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REVIEW

Novel insights into the genetic and epigenetic paternal contribution to the human embryo

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The integrity of the sperm genome and epigenome are critical for normal embryonic development. The advent of assisted reproductive technology has led to an increased understanding of the role of sperm in fertilization and embryogenesis. During fertilization, the sperm transmits not only nuclear DNA to the oocyte but also activation factor, centrosomes, and a host of messenger RNA and microRNAs. This complex complement of microRNAs and other non-coding RNAs is believed to modify important post-fertilization events. Thus, the health of the sperm genome and epigenome is critical for improving assisted conception rates and the birth of healthy offspring.

KEYWORDS: Messenger RNA (mRNA); DNA damage; epigenome; DNA integrity; telomere.

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INTRODUCTION

The paternal contribution to an embryo plays an important role in understanding early developmental processes and their effect on the health of a child. Sperm cells are highly differentiated, polarized and specialized, containing only the constituents required during and after fertilization (early embryonic development). However, the contribution of the male gamete to embryogenesis has not been well investigated. For quite some time, sperm have been considered mere vectors that carry the paternal genetic component to the oocyte. However, the contribution of sperm to the embryo has recently been better elucidated, with accumulating evidence suggesting that various spermatozoal components actively participate in early human development (1-4). During fertilization, the sperm transmits not only nuclear DNA but also oocyte activation factor (OAF) (critical for fertilization), centrosomes (critical for cell division) (5), and a population of messenger RNA (mRNA) that are of critical developmental importance (1,6). Studies investigating the epigenetic modifications in the developing sperm cell have provided new insights that may establish a more critical role for the sperm epigenome in the developing embryo. These non-genetic modifications include DNA methylation, histone tail modifications, targeted histone retention and protamine incorporation into the chromatin, all of which have a significant influence on the developing sperm cell. Sperm require these changes not only to shield the DNA throughout spermatogenesis but apparently, they also require these changes to contribute to the developmental program of the future embryo. Damage to genetic constituents and perturbations in the maintenance of these epigenetic changes have been demonstrated to affect fertilization potential and the early development of the embryo (7). This chapter will focus on the possible genetic and epigenetic contributions of sperm to the development of the human embryo.

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SPERM GENETIC FACTORS

Spermatozoal chromatin: effects on the developing embryo

The maturation of spermatozoa consists primarily of three important phases: the initial proliferative phase, the meiotic division of the chromosomes, and the final maturation step (spermiogenesis). Of the 280×10^6 human sperm normally ejaculated into the vagina, only 200 reach the ampullary region of the oviduct where fertilization takes place (8). Fewer than 1 in 10,000 sperm get close enough to the egg to complete the process of fertilization. However, even these highly competent sperm are frequently not sufficient for sustaining the later development of the embryo. Sperm cells are highly specialized vehicles for transporting chromatin cargo, which consists of DNA and its associated proteins. The chromatin in mammalian sperm can broadly be divided into three major structural domains: (1) the vast majority of sperm DNA is coiled into toroids by protamines, (2) a much smaller percent (5-15%) remains bound to histones and thus retains its nucleosomal structure, and (3) DNA that is attached to the sperm nuclear matrix at MARs (matrix

