Epigenetic reprogramming during spermatogenesis and male factor infertility

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Abstract

Infertility is an often devastating diagnosis encountered by around one in six couples who are trying to conceive. Moving away from the long-held belief that infertility is primarily a female issue, it is now recognised that half, if not more, of these cases may be due to male factors. Recent evidence has suggested that epigenetic abnormalities in chromatin dynamics, DNA methylation or sperm-borne RNAs may contribute to male infertility. In light of advances in deep sequencing technologies, researchers have been able to increase the coverage and depth of sequencing results, which in turn has allowed more comprehensive analyses of spermatozoa chromatin dynamics and methylomes and enabled the discovery of new subsets of sperm RNAs. This review examines the most current literature related to epigenetic processes in the male germline and the associations of aberrant modifications with fertility and development.

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Introduction

Male infertility is a complex disease that, despite being extensively researched, still remains poorly understood. Although infertility can be attributed to decreased semen quality, testicular dysfunction, infections of the genital tract and known genetic disorders (such as single-gene alterations and chromosomal abnormalities) (Kraus *et al.* 2015), a number of male patients suffer from a condition known as idiopathic or unexplained male infertility (UMI), in which the aetiology is poorly understood (Urduingio *et al.* 2015). Given the major genome reprogramming events that occur during gametogenesis and early development, in recent years, a number of studies have pointed towards aberrant epigenetic reprogramming of the genome as a potential contributory factor to male infertility (O'Doherty & McGettigan 2014, Laurentino *et al.* 2015, Stuppia *et al.* 2015, Urduingio *et al.* 2015, Schagdarsurengin & Steger 2016, Kropp *et al.* 2017, Laqqan *et al.* 2017a, Nasri *et al.* 2017). It should be stressed that, at present, direct associations between epigenetic changes and infertility are unclear – it is indeed possible that they are simply correlated with changes in infertility and not causal.

There are currently a variety of definitions and opinions on the term 'Epigenetics', which can often stir debate. A widely used and accepted definition of epigenetic processes is one that defines them as heritable changes influencing gene expression that are not caused by changes to DNA sequence (Holliday 1987). These

processes lead to the establishment of specialised chromatin states that are permissive or repressive to gene expression, can be influenced by the maternal or paternal environment due to their plasticity and are involved with determining cell identity (Schagdarsurengin & Steger 2016, Stewart *et al.* 2016). To date, DNA methylation has been the most comprehensively studied epigenetic mark (Smith & Meissner 2013), however, post-translational modifications to histone tails and the role of non-coding RNA molecules (both widely regarded as epigenetic processes/modifications) are gaining considerable attention.

Determining the underlying causes of unknown male factor infertility still remains a major challenge in reproductive medicine (Salas-Huetos *et al.* 2016). In the clinical setting, widely used semen analysis methods for diagnosing male factor infertility, such as microscopic examination and DNA fragmentation analyses, do not tell the whole story. These approaches are mostly ineffective for diagnosing UMI, where spermograms of normozoospermic infertile patients are indistinguishable from those of normozoospermic fertile patients (Hamada *et al.* 2012). However, recent advancements in sequencing technologies have allowed researchers to evaluate the sperm epigenome and build a bigger picture of their underlying molecular profiles, helping to determine why some couples experience idiopathic infertility.

In this review, we will focus on the potential role of aberrant epigenetic reprogramming of the sperm

genome on male fertility and early development, with particular emphasis on DNA methylation, histone tail modifications and non-coding RNA molecules (Fig. 1).

Epigenetic reprogramming during sperm development

The genome is extensively remodelled during mammalian germ cell development in both male and female germlines, and this epigenetic reprogramming is critical for imprinting and reprogramming in early embryos (Reik *et al.* 2001). Chromatin organisation in sperm and oocytes is markedly different: sperm DNA is tightly packed, histones are largely replaced with protamines

and there is a large degree of DNA methylation while oocytes have less DNA methylated and prevalently open chromatin (O'Doherty & McGettigan 2014, Bao & Bedford 2016, Hanna & Kelsey 2017). Given that the emphasis of the current review is on epigenetic mechanisms during spermatogenesis and male fertility, we will be focusing solely on the paternal germline. For a comprehensive review on genome reprogramming in the female germline, see Messerschmidt *et al.* 2014, Stewart *et al.* 2016.

