Published in final edited form as:

Fertil Steril. 2013 November ; 100(5): . doi:10.1016/j.fertnstert.2013.09.017.

Biomarkers of Chemotherapy-Induced Testicular Damage

Edward Dere, Ph.D.^{a,b}, Linnea M Anderson, M.Sc.^b, Kathleen Hwang, M.D.^{a,c}, and Kim Boekelheide, M.D., Ph.D.^b

^aDivision of Urology, Rhode Island Hospital, Providence, RI

^bDepartment of Pathology and Laboratory Medicine, Brown University, Providence, RI

^cThe Warren Alpert Medical School of Brown University, Providence, RI

Abstract

There are increasing numbers of men having or wanting children after chemotherapy treatment, a result that can be attributed to improvements in cancer therapies that increase survival. However, a side effect of most chemotherapy drugs is disruption of spermatogenesis and a drastic reduction in sperm count and quality. While a large number of men eventually recover reproductive function as indicated by normal semen analyses, there is no clinical test that can assess sperm quality at a high level of sensitivity. Sperm FISH and several different tests for DNA fragmentation have been used infrequently in clinical assessment. Animal models of chemotherapy-induced testicular damage are currently being used to identify potential molecular biomarkers that may be translatable to humans; these include sperm mRNAs, microRNAs, histone modifications, and DNA methylation patterns. Changes in these molecular measurements are both quantitative and sensitive, potentially making them important clinical biomarkers of testicular function following chemotherapy treatment.

Keywords

chemotherapy; biomarkers; testicular damage; transgenerational

Traditional endpoints used to monitor reproductive function in men, including semen and hormone analyses, are highly variable and insensitive to small but potentially meaningful alterations in testicular function. The sensitive histopathological endpoints used to measure reproductive toxicity in animal studies are not easily translatable to clinical practice. It is therefore necessary to develop sensitive, reliable, and easily attainable biomarkers of testicular injury that could be used to monitor human reproductive function, particularly after treatment with gonadotoxic chemotherapy drugs. Such biomarkers would not only help physicians monitor the reproductive capacity of their patients, but may allow them to better

© 2013 American Society for Reproductive Medicine. Published by Elsevier Inc. All rights reserved.

[†]Corresponding Author: Kim Boekelheide, Department of Pathology and Laboratory Medicine, Brown University, Box G-E, Providence, RI 02912, kim_boekelheide@brown.edu.

Disclosures: K. Boekelheide is an occasional expert consultant for chemical and pharmaceutical companies, including Dow Chemical, Akros, Pfizer, Zafgen, and Bristol Myers Squibb. K. Boekelheide owns stock in, and is a consultant for, CytoSolv, an early stage biotechnology company developing a wound healing therapeutic based on growth factors. These activities are unrelated to the current work but are mentioned in the spirit of full disclosure.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

counsel patients about their present and future fertility and the health of offspring conceived after chemotherapy treatment.

Animal Models of Chemotherapy-Induced Testicular Injury

The use of animal models of pharmaceutical drug exposure is a common and informative way to evaluate the toxicity of a compound, either on its own or in combination with other pharmaceuticals. Moreover, animal research allows researchers to examine the effects of pharmaceuticals on both target and non-target tissues and cell types at a level of detail that is generally not possible in human studies. Extensive research with chemotherapeutics in rodents has been conducted to establish safe doses and identify unanticipated toxicity prior to implementation in human clinical trials. However, concerns such as effects of chemotherapy on long-term fertility have driven further research on some of these drugs.

In particular, exposures of young men to chemotherapy drugs, largely for treatment of testicular or blood cancers, have raised concerns about the adverse effects on testicular function and the reproductive health of these men. The drugs most commonly studied in animal models of these human treatments are cyclophosphamide, an alkylating drug common to chemotherapy regimens, and the combination of bleomycin, etoposide, and cis-platinum (BEP), which is commonly used for the treatment of testicular cancer and Hodgkin's lymphoma. Animal exposures mimicking these regimens in both dose and treatment time are used to examine effects on testis histopathology and sperm production, as well as molecular changes in the testis and sperm. Rats sub-chronically treated with low-dose cyclophosphamide display few changes in standard male reproductive endpoints including organ weights, serum hormone levels (testosterone, follicle stimulating hormone [FSH], and luteinizing hormone [LH]), and testicular or epididymal sperm counts (1–3). However, despite the absence of changes in “traditional” reproductive endpoints, these animals demonstrate significantly worse progeny outcomes after mating, suggesting that the history of paternal exposure are propagated to the progeny through the sperm.