attachment regions) at intermediate intervals of approximately 50 kb throughout the genome (9). Sperm chromatin is essential for sperm function and subsequent embryonic development; defects in sperm chromatin have been linked to natural reproductive malfunctions, such as spontaneous abortion and assisted reproductive failure (10-12). These defects can include disrupted DNA integrity, which may affect fecundity and embryo growth, leading to embryo loss (13-15). Furthermore, the alteration of chromatin-associated proteins contributes to decreased fertility and poor embryonic growth (16). Most of the techniques used to detect sperm chromatin defects only detect gross defects in DNA integrity (17), while the roles of the associated proteins remain a mystery. Here, we will first discuss the role of sperm DNA and its associated proteins during embryonic growth. DNA damage (also referred to as DNA denaturation or fragmentation) is a common feature of human spermatozoa that affects DNA quality. Sperm DNA damage is thought to be induced by several mechanisms: (1) apoptosis during the process of spermatogenesis; (2) DNA breaks generated during the remodeling of sperm chromatin during the process of spermiogenesis; (3) post-testicular DNA fragmentation, which is induced by oxygen radicals, including the hydroxyl radical and nitric oxide, during sperm transport through the seminiferous tubules and the epididymis; (4) DNA fragmentation induced by endogenous caspases and endonucleases; (5) radiotherapy and chemotherapy; and (6) environmental toxicants and xenobiotics (18,19). Much concern has been expressed regarding the influence of sperm DNA integrity on abnormal reproductive outcomes (20,21). However, one of the well-established and extensively studied causes of DNA damage is oxidative stress, which causes the oxidation of the DNA bases and leads to the formation of various types of DNA adducts. These DNA adducts alter the function of the sperm genome, ultimately influencing *in vivo* or *in vitro* conception and the subsequent events that occur during early development (22). DNA fragmentation is more frequent in caudal epididymal and ejaculated sperm than testicular sperm (23-25). The persistence of high rates of DNA damage in ejaculated sperm is obvious, as mature spermatozoa possess a limited capacity to repair oxidative DNA lesions (26). It has been suggested that mammalian spermatozoa contain a mechanism by which they can digest their own DNA upon exposure to a stressful environment (27); however, the question of whether this mechanism is sufficient to cope with extensive damage arises. The answer lies in several studies postulating that the presence of a critical level of unrepaired DNA damage in embryos generated *in vivo/in vitro* explains the block in embryo development (28). The authors of these studies have arrived at the conclusion that a ''*late paternal effect*'' is responsible for blocking embryo development, which is evident from the outcomes of various assisted reproductive techniques (ARTs). The fragmentation of sperm DNA affects post-implantation embryonic development in ICSI procedures; specifically, high levels of sperm DNA fragmentation can compromise the viability of an embryo, resulting in pregnancy loss (21,29). Sperm carrying damaged DNA can complete the initial process of fertilization; however, the developmentally necessary genes in the damaged sperm DNA may hinder embryonic development upon activation of the embryonic genome at the 3-cell stage. Furthermore, sperm DNA damage has been linked to delayed chromosomal instability in blastocysts and post-implantation developmental abnormalities (30,31). However, very little is currently known regarding the nature of germline DNA damage and the extent to which germ cells are capable of eliminating damaged DNA and completing the process of DNA repair. Oocytes have been shown to possess different repair pathways to handle a certain level of DNA damage in sperm (32). There is little information regarding the fidelity and nature of this oocyte repair mechanism, but these mechanisms are negatively impacted by age. The majority of couples with reduced reproductive fitness may be of advanced age, and women above 35-40 years of age may have a defective DNA repair system that may not be able to repair extensive DNA damage. Oocyte repair mechanisms may also be inhibited by the accumulation of oxidized bases, such as ethenonucleosides, in the sperm genome. The accumulation of such DNA adducts is mutagenic and, if not corrected, may increase the mutagenic load in early embryos.

The analysis of sperm DNA fragmentation is a potentially valuable method for explaining the paternal origin of some unexplained and repeated ICSI fertilization and implantation failures (29). Such analysis may even help to determine the most efficient ART procedure (33) to reduce the probability of negative paternal contribution. A recent systematic review and meta-analysis of 2,969 couples showed a significant association between the level of DNA damage and pregnancy loss in both ART and spontaneous conceptions (34). These data affirm the clinical indication for the evaluation of sperm DNA damage prior to infertility treatment and the need to investigate the association between sperm DNA damage and recurrent pregnancy loss (10,35).

The intact structural framework of chromatin, which consists of molecular regulatory factors, is also required for proper embryonic development (9). Among the three types of sperm chromatin structures discussed earlier, histone-bound sperm DNA and MARs are inherited by the embryo and are most likely required for proper development. At least 2-15% of mammalian sperm chromatin is bound to histones rather than protamines (36-38). Histones are interspersed throughout the genome, primarily located at gene promoters (39). Another independent study also concluded that entire gene families important for embryo development are preferentially associated with histones in human spermatozoa (38). However, it will be interesting to know whether sperm histones are transmitted to the developing embryo. van der Heijden et al. (40,41) demonstrated that histones with specific modifications in the sperm cell are present in the paternal pronucleus, suggesting that these histones were never replaced. While the protamines in sperm chromatin are replaced with histones supplied by the oocyte after fertilization (42,43), this may not be necessary in regions where histones are already present in the sperm DNA.

