REPRODUCTION

REVIEW

The sperm epigenome and potential implications for the developing embryo

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Abstract

Recent work in the field of male fertility has yielded significant increases in our understanding of the sperm epigenome and its potential role in embryonic development. These new findings have enabled a broad classification of a normal epigenetic state in the male gamete and have provided insight into the possible etiologies of some idiopathic male infertility cases. Histone retention and modification, protamine incorporation into the chromatin, DNA methylation, and spermatozoal RNA transcripts appear to play important roles in the epigenetic state of mature sperm. These epigenetic factors may reveal a historical record of spermatogenesis, portend future functions in embryogenesis, and help to elucidate mechanism of pluripotency. In contrast to the once held dogma regarding the importance of the paternal epigenome, the unique epigenetic landscape in sperm appears to serve more than the gamete itself and is likely influential in the developing embryo. In fact, growing evidence suggests that mature sperm provide appropriate epigenetic marks that drive specific genes toward activation and contribute to the pluripotent state of the embryonic cells. Although not definitive, the current literature provides evidence for the role of the sperm epigenome in the embryo. Future work must be focused on the characterization of epigenetic abnormalities commonly found in individuals with compromised fertility to further establish this role. Additionally, studies should target the effects of environment and aging on the sperm epigenetic program and subsequent fertility loss to determine the etiology of aberrant epigenetic profiles.

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Introduction

The sperm epigenetic program is unique and tailored to meet the needs of this highly specialized cell. Chromatin changes in sperm contribute to virtually every function that the male gamete must perform throughout spermatogenesis and in the mature cell. One requirement of sperm is that of transportation through both the female and male reproductive tracts, which demands a highly motile cell type. The unique nuclear protein landscape in sperm creates a chromatin structure that is between six and 20 times more dense than nucleosome-bound DNA, ultimately resulting in tightly condensed nucleus (Ward & Coffey 1991, Balhorn 2007). The compacted sperm head is proposed to provide enhanced motility and protection from DNA damage in a cell type that lacks robust repair mechanisms (Oliva & Dixon 1991). This is particularly important as sperm encounter many harsh environments during transport through the female reproductive tract.

The unique chromatin structure in sperm is essential for the safe delivery of paternal DNA to the oocyte, but the requisite replacement of canonical histones with sperm-specific protamine proteins has called into question the utility of the paternal epigenome in embryonic development. The stepwise transition of sperm nuclear proteins first involves the replacement of canonical histones with transition proteins. Next, two isoforms of protamine proteins, protamine 1 (P1) and P2, take the place of transition proteins in the sperm chromatin. The ratio of incorporated P1 and P2 is tightly regulated at ~1:1 ratio in the mature sperm (Balhorn *et al*. 1988, Hecht 1990, Oliva & Dixon 1990, Dadoune 1995). Aberrations in this ratio have been correlated with general infertility and poor fertilization ability (Aoki *et al*. 2005, 2006, Zhang *et al*. 2006). The protamination of sperm chromatin provides the compaction necessary for safe delivery to the oocyte but removes histones, which are capable of eliciting gene activation or silencing via tail modifications (methylation, acetylation, etc.). In effect, protamination removes a potentially informative epigenetic layer from the paternal chromatin, leading to the previously held belief that sperm are incompetent to

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drive epigenetic changes in the embryo and suggesting that their utility is found only in the delivery of an undamaged DNA blueprint. However, recent data challenges this dogma.

It is known that the replacement of histone with protamine is incomplete, with about 5–15% of the chromatin remaining nucleosome-bound (Tanphaichitr *et al*. 1978, Wykes & Krawetz 2003). Interestingly, this incomplete replacement was found to be programmatic and not simply a result of inefficient machinery, suggesting that retained histones may contain modifications that play a role in epigenetic regulation (Arpanahi *et al*. 2009, Hammoud *et al*. 2009). In known fertile patients, histone retention is found at the promoters of genes important in the embryo including developmental gene promoters, microRNA clusters, and imprinted loci, suggesting that the nucleosome retention is programmatic in nature (Hammoud *et al*. 2009). Taken together, these data suggest that the limited view of the sperm epigenome in developmental regulation is incomplete.