There are two major reprogramming events that involve genome-wide erasure and re-establishment of DNA methylation patterns during mammalian development; the first occurs in primordial germ cells

Figure 1 Reconfiguration of the sperm epigenome during spermatogenesis. The genome of PGCs is demethylated prenatally during the colonisation of the genital ridge with migratory PGCs. Establishment of global DNA methylation occurs in mitotically arrested prospermatogonia and is completed prior to birth (Li *et al.* 2004, Stewart *et al.* 2016). *De novo* DNA methylation in prospermatogonia coincides with an early redistribution of the histone tail marks H3K4me2/3 and H3K36me3 (Singh *et al.* 2013, Morselli *et al.* 2015). Other histone tail modifications, such as acetylation of histone 4 lysine residues, occur later in elongating spermatids during spermiogenesis (Oliva & Mezquita 1982, Lahn *et al.* 2002, Awe & Renkawitz-Pohl 2010). Testis-specific histone variants are expressed throughout spermatogenesis until the elongated spermatid stage when they are, along with the majority of the core histones, sequentially replaced by transitional proteins prior to histone-to-protamine transition and hyper-compaction of the chromatin (Zalensky *et al.* 2002, Bao & Bedford 2016, Ueda *et al.* 2017). RNA molecules that are expressed throughout spermatogenesis and stored in mature spermatozoa may reflect sperm maturation, fertility potential and the paternal contribution to the development of the offspring (Meikar *et al.* 2011, Godia *et al.* 2018). An example of some of the factors that have been shown to be associated with aberrant reprogramming during spermatogenesis (e.g. diet and smoking), discussed in this review, are highlighted. This figure was prepared using the Biomedical PPT toolkit suite (www.motifolio.com) and Biorender (<https://biorender.io/>). PGCs,

(PGCs) and the second following fertilisation in the pre-implantation embryo (Prokopuk *et al.* 2015). These events have been mostly characterised using the murine model and, although epigenetic profiles have been assessed in spermatozoa and embryos of other species (Dean *et al.* 2001, Beaujean *et al.* 2004, Deshmukh *et al.* 2011, O'Doherty *et al.* 2012, 2015, Guo *et al.* 2014b), detailed comparative studies are still lacking. Firstly, during proliferation and migration of PGCs from the epiblast to colonisation of the genital ridge (~E6.5–E13.5), the PGC genome is demethylated in a biphasic manner that results in their genomes displaying very low levels of DNA methylation (Messerschmidt *et al.* 2014). From here, PGCs are divergently remethylated in males and females in a germ cell-specific fashion (Sasaki & Matsui 2008, Seisenberger *et al.* 2012). Male germ cells gain as much as 50% global methylation by E16.5 and are almost completely methylated at birth. In contrast, remethylation in the female germline occurs after birth, during the oocyte growth phase (O'Doherty *et al.* 2012, Tomizawa *et al.* 2012). Secondly, following fertilisation, male pronuclei are actively and rapidly demethylated in the zygote while the maternal genome is passively demethylated in a replication-dependent manner during cleavage (Lees-Murdock & Walsh 2008). Until recently, it was widely accepted that the maternal genome and paternal genome were not actively demethylated or passively demethylated, respectively. It should be noted, however, that active and passive demethylation do not occur exclusively on the paternal and maternal genomes, as it has been demonstrated that both genomes undergo widespread active and passive demethylation in the zygote prior to the first mitotic division (Gkoutela & Clark 2014, Guo *et al.* 2014a).

These periods of reprogramming occur during crucial developmental time points and represent windows of susceptibility for epigenetic errors to be introduced, that may impact fertility and embryonic competence. Over four decades ago, it was reported that a Fathers occupation, and potential exposure to (spermatogenesis impairing) environmental toxins, was associated with carcinogenic defects being transmitted to their children (Fabia & Thuy 1974). Since then, a number of other studies have shown links between paternal exposure to environmental toxins (e.g. paints, hydrocarbons, pesticides) and offspring health; reviewed in (Soubry *et al.* 2014). Given the continuous cycles of mitosis and meiosis that occur during spermatogenesis in adult males, the chances of accumulating environmentally induced epigenetic errors during this protracted period of replication and cell division are much greater in males than in females (Messerschmidt *et al.* 2014).

