Chapter 4
The Sperm Epigenome: Implications for the Embryo

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Abstract Recent advances, including the human genome project and numerous studies of cancer and other diseases, have shown that the genetic code is not simply limited to the sequence of the four bases of DNA but also includes epigenetic programming, heritable changes that affect gene expression [Riggs A, Martinssen R, Russo V (2007) Introduction. In: Riggs A, Martinssen R, Russo V (eds) Epigenetics mechanisms of gene regulation. Cold Spring Harbor Press, New York]. The science of epigenetics is important in understanding many diseases and biological processes, including in identifying the causes of disease and better understanding the mechanisms by which the environment can affect gene expression [Carrell Fertil Steril 97 (2):267–274, 2012]. This chapter will focus on the epigenome of sperm and particularly highlight the potential role of the sperm epigenome in embryogenesis.

The sperm epigenome is unique and highly specialized because of the unique nature and function of sperm and because of the diverse requirements for successful fertilization. Due to the need for motility, sperm chromatin must be compacted and highly organized. During spermiogenesis the chromatin is packaged tightly into the sperm head by the replacement of most histones with protamines. This allows for protection of the DNA from the hostile environment in the female reproductive tract. Remaining histones can have chemical modifications to the tails of the protein that either facilitate or repress gene transcription. Sperm, like embryonic stem cells, have a unique pattern of histone modifications that includes both activating and silencing marks in the promoters of genes associated with development. These bivalent

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marks, along with DNA hypomethylation, comprise a unique state in which the key genes are “poised” for possible activation in embryogenesis. Sperm epigenetic abnormalities have been linked with multiple diseases including male factor infertility and poor embryogenesis.

Keywords Epigenetics • Protamine • Histone • DNA methylation • Embryogenesis

Introduction

Embryogenesis, the process by which an embryo develops, includes a complex uniting and interplay of the maternal and paternal haploid genomes (Ostrup et al. 2012). It is well established that the haploid genomes include the DNA sequence coding for genes and unique “marks” on both the DNA and the histones that comprise the nucleosomes required for packaging the chromatin (Rivera and Ross 2013). These marks, which regulate gene activation and transcription via chemical modifications to the DNA or histones, are termed epigenetic modifications. Epigenetic programming helps define the cell function, and abnormalities are often associated with diseases, such as cancer.

The sperm nucleus is a highly organized and complex structure and unique compared to all other cell types (Ward 2010). The DNA in somatic cells is wrapped around octamers of histones, the protein responsible for complex packaging and organization of DNA. Histones function to regulate, protect, and organize DNA in both somatic and germ cells. Male germ cells are the only cells that express protamines, a secondary nuclear compaction protein that provides an even higher order of chromatin compaction. In the human, most of the sperm genome is packaged by protamines rather than histones (Brewer et al. 2002). However, approximately 5–15 % of the genome remains bound to histones (Hamatani 2012). The retention of sperm histones allows the possibility that the histones may have a programmatic function through the epigenetic modification of the histones as well as methylation of the DNA itself. This concept has initiated an exciting field of study to describe and evaluate the sperm epigenome and its possible role in embryogenesis. This chapter will explore those studies.

Histones, Protamination, and the Nuclear Matrix

The evaluation of the sperm genome must begin with the unique manner in which sperm DNA is packaged. Three structural elements in sperm function to create sperm chromatin, the protamines, retained histones, and the nuclear matrix (Conaway 2012). Each element has a specific and distinct role in paternal genetic influences on fertilization and embryogenesis. Sperm chromatin is by necessity a tightly bound, highly organized structure, but the DNA must also be packaged in a

manner that allows rapid decondensation and integration of the DNA into the zygotic genome, including the possibility of gene expression in the early stages of embryogenesis (Rivera and Ross 2013).

