3 The Sperm Epigenome: Implications for Assisted Reproductive Technologies

Douglas T. Carrell

Abstract

Compared to other cells, sperm undergo dramatic remodeling of their chromatin during late spermiogenesis in which approximately 95% of histones are removed and replaced with protamines. Despite this large-scale remodeling, key developmental genes, some miRNA genes, and imprinted genes retain their association with histone. The developmental genes have a unique epigenetic signature, termed bivalency, that poises the genes for embryonic activation. Anomalies in that epigenetic poising signature, either in the form of DNA methylation aberrations, improper protamination, or altered histone modifications, are associated with infertility and reduced embryogenesis capability. Additionally, some small noncoding RNAs are retained, while others are actively added to the sperm and appear to affect embryogenesis. Therefore, initial studies have begun to formulate pathways by which the sperm epigenome can be used as a diagnostic tool in the clinic. While in their infancy, these assays likely portend improved diagnostics and added information for patients and clinicians. Recent studies also highlight the possibility that the sperm epigenome can be used to evaluate lifestyle and environmental risks to the patient and potentially to the offspring.

Keywords

ART · Embryogenesis · Epigenetics · DNA methylation · Histones · Small RNAs · Environment

Introduction

Epigenetics, a term first coined by Conrad Waddington in the 1940s, is generally defined as having three major components. First, epigenetic "marks" are stable, non-DNA coding (polymorphisms or mutations) changes that, second, affect gene expression. Third, epigenetic alterations are heritable, generally defined as being passed at least to the F3 generation (Waddington 1942; Holliday 2006). Epigenetics is both practically and historically linked to the field of reproductive biology, since such gene expression changes are the key to cellular differentiation during embryonic development and since the term "epigenetics" is derived from "epigenesis," the embryological concept of "stepwise" development of the embryo that has been discussed since the times of Aristotle, but particularly debated in the seventeenth and eighteenth centuries during

D. T. Carrell (✉)
Andrology and IVF Laboratories, Department of Surgery, and Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, UT, USA
e-mail: douglas.carrell@hsc.utah.edu

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the debates of the "preformation theory" of development versus the epigenesis theory of development (Harvey 1651, 1653). Since the term epigenetics was first coined, epigenetics has moved into a much broader realm, including the study of environmental influences on gene expression and disease etiologies (Jirtle and Skinner 2007; Deans and Maggert 2015).

Numerous studies have now demonstrated the powerful effects of alterations of the epigenome due to environmental or lifestyle factors on subsequent health. Among the best examples are studies evaluating famine or diet alterations at specific developmental time periods, such as the prenatal, perinatal, and peri-pubertal periods. For example, children born during the 1944–1945 Dutch famine period have been shown to have an increased risk of heart disease and obesity if their mother was exposed to the famine, apparently due to a change in DNA methylation to the insulin-like growth factor 2 (ILGF2) gene (Painter et al. 2005; Heijmans et al. 2008). In another example, paternal grandsons of prepubertal boys exposed to famine in the Overkalix region of Sweden have been shown to have increased mortality, although no effects were seen for the paternal grandmother or maternal grandparents (Pembrey et al. 2006). These studies highlight the ability of environmental changes, diet in these cases, to alter the epigenome through sperm, as well as highlighting the potential of abnormal epigenetics to affect the health of offspring and progeny.

As is often the case, the first studies of the sperm epigenome were not aimed at defining the normal sperm epigenome in normal development, rather the result of a possible associated disease risk. The earliest studies of the sperm epigenome were the result of a reported increase in the incidence of imprinting diseases, a form of epigenetic abnormality, in the offspring of individuals conceived using intracytoplasmic sperm injection (ICSI) during assisted reproductive therapy (ART) (Cox et al. 2002; Kobayashi et al. 2007; Le Bouc et al. 2010). Imprinting diseases are the result of improper sex-determined allelic methylation, and some studies demonstrated that the sperm of men undergoing ICSI had increased levels of abnormal DNA methylation, associated with decreased sperm counts. These studies opened the door to a deeper evaluation of the sperm epigenome and the role it may play in embryogenesis and the health of the offspring. While still in its infancy, the role of the sperm epigenome in embryogenesis is becoming better understood, as well as the role of certain lifestyle and environmental factors in altering it. This chapter explores these advances in terms of the potential to offer improved diagnosis and treatment of infertility through ART and in terms of ultimately better understanding the transmission of health risk to offspring through the sperm epigenome.