In the sperm nucleus, the chromatin is organized into loop domains that are attached to a proteinaceous structure, termed the nuclear matrix, every 20-120 kb. This organizes the chromatin into functional loops of DNA that help regulate DNA replication and gene transcription (9). Several studies have demonstrated a functional role for the sperm nuclear matrix during early embryogenesis. These data suggest two roles for the sperm nuclear matrix (44-47): to facilitate the proper association of DNA with the nuclear matrix (required for paternal pronuclear DNA replication in

the one-cell embryo) and to serve as a checkpoint for sperm DNA integrity after fertilization.

SPERMATOZOAL TRANSCRIPTS: HIDDEN MESSENGERS

In the previous section, the importance and contributions of sperm chromatin were discussed. In this section, we will further elaborate the role of sperm RNAs and their indispensability to the spermatozoa and embryo development. In the late 1950s and early 1960s, controversy regarding the role of sperm RNAs arose, and the presence of these molecules was questioned. Various landmark studies in the 1970s concluded that bovine spermatozoa were transcriptionally active but this activity was localized to the mitochondria (48-50). Contributing to these controversies, Pessot et al. demonstrated the presence of nuclear RNA in rat and human sperm. The RNA was extracted from sperm and analyzed by electrophoresis on a 10% polyacrylamide gel and 7 M urea. The electrophoretic profile revealed a complex set of bands ranging in size from tRNA to high-molecular-weight components. On average, an RNA content of approximately 0.1 pg per rat or human sperm was found (51). The repackaging of DNA into a nucleotorroidal conformation, which is approximately 20 times more condensed, enables the complete shutdown of the spermatid nucleus (52,53). The gradual shutdown of RNA transcription begins during meiosis, when the paired sex chromosomes are accommodated in the male germ cell, leading to the repression of gene expression on the X and Y chromosomes (54,55). The shutdown of transcriptional activity in human spermatozoa has been confirmed (56), strengthening the previous observations. The retention of mRNAs in spermatozoa begins to occur during the early stages of spermatogenesis. In the late 1990s, various studies documented the presence of different types of RNAs in human spermatozoa. Kumar et al. first documented the presence of c-MYC mRNA in the mid-piece and tail region of human spermatozoa (57). mRNAs coding for the following molecules have been found in human ejaculate spermatozoa: HLA (58), integrins (59), cyclic nucleotide phosphodiesterases (60), the L-type calcium channel and N-cadherin (61,62), estrogen and progestin-like receptors (63,64), nitric oxide synthase (NOS) (65), and, surprisingly, insulin (66), among others (67). Complex RNA populations have also been reported in the sperm of cows (68,69) and human spermatozoa (1,70,71). These mRNAs were assumed to be residues left over from spermatogenesis, but there is evidence that the spermatozoa deliver a unique set of mRNAs to the oocyte. Three different types of sperm mRNA have been discovered (70). The first group of mRNAs has a specific function during spermatogenesis but does not exhibit an obvious function post-fertilization. The presence of this set of remnant mRNAs could serve as a diagnostic tool with which to follow the fidelity of the later phases of spermatogenesis (71). A second group of RNAs (e.g., mRNA coding for PLC-z) also originates from the testicular germ cells and may have an additional role in the fertilized oocyte. A third not yet extensively studied group of sperm mRNAs (e.g., mRNA coding for clusterin) may originate from a non-testicular source and, after incorporation into the sperm, could be introduced into the oocyte during fertilization. It is of considerable interest that foreign RNA constructs can be introduced into the sperm cell to be expressed by the oocyte after fertilization. These non-testicular RNA-containing sperm can take up DNA and RNA in vitro; these molecules are not only delivered to but also expressed in the fertilized oocyte (72,73). This in vitro experiment showed that, in theory, foreign sperm RNA could play a role in early embryogenesis. Furthermore, the fact that some of these spermatozoal mRNAs are also found in zygotes indicates that these transcripts may be functionally important (74). Rassoulzadegan et al. (75) were the first to prove that sperm RNA can influence embryo development by studying the Kit gene-derived heritable effect, which appeared to be affected in heterozygous mice carrying a wild-type Kit allele and a silent Kit allele. This work was followed by a study showing that the pathological overexpression of Cdk9, a key regulator of cardiac growth in the mouse, could be induced and heritably transmitted following the intraooplasmic injection of RNAs targeting the gene, including the sequence-related expression of miR-1 miRNA. miR-1 is among a number of small inhibitory RNAs that are present in sperm (76). A recent report also demonstrated that some mRNAs can be translated de novo, supporting the hypothesis that a population of mRNAs may have a function during or beyond the process of fertilization (77). As there is no evidence that the proteins encoded by the majority of mRNAs found in mature spermatozoa are also present in sperm (67), these mRNAs may be viewed as potential contributors to early embryogenesis. In addition to mRNA, spermatozoa are enriched in antisense RNAs and microRNAs (miRNAs). These miRNAs have been detected in human spermatozoa, raising the possibility that these molecules may play a role in early fertilization events (6) and embryo development by regulating the expression of various genes. For example, sperm-borne microRNA-34c is required for the first cleavage division in mice (78). A recent survey of small RNAs in sperm also revealed a complex population of male-derived sncRNAs (small, non-coding RNAs) that are available for delivery upon fertilization (79). In addition to finding the miRNA class previously detected in mature mouse, porcine, and human spermatozoa (6,80,81), this survey also identified piRNAs for the first time (79). Any alteration in the amount or composition of sperm mRNAs may indicate abnormalities in spermatogenesis, which may later affect embryo development. The mRNA fingerprints of normozoospermic and teratozoospermic men have been shown to differ (82). Additionally, variations in the expression of two sperm RNAs coding for LDHC transcript variant 1 and TPX1 have been reported in men with poor sperm motility (83). Ostermeier et al. detected over 3,000 mRNA species in ejaculated spermatozoa through microarray analysis (84). The expression profiling of human spermatozoa by serial analysis of gene expression (SAGE) revealed 389 clustered genes, with a highly selective grouping among the most abundant SAGE tags (85). Approximately 25% of these tags (96) were related to DNA-dependent transcription or transcriptional regulation. In addition, a comparison of the spermatozoa used in homologous intrauterine insemination showed a difference in the transcripts of the two groups (patients achieving pregnancy versus those who did not; both fresh and frozen spermatozoa were used) (86). The authors found 741 exclusive transcripts that were expressed only in the pregnant group and 976 transcripts that were expressed only in the non-pregnant group. Several studies have reported that certain sperm transcripts do have an important role in

