigger changes in the transcriptome and impact the development of pathologies or phenotypic variation. As described above, epigenetic factors are the molecular mechanisms an organism uses to respond to an environmental change with modifications in gene expression. Most environmental factors and toxicants do not possess the capacity to alter DNA sequence or promote genetic mutations (67). However, the environment is able to dramatically influence epigenetic processes which then affect gene expression and development. In human and animal models, several studies have demonstrated that exposure to certain environmental toxicants at a specific window of development, especially when the epigenome is reprogramming, can affect the mechanisms involved in the establishment of the sperm epigenome. Since the sperm epigenome has been shown to be crucial for the fertility of the individuals, any variations could be related to male infertility (68). Some epimutations have also been shown to be transmitted via the sperm to the offspring and subsequent generations which has been defined as the concept of epigenetic transgenerational inheritance (4, 6, 69). Therefore, epigenetics provides a molecular mechanism for the environment to directly alter the biology of an organism (70). The presence of an altered epigenetic factor at a specific chromosomal location in response to an environmental factor is called an "epimutation" (71).

These environmentally induced epigenetic changes can be passed to subsequent generations and can result in changes in gene expression and phenotype in the offspring, even when there is no continued direct environmental exposure. This transgenerational inheritance is defined as: "Germline (sperm or egg) transmission of epigenetic information between generations in the absence of any continued direct exposures or genetic manipulations" (71). In 2005, Anway et al. studied the toxicant actions of the agricultural fungicide vinclozolin and showed its epigenetic transgenerational inheritance phenomenon (Figure 7.2) (6). F0 gestating female rats were exposed to an environmental toxicant, vinclozolin, during the fetal sex determination windows. The direct effects on the offspring from the F1 generation

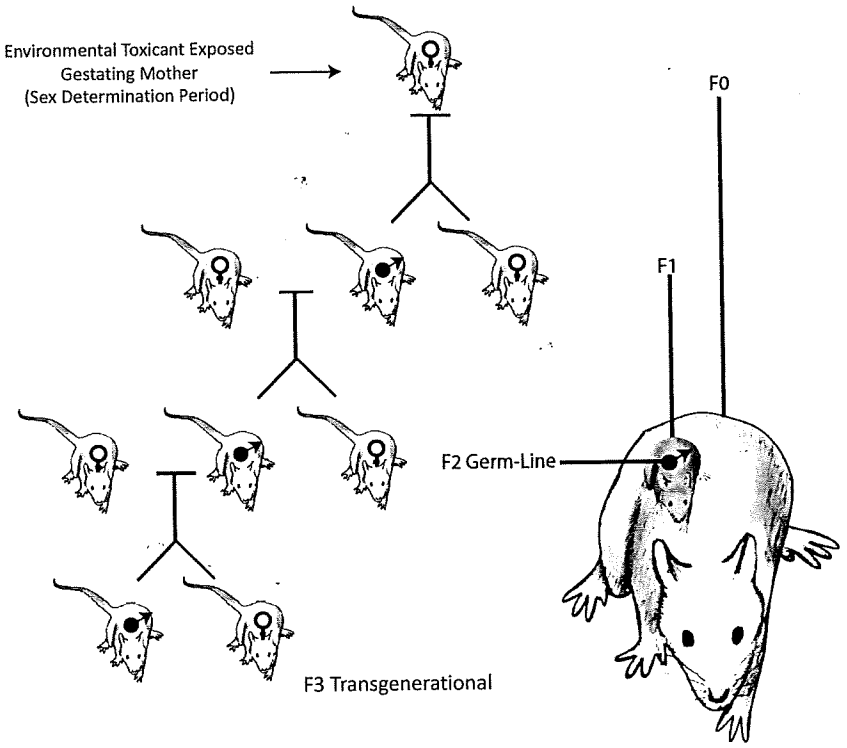

FIGURE 7.2 Epigenetic transgenerational inheritance. Summary of environmentally induced epigenetic reprogramming of primordial germ cells in the fetus that leads to the germline transmission of epimutations, resulting in all somatic cells having altered gene expression. This can result in changes in phenotype or increased disease susceptibility in the F1, F2, or transgenerational F3 generation. The F0, F1, and F2 generations have direct exposure so are multigenerational, while the F3 generation has no direct exposure so is transgenerational. Modified from (4).