The Sperm Epigenome: Protamination and Histone Modifications

During late spermiogenesis, sperm undergo a dramatic remodeling of the sperm chromatin in which approximately 95% of the histones are sequentially replaced, first by transition proteins and then by protamines (P1 and P2) (see Fig. 3.1). The replacement of histones facilitates a higher order of DNA packaging, up to 20 times more than DNA in somatic cells, and is useful in providing a compact sperm head consistent with sperm motility requirements. Lastly, it is believed that the compaction of the DNA into toroidal structures also protects the DNA from oxidative stress while the sperm traverse the female reproductive tract. In fertile men, P1 and P2 replace histones with an approximate ratio of 1:1 (Carrell and Liu 2001), and alterations in the P1/P2 ratio reflect abnormal protamination and are associated with reduced semen quality, increased sperm DNA fragmentation, and reduced fertilization capabilities and embryo implantation in couples undergoing IVF (Aoki et al. 2005, 2006a; Carrell et al. 2008; Hammoud et al. 2009a; Carrell 2012).

Using a mouse model, intracytoplasmic sperm injection (ICSI) of sperm containing altered histone/protamine ratios and high DNA fragmentation resulted in embryos with a lower competency (Cho et al. 2003). Generally, there is a consensus

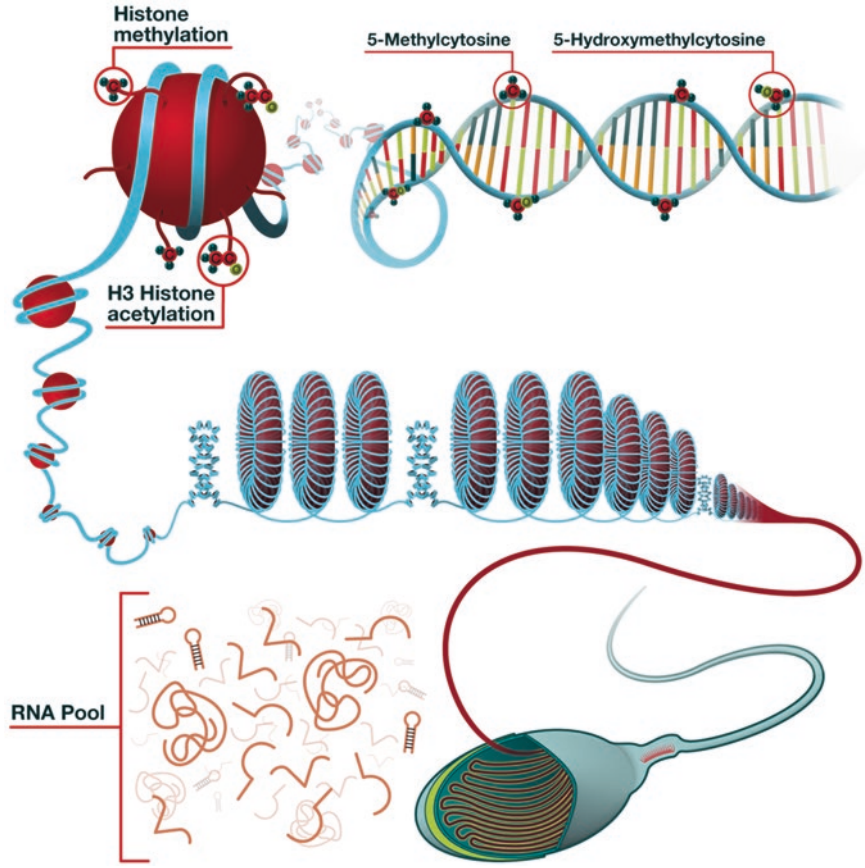

Fig. 3.1 An overview of the sperm epigenome. This figure highlights the repackaging of the sperm chromatin with protamines, with interspersed histones at key loci, including developmental gene promoters. The packaging of the genome with protamines facilitates higher-order chromatin compaction, including the formation of toroids. The figure shows the three key components of the sperm epigenome: histone modifications, DNA methylation, and the pool of RNAs, some of which are miRNAs and tRFs

that aberrant protamination is associated with increased DNA fragmentation and reduced embryo quality (Carrell and Liu 2001; Aoki et al. 2006a, b, c; Cho et al. 2003).