DNA methylation

As mentioned earlier, DNA methylation is the most widely investigated epigenetic mark. This epigenetic

modification is essential for male and female gametogenesis and mammalian development (Okano *et al.* 1999, Bourc'his *et al.* 2001, Kaneda *et al.* 2004). In the mammalian genome, DNA methylation mostly occurs on the fifth position of cytosine bases in the context of cytosine followed by guanine (CpG dinucleotides): this is referred to as 5-methylcytosine (5mC) (Yoder *et al.* 1997). It can also occur to a much lesser extent at cytosine bases in a non-CpG context (Jang *et al.* 2017). Additionally, 5mC can be oxidised by members of the ten-eleven translocation family of proteins to form 5-hydroxymethylcytosine (5hmC), 5-formylcytosine and 5-carboxylcytosine (Ito *et al.* 2011, Wu & Zhang 2014, Neri *et al.* 2016). These intermediary modifications are much less studied compared to 5mC or 5hmC and are yet to be characterised during male gametogenesis.

Aberrations in sperm DNA methylation have also been shown to be associated with abnormalities in semen parameters (low sperm count, decreased volume of semen, reduced progressive motility, high percentage of immotile sperm lowered sperm vitality) of subfertile males that were trying to conceive for at least 10 years (Laqqan *et al.* 2017a). The authors of this study identified that DNA methylation was consistently different at a small number of CpGs between proven fertile males and subfertile case subjects, using Infinium Human Methylation 450K BeadChip arrays, validating previous findings using local deep bisulfite sequencing (Gries *et al.* 2013). This study builds on earlier investigations, and recent studies by Nasri *et al.* (2017) and Kobayashi *et al.* (2017), showing that aberrant methylation, particularly at imprinted loci, is associated with poor-quality sperm (Marques *et al.* 2004, 2008, Kobayashi *et al.* 2007, Poplinski *et al.* 2010, Pacheco *et al.* 2011, Laurentino *et al.* 2015). However, the methylation differences in Laqqan *et al.* study were very small and restricted to a small number of CpGs; therefore, whether these differences are related to the subfertile phenotype remains to be fully determined. In a previous study using the same 450K DNA methylation array, Urdinguio *et al.* revealed that DNA methylation patterns in the male partners of couples with UMI were measurably different at specific loci and Alu Yb8 repetitive elements (Urdinguio *et al.* 2015). Given that no abnormalities in semen parameters are distinguishable between UMI patients and fertile control patients, and that female factor infertility was ruled out, this study provides encouraging evidence to show that aberrant sperm DNA methylation may be associated with fertility impairment in couples with UMI. This is further supported by a study by Aston and colleagues showing that, in similar cohorts of patients, sperm DNA methylation may be used to predict fertility and potentially predictive of embryo quality during IVF (Aston *et al.* 2015).

It has been reported that smoking has a strong correlation with CpG methylation and sperm count, morphology and motility (Laqqan *et al.* 2017b). The authors of this study also identified that smoking is associated with changes in sperm DNA methylation patterns, specifically at CpGs located in regions related to *MAPK8IP* and *TKR* genes. A link between smoking, male fertility and methylation at imprinted loci has been highlighted in a study by Dong *et al.* (2016). In this study, they revealed that hypomethylation of the *H19* imprint control region (ICR) and hypermethylation of the *SNRPN*-ICR were associated with infertility and that the risk was potentiated by smoking. In addition to smoking, alcohol consumption has been shown to be associated with DNA methylation at regulatory regions of the imprinted gene, *H19*, in spermatozoa of humans and mice; however, the impact of alcohol-induced alteration to DNA methylation on fertility remains to be determined (Ouko *et al.* 2009, Stouder *et al.* 2011, Lim & Song 2012).

The effect of paternal diet on sperm quality, offspring health and epigenetics has been discussed previously (Schagdarsurengin & Steger 2016). In an early study, it was demonstrated that offspring from male mice fed a low-protein diet exhibited differential expression of key lipid and cholesterol biosynthesis-related genes in the liver that may be related to altered DNA methylation patterns inherited through the male germline (Carone *et al.* 2010). In a similar study, differential methylation was observed at genes implicated in development, chronic diseases, diabetes, autism and schizophrenia in sperm isolated from mice fed a folate-deficient diet, providing evidence for a possible link between diet, the sperm methylome and offspring health (Lambrot *et al.* 2013). However, it was reported in a subsequent study from Rando and colleagues that, although paternal diet has an effect on offspring phenotype, no consistent effects of diet on the sperm methylome are observable and that variation in offspring DNA methylation is due to genetic and epigenetic variations (Rando & Simmons 2015, Shea *et al.* 2015, Whitelaw 2015). Although the precise epigenetic mechanism/s of how the paternal diet can affect fertility and offspring health remain to be fully unravelled, the importance of the paternal (and maternal) diet on fertility should not be disregarded and needs to be investigated further.