The growing evidence in support of the hypothesis that the paternal epigenome plays an important role in the developing embryo is not limited to nucleosome retention data. Recent studies analyzing sperm DNA methylation, histone modifications, and spermatozoal RNA transcripts further establish the role of the sperm epigenetic program in the developing embryo. This review will describe the current literature regarding the paternal epigenome and its influence on embryogenesis and will additionally address critical future research directions.

DNA methylation

DNA methylation is a common regulatory mark found on the 5 carbon of cytosine residues (5-mC) at cytosine–phosphate–guanine dinucleotides (CpGs; Fig. 1), which exert strong epigenetic regulation in many cell types (Portela & Esteller 2010). DNA methylation is essential in genomic imprinting, gene expression regulation, X chromosomal inactivation, and embryonic development (Jaenisch & Jahner 1984, Surani 1998, Ng & Bird 1999). This epigenetic mark can activate or repress gene transcription at specific sites based on the methylation levels at promoter regions. Hypermethylation at promoters blocks access of transcriptional machinery and thus inhibits gene expression. Conversely, hypomethylation facilitates gene activation as a result of increased accessibility of DNA by polymerase (Fig. 2).

The regulation of DNA methylation is essential to normal cell function in somatic cells, gametes, and the embryo (Jaenisch & Jahner 1984, Surani 1998, Ng & Bird 1999). The DNA methyltransferase (DNMT) family of proteins helps to facilitate *de novo* methylation and methylation maintenance (Eden & Cedar 1994). The enzymes directly responsible for *de novo* methylation include DNMT3A (DNMT3a), DNMT3B (DNMT3b), and DNMT3L (DNMT3l). Both DNMT3A and DNMT3B contain catalytic domains, which allow them to directly lay down new methylation marks. DNMT3L is essential in directing the proper placement of marks by working in concert with DNMT3A and DNMT3B (Okano *et al*. 1999). Once methylation marks have been established, DNMT1 maintains those marks through cell division (Bestor 1992, Lei *et al*. 1996).

The importance of DNA methylation has been demonstrated globally, regionally, and at the single locus level in both humans and animal models. Of great value in the effort to increase our understanding of the role of DNA methylation is recent data describing the human sperm methylome, which provides a general classification of a 'normal' methylation status at 96% of genomic CpGs (Molaro *et al*. 2011). Recent data demonstrate that aberrant methylation of promoters for specific genes (e.g. *DAZL* and *MTHFR*) and general gene classes, such as imprinted loci, are strongly associated with various forms of infertility and sperm defects in men

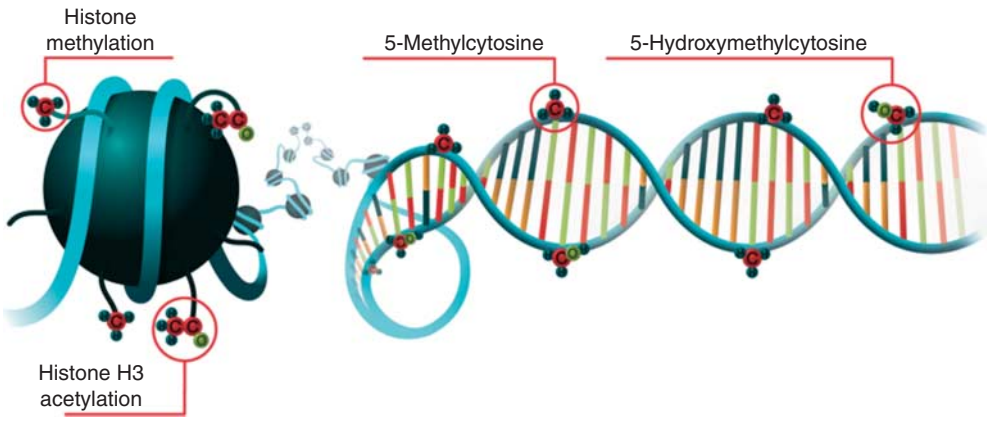

Figure 1 Epigenetic modifications (both histone modifications and DNA methylation) commonly found in sperm. Depicted are histone tail modifications (methylation and acetylation) as well as 5-methylcytosine (5-mC) and the demethylation intermediate, 5-hydroxymethylcytosine (5-hmC). Each modification is believed to play a regulatory role in gene expression.