In addition to the role of protamination on protecting sperm DNA, the replacement of 95% of histones begs the question of if there is a role for the remaining histones—perhaps an epigenetic role? Stated differently, for such an evolutionary important aspect of reproduction, why would protamination be so inefficient as to leave 5% of the genome non-protaminated? Lastly, one would also hypothesize that if there were a role for retained histones, their loci of retention would be consistent and may suggest a biological role. Studies by Hammoud et al. were initially undertaken to help answer those questions and included genome-wide analysis of the loci of histone retention, specific histone modification analysis, and evaluation of DNA methylation status genome-wide and found that retained histones are found at consistent and deliberate locations throughout the sperm genome, including key developmental genes, poising these genes for activation during early embryogenesis (Hammoud et al. 2009b). These findings imply that proper protamination, and likewise normal retention of specific histones, is not only important in regard to a reflection of male-factor (MF) infertility status, as a reflection of abnormal spermatogenesis, and in regard to protecting the genome from DNA damage but also suggested a

role in paternal contributions to normal embryogenesis.

The hypothesis that retention of sperm histones at key developmental loci is of biological significance is also dependent on proper histone modifications, since specific modifications can either facilitate or preclude transcription by making gene promoters accessible or inaccessible to transcription factors. Histone tail modifications are a major class of epigenetic regulators in somatic cells. Briefly, acetylation of H3 and H4 as well as methylation of H3K4 results in an "open" state of genes that facilitates transcription. Conversely, methylation of H3K9 and H3K27 and deacetylation of H3 and H4 drive a chromatin state which silences genes at those loci (Jenkins and Carrell 2011; Jenuwein and Allis 2001). Hammoud et al., and subsequently others, demonstrated that in human sperm, the modifications of histones, associated with developmental genes, are unique in that there is bivalency as both marks containing both H3K4me3 activation marks and H3K27 silencing marks are present, similar to what is found in some embryonic stem cell gene loci (Hammoud et al. 2009b). This arrangement suggests a "gene poising" of key genes involved in embryonic development. Interestingly, many IVF patients with altered embryogenesis capability have been shown to exhibit defects in this poising pattern (Hammoud et al. 2011). Furthermore, this unique poising pattern of embryonic developmental genes has been confirmed in zebrafish, an evolutionarily distant species (Murphy et al. 2018a).

The Sperm Epigenome: DNA Methylation

DNA methylation is the major regulator of gene transcription and technically easier to evaluate than histone modifications; therefore, more studies have reported DNA methylation status of the human sperm than those evaluating histone modifications. While targeted sequencing studies can be employed following bisulfate conversion, many studies have used arrays to screen many loci and evaluate possible associations between DNA methylation alterations and various phenotypes of male infertility. The arrays offer the advantages of ease of use, as well as screening many possible CpG loci. Hammoud et al. and others have demonstrated that the normal male sperm epigenome has variable methylation at the key developmental loci described above that contain bivalent histone modifications, thus strengthening the poising hypothesis (Hammoud et al. 2009b). Furthermore, several early studies have observed DNA methylation aberrations in sperm with abnormal chromatin packaging, sperm from men who generate embryos of poor quality while undergoing in vitro fertilization, as well as infertile men (Hammoud et al. 2010, 2011; Aston et al. 2012, 2015; Nanassy and Carrell 2011).