Chromatin structure and modifications

During the final stages of male gametogenesis (spermiogenesis), motile spermatozoa develop from haploid round spermatids (Bao & Bedford 2016). The chromatin undergoes a dramatic reconfiguration during this time, when the vast majority (90–95%) of nucleosomal core histones (H2A, H2B, H3 and H4) are first replaced by transitional proteins and then by protamines (Balhorn 1982, Meistrich *et al.* 2003,

Eirin-Lopez *et al.* 2006, Rathke *et al.* 2014). This process, known as histone-to-protamine transition, facilitates a tight packaging of sperm DNA enabling condensation of sperm heads and protection of DNA from damage and mutagenesis (Ward & Coffey 1991, Rathke *et al.* 2014). The ratio of remaining nuclear histones to protamines has been shown to play a role in male fertility (Zhang *et al.* 2006). Findings from this study suggested that infertile men possess a higher proportion of spermatozoa with an increased histone-to-protamine ratio, compared to fertile controls. Histone-to-protamine transition and links with fertility have also been reported elsewhere (Carrell & Liu 2001, Aoki *et al.* 2006, Carrell *et al.* 2007). These studies showed that altered expression of protamine, protamine 1 and protamine 2 are related to diminished spermatogenesis and fertilisation capacity. In addition, cryopreservation, a routinely used process for sperm storage in fertility clinics, has been shown to impact chromatin integrity (Fortunato *et al.* 2013). The authors of this study reported that cryopreservation influenced chromatin decondensation and may lead to sperm chromatin cryoinjury.

The small proportion of histones (5–10%) that are retained during histone-to-protamine transition and the post-translational modification of these remaining histones and their tails is an expanding area of biological research (Carrell & Hammoud 2010, Krejci *et al.* 2015). Retained histones are modifiable and thought to occur at specific genomic loci that are involved with regulating transcription following fertilisation (Hammoud *et al.* 2009, Miller *et al.* 2010). However, the number of studies investigating the relationship between chromatin structure in sperm and fertility remains substantially lower, relative to studies involving DNA methylation or abundance of RNA molecules (discussed later). One potential hurdle may be that pinpointing histone modifications that are directly associated with fertility may be difficult due to the plethora of post-translational modifications (e.g. acetylation, methylation, SUMOylation, phosphorylation) that can be present on the various histone isoforms (Luense *et al.* 2016). However, studies have shown links between fertility and chromatin structure and are discussed below.

Several recent studies investigating the relationship between chromatin structure and male fertility have been carried out using the bovine model. An early study evaluated the differences in bull fertility, histone retention and expression of a histone variant (H3.3) and two core histones (H2B and H4) using immunoblotting, immunocytochemistry and staining techniques (de Oliveira *et al.* 2013). Although no differences were detected for H3.3, H2B or H4 between sperm from the low-fertility (LF) group and the high-fertility (HF) group, there were differences in chromatin condensation between the groups that were associated with *in vivo* bull fertility. Interestingly, using transgenic mice carrying null mutations at two genes encoding the histone variant

H3.3, Tang and colleagues revealed that H3.3 is heavily involved in viability and male fertility (Tang *et al.* 2015). Using immunocytochemistry and flow cytometry techniques, it has been shown that acetylation and methylation of sperm histone 3 lysine 27 (H3K27ac and H3K27me3) are associated with bull fertility (Kutchy *et al.* 2018). Furthermore, flow cytometry, immunofluorescence and western blotting have been used to demonstrate that expression of the testis-specific histone variant 2B (TH2B) in sperm isolated from bulls with contrasting fertility scores can be potentially used to evaluate semen quality and male fertility (Kutchy *et al.* 2017). In another *in vitro* study comparing HF and LF bulls, Castro and colleagues assessed the potential contribution of chromatin integrity to fertility using three separate approaches to evaluate chromatin deficiency (CMA3), chromatin stability (SCSA; AO+) and DNA fragmentation (COMET assay) (Castro *et al.* 2018). Although differences in chromatin deficiency were observed between the HF and LF groups, the authors concluded that protamine deficiency in bovine spermatozoa may not have a strong biological impact on *in vitro* fertility.