Rat models of BEP treatment, unlike cyclophosphamide, exhibit severe defects at doses and lengths of exposure that mimic those used in human patients. These defects include those consistent with traditional infertility assessments: decreased body, testis, and epididymis weights; decreased sperm counts and motility with increased morphological defects; and grossly altered testis histology (4, 5). Many of these effects are reversible and resolve after a recovery period equal to the length of treatment. However, testis histology remains altered, suggesting a long-lasting effect of these drugs on male reproduction (5). In addition to changes in the tissues of treated animals, mating studies have also identified deficits in implantation and development, which may persist even after a recovery period (4, 5), further demonstrating the effects on the developing sperm.

In addition to these effects, most studies examining the reproductive effects of chemotherapeutic agents have documented an increase in germ cell apoptosis. Exposure to BEP produces an increase in the percentage of seminiferous tubules containing apoptotic germ cells (5), while both cyclophosphamide (6) and etoposide (7) induce germ cell apoptosis in a stage-dependent manner.

Chemotherapy-Induced Testicular Injury In Humans

The Prepubertal Testis

Changes in hormone secretion and receptor expression alter the functional activity of the hypothalamic-pituitary-gonadal axis throughout male development. Circulating levels of gonadotropins and testicular hormones vary with developmental phase, so the ability of

hormonal biomarkers to accurately assess testicular function is highly dependent on an individual's current phase of life (8). During the first three to six months of life, basal levels of luteinizing hormone (LH), testosterone, and insulin-like peptide 3 (INSL3) are useful markers of the pituitary-Leydig cell axis, whereas follicle stimulating hormone (FSH), anti-Müllerian hormone (AMH), and inhibin B are useful markers of the pituitary-Sertoli cell axis (9). For the remainder of infancy and childhood, AMH and inhibin B are the most useful markers of gonadal function in basal conditions, and are particularly informative in response to FSH treatment (10).

While the elevation of serum gonadotropins may be a reliable sign of primary testicular failure during childhood, it is inconsistent when testicular failure is established after six months of age (11). After nine years of age, the tubular compartment function can be assessed by inhibin B levels, which reflect both FSH and germ cell activities; basal or stimulated gonadotropins and basal testosterone levels become useful during this time as well (12). Furthermore, AMH levels should begin to decline, reflecting adequate androgen action on Sertoli cells and spermatogenic development (13). Overall, gonadotoxic treatment with chemotherapy has demonstrated a more significant impact on germ cells than Leydig cells, such that cancer survivors who are azoospermic after treatment may maintain adequate testosterone production (14).

The Adult Testis

Pubertal development of the gonads is driven by the increase of gonadotropin pulse frequency and amplitude (15). Clinically, the onset of puberty is defined by a testicular volume ≥ 4 mL, and with normal spermatogenic development, the testis will reach a final volume of 15–25 mL. Testosterone concentration increases within the testis to stimulate Sertoli cell maturation and down-regulate AMH prior to an increase in circulating testosterone levels. Normal spermatogenesis is qualitatively and quantitatively reflected by adequate sperm output. In addition, FSH and inhibin B function as endocrine markers of spermatogenesis (16, 17); however, they are both highly variable and therefore have limited predictive value for clinical endpoints. Standard semen parameters are also limited in predicting reproductive success and may not be sensitive or specific to environmentally relevant levels of gonadotoxic exposure (18). Furthermore, traditional markers exhibit marked intra-individual variability that can be influenced by potential confounding factors.

Although Leydig cells are generally more resistant to cytotoxic damage than the cells of the germinal epithelium, they are still affected by chemotherapy treatment. Elevated LH levels and normal or subnormal testosterone levels have generally been associated with chemotherapy treatment, suggestive of compensatory Leydig cell failure (19–25). However, this evidence is contradicted by a recent study where levels of testosterone were decreased in the absence of changes in LH levels following cyclophosphamide treatment (26). Mild testosterone deficiency may have important clinical implications in long-term cancer survivors and could be used to determine the need for testosterone replacement therapy. Studies have found correlations between testosterone deficiency in survivors with reduced sexual interest and activity (27), increased serum cholesterol (28), increased cardiovascular risk (29, 30), and decreased bone density (31). Overall, these studies highlight the current lack of knowledge regarding the mechanism of chemotherapy-induced Leydig cell impairment.