Histone-bound DNA is associated with the nuclear matrix in both somatic and gamete cell lines. Histones are alkaline proteins, which serve as a structure for DNA to wrap around, which they do approximately 1.6 times. Histones have five different subtypes (H1/H5, H2A, H2B, H3, and H4) that, when bound together, are known as a nucleosome (Conaway 2012). Nucleosomes are linked together by short stretches of linker DNA consisting of approximately 50 nucleotides. Human sperm are unique in that during the late stages of spermiogenesis, most of the histones are replaced by protamines (Fig. 4.1). Protamination is a multistep process that involves numerous enzymes and regulators, but it is essential for normal fertility.

Protamines are large, charged proteins containing positively charged arginine amino acids that can bind to the negatively charged phosphorus in DNA (Hud and Vilfan 2005). The interaction between the positively charged arginine fragments and the DNA backbone lead to tight coiling of the DNA, causing it to nearly appear hidden in the protamine; this structure is known as a toroid (Hud and Vilfan 2005) (Fig. 4.1). Protamine compaction is further achieved via disulfide bonds between cysteine bases. This further arranges the protamine into a tightly packed structure of toroids "stacked side to side like a package of Life Savers" (Hud and Vilfan 2005; Mudrak et al. 2009). The highly compacted protamine structure of sperm chromatin facilitates protection of the sperm chromatin. The protamines in mature spermatozoa are not found in any other type of somatic cell. When compared to the more loosely bound histone segments of the paternal DNA, the protamine structure appears more stable and less prone to modifications.

In fertile males between 5 % and 15 % of the spermatozoa chromatin remains bound to histones rather than protamines (Carrell 2012; Hammoud et al. 2011; Ward 2010; Jenkins and Carrell 2012). Through the process of specific histone modification that either facilitates or represses gene expression, histone retention in sperm may facilitate important epigenetic programming for regulation of the paternal genome during early embryogenesis. It is now known that histone placement and retention in the sperm is nonrandom (Hammoud et al. 2011). The work done by Hammoud et al. in 2009 and 2011 showed that in mature sperm histones were retained in regions of the genome that were related to gene promoters of developmentally important genes, including developmental genes, transcription factors, miRNAs, and imprinted genes. Furthermore, specific modifications to those histones correlated with an increased facility to be "accessible" to transcription factors, thus potentially poising the paternal genome for activation in the early stages of embryogenesis. Histones, specifically H3 and H4, have long tails that may be modified with covalent bonds; these modifications include methylation, acetylation, ubiquitination, and other chemical modifications that will be discussed in more depth throughout this chapter. The modifications have the ability to activate or suppress the activity of the DNA bound to the histone (Zentner and Henikoff 2013). When specific segments of the paternal DNA are activated or suppressed, we can see epigenetic effects on the offspring.