Using a more targeted approach with a custom ChIP-on-chip array, Verma *et al.* investigated di-methylated H3K4 (H3K4me2) and tri-methylated H3K27 (H3K27me3) in spermatozoa of water buffalo bulls (*Bubalus bubalis*) identified as having wide differences in fertility (Verma *et al.* 2015). For H3K4me2 and H3K24me3, the authors identified 84 and 80 genes, respectively, that were differentially enriched between mature sperm from high and sub-fertile bulls. Gene ontology analysis of these differentially marked genes revealed enrichment for processes such as germ cell development, spermatogenesis and embryonic development—demonstrating that appropriate chromatin configuration at functionally relevant genes is related to bull fertility. Similarly, it has recently been shown that epigenetic marks in the sperm of *Xenopus laevis*, a model organism for developmental biology research, regulate embryonic gene expression (Teperek *et al.* 2016). Specifically, this work showed important regulatory roles for loss of methylation of histone H3 on lysine 4 (H3K4) and retention of methylation of histone H3 on lysine 27 (H3K27) and demonstrated that epigenetic cues delivered by the sperm are required for correct embryonic gene expression. Work by Öst and colleagues, using the model organism *Drosophila*, provided a link between paternal sugar intake, H3K9/K27me3-dependent reprogramming of metabolic genes and offspring adiposity (Öst *et al.* 2014). In humans, acetylation of histone H4 at lysine 12 (H4K12ac) has been implicated at developmentally important gene promoters in subfertile men (Vieweg *et al.* 2015).

Work by Dumasia and colleagues, looking at the roles of oestrogen and the oestrogen receptors (ESRs) during rat spermatogenesis, has shown that ESR are involved

with spermatogenesis and fertility (Dumasia *et al.* 2015, 2016, Kumar *et al.* 2017); oestrogen signalling regulates DNA methylation (Dumasia *et al.* 2017a) and that treating adult rats with selective ESR agonists resulted in altered chromatin modifications in the testis (Dumasia *et al.* 2017b). More specifically, they found that ESR agonist treatment caused increased histone retention, protamine deficiencies, and altered levels of histone modifications that correspond with active and repressed chromatin states, and altered heterochromatin marks (Dumasia *et al.* 2017b). In another study investigating the impact of oestrogens in the environment, Filby *et al.* analysed *Pimephales promelas*, a model fish species, after exposure to differing concentrations of oestrogen as a single chemical and as part of an oestrogenic effluent from waste water treatment work (WwTW) (Filby *et al.* 2007). They revealed an association between oestrogens in the environment and some adverse and sex-related health effects observed in the fish including genotoxic damage, modulated immune function, and altered metabolism, in addition to endocrine disruption (Filby *et al.* 2007). Future studies investigating the impact of environmental oestrogens will allow us to determine how they affect chromatin remodelling during spermatogenesis and if they negatively impact male fertility.

Sperm-borne coding and non-coding RNA molecules

Until recently the presence of RNA in sperm was largely thought to be due to carryover or contamination with non-sperm cells (discussed in O'Doherty & McGettigan 2014). The presence and characterisation of RNA molecules in sperm has been confirmed and greatly facilitated in recent years through technological advances in sequencing platforms and the focus has now shifted to elucidating their function. Although transcriptionally quiescent, sperm have been shown to contain a wide variety of RNA molecules, both coding and non-coding, reported to be required for spermatogenesis, early development and epigenetic inheritance (Schuster *et al.* 2016). Transcriptomic analysis of sperm isolated from men of known fertility, idiopathic infertility (normozoospermic patients), and asthenozoospermia (reduced motility) has identified different RNA profiles between the patient cohorts, highlighting the potential importance of these molecules in male fertility (Bansal *et al.* 2015).

It has been reported in humans that as little as 2% of transcriptional output is translated into protein, thus the remaining 98% is non-coding (Mattick 2001). The inclusion of ncRNA as epigenetic regulators of gene function remains somewhat divisive and evokes discussions on whether it is appropriate to define them as such. The focus of this review is not to determine whether or not ncRNAs should be included under the

epigenetic umbrella. Therefore, we will take the wider view that they are involved in epigenetic regulation and will report the most recent findings linking them with male infertility. Isolation of sperm RNAs remains methodologically challenging due to their very low abundancies and affinity of certain RNAs to chromatin (Goodrich *et al.* 2007, 2013). Nevertheless, many investigations, summarised hereafter, have reported associations between sperm-borne RNA molecules and fertility and shown that they have key roles in subsequent embryonic development.