Potential Biomarkers of Persistent Effects

Genetic Testing of Sperm

Sperm FISH—The routine semen analysis, measuring sperm concentration, motility, morphology, and the presence of other cells, is one of the first tests ordered in the evaluation of potentially infertile males. However, this analysis only provides clues that may indicate the need to perform additional evaluation. The incidence of chromosomal aberrations may be increased in sperm from men with an abnormal semen analysis (32); indeed, gametes of infertile men show a higher rate of chromosome abnormalities than the general population (33). Nevertheless, the selection of sperm with normal morphology does not guarantee a normal haploid chromosomal complement (34), and as a result, the use of fluorescent *in situ* hybridization (FISH) has become very useful for the detection of sperm aneuploidy

Spermatozoa consist of the head, with a nucleus containing the highly compacted male haploid genome, and the flagellum, which is responsible for sperm motility. Sperm chromatin is a highly organized and compact structure consisting of DNA and heterogeneous nucleoproteins, which are essential for the accurate transmission of genetic information to offspring. Sperm FISH is most commonly used to determine the proportion of aneuploidy present in sex chromosomes and autosomes of infertile men (Figure 1). Additionally, it can quantify the probability of transmitting aneuploidies and complex chromosomal rearrangements, such as translocations and inversions, to the offspring (35).

FISH is also used in rodent models to define numerical chromosomal aberrations after administration of potentially gonadotoxic compounds, and provide insight into potential adverse effects in humans. Administration of the chemotherapy agents etoposide and merbarone induced disomy and diploidy (36), while vinblastine (37) caused either borderline significant increases in disomic sperm or inconsistent increases in disomic sperm, respectively. More recently, amsacrine and nocodazole were similarly shown to increase sperm disomy (38). In the rat model, chronic low-dose cyclophosphamide significantly increased autosome disomy or nullisomy, but did not increase Y chromosome disomy or diploidy (39).

Sperm aneuploidy can occur following chemotherapy in human patients as well. BEP administration to men treated for testicular cancer elicited an increase in sperm aneuploidy that typically returned to baseline within two years (40, 41). Similarly, patients treated for Hodgkin's lymphoma receiving bleomycin, doxorubicin, vincristine, and dacarbazine (ABVD) or novantrone, oncovin, velban, and prednisone (NOVP) had an increased incidence of sperm disomy and diploidy after therapy; this aneuploidy typically returned to baseline levels months after treatment, although elevated frequencies persisted in some chromosomes for up to two years (42). While a definitive clinical guideline has yet to be established, investigators suggest using the 18–24 month post-treatment interval in counseling patients on the safety of conception to allow for aneuploidy rates to return to baseline frequencies (42).

DNA Fragmentation—Damage to the spermatid DNA can occur either during intratesticular spermatogenesis or during sperm maturation and transport. Sperm from subfertile men have considerably more DNA damage than sperm from fertile controls (43). Evidence suggests that DNA fragmentation in spermatozoa is associated with poor pregnancy outcomes, including early embryo death, poor embryo development, and poor implantation (44). DNA damage increases with elevated levels of reactive oxygen species and with the presence of varicoceles, although subsequent surgical correction of the varicocele can reduce this damage (45–47). The precise normal value for DNA damage will depend on the particular assay used, but most laboratories set the normal range as <30%

DNA fragmentation (48, 49). Evaluating and defining an acceptable level of DNA damage has been clinically helpful in patients with unexplained recurrent early gestational loss.

The causes of DNA damage are largely unknown, although there is evidence suggesting that genetic defects may underlie some sperm DNA damage (50, 51). Spermatogenesis is controlled by selective apoptosis. Abnormal sperm are tagged for apoptosis in the same manner that all other cells are marked for programmed cell death. Protamine deficiency has been identified as another primary testicular cause of sperm DNA damage, and this deficiency is frequently seen in infertile men (52). In addition, certain polymorphisms in the protamine gene have been implicated in male infertility and sperm DNA (53). Evidence suggests that a malfunction in this process allows sperm with DNA damage to be transported in the ejaculate, a process referred to as abortive apoptosis (54). A variety of different tests are available to evaluate sperm DNA damage: the acridine orange-staining test, the sperm chromatin structural assay, terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL), and the comet assay (a single cell gel electrophoresis).