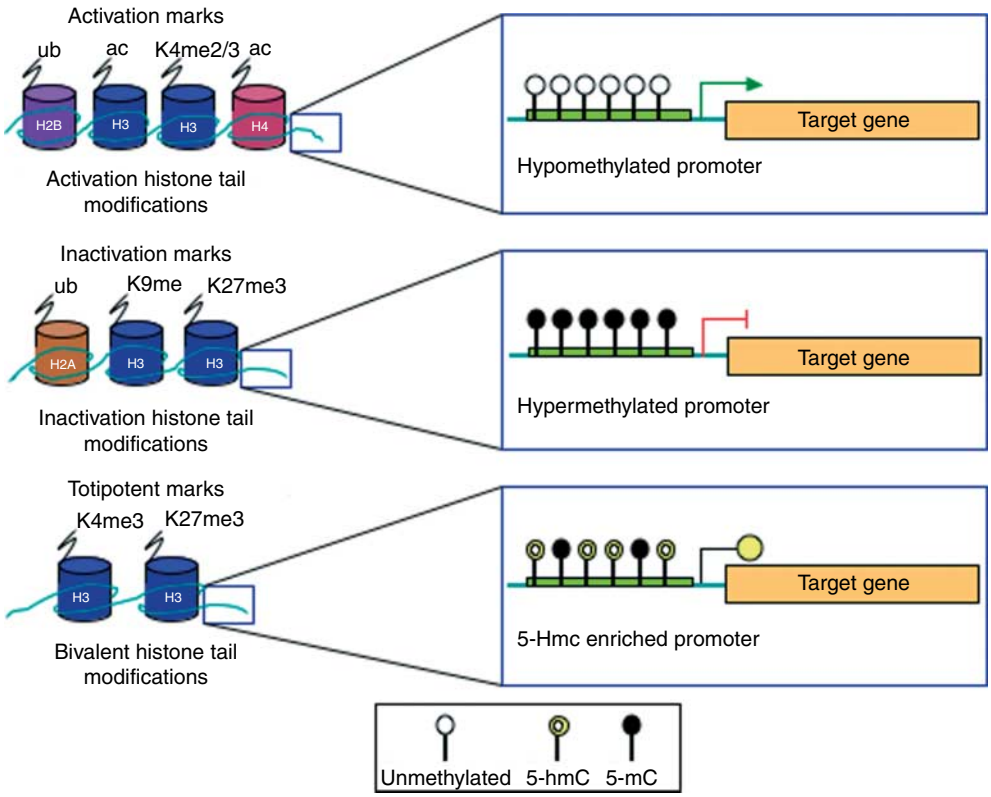

Figure 2 This figure depicts epigenetic marks that contribute to gene activation or inactivation, as well as bivalent modifications. Epigenetic regulators that promote gene transcription include DNA hypomethylation, histone H2B ubiquitination, H3 acetylation, H3K4 methylation, and H4 acetylation. Marks that tend to inhibit transcription include DNA hypermethylation, H2A ubiquitination, H3K9 methylation, and H3K27 methylation. Bivalent histone modifications (H3K27 methylation and H3K4 methylation) and enrichment of 5-hmC at gene promoters appear to be a hallmark of pluripotency.

(Navarro-Costa *et al*. 2011, Pacheco *et al*. 2011, Wu *et al*. 2011). Additionally, El Hajj *et al*. (2011) suggest that improper methylation of repetitive elements may be linked to recurrent pregnancy loss. Methylation abnormalities at the promoter of CREM were seen in a subset of patients with coinciding protamine ratio abnormalities as well as in patients presenting with various forms of male factor infertility (Nanassy & Carrell 2011*a*, 2011*b*). Additionally, Benchaib *et al*. (2005) reported that among IVF patients, global sperm DNA hypomethylation is correlated with poor pregnancy outcomes. Interestingly, the data revealing the importance of DNA methylation are not only limited to studies on patients with reduced fertility but has also been suggested on known fertile donors. These data reveal an epigenetic landscape that appears programmed for use in the embryo with hypomethylated DNA at developmental promoters (Hammoud *et al*. 2009). Taken together, these data suggest that DNA methylation may play an essential role in both the sperm and the embryo.