A 2015 study suggested that analysing sperm RNA elements (SREs) could be an efficient predictor of effective fertility treatments on an individual level (Jodar *et al.* 2015). This study revealed that idiopathic infertile couples who had an incomplete set of necessary SREs had a significantly reduced probability of achieving a live birth by intrauterine insemination (IUI) or timed intercourse (TIC) from 73 to 27%. When using assisted reproductive technologies (ART), such as IVF or ICSI, the absence of SREs did not seem to be critical. Furthermore, this study showed that the infertility of idiopathic infertile couples without a complete set of SREs is most likely due to a male component. While the infertility of couples that failed to achieve a live birth, but have a complete set of SREs, could be due to female factor infertility.

Since the discovery of epigenetic reprogramming during gametogenesis and embryogenesis (Reik *et al.* 2001), and recent studies showing that environmentally induced DNA methylation alterations in the F1 generation are not maintained in the F2 generation (Radford *et al.* 2014) and that sperm methylation errors are corrected by reprogramming (de Waal *et al.* 2012, Iqbal *et al.* 2015), epigenetic researchers have shifted their focus to other molecules and mechanisms that have been proposed to play a role in maintaining transgenerational transfer of acquired traits, such as sperm RNAs and sperm RNA modifications (Chen *et al.* 2016). The first major piece of evidence supporting a role for sperm RNAs in the transgenerational inheritance of acquired traits came from a 2014 study which demonstrated that by injecting total sperm RNAs from mice that were exposed to mentally stressful conditions into normal zygotes, behavioural patterns and alterations in metabolism that were observed in the father can be recapitulated in the offspring, and maintained through subsequent generations (Chen *et al.* 2016). Subsequent studies showed that mice who were fed a high-fat diet (HFD) or high-fat-high-sugar diet also contained sperm RNAs that, when injected into normal zygotes, would produce offspring with similar paternal metabolic disorders (Chen *et al.* 2016). These studies have led many researchers to question which RNAs are potentially responsible for transmitting the paternal phenotypes. Recent evidence has turned researchers' attentions to sperm tsRNAs and miRNAs, which are up regulated in

mature sperm after HFD, low-protein diets or after other environmental exposures (Chen *et al.* 2016).

The ncRNA transcriptome can be broadly divided into two main groups based on their length. The first group is comprised of short ncRNAs ranging between 20 and 30 nucleotides and the second group is made up of lncRNA transcripts that are >200 nucleotides in length. The role of these two groups of RNAs in male factor infertility is discussed below.

Short non-coding RNAs

Short ncRNAs have been investigated extensively during the past decade (Huang *et al.* 2013). Two short ncRNA classes, sperm piRNA and microRNA (miRNA), which play an essential role in spermatogenesis, have been implicated in male fertility disorders (Carmell *et al.* 2007, Houwing *et al.* 2007, Das *et al.* 2008). Capra *et al.* recently investigated piRNAs and miRNAs derived from high motile and low motile cryopreserved bovine spermatozoa populations, through small RNA sequencing, and found that a number of miRNAs were differentially expressed in the two populations (Capra *et al.* 2017). Moreover, they found that these miRNAs targeted genes involved in apoptosis, mitochondrial membrane integrity and spermatogenesis alteration, indicating a possible role in bull fertility. In a study by Pantano *et al.*, small RNA sequencing revealed an abundance of piRNAs derived from pseudogenes (Pantano *et al.* 2015). The authors proposed that these pseudogene-derived piRNAs may regulate their parent gene expression during sperm development and therefore may be required for fertility. Analysis of spermatozoa from normozoospermic fertile and normozoospermic infertile patients, using the TaqMan Array Human MicroRNA, has revealed that normozoospermic fertile and infertile individuals convey distinct miRNA cargos that may have implications in male reproductive performance (Salas-Huetos *et al.* 2016).

Recent studies have shown that the injection of specific subsets of miRNAs into normal zygotes can reproduce paternal phenotypes which can be followed into subsequent generations, suggesting that these miRNA can induce changes that are maintained in the germline. tsRNAs, which are derived from the 5' end of tRNAs, have also been shown to be involved in epigenetic inheritance of acquired traits. By injecting sperm tsRNA from mice that were on a HFD into normal zygotes, researches have shown that metabolic disorders observed in the father can be recapitulated in the offspring (Chen *et al.* 2016).

In non-mammalian species, the recently discovered phase small-interfering RNAs (phasiRNA), that bear similarities to mammalian piRNAs (Zhai *et al.* 2015), have been shown to be important for male meiosis and fertility in plants (Dukowic-Schulze *et al.* 2016, Fan *et al.* 2016). A study using photoperiod-sensitive

male sterility (PSMS) rice (*Oryza sativa* L.) demonstrated the potential biological importance of (phasiRNA), which are an emerging class of 21- or 24-nt small RNAs generated from precursor RNA. This study showed that accumulation of a 21 nt *PMS1T*-phasiRNA eventually caused male sterility in the rice, although further studies are needed to elucidate the mechanistic pathway involved (Fan *et al.* 2016).