early embryogenesis. The presence of mRNA transcripts encoding PSG-1 and HLA-E in human spermatozoa has previously been confirmed (84,85). Conversely, microarray analysis of the transcriptome of the human oocyte did not demonstrate the presence of PSG-1 mRNA and showed that HLA-E mRNA was down-regulated 87). An investigation of these transcripts showed significantly higher levels of PSG1 and HLA-E mRNA in the fertile group than the infertile group (88). In accordance with this study, a preliminary examination of the *PSG1* gene also showed a lower level of *PSG1* expression in the male partners of couples experiencing recurrent pregnancy loss than in fertile men (*in press*). It has been speculated that the PSG1 protein may play a crucial role in supporting early gestation and protecting the fetus from the maternal immune system. This hypothesis is supported by the fact that PSG1 is able to modulate monocyte/macrophage metabolism to regulate T-cell activation and proliferation. In addition, PSG1 induces the secretion of anti-inflammatory cytokines by monocytes (89). We recently investigated the expression of a few important genes (*WNT5A, HSP90,* and *PRM2*) that have been postulated to have a critical role in early development (unpublished). The *PRM2* and *HSP90* expression patterns were significantly altered in the male partners of couples with idiopathic recurrent pregnancy loss, while no association with recurrent pregnancy loss was found for *WNT5A*. Scientists investigating the heat-shock response (HSR) have focused on developmental processes because of the remarkably unusual characteristics of heat shock protein (Hsp) expression in pre-implantation embryos and gametogenesis. A striking Hsp expression pattern is exhibited in embryos during gametogenesis and in stem cell and differentiation models, and the expression of these proteins was shown to be stage-specific in both tissue models and male germ cells, the latter of which exhibited impaired abilities to mount a classical HSR. In addition, spermatogenesis and pre-implantation embryos showed extreme sensitivity to heat stress. The basal levels of HSFs and, even more interestingly, the ratios between different HSFs, which could vary from one individual to another, could contribute to reproductive success versus infertility or developmental success versus failure in humans. Our study revealed significantly higher levels of *HSP90* and significantly lower *PRM2* levels in the male partners of couples with idiopathic recurrent pregnancy loss. This result may be due to higher levels of free radicals causing oxidative stress, which is compensated for by increased *HSP90* expression. High free radical levels in spermatozoa may cause a pronuclear block, impair cleavage and lead to blastomere fragmentation and poor-quality blastocysts. The glutathione system (GS) is an oxidative stress defense system in sperm that is specifically controlled by GPX family members and has been correlated with embryo morphology on day 3. The results of this study indicated that sperm-derived mRNA may condition the human embryo and persist to the cleavage stage (90).