Multiple targeted studies have been performed in animal models to establish a clear role of DNA methylation in the sperm and embryo. Knockouts and mutations of *Dnmt1* in mice caused severe global hypomethylation resulting in biallelic expression at imprinted differentially methylated regions (DMRs), transcription of retrotransposons, loss of chromosomal inactivation, retarded gestational growth, and, in turn, embryo lethality (Li *et al*. 1992, Panning & Jaenisch 1996, Walsh *et al*. 1998). Conditional knockouts of DNMT3A and DNMT3L were hypomethylated at imprinted DMRs and resulted in severe decreases in spermatogenesis with DNMT3L loss also resulting in global decreases in methylation (Kaneda *et al*. 2004, La Salle *et al*. 2007). The loss of DNMT3B did not result in severe phenotypes, suggesting that there is redundancy in its function. However, the loss of DNMT3B did prove to affect *Rasgrf1* promoter methylation, which was not seen in the DNMT3A conditional knockout (Kaneda *et al*. 2004, Kato *et al*. 2007). These data suggest that there are regions where DNMT3A and DNMT3B act independently, as well as regions of redundancy.

In addition to studies using targeted genetic approaches in the study of DNA methylation are those using 5-azacytidine and 5-aza-2-deoxycytidine, both potent DNA methylation inhibitors (Egger *et al*. 2004). Short-term 5-aza-2-deoxycytidine exposure in neonatal male mice reduced overall fertility status by halting spermatogenic differentiation (Raman & Narayan 1995). Adult male mice treated with 5-azacytidine before

mating had decreases in fertility and increased incidence of embryo mortality (Seifertova *et al*. 1976). Additionally, 5-aza-2-deoxycytidine treatment in both mouse and rat resulted in decreased fertilization rates and/or increased preimplantation loss (Doerksen & Trasler 1996, Kelly *et al*. 2003, Oakes *et al*. 2007). It should be noted that while global demethylation is observed in the sperm with this treatment, the effects on embryogenesis may ultimately be a result of the cytotoxic effects of the drug and not solely the result of methylation defects (Oakes *et al*. 2007).

Along with the current interest in CpG methylation, recent data has suggested that intermediates formed during DNA demethylation may be important epigenetic regulators. Most prominent among these intermediates is 5-hydroxymethylcytosine (5-hmC; Fig. 1). This DNA modification is formed via the TET family of proteins (Tahiliani *et al*. 2009) just before complete removal of methylation marks. Because it is an intermediate, 5-hmC can achieve full demethylation more quickly than 5-methylcytosine, and as a result, gene promoters with enriched 5-hmC are considered easier to activate and thus may play a role in epigenetic regulation (Fig. 2; Pastor *et al*. 2011). Interestingly, recent work from Pastor *et al*. (2011) has revealed an enrichment pattern of 5-hmC at poised genes in the stem cell. This unique localization suggests that 5-hmC has utility in the embryonic stem cell and possibly the epigenome of multiple other cells types. As nucleosome retention was found at similar poised loci in sperm (Hammoud *et al*. 2009), identification of regional 5-hmC enrichment must be analyzed in the male gamete. These data suggest that 5-hmC may have real impacts on the epigenome and must be a focus of future studies to fully elucidate its regulatory role.

The current literature provides strong evidence for the importance of appropriate DNA methylation. This essential epigenetic mark has tremendous utility in the maintenance of gene activation and repression. Inappropriate methylation clearly results in abnormal phenotypes and as such, efforts should be made to determine the etiology of these aberrant profiles so that proper preventative steps and treatments can be established.

Retained histones, their modifications, and spermatozoal RNA transcripts

One of the most unique epigenetic features in the male gamete is the replacement of DNA-bound histones with protamines. As mentioned earlier, this protamination creates a highly condensed nuclear structure that helps to enable proper motility and protects DNA from damage. Although incorporation of this unique, sperm-specific protein results in a quiescent chromatin structure, some regions retain histones and their associated modifications. Recent studies have found this nucleosome retention to be programmatic and not a result of random distribution (Arpanahi *et al*. 2009, Hammoud *et al*. 2009). The mechanism that directs this selective nucleosome retention remains largely uncharacterized, but there is evidence to suggest a role for RNA transcripts in the process (Rassoulzadegan *et al*. 2006, Dadoune 2009). It appears that spermatozoal RNA transcripts are capable of inhibiting the protamination process and maintaining a histone-bound chromatin structure (Miller *et al*. 2005). RNA transcripts colocalize with nucleosome-bound chromatin near the nuclear envelope in the mature sperm, as is the case with the insulin-like growth factor 2 (IGF2) locus (Wykes & Krawetz 2003, Miller *et al*. 2005). There remains controversy in the role of RNA in this process, but the colocalization with regions of retained nucleosomes does provide a possible mechanism to explain the regulation of histone retention. In theory, this selective retention in sperm could allow for targeted gene activation or silencing in the embryo.