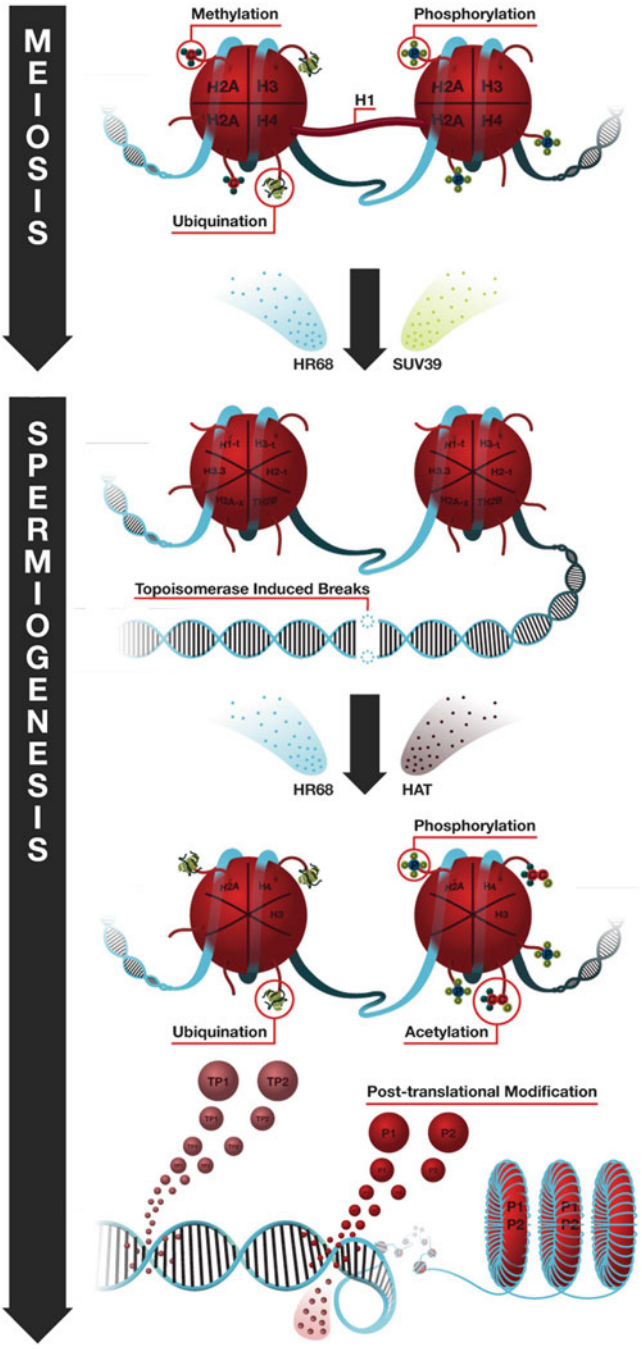

Fig. 4.1 (continued)

After successful fertilization of oocytes, sperm protamines are replaced with histones. Studies have shown that replacement of paternal protamines with maternal histones is achieved within the first 2–4 h after sperm penetration of the oocyte, with minimal initial activity of these particular encoding regions of DNA (Hammoud et al. 2011; van der Heijden et al. 2006). Paternal histone-bound segments are largely not initially replaced in the maternal oocyte, unlike the protamine segments (Ward 2010).

In addition to protamine and histone structure, there is a third method by which the paternal chromatin is organized within the nucleus. DNA not involved in a protamine toroid or histone solenoid is commonly referred to as a linker segment and is attached to the sperm nuclear matrix at matrix attachment regions at 50 kb intervals (Ward 2010). These linker segments are known as matrix attachment regions (MARs). The MARs appear to be involved as a checkpoint for sperm DNA integrity since sperm devoid of MARs fail DNA replication after intracytoplasmic sperm injection (ICSI) in the mouse model (Yamauchi et al. 2007a; Shaman et al. 2007). In addition to this role, MARs appear to act as promoters for the formation of the paternal pronucleus after fertilization (Ward 2010). The nuclear-matrix-attached regions serve as a required organizational step as the fertilized oocyte begins to divide (Shaman et al. 2007).

The nuclear matrix is a functional proteinaceous scaffold that also plays a role in gene expression (Pederson 2000). The scaffolding properties of the matrix are an intertwining of cytoskeletal elements such as actin and vimentin (Capco et al. 1982). The matrix is attached to the nuclear lamina and adds structural support to the chromatin and nucleus as a whole, but further investigation into the nature of MARs, chromatin organization, and matrix-associated mRNAs has shown a functional component as well (Cockerill and Garrard 1986). Recent studies indicate that this structural arrangement allows transcription machinery access to the open configuration of DNA within the nucleus; this has also been demonstrated in sperm (Kramer and Krawetz 1996; Ward et al. 1989). The loop organization of the chromatin is anchored to the matrix at the MARs every 20–120 kb (Vogelstein et al. 1980). This structure allows for one protamine-bound toroid per loop domain (Ward 1993). The strongest evidence for the functional nature of the sperm nuclear matrix in sperm is from a 1999 study by Ward et al. (1999) where mammalian sperm with a disrupted nuclear matrix were unable to support embryonic development. This study, and other

Fig. 4.1 The function of mature sperm requires extensive nuclear remodeling. The removal of many somatic canonical histones and the replacement with testis-specific histone variants is required for the early stages of germ cell differentiation, termed spermatogenesis. These testis-specific substitutions are also hallmarked by changes in expression regulation (epigenesis), such as the modification of histone tail marks and DNA methylation. The sperm morphology and chromatin modification are even more dramatic during spermiogenesis (late-stage spermatogenesis), where there is a stepwise replacement of the majority of canonical and testis-specific histones with transition proteins. Transition proteins are completely removed and replaced with protamines as the chromatin is wound into tightly packaged toroids. The toroid structure facilitates DNA protection, transcriptional quiescence, and efficient sperm motility. The DNA will remain in this state until nuclear decondensation during fertilization

functional studies (Yamauchi et al. 2007a, b), have shown that the sperm nuclear matrix and an organized chromatin configuration are indeed required for normal embryogenesis in mammalian models.