As explained earlier, protamines play a crucial role in the condensation of sperm chromatin and the protection of the paternal genome from internal and external environmental insults. Altered *PRM2* expression is reported among men with poor fertilizing capacity (91), and a lack of *PRM2* leads to sperm DNA damage and embryo death in mice (92).

While high-throughput technologies have provided a glance at the mRNA population contained in spermatozoa, future studies should focus on the functional aspects of these RNAs in the growing embryo. The results from such studies will further strengthen the correlation between the mRNA fingerprint of sperm and embryogenesis.

Telomeres are evolutionarily conserved tandem hexameric repeats at the ends of chromosomes that maintain genomic integrity and chromosome stability. Telomeres serve as a biological clock and undergo attrition at a rate of 50-200 bp per cell division. The telomeres in germ cells are 10-20 kb in length, compared with 5-10 kb in somatic cells. The inheritance of telomere length in the embryo is a complex trait codetermined by the length of the telomeres in the sperm and ovum, the age of the father, free radical levels and gender. Tandem telomere repeats are rich in guanine, the nucleotide with the lowest oxidative potential and, thus, the most susceptibility to oxidative damage. We have previously shown high levels of free radicals to be associated with DNA damage in the semen and sperm, as well as shorter telomeres in the male partner of infertile couples and couples experiencing idiopathic recurrent pregnancy loss. As telomeres are histone-bound and located in the periphery of the sperm nucleus, telomeres are highly susceptible to oxidative damage. This induces GC to TA transitions, single- and double-strand breaks and accelerated telomere shortening. Inheriting shortened telomeres from the father may thus result in impaired cleavage and embryonic development. Zalenskaya et al. reported that sperm telomeres are the first structures to respond to the oocyte signal for pronucleus formation (93), and Rodriguez and colleagues (94) later reported that shortened sperm telomeres are associated with sperm DNA fragmentation and abnormal embryonic development. Concordance in telomere length is required for synapsis, homologous recombination, and normal chromosome segregation. Sperm with shortened telomeres show segregation abnormalities and nondisjunction, thus giving rise to aneuploid sperm after meiosis. Shortened telomere can increase the incidence of offspring with major or minor congenital malformations, childhood cancers, perinatal morbidity, developmental delay, and failure to thrive. In an ongoing study in our laboratory, we have found significantly shortened telomeres in the male partners of couples experiencing idiopathic recurrent pregnancy loss. We did not find an association with high levels of reactive oxygen species in this pilot study; however, this was a very clinically significant finding, and studies are still ongoing to further validate this result.

THE SPERM EPIGENOME

Genomic imprinting is a parental, origin-specific, gene-marking phenomenon that is crucial for normal mammalian development. Imprinted genes are characterized by epigenetic modifications (95,96), including DNA methylation, and are associated with differentially methylated regions (DMRs) that are methylated on either the paternal or maternal allele. Epigenetics refers to phenotypic changes that are caused by mechanisms other than changes in the DNA sequence (thus the name epi- (above or over) genetics). The methylation of primary DMRs is presumed to be maintained throughout embryonic development, including the pre-implantation stages, during which extensive demethylation of the genome takes place. Recent studies have demonstrated that sperm have unique and potentially important epigenetic modifications.

Male germ cells undergo unique and extensive chromatin and epigenetic remodeling soon after their specification (determination to become a spermatocyte) and during the differentiation process to become a mature spermatozoon (97). The epigenetic program of sperm is unique and tailored to meet the needs of this highly specialized cell. The unique nuclear protein landscape in sperm creates a chromatin structure that is between six and 20 times more dense than nucleosome-bound DNA, ultimately resulting in a tightly condensed nucleus (53,98). Although the mechanisms regulating and orchestrating specification and spermiogenesis remain poorly understood, some progress has been made in elucidating the molecular changes associated with these complex cellular changes. Recent studies analyzing sperm DNA methylation, histone modifications and the RNA transcripts in spermatozoa have further established the role of the sperm epigenetic program in the developing embryo.