were identified where a testis abnormality was observed. In the F2 generation grand offspring, the males demonstrated the same testis defect as their parents. When the F2 animals were bred to the transgenerational F3 generation, the testis disease appeared in over 90% of the male progeny (6). The abnormality incidence did not decrease through the generations but stayed high, suggesting a non-Mendelian phenomenon not relying on classic genetic processes. When the F3 generation vinclozolin males were outcrossed to wildtype females, the transgenerational phenotype was maintained at the same frequency. When the F3 generation vinclozolin females were outcrossed to wildtype males, the phenotype was lost (6). The authors concluded that the transgenerational phenomenon was transmitted through the male germline (sperm). This phenomenon has been shown to occur in females with different toxicants, such that the transgenerational phenotype was transmitted in a parent of origin allelic manner, similar to imprinted genes (72) (Figure 7.3).

It is critical to distinguish direct environmental exposure versus germline-mediated transgenerational events to understand the transgenerational inheritance phenomenon. When an F0 generation gestating female is exposed, the F1 generation fetus and its germ cells that will provide the F2 generation, are also directly exposed. Therefore, the effects observed in the F0, F1, and F2 generations are due to direct exposure toxicity as well as to environmentally induced epigenetic changes. The first generation displaying transgenerational epigenetic effects without any direct exposure toxicity is the F3 generation (Figure 7.2) (69). Because these transgenerational events require the sperm or the oocyte to be transmitted to the subsequent generations, they are mediated through the germ cells.

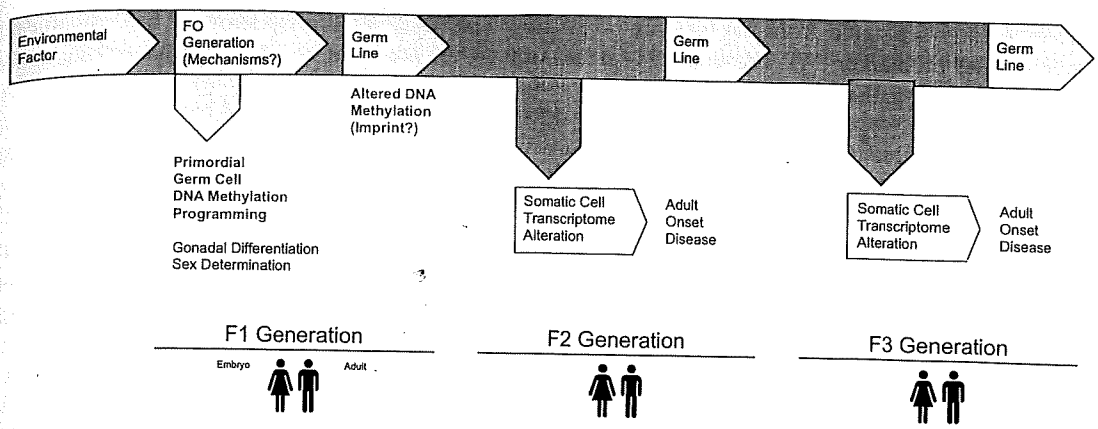

FIGURE 7.3 Role of germline in epigenetic transgenerational inheritance. Summary of environmentally induced epigenetic reprogramming of primordial germ cells that leads to the germline transmission of epimutations resulting in all somatic cells having an altered transcriptome that results in disease susceptibility. Modified from (4).