Sperm DNA methylation is also important in terms of genomic imprinting, a system in which certain genes are methylated or demethylated based on whether the locus is inherited from the father or mother. Prior to zygotic genome activation in the early embryo, DNA methylation patterns acquired from the sperm and oocyte are actively and passively demethylated and then reset later in the primordial germ cells (Messerschmidt et al. 2014). This process has led some to minimize the potential importance of DNA methylation in epigenetic inheritance; however, it is known that not only imprinted genes but other regions of the genome escape this reprogramming event in early embryos, and at least in the case of imprinted loci, the methylation signature provided to the embryo by sperm is maintained (Messerschmidt et al. 2014; Reik and Walter 2001). Some methylation signatures beyond imprinted regions are retained in the embryo and are involved in modulating development and affecting phenotype transgenerationally. Such signatures have been identified, including methylation patterns inherited via sperm (Guibert et al. 2012; Illum et al. 2018; Seisenberger et al. 2012). One study found that the female offspring of male rats consuming a high-fat diet displayed multiple characteristics consistent with metabolic phenotypes, including reduced birthweight, decreased pancreatic beta-cell mass, and glucose intolerance (Barbosa et al. 2015). DNA methylation analysis was conducted

on the sperm from the F0 high-fat-fed rats and their F1 male offspring, and multiple methylation alterations were observed when compared to control rats, and in fact many differentially methylated regions were concordantly observed in both the F0 and F1 male sperm, suggesting a possible mechanism for transgenerational inheritance of metabolic disease.

Given the early data described above in which methylation errors at imprinted loci were found to be more common in men with abnormal spermatogenesis, and particularly men with low sperm counts, it was imperative to evaluate the methylation status of DNA from sperm of men with broader types of male-associated infertility. In one such early study, Aston et al. evaluated several thousand loci, using an early array system, in sperm from men with either aberrant protamination or men with unexplained poor embryogenesis while undergoing IVF[27]. Interestingly, more than 7% of all loci evaluated were abnormally methylated in these patients, and more than 60% of imprinted loci were aberrantly methylated. This study was supported by numerous other studies and focused attention on the potential use of sperm DNA methylation analysis as a potential screen for IVF embryogenesis outcome, as will be discussed below (Jenkins et al. 2016a; Karaca et al. 2017; Laqqan and Hammadeh 2018; Santi et al. 2017).

The Sperm Epigenome: Small, Noncoding RNAs

Although gene transcription does not occur in a mature sperm, sperm contain many RNA species that are stable in the embryo following fertilization (Ostermeier et al. 2004; Pessot et al. 1989). Sperm RNAs include remnant mRNAs from spermatogenesis (Ostermeier et al. 2002, 2004), mRNAs that may be functionally important to the developing embryo (Ostermeier et al. 2002; Jodar et al. 2015; Sendler et al. 2013), and a variety of noncoding RNAs (Krawetz et al. 2011). Recently, much of the research on sperm epigenetic factors and epigenetic-mediated inheritance has focused on the small, noncoding RNAs (Conine et al. 2018; Liu et al. 2012; Chen et al. 2016; Sharma et al. 2016; Zhang et al. 2018).

Studies in the mouse have shown that there are two major sources of noncoding RNAs in sperm. First, sperm contain a large number of piRNAs that are remnants of spermatogenesis. Second, during epididymal transit, there appears to be a significant remodeling of the RNAs, with some RNAs apparently removed in the caput epididymis and then subsequently replaced, along with other species of RNAs. This replacement and remodeling appears to occur largely through epididymosomes, exosomes that are secreted in the epididymis and attach to sperm and transfer their contents. These epididymosomes transfer a large contingent of miRNAs and tRNA fragments (tRFs), as well as proteins necessary in the acquisition of sperm motility and fertilization ability. Interestingly, the RNA payload of a sperm isolated from the cauda epididymis is similar to a sperm isolated from the testis, but RNAs are removed in the caput epididymis and then are subsequently replaced via the epidiymosomes (Sharma et al. 2018).