High levels of DNA damage are commonly seen during and directly following chemotherapy treatment, however, the persistence of these genetic effects post-treatment has not been well established. More recently, a large, multi-center, prospective study serially assessed men undergoing gonadotoxic treatment with either chemotherapy or radiation therapy (55). While the research did not find a higher proportion of patients with increased sperm DNA fragmentation following treatment, it did report a higher incidence of chromatin defects at six months post-treatment. Other studies have demonstrated significantly higher levels of sperm DNA damage compared to controls that have persisted for up to two years following chemotherapy treatment (56, 57).

The use of sperm FISH and many other techniques of assessing DNA damage can provide insight into the genetic integrity of sperm after chemotherapy. However, clinically, these techniques have so far been used infrequently to examine sperm quality after chemotherapy treatment.

Sperm Molecular Biomarkers of Residual Effect

Sperm mRNAs

Molecular components of sperm have the potential to be important indicators of reproductive toxicity. This is especially important for long-term exposures, such as those experienced in chemotherapeutic regimens, in which cytotoxic drugs are often given for months rather than days. Human spermatogenesis takes 64 days (58), therefore, mature sperm in the ejaculate reflect the testicular conditions over that entire time period. Exposures that target germ cells for extended periods of time, as chemotherapy drugs do, may produce changes in the molecular contents of the sperm that can serve as biomarkers of testicular dysfunction.

Measurement of mRNA alterations is perhaps the simplest form this may take. While the mRNA content of sperm is low relative to many human or animal tissues, it is sufficient to detect exposure-related changes. Moreover, a panel of sperm mRNA transcripts has been developed that can detect and predict low-level exposures to Sertoli cell toxicants in rats (59). A similar approach may be used to identify the effects of chemotherapy-induced testicular or sperm damage at the molecular level. A recent study in rats investigated the effects of BEP chemotherapy regimen on drug metabolizing enzymes and found elevated levels of transcripts for glutathione S-transferases and aldehyde dehydrogenases in round spermatids (60). These findings suggest that phase II metabolizing enzymes, in particular the

families of glutathione S-transferases and aldehyde dehydrogenases, may serve as predictive biomarkers of chemotherapy-induced testicular toxicity.

Evaluation of mRNA present in ejaculated human sperm has shown many different RNAs with varying functions to be consistently present. Molecular profiles of testicular and ejaculated sperm from normozoospermic men suggest that the spermatozoal mRNA profile is reflective of past spermatogenic events, and the mRNAs present may function in future embryo development (61–64). Sperm mRNAs have also been correlated with motility in normozoospermic men, and the results demonstrate that mRNAs associate with motility in a way that makes functional sense (65). This is significant, as motility is an important measurement in the routine semen analysis, and may be more predictive of fertility than the other endpoints measured in that clinical test (66). While little research has been done on the sperm profiles of post-chemotherapy men, results from analyses of fertile and infertile men suggest that sperm mRNAs are important potential biomarkers in humans. Studies of residual sperm mRNAs in animals will guide development of human-specific mRNAs that may function as biomarkers of fertility and infertility in post-chemotherapy patients.

Sperm Proteins

Tremendous advancements in proteomics have improved the detection of proteins in human sperm and are capable of distinguishing between different isoforms and post-translationally modified gene products (67–70). Although the field of sperm proteomics is still in its infancy, it will benefit from the previous successes in other cell types (71) and establish a foundation for the current understanding of the sperm proteome. Alterations in the sperm proteome have already been explored as a potential diagnostic biomarker of asthenozoospermia (72, 73). Furthermore, proteomic approaches were able to identify post-translational protein modification in human sperm following *in vitro* nitric oxide exposure (74). These studies are promising examples of the potential diagnostic utility of the sperm proteome as biomarkers of testicular injury following chemotherapy treatment.

Sperm Epigenetic Modifications

The term “epigenetics” was originally coined by Conrad Waddington to describe how a phenotype arises from a given genotype (75). Over the years and as our scientific knowledge has expanded, the term has grown in scope. Specifically, epigenetics is now used to describe the study of heritable changes in gene expression that are independent of the underlying DNA sequence (75–77). It is now known that epigenetic regulation is crucial for normal development and cellular differentiation, and is involved in numerous processes including genomic imprinting and X-chromosome inactivation (78–80). The cellular epigenetic states are susceptible to environmental influences and aging, and aberrant epigenetic changes are linked with various disease states (81). Furthermore, these studies in rats have reported transgenerational effects; embryonic rats treated with anti-androgens exhibit disrupted spermatogenesis in later generations (i.e. F₃) (82). The stability and persistence of these epigenetic changes suggests that they can be used to monitor the long-lasting effects of chemotherapeutic treatment in men.