Sperm DNA Methylation and Histone Modifications

Methylation in the mature sperm nucleus is greatly reduced from other mammalian somatic cell types and cell lines. Methylation can regulate gene transcription by direct modification of DNA at cytosine residues (5-mC) that are located within gene regulatory regions, termed cytosine-phosphate-guanine dinucleotide islands (CpG islands) (Portela and Esteller 2010) or by histone tail modification (Jenuwein and Allis 2001). These types of marks are strong epigenetic regulators that can poise a gene or gene region for activation or suppression.

DNA hypomethylation (i.e., low levels of 5-MC) is a gene activation mark, while DNA hypermethylation causes interference of gene transcription machinery. Therefore, removal of methylation is necessary, but not sufficient, for gene expression. Enzymes that can actively remove DNA methyl marks in sperm have been surmised due to the temporal progression of demethylation during spermatogenesis, but these enzymes have yet to be elucidated (Ooi and Bestor 2008). The DNA methyltransferase 1 (DNMT1) is found in spermatogenesis and is responsible for de novo methylation and maintenance 5-MC (Eden and Cedar 1994).

While the majority of the sperm nucleus has been remodeled with sperm-specific protamines, 5–15 % of the chromatin remains histone-bound (Tanphaichitr et al. 1978; Wykes and Krawetz 2003). This fraction of the genome has been shown to be relevant to gene poising in the embryo and contains methylated histone marks for activation, inactivation, and gene poising (Hammoud et al. 2009). The most abundant histone methylation mark for gene activation is H3K4, while H3K9 and H3K27 modifications are gene repressors (Fig. 4.2). It has been shown that, in sperm, bivalent histone methylation (H3K9 and H3k27, both found in the same region) causes gene poising (Hammoud et al. 2009). Gene poising is the notion that genes can be *preprimed* for gene expression by setting up gene promoters for activation (Orford et al. 2008).

RNA in Sperm: An Epigenetic Factor?

There are approximately 10–400 femtograms of RNA present in mature spermatozoa (Miller and Ostermeier 2006). These cytoplasmic mRNAs are not actively translated due in part to the fact that there is an irregular distribution of the ribosomal subunits needed for mRNA translation within the mature spermatozoa cytoplasm (Miller and Ostermeier 2006). The mRNAs present in mature sperm appear to be selectively retained, and the profile of retained mRNAs has been shown to be

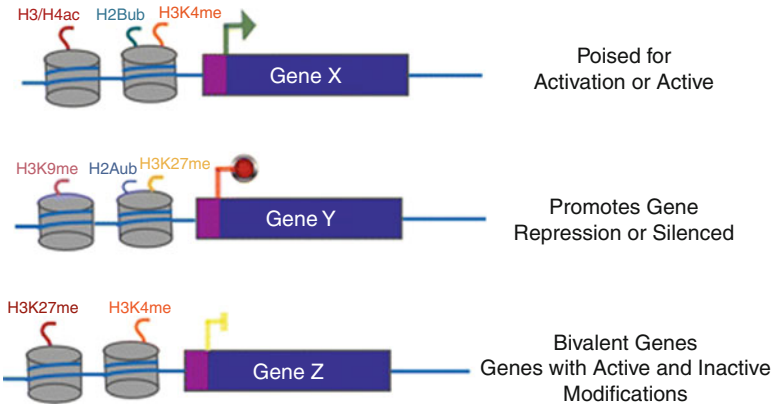

Fig. 4.2 Role of histone tail marks in epigenetic regulation. Histones play a critical role in gene activation, repression, and gene poising. This figure summarizes specific histone modifications that repress or activate transcription