In an elegant study by Conine et al., it was shown that some of the RNA species that are lost and subsequently regained by sperm cells transiting the epididymis are associated with improper embryonic implantation as well as gross defects in embryonic development[47]. Using caput and cauda sperm to generate mouse embryos, Conine et al. reported that caput-derived embryos showed significantly reduced rates of successful implantation, gross embryo morphology defects, and a reduced number of viable offspring (Conine et al. 2018). They then showed that the embryos could be "rescued" by injection of the miRNA fraction of epididymosomes. These results suggest that the miRNAs delivered to sperm during epididymal transit are required for proper preimplantation gene expression in the mouse (Conine et al. 2018). The miRNAs injected included miR-34c, which has previously been shown by another group to be essential for the first cleavage division in mouse embryos (Liu et al. 2012). These two studies strongly suggest a role for spermatozoal RNAs, specifically miRNAs, in embryogenesis.

Studies evaluating the effects of diet changes have implicated spermatozoal RNAs in epigenetic inheritance of metabolic disease from fathers. Altered metabolic phenotypes have been observed in the offspring of male mice consuming high-fat or low-protein diets. These offspring display a phenotype characterized by glucose intolerance and impaired insulin secretion (Barbosa et al. 2015; Chen et al. 2016; Sharma et al. 2016; Carone et al. 2010; Ng et al. 2010). Spermatozoal RNAs, especially tRFs whose effects appear to be mediated by DNA methyltransferase 2 (DNMT2), have been implicated in this form of epigenetic inheritance (Chen et al. 2016; Sharma et al. 2016; Zhang et al. 2018). Jodar et al. rescued diet-affected zygotes by microinjection with RNAs isolated from control-diet sperm (Sharma et al. 2016). These studies highlight the ability of spermatozoal RNAs to affect gene expression patterns in the embryo and the possible role of sperm RNAs in epigenetic inheritance.

Using the Sperm Epigenome to Predict Fertility or ART Outcome

The bivalent poising of the human sperm epigenome strongly suggests a role in normal embryogenesis, and similar poising motifs are observed in diverse species, again suggesting an evolutionary role of importance (Hammoud et al. 2009b; Wu et al. 2011). Additionally, numerous studies have reported associations among abnormal sperm DNA methylation, histone replacement, histone modifications, or RNA complements with reduced male fertility, altered spermatogenesis, and altered embryogenesis capability during IVF (Jenkins et al. 2017; Gannon et al. 2014). Therefore, the possible use of the sperm epigenome as a diagnostic tool for couples undergoing infertility evaluation is apparent and has begun to be the focus of some researchers (Carrell 2012).

Aston et al. initially set out to determine the predictive role of sperm methylation patterns in patients who had undergone IVF treatment (Aston et al. 2015). Previous IVF patients were classified on whether their sperm generally generated good-quality embryos (normal blastocyst morphology) and positive pregnancies or generated an unusually high rate of poor morphological quality embryos. Sperm from these two groups were compared to sperm from men of known fertility and analyzed using machine-learning techniques to develop predictive algorithms. Surprisingly, this study found that predictive models based on methylation array data from these groups were highly predictive of male fertility status. In other words, IVF patients, who were not exclusively classified as having male-factor infertility, could with good accuracy be identified from men of known fertility. Interestingly, the most accurate algorithm was able to predict fertility status using a relatively low number of CpG loci, which were enriched for imprinted loci. This finding was surprising but strengthened by a concurrent study in which time to pregnancy was evaluated in young couples not presumed to be experiencing infertility and which also identified DNA methylation markers apparently predictive of fecundity (Jenkins et al. 2016b).

Additionally, hierarchical clustering was capable of identifying clusters containing IVF patients and poor embryo quality samples based on methylation array data, and a predictive algorithm was developed (Aston et al. 2015). While the methylation changes observed between these groups were not biased toward genomic regions of any particular annotation category, such as imprinted regions, these data show that global alterations in sperm methylation can be predictive of male fertility status and potentially embryo quality during IVF treatment (Aston et al. 2015). The data also imply that, as is well understood, embryogenesis includes a broad complement of gene pathways, and it is likely that poor embryogenesis is not the result of a dominant defective pathway, but rather may include a diverse set of defects in a myriad of pathways in a cohort of patients. The initial loci reported by Aston et al. were used by Abbasi et al. to develop a simplified and accurate testing platform for possible patient testing (Abbasi et al. 2018). The utility of such platforms will become apparent with further usage.