The importance of DNA methylation has been demonstrated globally, regionally, and at the single locus level in both humans and animal models. Multiple targeted studies have been performed in animal models to establish a clear role of DNA methylation in sperm and embryos. El Hajj et al. (99) suggested that the improper methylation of repetitive elements may be linked to recurrent pregnancy loss. Methylation abnormalities in the CREM promoter were observed in a subset of patients with protamine ratio abnormalities, as well as in patients presenting with various forms of male factor infertility (100). Recent data demonstrate that the aberrant methylation of promoters for specific genes (e.g., DAZL and MTHFR) and general gene classes, such as imprinted loci, is strongly associated with various forms of infertility and sperm defects in men (101-103).

Twin studies have played an essential role in enabling the estimation of phenotypic heritability, and these studies now offer an opportunity to study epigenetic variation as a dynamic quantitative trait. Monozygotic (MZ) twin studies have proven to be very effective in answering key questions ranging from the genetics of social behavior and the nature versus nurture question to the heritability of phenotypic variation and disorders (104,105). Several studies have examined DNA methylation patterns in twins, and a recent study found that MZ twins exhibit a considerable degree of variability in DNA methylation patterns, which may impact the variability of gene expression and possible differences in disease susceptibility.

Along with the current interest in CpG methylation, recent data have suggested that the intermediates formed during DNA demethylation may be important epigenetic regulators. Most prominent among these intermediates is 5-hydroxymethylcytosine (5-hmC). A recent study by Pastor et al. revealed a pattern of 5-hmC enrichment in transcriptionally poised genes in stem cells (106). This unique localization suggests that 5-hmC has a role in embryonic stem cells and possibly the epigenome of multiple other cell types.

The timing of the establishment and removal of methylation is critical to normal spermatogenesis. During cell division, the DNA in male germ cells is packaged in nucleosomes comprised of histone 2A (H2A), histone 2B (H2B), histone 3 (H3) and histone 4 (H4), all of which are susceptible to covalent modifications. Histones are basic proteins in eukaryotic nuclei that package DNA into nucleosomes. The H2A, H2B, H3 and H4 histones are integral components of nucleosomes. Histone modifications, such as acetylation, methylation, ubiquitylation and phosphorylation, have emerged as the main players regulating epigenetic modifications. Each of these chemical modifications to histones can influence gene repression and/or activation.

In post-implantation mammalian embryos, pluripotent cells in the epiblast give rise to primordial germ cells (PGCs). Germ cells undergo several changes in their epigenetic profile during the different stages of meiosis. PGCs enter mitotic arrest in males, whereas PGCs are arrested in prophase of meiosis I in females. Global nuclear remodeling occurs in haploid round spermatids, although some histone marks, such as H3K9me2, on the inactive X chromosome are retained (107,108). The testis-specific linker histone variant H1T2 appears at this stage and plays a crucial role in chromatin condensation during spermiogenesis (108). Later, the linker histone variant HIls1 (histone-1-like protein in spermatids 1) is expressed in elongated spermatids. In the histone-protamine exchange process, nuclear histones become hyperacetylated during spermiogenesis and disassemble shortly thereafter, replaced by transition proteins (TP1 and TP2). In the final stage of spermiogenesis, the transition proteins are removed and replaced by protamines (109). The incorporation of protamines into sperm chromatin induces DNA compaction, which is important for the formation of spermatozoa and for providing a safe environment for the genome. The presence of somatic-like chromatin in the sperm nucleus could transmit different epigenetic information to offspring. Oakes et al. (110) suggested that the genome-wide DNA methylation pattern observed during spermiogenesis changes little after the pachytene spermatocyte stage.

Many epigenetic modifiers, including DNA methyltransferases, histone modification enzymes and their regulatory proteins, play essential roles in germ cell development. Some of these modifiers are specifically expressed in germ cells, whereas others are more widely expressed. The crucial roles of germ-cell-specific genes, such as Dnmt3L and Prdm9, were revealed by conventional knockout studies (111-113). A recent report showed numerous intra- and inter-individual differences in the DNA methylation of human sperm samples, which could contribute to phenotypic differences in offspring. Furthermore, it has been reported that sperm samples from oligospermic patients often contain DNA methylation defects at imprinted loci (114,115). Kobayashi and his associates reported methylation defects in 17 of 78 embryos conceived by ART. He found that seven of these 17 embryos had inherited these altered methylation patterns paternally, while the rest were believed to have resulted from the ART process. The altered hormonal milieu associated with ART and the retrieval of epigenetically immature oocytes may result in an increase in epigenetic/imprinting defects in children conceived through ART.