SPERM EPIGENETIC BIOMARKERS/ DIAGNOSTICS FOR PATHOLOGY

Sperm Epigenetic Diagnostics for Male Infertility

Molecular diagnostics are shifting the evaluation and treatment of human diseases. In reproductive health, male factors are involved in the couple's infertility in 50% of cases (73). The diagnosis to establish a male infertility is currently primarily through semen analysis, which evaluates sperm concentration, motility, and morphology using light microscopy (74). However, semen analysis is a poor predictor of a male fertility except in cases of oligospermia, azoospermia, or oligozoospermia (75, 76).

Several independent studies have shown that alterations in human sperm DNA methylation profiles were correlated with a decreased fecundity and an increased risk of abnormal embryo development (6, 77, 78). In 2015, Aston et al. showed that aberrant sperm DNA methylation patterns could be used to evaluate male fertility status and embryo quality (76). The Illumina Infinium array fertility test was developed as a way to assess male fertility potential and embryo development quality by measuring DNA methylation at a limited number of locations (CpG island sites) across the genome and comparing these methylation levels to those of the average fertile male (the average taken from 156 semen samples from men with normal semen parameters and a history of normal pregnancies). Abbesi et al. expanded this technique by identifying these aberrant DNA methylation patterns in sperm based on bisulfite sequencing combined with Illumina's Infinium technology for profiling human methylation levels which makes it suitable for clinical epigenetic work, as long as appropriate quality control procedures are established and followed (18). A recent genome-wide analysis for DNA methylation alterations in male infertility patients was found to generate a more robust and accurate diagnostic for male infertility (51). Future studies and diagnostics will need to use genome-wide analysis to develop the optimal diagnostics for disease.

A promising approach for the clinical therapy of male infertility is the use of endocrine therapeutics such as FSH, similar to what is currently used in the female (79). While this treatment is successful to stimulate oogenesis in the female, the response in stimulating spermatogenesis is much more variable within the infertile population (80). A recent study demonstrated a genome-wide analysis of DNA methylation identifying a male infertility signature of DMRs present in male infertility patients. Fertile patients

were efficiently separated from the infertile population with minimal overlap showing the potential of this molecular biomarker (51). These results further support the potential use of an epigenetic biomarker diagnostic tool for patients with male infertility.

Sperm Epigenetic Diagnostic for Transgenerational Disease

Various environmental exposures such as stress, chemical exposures, and nutrition have been demonstrated to promote the epigenetic transgenerational inheritance of adult onset disease in a wide variety of organisms from plants to humans (4). These epigenetic changes could be used as potential biomarkers of exposure and disease (81). Several environmental exposures and toxicants have been shown to promote the epigenetic transgenerational inheritance of disease (6–11). Recent studies have also shown that ancestral environmental exposures could promote alterations in differential DNA methylated regions (DMRs), differential retention sites (DHRs), and non-coding RNA (2, 3). The agricultural fungicide vinclozolin (82, 83), the pesticide DDT (dichloro-diphenyl-trichloroethane) (13, 84), the herbicide atrazine (8), and herbicide glyphosate (85) have all been shown to promote the epigenetic transgenerational inheritance of disease. Moreover, unique epigenetic signatures of differential DNA methylation were associated with the pathologies observed in the transgenerational F3 generation males and females (8, 13, 82).

Studies have further investigated the individual animals and the specific pathologies, such as testis disease, observed in the transgenerational F3 generation were associated with specific DMRs for each disease and environmental exposure. Disease specific DMRs were identified for a number of these transgenerational pathologies which demonstrates that the establishment of an epigenetic biomarker for a specific disease and a specific exposure is possible (8, 13–17, 85). Transgenerational testis disease biomarkers/diagnostics were identified with most of these studies. Observations suggest male infertility and testis disease include transgenerational components which potentially can be detected with epigenetic diagnostics.