Recently, Denomme et al. studied on the epigenetic evaluation of embryos derived from male-factor (MF) infertility patients compared to controls and reported a dysregulation of DNA methylation in the embryos of the MF-derived embryos, including in genes involved in regulation of cellular metabolic processes (Denomme et al. 2018). While the overall pregnancy rates were similar for the two groups, the MF-derived embryos with altered methylation were associated with an increased miscarriage rate. In a separate study that eliminated female-factor confounders by using only couples employing donor oocytes, this group also found differences in sperm DNA methylation and miRNAs, as well as embryonic gene expression, in "good embryo quality" patients versus "poor embryo quality" patients (Denomme et al. 2017). These studies strengthen the potential use of methylation data to predict IVF outcome and fertility.

At present, several studies are underway with the intent of validating the above studies. Similarly, other aspects of reproduction and infertility are also being evaluated. For example, the role of inherited DNA methylation defects in unexplained, recurrent miscarriage is one area of intense interest (Ankolkar et al. 2012; Rotondo et al. 2012; Spinelli et al. 2019). Additionally, studies are beginning to identify abnormalities in sperm DNA methylation associated with environmental exposures, including bisphenol A (Dere et al. 2018), mercury (Lu et al. 2018), pesticides (Pallotta et al. 2019; Skinner et al. 2018), phthalates (Tian et al. 2018), vinclozolin (Beck et al. 2017), tobacco (Murphy et al. 2018b), and other chemicals (Siddeek et al. 2018). Interestingly, studies have also reported changes in the sperm methylome associated with paternal aging, including a stepwise increase in the number of abnormally methylated loci as during aging, beginning in approximately the mid-30s (Jenkins et al. 2013, 2014). This issue is of clinical relevance due to the increasing societal trend of delaying pregnancy until later paternal ages.

The concept that the sperm epigenome is altered by environmental exposures, age, and life events and decisions, coupled with the emerging understanding that epigenetic defects can be transmitted transgenerationally, suggests that in the future it may be able to screen potential fathers for sperm epigenetic abnormalities with the objective of identifying potential risks to offspring and progeny, in addition to infertility risk, and motivating preconception lifestyle changes to mitigate that risk (Jenkins et al. 2018). Such uses of the sperm epigenome are distant but may provide additional benefits to patients and offspring.

Conclusions

This chapter has provided a brief overview of the sperm epigenome and its potential use as a diagnostic tool to predict male infertility, to assess sperm competency in developing normal embryos, and possibly as a means to assess environmental and lifestyle risks. With the growth of the field in the past 10 years, it is likely that the future is bright in regard to meaningful advances that will directly benefit patients and clinicians, as well as aid society at large.

The existing data clearly show an implied mechanism for the sperm epigenome in regulating or facilitating early embryogenesis. Additionally, the studies described above clearly show associations with aberrant sperm epigenomes and diminished embryogenesis capacity. Lastly, early studies have demonstrated an ability to predict embryogenesis ability. At this time, the field awaits independent, large-scale validation studies before these technologies can be implemented in the clinic. Similarly, the sperm epigenome also provides a historical record of spermatogenesis, and numerous studies have shown that altered spermatogenesis is associated with altered sperm DNA methylation, particularly of imprinted genes. However, validation studies are also needed in this regard before sperm epigenetic testing can be used as a screen of fertility status. The use of the sperm epigenome as a toxicology tool and as a means to assess risk to progeny is a more distant goal, but very intriguing. While associations are strong, studies are needed to better understand the biology involved in DNA reprogramming in the embryo and fetus and the means of transmission

to progeny. Most importantly, it will be imperative that such assays quantify risk in a clinically useful manner.

To this point, sperm DNA methylation has been the major focus of studies evaluating the possible development of diagnostic tools. This is due to cost and technical feasibility issues. However, it is important that evaluation of histone modifications and RNAs continue to be a focus, since it is likely that epigenetic pathways using these markers are of high relevance to reproduction.

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