Imprinting errors in the developing fetus have been identified and shown to cause severe pathologies. Evidence suggests that Prader-Willi syndrome (PWS) and Angelman syndrome (AS) arise from the functional loss of several paternally expressed genes. The Prader-Willi/Angelman imprinted domain on human chromosome 15q11-q13 is regulated by an imprinting control regions (ICRs). Imprinting defects affecting the PWS/AS region can arise from the failure to demethylate the PWS-SRO in the male germ line, the failure to methylate the maternal PWS-SRO,

or the failure to maintain PWS-SRO methylation after fertilization (116). Some studies have also suggested that the use of ART increases the risk of imprinting diseases, as the germ cells of infertile couples are prone to epigenetic instability. A pattern of decreased genome-wide methylation in sperm has also been associated with poor embryo quality in rats and decreased IVF pregnancy rates in humans (117). Benchaib et al. (117) used 5-methyl-cytosine immunostaining as an indicator of the genome-wide methylation pattern in sperm. He showed that decreased global methylation in semen samples from normozoospermic men was related to a poor pregnancy outcome from IVF (118), suggesting that global methylation status independently affects embryogenesis. 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. It has been proposed that the level of DNA methylation in human sperm could be linked to their ability to initiate a pregnancy in an assisted reproduction procedure (118). A defect at any one step may manifest as a syndrome of male infertility.

The epigenome cycles through a series of precisely timed methylation changes during development, making the epigenome vulnerable to interference from environmental exposure (119). As the embryo grows, these parent-of-origin imprints are maintained in somatic tissues but erased in primordial germ cells so that imprints can be re-established in a sex-specific manner during gametogenesis. Histone modifications are thought to play a role in this sex-specific mark establishment, as the extensive loss of histone methylation and acetylation occurs along with the loss of DNA methylation (120). The methylation marks are then sustained throughout the individual's lifetime until methylation marks are erased and re-established following fertilization of the next generation (120). Epigenetic programming plays an important role in an organism's response to environmental stress during critical developmental periods (121).

Epigenetic changes are not only heritable in somatic cells but can also be maintained during meiosis. Transgenerational epigenetic inheritance is governed by chromatin remodeling in *Drosophila melanogaster* (122), and the inheritance of coat color in successive generations of the *Agouti* viable yellow mouse is controlled by epigenetic mechanisms associated with the *Agouti* allele (123). The *Agouti* viable yellow (A^{vy}) and *Axin-Fused* ($Axin^{Fu}$) mice are unique animal models that carry the A^{vy} and $Axin^{Fu}$ metastable epialleles, respectively. Researchers have used these mice to show that several nutritional and environmental exposures can alter epigenetic programming during gestation. For instance, exposure to methyl donors, such as folic acid, can hypermethylate the A^{vy} and $Axin^{Fu}$ alleles, leading to offspring mice that are brown (pseudoagouti) and to mice with straightened tails, respectively (124,125).

The sperm cell has a highly differentiated and specialized morphology, and the epigenome of human sperm is unique, elegant and essential to embryogenesis. Epigenetic factors suggest that sperm play diverse and critical roles in regulating embryogenesis. Understanding the epigenetics of sperm and spermatogonia may be key in understanding the mechanisms of pluripotency, which has broad implications for potential therapies. Efforts should be aimed at identifying select candidate alleles that are key factors in embryogenesis or that are representative of the genome at large and are predictive of abnormal epigenetic modifications. Future studies will likely focus on the epigenetics of both gametes, as well as on the changes observed throughout embryogenesis. Thus, investigating the effects of genetic and epigenetic alterations in sperm and how these modifications arise and affect embryonic genes will be important in the prevention, diagnosis and treatment of disease.

EXPERT COMMENTARY

Sperm are highly polarized cells that are both transcriptionally and translationally silent. At the time of fertilization, sperm transfer not only nuclear DNA but also oocyte activation factor, centrosomes, long-lived mRNAs, and small non-coding RNAs. It is believed that these mRNAs are transcripts for key developmental genes and that the small non-coding RNAs modify post-fertilization events.