Long non-coding RNAs

lncRNAs have gained considerable attention in recent years. They are, generally, polyadenylated non-coding RNA molecules that are RNA Polymerase II transcribed. Although classified as non-coding, it should be noted that a number of lncRNAs have been shown to harbour short open reading frames (sORFs) that can be translated into short peptides (Rohrig *et al.* 2002, Galindo *et al.* 2007, Kondo *et al.* 2007, 2010, Magny *et al.* 2013, Anderson *et al.* 2015). lncRNAs have been shown to possess a variety of functions such as transportation of mRNA from the nucleus (Chen & Carmichael 2009), acting as competing endogenous RNAs for miRNAs (Cheng & Lin 2013) and decoys for DNA-binding proteins (Kino *et al.* 2010). Some well characterised lncRNAs are involved in regulating imprinted gene expression and X chromosome inactivation and include *Xist* (Brockdorff *et al.* 1992), *H19* (Brannan *et al.* 1990) and *AIRN* (Sleutels *et al.* 2002). For an in-depth review on the evolution, genomic contexts, biological functions, and mechanisms of action of lncRNAs (Kung *et al.* 2013). lncRNAs have pivotal roles during animal sperm development, for a comprehensive reviews on lncRNA during spermatogenesis (Luk *et al.* 2014, Zhang *et al.* 2016).

It has been reported that type 1 and type 2 diabetes mellitus can negatively impact fertility (Singh *et al.* 2014, Wiebe *et al.* 2014) and there is a great amount of data indicating that lncRNAs are involved in diabetes mellitus, reviewed in (He *et al.* 2017). A large number of lncRNAs (and mRNAs) are differentially expressed in sperm isolated from diabetic and non-diabetic mice models (Jiang *et al.* 2016), therefore it is possible that some of these lncRNAs are related to diabetes-related male infertility – however larger studies are needed to confirm or rule this possibility out. lncRNAs have also been shown to play a role in sperm motility (Liu *et al.* 2017). Using Beijing-you cocks with divergent sperm motility scores, Liu and colleagues identified several lncRNAs that were differentially expressed in the testis between animals with high and low sperm motility. Although this study provides some novel data linking lncRNAs to sperm motility (and potentially fertility), analysis of lncRNA in the high and low motility sperm, and the fertility of said sperm, would aid in the identification of motility-associated lncRNAs.

Although having important roles during spermatogenesis and potentially being important for male fertility, some lncRNAs have been shown to be indispensable for fertility. Despite being enriched in the sperm nucleus, transgenic mice carrying homozygous null mutations for the lncRNA, *Malat1*, have no identifiable defects in fertility (Zhang *et al.* 2012, Johnson *et al.* 2015). Similarly, knockout of the testis-specific lncRNA, *Tsx*, results in viable fertile offspring (Anguera *et al.* 2011). These studies show that although lncRNAs can be important for germ cell development they may not be required for fertility; and thus highlight the requirement for further studies aimed at identifying the role of lncRNAs in male fertility.

Modification of RNA

In addition to the involvement of a variety of RNA molecules in male fertility, modifications to RNA itself have also been implicated in fertility. Although there are in excess of 140 modified RNA nucleotide variants, one RNA modification that is getting a lot of attention recently is N⁶-methyladenine (m⁶A), in which an adenine base is methylated at the 6' position (Fu *et al.* 2014). m⁶A is the most abundant mRNA/lncRNA modification discovered thus far, and is the only modification that has exhibited characteristics of epigenetic regulation including readers, writers and erasers (Dominissini *et al.* 2012, Meyer & Jaffrey 2014, Liu & Pan 2015). m⁶A has been implicated at virtually every level of mRNA regulation including pre-mRNA splicing, mRNA export, and stability, and translation of mRNA (Lin *et al.* 2017). Due to m⁶A's extensive involvement in many biological processes, it has been associated with cancer progression (Batista *et al.* 2014, Geula *et al.* 2015) and many diseases including obesity and diabetes (Zeggini *et al.* 2007, Jia *et al.* 2011).