Multiple histone variants found in sperm play an essential role throughout spermatogenesis as well as in the mature spermatozoa. Among these, important nuclear proteins are histone 2A and B (H2A and H2B), histone 3 (H3), histone 4 (H4), and the testes variant (tH2B) (Gatewood *et al*. 1990, Jenuwein & Allis 2001, Fenic *et al*. 2004, Baarends *et al*. 2005, Zhu *et al*. 2005, Okada *et al*. 2007). Histone proteins have the distinct capability of driving epigenetic changes based on tail modifications. As a result, those histones retained through the protamination process are likely competent to exert similar regulatory effects. Targeted gene activation or silencing in many different cell types can be driven by these tail modifications found at lysine (K) and serine (S) residues of histones. The main forms of modifications in sperm include methylation, acetylation, ubiquitination, and phosphorylation, which can act alone or in concert to ensure the proper state of activation or suppression at any given gene or gene promoter (Fig. 1). H3K4 methylation, H3 and H4 acetylation, and H2B ubiquitination drive the genes toward activation while H3K9 and H3K27 methylation, deacetylation at H3 and H4, and H2A ubiquitination enrichment support gene silencing (Fig. 2; Jenuwein & Allis 2001, Lachner & Jenuwein 2002, Baarends *et al*. 2005, Zhu *et al*. 2005). These modifications are established and regulated by a variety of enzymes. The histone methyltransferase and demethylase family of proteins catalyze methylation and demethylation (Lachner & Jenuwein 2002). Acetylation establishment and removal are regulated by histone acetyltransferase and deacetylase respectively (Jenuwein & Allis 2001).

As a result of protamination, few histones remain to function as epigenetic regulators. However, as previously mentioned, the few loci that have been shown to retain histones in the mature sperm are known

to be important in developmental processes (Hammoud *et al*. 2009). This suggests that histones in these select regions are able to provide some degree of retained regulatory competence via histone tail modifications. Indeed, recent studies have implicated aberrant histone methylation and/or acetylation in the mature sperm in various forms of infertility. Loss-of-function mutation of JmjC-domain-containing-histone demethylase 2A (JHDM2A), an enzyme with known H3K9 demethylase activity, revealed decreased transcription of transition protein 1 and P1 during spermatogenesis (Okada *et al*. 2007). Additional studies have demonstrated that varied degrees of infertility, including sterility, are correlated with perturbations in histone methylation (Lee *et al*. 2005, Glaser *et al*. 2009). Deacetylase inhibitors, such as trichostatin-A, have been used in the study of histone modification and epigenetic regulation in mice. Fenic *et al*. (2004, 2008) demonstrated that s.c. injection of trichostatin-A in male mice resulted in a dose-dependent decrease in spermatogenesis and testis weight as well as decrease in histone deacetylase activity and subsequent alterations in histone acetylation. On a more broad level, recent work from Hammoud *et al*. (2011) has shown that histone retention is not programmatic but random in some patients with two different classes of infertility. A decreased enrichment of H3K4me or H3K27me at select regions important to development was also identified. These studies provide additional evidence that suggests a regulatory role of histone modifications in sperm.

In normal human sperm, histone modifications and their enrichment patterns suggest a highly regulated epigenetic landscape. H3K4 dimethylation (H3K4me2) and H3K4 trimethylation (H3K4me3) were found enriched at developmental promoters important in the embryo. Additionally, H3K27 trimethylation (H3K27me3) is enriched at gene promoters that are silenced in the early embryo (Hammoud *et al*. 2009). Further data describe a pluripotent-like state that some genomic regions important to the developmental program show in both human and mouse (Hammoud *et al*. 2009, Brykczynska *et al*. 2010). In these regions, bivalent histone modifications, H3K4me3 and H3K27me3, reflect marks found in stem cells (Fig. 2). These findings provide additional evidence that the paternal epigenome may be needed for proper embryonic development and may additionally contribute to the pluripotent state of embryonic stem cells.