Due to its high polyunsaturated fatty acid content and the loss of the majority of cytosolic antioxidants, the mitochondrial and nuclear DNA of sperm are highly susceptible to oxidative damage. Mitochondria are the first site of free radical production and free radical-induced DNA damage. Thus, the sperm mtDNA is reduced to disposable elements at the time of fertilization. However, mtDNA accumulates sequence variations that produce high levels of free radicals, which damage both the mitochondrial and nuclear genomes. The mtDNA copy number in healthy sperm is 1-1.4, compared with approximately 5-10-fold more in sperm with morphological abnormalities and impaired motility. This high copy number of mtDNA when aging oocytes with a defective genetic filter are fertilized. The mtDNA copy number has a profound impact on the methylation pattern of nuclear genes.

In a previous study from our laboratory, we established sperm DNA fragmentation indices of 30 and 26% in infertile males and the male partners of couple experiencing idiopathic recurrent pregnancy loss, respectively. The sperm genome is partitioned into a peripheral compartment that has histone-bound DNA, retains the nucleosomal structure and thus maintains imprints. This region of the genome contains promoters for developmentally important genes, transcription factors, signaling factors and microRNAs. The rest of the genome is packaged into a crystalline toroid by protamines. The retention of histones is not random but is significantly enriched in many developmentally important loci.

Recent studies have also shown that the mtDNA copy number has a significant impact on the nuclear methylation pattern. Thus, one cannot study the nuclear genome in isolation because of the cross-talk between the mitochondrial and nuclear genomes of sperm.

Telomeres are tandemly repeating hexameric units that cap chromosomal ends and are vital for genomic integrity and chromosomal stability. Concordance in telomere length in germ cells is necessary for synapsis, recombination and chromosome segregation. Rapid telomere attrition due to oxidative stress may result in meiotic recombination defects and segregation errors. This results in meiotic arrest and the generation of gametes with increased disjunction errors and aneuploidy. The inheritance of short telomeres from sperm may also impair cleavage, as optimal telomere length is a prerequisite for cell division. Short telomeres can also result in blastocysts with poor morphology. This may be one of the

key factors affecting pre- and post-implantation losses and the birth of offspring with major and minor congenital malformations and childhood cancers. Thus, the sperm genome is highly vulnerable to oxidative stress-induced DNA damage. Various lifestyle modifications (such as the increased intake of fruits and vegetables, exercise, meditation, yoga, cessation of smoking, and reduction in alcohol intake) can improve the health of the sperm genome and result in normal embryonic development and the birth of healthy offspring.

KEY ISSUES

- The integrity of the sperm genome and epigenome is critical for the birth of healthy offspring.
- The sperm nuclear genome is uniquely partitioned into compartments. These compartments include a central compact toroid, in which DNA is protamine-bound. This portion of the genome is transcriptionally and translationally inert. The peripheral compartment contains histone-bound DNA (5-15%) that retains the nucleosomal structure. This region contains promoters for developmentally important genes, microRNAs, and signaling factors.
- The histone-bound DNA is highly susceptible to environmental insults, especially oxidative damage.
- Telomeres, the nucleoprotein structures that constitute the biological molecular clock, cap the chromosomal ends and maintain chromosomal and genomic integrity. These guanine-rich repeats are highly susceptible to free radical-induced DNA damage. We believe that the rapid alteration of telomeres in the sperm genome underlies the etiology of infertility, which occurs as a result of accelerated telomere aging. Because the inheritance of telomere length is a complex trait, a short telomere length adversely affects cleavage and results in the generation of blastocysts with poor morphology.
- Sperm not only transfer the nuclear genome to oocytes but also transfer a stable population of developmentally important mRNAs.
- The sperm epigenome is maintained through the retention of histones, the compaction of major portions of the genome by protamines, DNA methylation, and covalent histone modifications.
- Because sperm lose the majority of cytosolic antioxidants at the time of spermiogenesis, sperm cells are highly vulnerable to free radical-induced DNA damage. Lower levels of key DNA repair enzymes have also been found in sperm (unpublished study from our laboratory). This finding may explain the persistence of DNA damage in sperm. The fertilization of oocytes by such sperm, either spontaneously or through assisted reproductive techniques, may help us to understand the pathogenesis of congenital malformations, childhood cancers and perinatal morbidity.

AUTHOR CONTRIBUTIONS

Dada R designed the study and the data acquisition procedure for determining telomere length, performed the DFI analysis, and drafted and revised the manuscript. Kumar K contributed to the experimental work and the drafting and revision of the manuscript. Kumar M contributed to the drafting and revision of the manuscript. Jain S performed the experimental work and helped in the manuscript drafting. Hassan T helped in the manuscript drafting.

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