Work by Yang and colleagues has identified an association between levels of m⁶A in human sperm and fertility (Yang *et al.* 2016). The authors revealed that individuals with asthenozoospermia have increased levels of m⁶A compared to controls and suggested that increased levels of spermatozoal m⁶A is a potential risk factor for decreased sperm motility (Yang *et al.* 2016). The m⁶A modification has also been shown to be functionally vital for the survival of cells in culture in a study demonstrating that siRNA knockdown of *METTL3*, the methyltransferase known to be involved in m⁶A methylation, results in apoptosis (Lin *et al.* 2016). Another study showed that inactivation of either *Mettl3* or *Mettl14* in a male germ-cell specific manner resulted in a lack of m⁶A, which results in translational dysregulation and subsequently, a depletion of spermatogonial stem cells (SSC), although normal spermatogenesis was observed (Lin *et al.* 2017). However, this study also showed that double deletion of *Mettl3* and *Mettl14* results in impaired spermiogenesis due to translational dysregulation of

factors that are key to spermatogenesis (Lin *et al.* 2017). m⁶A has been shown to be dynamically regulated at different developmental stages during spermatogenesis, with m⁶A enrichment occurring in pachytene/diplotene spermatocytes and round spermatids (Lin *et al.* 2017). These studies suggest that mRNA m⁶A modifications are critical to the temporal regulation of translation during spermatogenesis by marking genes that are key factors during spermatogenesis, stabilising those transcripts, increasing their translation, and preventing translation of genes that would be deleterious by erasing the m⁶A modification.

Mutation of a gene that is involved in the regulation of RNA modifications has also been shown to potentially influence male fertility (Zheng *et al.* 2013). A mutation in the gene that encodes ALKBH5, an m⁶A demethylase, causes increased levels of m⁶A, which also affects the export and metabolism of mRNA, and results in aberrant spermatogenesis, low sperm count, poor quality sperm and impaired fertility (Zheng *et al.* 2013). ALKBH5-deficient mice have been shown to have decreased testes size (Tang *et al.* 2018) and a reduced breeding success rate (Zheng *et al.* 2013), which has steered researchers focus to ALKBH5's role in spermatogenesis. Subsequent studies by an independent group have shown that ALKBH5 is required for late meiotic and haploid phases of spermatogenesis (Tang *et al.* 2018). To study m⁶A modification in mRNA and lncRNA researchers use a method referred to as m⁶A-seq or MeRIP-seq in which an m⁶A antibody is used to immunoprecipitate RNAs with the m⁶A modification, followed by high-throughput sequencing (Tang *et al.* 2018). Due to the advancement in deep sequencing technologies, researchers have been able to map m⁶A modification using this method at a single nucleotide resolution and have found these modification to be enriched around stop codons and internal exons (Liebers *et al.* 2014, Linder *et al.* 2015). These advancements will help researchers to discovery novel roles of m⁶A modification and other RNA modifications alike.

Concluding remarks

Deciphering the underlying cause of male infertility, and determining the key molecular players, still remains a major challenge in reproductive medicine. This review highlights that there are many epigenetic factors involved with multiple layers of gene regulation at multiple genomic loci, related to sperm function and potentially important for fertility. As with any field of biological research, technical and biological limitations need to be carefully considered, the utility of sperm epigenetics and potential hurdles have been discussed in-depth recently by Jenkins and colleagues (Jenkins *et al.* 2017). However, carefully designed future studies investigating epigenetic modifications of the sperm genome and the vast suite of sperm-borne RNA molecules, and their

modifications, are warranted and will help to shed more light on their involvement (cause or correlation) with male infertility and help with the development of new diagnostics and possibly therapeutics. Male infertility research will greatly benefit from studies using larger sample sizes that integrate multiple epigenomic analyses (integromics). This will help in the identification of robust epigenetic markers of fertility/infertility.

The majority of the studies examining the contribution of epigenetic processes to infertility discussed in this review have used samples consisting of large numbers of sperm. Given that sperm are one of the most diverse cell types (Ramon *et al.* 2014) and that sperm have been reported to be epigenetically heterogeneous (Laurentino *et al.* 2016), future investigations using high-throughput single cell epigenomics will be important to help determine whether aberrant infertility-related epigenetic marks are present in all spermatozoa of the ejaculate. Finally, advising couples suffering from UMI, and males in general, on known risk factors such as smoking (Dong *et al.* 2016, Laqqan *et al.* 2017b), obesity (Katib 2015, Craig *et al.* 2017) and alcohol consumption (Ouko *et al.* 2009, Lim & Song 2012) may help to reduce infertility-related epigenetic aberrancies in sperm and go some way on the long road to understanding and potentially treating male factor infertility.

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