Genome-wide association studies (GWAS) have found specific genetic mutations associated with these human pathologies; however, these genetic mutations typically appear in less than 1% of the diseased population. In contrast, in a rodent model, the epigenetic alterations seem to have a higher frequency and appear in most individuals with the disease (8, 13–17, 85). Future studies in humans are now required to translate these animal studies. These studies have shed some light on potential new approaches to diagnose and prevent pathologies or diseases that may be transmitted through the transgenerational epigenetic inheritance phenomenon (Figure 7.3). Epigenetic biomarkers have a high frequency of association with pathologies, and their incorporation into medical diagnostics will facilitate preventative medicine not only for infertility diagnostics but for a number of different diseases.

ASSISTIVE REPRODUCTIVE TECHNOLOGIES, INFERTILITY, AND EPIGENETICS

Decreasing male infertility has been observed for the past 60 years (86–90). Several parameters of the male reproductive system have been affected such as a decrease in sperm counts and quality (87), an increase in the incidence of testicular cancer (88), an increase in hypospadias, and cryptorchidism cases (86). Most recent studies have linked this decline with environmental exposures rather than genetic factors due to the rapid pace of the decrease of semen quality and the increase in occurrence of male infertility (87, 91, 92). Abnormal methylation patterns and aberrant protamine insertions have been shown to influence male fertility. CpG islands at gene promoter regions which are usually hypomethylated are especially susceptible to aberrant methylation for specific genes such as DAZL and MTHFR or imprinted loci. Various forms of infertility have been linked and sperm defects in men have been associated with epigenetic abnormalities in these regions (93). Abnormal DNA methylation is detrimental in imprinted genes as

they are known to be directly inherited from the parental germline and they are thought to be involved in part in the transgenerational effect phenomenon. Oligospermic patients (men with less than 10 million spermatozoa per 1 milliliter of semen) have been shown to contain a greater number of DNA methylation anomalies compared to men containing normal sperm counts (94). Interestingly, these imprinted loci are suspected to be inherited by the offspring during *in vitro* fertilization (IVF) treatments (95).

Epigenetic dysfunction can also occur during spermatogenesis and may be a contributing factor in male infertility. During the crucial step of histones replacement by protamines, many epigenetic regulators work together to facilitate the paternal genome reorganization and packaging through histone variation, specific histone modification, and their related chromatin remodelers. Any defects during this step are thought to be linked to male infertility (96). Besides, the differential DNA methylation patterns observed with male infertility are often accompanied by an altered protamine ratio and indicate the interaction between epigenetic phenotypes. Histone retention has been shown to occur sporadically throughout the genome, and loss of histone retention at imprinted loci is also associated with male infertility (52). The use of assisted reproductive technologies has increased in recent years. Understanding the epigenetic processes underlying male factor infertility will aid proper diagnosis, as well as help develop sperm selection techniques used for IVF (97).

SUMMARY

Our knowledge of epigenetics has considerably increased in the past two decades. Both DNA methylation and histone modifications are two well-studied epigenetic modifications in the germline. However, histone retention, ncRNA, and RNA methylation are also emerging as potentially important factors that could help understand the epigenetic mechanisms. The novel aspect brought by transgenerational inheritance involves a non-genetic form of inheritance, a non-genetic etiology of disease etiology, a molecular mechanism of how environmental factors (diet, stress, or chemical insults) can indirectly influence genome activity and disease, and the existence of the epigenetic transgenerational inheritance of disease and phenotypic variation. While many environmental conditions (such as toxicants or obesity) might explain the increase in male infertility, evidence suggests that the molecular mechanisms driving this increase involve epigenetic alterations. The constant advancement of molecular biology is anticipated to enhance the diagnosis and management of male infertility patients, as well as improve general therapeutic options and development. Many studies have shown that epigenetic diagnostics could be developed and applied to pathologies and disease. Expanded clinical trials are now needed to help validate and apply this novel technology to the management and treatment of male infertility.

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