reproducible when repeat analyses have been conducted on the same patient. Numerous studies in both hamsters and mice have demonstrated the inheritance and activity of spermatozoa-specific mRNAs in the early embryo and in offspring. This has perhaps best been elucidated by studies of the *Kit* locus, which codes for a tyrosine kinase receptor. When this was present, or inserted into the pool sperm mRNA, there was a clear and demonstrated inherited phenotype based upon the mRNA present while the DNA code was unchanged (Rassoulzadegan et al. 2006). This and other studies have shown that the select mRNA present in mature spermatozoa may have an epigenetic effect on the embryo. However, unlike other types of epigenetic regulation, RNA effects would not be heritable.

The exact epigenetic role mRNA plays in concert with the sperm chromatin is unclear. Whether they have an active role, helping to stabilize and promote the remaining histone-bound portions of the sperm chromatin, or they are passive bundles awaiting translation in the fertilized oocyte has not been clearly elucidated (Hamatani 2012). Further research on smaller noncoding segments of RNA, including miRNA and piRNA, have shown that they additionally may play a role as promoters or regulators of early embryogenesis. This is an important area for further development and research.

Postfertilization Epigenetic Remodeling

Following successful fertilization of the oocyte in the fallopian tube dynamic changes take place in both paternal and maternal chromatins in order for embryonic development to proceed. These dynamic and rapidly evolving changes transform two haploid cells into a diploid embryo. This transformation requires protamine

removal from the paternal chromatin and reestablishment of nucleosome-bound DNA with maternally derived histones. This complex transition is poorly understood, though we are beginning to gain intriguing insights into the process. Embryonic epigenetic reprogramming is essential in facilitating tissue-specific gene expression profiles requisite for proper embryo development (Jenuwein and Allis 2001).

Protamines located in the paternal chromatin are replaced by maternal histones soon after the fertilization event. Many studies have placed this timing within the first 4 h after fertilization, though the data are difficult to interpret due to the impossibility of directly studying healthy human embryos immediately following fertilization. As a result, our current understanding of the dynamics of human paternal pronuclear deprotamination in the early zygote is derived from mammalian studies of these events as well as heterologous (ICSI) with human sperm (Rodman et al. 1981; Nonchev and Tsanev 1990; Shimada et al. 2000). Though the precise timing is still controversial and poorly characterized, we do know that paternal chromatin relaxation (decondensation) occurs rapidly following fertilization and is likely driven directly by the removal of protamines. This event is essential in the future development of the embryo and is completed prior to syngamy (Wright and Longo 1988; Jones et al. 2011). Interestingly, the protamination of the paternal pronucleus, resulting in a quiescent chromatin structure, is incomplete as a result of events during spermatogenesis (Hammoud et al. 2009). The result is a few select regions of paternal histone retention. It is now believed that, because these regions of chromatin are in a more "relaxed" state, they may have the potential to be of great consequence in the early embryo. In fact, it has been demonstrated that the histone modifications in these regions of retention are in a poised state, similar to that of embryonic stem cells (Hammoud et al. 2009; Arpanahi et al. 2009). Current studies are attempting to elucidate the early activity of histone- and protamine-bound segments. The decondensation of the paternal chromatin through the protamine-to-histone transition results in the formation of the paternal pronucleus.

In addition to the swift decondensation previously discussed, paternally derived DNA must also undergo a dramatic demethylation to facilitate normal embryonic development (Ooi and Bestor 2008). The maternal DNA also undergoes demethylation; however, this is done through a passive, replication-dependent process (Eden and Cedar 1994). Importantly, it should be noted that the active demethylation in the paternal pronucleus is incomplete (Abdalla et al. 2009a, b). Multiple regions are known to escape this active process, including imprinted clusters and retrotransposons (Abdalla et al. 2009b). This creates an environment where new methylation marks that are tissue specific can be laid down in each given cell type as fates are determined, but it also suggests that the paternal epigenome is of some consequence to the developing embryo and, likely, to the offspring. The transition from gamete-derived epigenetic landscapes to that seen in the embryo is a critical step required for the growth and success of the embryo.