Taken together, these data suggest a role of the paternal epigenome in early embryonic development. Nucleosome retention provides additional epigenetic competence to the paternal chromatin that was once considered to be void of such marks. It appears likely that histone retention and histone modifications are key to normal sperm function and ultimately normal embryogenesis. As our understanding of the role of histones and their modifications in sperm increase, we will be able to better classify, diagnose, and treat male factor infertility.

Conclusions and future directions

The role of the paternal epigenome in the embryo has long been considered to exert only limited influence on development. However, the recent literature described in this review provides evidence that the unique epigenetic landscape in sperm may play a larger role in development than previously believed. From these data, it is evident that proper establishment and maintenance of the paternal epigenetic program is associated with appropriate gamete and embryonic development, disruption of which is associated with varied degrees of infertility. While recent studies have been key in opening this relatively new area of study, many questions remain unanswered.

Further research is required to fully elucidate the paternal regulatory control in the embryo. One key will be to further investigate the epigenetic profiles of various classifications of infertility along with embryo quality data. Special caution must be taken to ensure that proper patient classification is used to ensure that results are clearly attributable to unique and definable etiologies as opposed to infertility in general. This will allow a determination of direct correlations, and possibly causation, to specific infertility classes.

In addition to observing aberrant epigenetic profiles common among specific populations of infertile men, the etiologies of these various abnormalities must be investigated. There are many likely candidates that may cause epigenetic alterations in sperm and resultant abnormal embryogenesis, the most prominent of which are environmental toxins and aging. Studies have evaluated various environmental agents and their effects on male fertility in general, but few have analyzed specific epigenetic alterations that may be occurring as a result of exposure. However, there is precedence for environmental impacts on the paternal epigenetic landscape. Recent studies provide intriguing evidence for the effects of heavy metals on sperm nuclear proteins, specifically P2. Quintanilla-Vega *et al*. (2000*a*, 2000*b*) reported that lead binds to P2 and inhibits its DNA binding, ultimately affecting chromatin compaction. Yauk *et al*. (2008) found that mice exposed to ambient air pollution had global DNA hypermethylation compared with animals housed with high-efficiency particulate arresting (HEPA)-filtered air. Additionally, exposure to endocrine disrupters has also been shown to disrupt heritable germ cell epigenetics (Anway & Skinner 2008). These data clearly suggest that environmental agents can affect sperm epigenetics. Environmental toxins must be targeted in future studies analyzing the etiology of epigenetic alterations in sperm to aid improvement in diagnostic and treatment approaches in men who present with infertility.

In addition to the effects of the environment on the sperm epigenome, there is also increasing concern regarding the effects of advanced paternal age on epigenetic alterations seen in male gametes. It is well established that advanced maternal age is associated with poor pregnancy outcomes, mainly as a result of increased incidence of chromosomal nondisjunction (Eichenlaub-Ritter 1998). Conversely, paternal aging has long been considered of little consequence in the development of functional sperm capable of generating normal offspring. However, the available data suggest that there are likely aberrations occurring in the sperm of aging males that may affect offspring. Recent work from Flatscher-Bader *et al*. (2011) has shown that copy number variations are more common in the offspring of older male mice than from younger fathers. Although the idea of epigenetic alterations occurring as a result of aging in males is still controversial, recent studies have yielded interesting data on this front as well. Changes in gene expression and chromatin compaction in sperm and testes tissue of older males have been described in recent studies while contradictory data have been presented in other studies (Wyrobek *et al*. 2006, Zubkova & Robaire 2006, Kokkinaki *et al*. 2010). Despite some controversy when taken together, these data still appear to suggest that advanced paternal aging may be accompanied by some degree of epigenetic alterations in the sperm. These changes are, however, poorly characterized.

It is evident that the paternal epigenome plays a role in sperm quality and likely in embryonic development; however, many unanswered questions remain. It will be critical for future studies to focus on common epigenetic abnormalities found in specific classes of infertility. Additionally, research should focus on the etiologies of such abnormalities. The effect of environmental exposures and aging are two major topics that have yet to be fully addressed in relation to the genesis of an aberrant sperm epigenetic program. As we learn more about the true effects of epigenetic alterations in sperm, how they arise and how they affect fecundity, we will be more capable of addressing the growing issue of male factor infertility in prevention, diagnosis, and treatment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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