It is known that gene regulation is highly governed by the epigenetic landscape in each cell type. It is also well established that the sperm epigenome is among the most unique found in any cell in the human body, due both to its unique DNA methylation marks and nuclear protein content. It follows, then, that many of these

unique epigenetic features must be removed, oftentimes by dramatic mechanisms, to enable the paternal pronuclei to be capable of contributing to embryonic totipotency. However, it must also be noted that while many of the sperm-specific epigenetic marks are removed, some remain, and of those already studied, it appears that these marks are important for the embryo and the offspring. This is in stark contrast to the previously held dogma that the sperm was limited in its capacity to effect change in the embryo due to its epigenetic specificity. The dynamic nature of embryonic epigenomes makes the study of epigenetics in the early embryo difficult. Much of our knowledge is therefore derived from failures of embryogenesis and the subsequently identified epigenetic abnormalities (Eden and Cedar 1994). Other aspects of our knowledge stem from recognized imprinting errors of the paternal epigenome and genome, which have been linked to several severe diseases including Beckwith-Wiedemann syndrome (Hammoud et al. 2011). These diseases appear to be more prevalent in fetuses derived from in vitro fertilization (IVF)/ICSI, which has led to in-depth study of epigenetic factors in this population (Reefhuis et al. 2009). Clearly, much work is still required to further our understanding of the epigenetics of embryonic development; however, we have gained a great deal of insight into these processes in recent history.

Sperm Epigenetics and Infertility

As described previously, successful fertilization requires dramatic changes in the sperm chromatin, including protamination, proper histone retention, specific histone modifications, and fidelity in the maintenance of DNA methylation, as well as the likely role that retained RNAs may play in embryogenesis. Complicating this process even further is the reprogramming that must occur to the male pronucleus following fertilization. The presence of the paternal epigenetic landscape is necessary for complete embryogenesis, and data are now beginning to demonstrate epigenetic abnormalities in infertile patients (Jenkins and Carrell 2012).

Numerous studies have demonstrated a strong association of abnormal protamination with male infertility (Aoki et al. 2006a; Depa-Martynow et al. 2012). In humans, protamination results in the placement of two protamines, protamines 1 and 2, at a ratio of 1:1. It has clearly been shown that fertile men exhibit a tight distribution of the protamine ratio around 1.0, while in infertile men the ration can vary dramatically but is generally associated with decreased sperm quality, diminished sperm fertilizing capacity, and poorer embryogenesis in patients undergoing IVF (Aoki et al. 2006b). Studies indirectly evaluating protamination via indirect staining with aniline blue or chronomycin A have demonstrated a similar relationship (Sakkas et al. 1998).

The underlying causes of abnormal protamination have not been well elucidated, but gene polymorphisms of the transition protein and protamine genes are not the cause of abnormal expression in most men with abnormal protamination (Aoki et al. 2006c; Hammoud et al. 2007). Numerous enzymes involved in protamination

have been shown to be essential and may be related to infertility in some men. For example, impaired spermatogenesis has been associated with aberrant acetylation of the histone 4 domain, and hyperacetylation of this domain has been documented in infertile men with Sertoli cell-only syndrome (Faure et al. 2003). Abnormal phosphorylation of the P2 domain has been linked to male infertility, DNA damage, and poor sperm quality (Wu and Means 2000; Balhorn et al. 1988). Further studies have shown that the downregulation or upregulation of protamine with epigenetic modifications caused failure of spermatogenesis, arrest of transcription, translation, and additional abnormalities in embryogenesis (Kleene 2003).

Hammoud et al. (2011) recently completed a genomewide analysis of seven infertile men evaluating the location of regions of the genome enriched for histones, as well as histone modification. This genomewide evaluation was performed in two types of selected patients, those with known abnormalities of protamination and those with unexplained, repeated poor embryogenesis. This seminal study clearly showed marked abnormalities in the retention of histones at some promoters of developmentally related genes in many of the patients. When specific histone modifications were evaluated, more subtle results were seen. This study is the only study thus far that has evaluated histone modifications in sperm from infertile men, but it highlights the potential role that defects in this process may play in infertility.

DNA methylation, facilitated by DNA methyltransferase (DNMT), is an essential sperm epigenetic mark. Knockout mice with no DNA methyltransferase activity have decreased male fertility (Kato et al. 2007). Additional studies from couples undergoing assisted reproductive technology have also shown that changes, specifically hypomethylation, in spermatozoa DNA methylation is linked with decreased pregnancy rates, decreased sperm maturation, and embryogenesis (Benchaib et al. 2005). Additionally, numerous studies have found abnormal methylation of imprinted genes in sperm from oligozoospermic men (Marques et al. 2008; Kobayashi et al. 2007). Additionally, aberrant methylation of specific CpG islands in sperm DNA has been found to be elevated in sperm from men with abnormal protamination (Hammoud et al. 2010). Initial studies attempting to screen for aberrant methylation in sperm have been met with variable success; however, specific patients can be identified with broad methylation abnormalities (Aston et al. 2012; Nanassy and Carrell 2011).

As described earlier, noncoding and mRNAs may represent another epigenetic factor that could be related to infertility. Since mRNA is responsible for encoding protein synthesis during the translation process, the lack of mature sperm proteins, or their lack of function, may lead to infertility. Studies published thus far have demonstrated consistent differences in the RNA profiles of some infertile men, which may lead to the possible use of mRNA screening in infertile men as a tool to determine the cause of infertility (Hamatani 2012).

Screening for mRNA or other epigenetic defects could be conducted using commercially widely available methods for genetic amplification, and microarray screening could be used to help identify specific mRNA sequences or deficiencies that correlate with infertility. Significant differences have been noted, aside from the

protamine ratios, when comparing the mRNA proteins of fertile and infertile men on microarray analysis (Garrido et al. 2009). Among the differences cited in this study and others is underexpression of mRNA sequences that code for enzymes responsible for DNA repair and embryogenesis. mRNA screening has been further emphasized by comparing the semen analysis of known fertile men with patients having a history of heavy cigarette smoking and cryptorchidism. In each of these analyses, patients with decreased fertility have been identified by an underexpression of mRNA genes (Hamatani 2012).

Numerous studies, some of which were briefly discussed and cited earlier, have shown that disruption of the paternal epigenetic landscape leads to infertility and an inability for the embryo to proceed with appropriate development. The paternal genome and its epigenetic factors are crucial in numerous steps that lead to the successful formation of a fertilized and growing embryo. As Dada et al. said, "Both the complex path of sperm production and the delicate balance of epigenetic and genetic factors during sperm maturation contribute to the formation of a mature sperm with the ability to fertilize an oocyte and contribute to the developing embryo" (Dada et al. 2012). Therefore, a change or disruption of this balance may then lead to male infertility, as the required epigenetic factors for successful embryogenesis would be absent.

Conclusions

The spermatozoa chromatin is a highly specialized and unique structure and appears to be critical in facilitating normal fertilization and embryogenesis. Protamination, a process unique to sperm, results in a greatly compacted and quiescent sperm nucleus. Alterations to protamination appear to affect other epigenetic processes, such as DNA methylation, and are associated with reduced fertility. Classic epigenetic modifications to the retained sperm histones are consistent with a programmatic role for poising developmentally related genes for early embryogenesis. These modifications include regulatory modifications to the retained histones and DNA demethylation at the poised sites. Together, these changes suggest a string role for the sperm epigenome in embryogenesis.

Maternal changes to the male genetic code occur after fertilization. These modifications include removal of the protamines during pronucleus formation, replacement with maternal histones, and an active demethylation of most of the paternal genome. How these changes interplay with the marks set in the sperm epigenome during spermiogenesis is a key area of interest for further study.

Preliminary studies have clearly demonstrated epigenetic abnormalities in many infertile patients, including abnormal DNA methylation and aberrant histone enrichment in developmentally relevant genes. However, studies thus far are almost entirely descriptive and not mechanistic. Therefore, caution must be urged in evaluating the data from this important field of study.

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