Investigations into the molecular basis of epigenetic regulation have largely focused on DNA methylation and histone modifications, and how these alterations influence chromatin structure. However, the discovery of non-coding RNAs, specifically microRNAs (miRNAs), has revealed their critical importance in epigenetics and epigenetic regulation (83).

Histone Modifications—Genomic DNA in the cell is tightly packaged and coiled as repeating units of nucleosomes, which consists of a core of histones intertwined with DNA. The core histones, H2A, H2B, H3, and H4, and histone linker H1 share a common structure,

consisting of a central “fold domain” and “tails,” NH₂- and COOH-terminals (84). The N-terminal tails of the four histones extend from the nucleosome core and are targets for modifications. The histone tails are commonly acetylated at lysines, methylated at lysines and arginines, and phosphorylated at serines and threonines (as reviewed in (85)). The tail modifications contribute to the epigenetic code that regulates chromatin remodeling between the transcriptionally active euchromatin and transcriptionally silent heterochromatin (86).

During spermiogenesis, the chromatin is further condensed by replacing the histones in the nucleosomes of elongating spermatids with protamines (87, 88). The process is facilitated by the acetylation of H3 and H4 to lessen the histone-DNA interaction and allow for protamine substitution (89–91). However, approximately 15% of the genomic DNA in human sperm remains bound to histones (92–95), and post-translational modifications of these remaining histones could have profound functional importance. Studies have found that the genomic locations of the residual histones are not randomly located (96–99), providing further evidence that their epigenetic modifications may have critical regulatory consequences. Moreover, H3K27me₃, H3K4me₂, and H3K4me₃ are strongly enriched in the regulatory regions of genes related to embryogenesis and development, suggesting that these modifications may be inheritable by subsequent generations (96, 100, 101). H4 acetylation is required for normal fertility, while impaired H4 acetylation in mice results in spermatogenic arrest and ultimately sterility (102, 103).

DNA Methylation—In mammals, DNA methylation occurs at cytosine bases that are positioned 5' to guanosine in a cytosine-phosphate-guanine (CpG) dinucleotide (104). However, short interspersed CpG-rich regions called CpG islands (CGIs), are generally unmethylated in normal cells (105). CGIs are found in the proximal promoter regions of nearly half the genes in the genome, and hypermethylation of CGIs is associated with gene silencing. Methylation of CpGs is facilitated by two different types of DNA methyltransferases (DNMTs): i) *de novo* and ii) maintenance methyltransferases. DNMT3 homologs are responsible for *de novo* methylation of non-methylated or hemi-methylated DNA (106), while DNMT1 maintains the methylation status of hemi-methylated DNA through cell division (107). The function of DNMT1 is crucial in conserving the methylation status of the parent DNA strand in the daughter strands, and provides a mechanism for which an epigenetic mark is transmitted by a dividing cell to its progeny.

DNA methylation is essential for normal development and cellular differentiation, and is involved in numerous processes including genomic imprinting and X-chromosome inactivation (78–80). Furthermore, methylation marks in the germline are erased and reestablished during embryonic development in a sex-specific manner and during spermatogenesis (108–111). Since DNA methylation remodeling occurs during spermatogenesis, various studies have investigated the association between altered testicular function and sperm DNA methylation marks, finding aberrant DNA methylation of both imprinted (112–120) and non-imprinted genes (114, 121, 122). Genome-wide DNA methylation profiling of men with poor *in vitro* fertilization-related embryogenesis and abnormal sperm chromatin compaction found a subset of men with aberrant methylation of imprinted genes (123). Furthermore, methylation analysis of low motility sperm found that 34% of the CpGs analyzed were predominantly hypomethylated and were associated with genes involved in spermatogenesis and epigenetic regulation (124).

microRNAs—MicroRNAs (miRNAs) are ~22 nucleotide, single stranded, non-coding RNAs that regulate gene expression via sequence-specific base pairing with target gene transcripts (83). They have been discovered to have critical regulatory roles across almost all biological processes and are implicated in a variety of disease states (125–128). In mammals, miRNAs are highly conserved and inhibit transcript translation by binding in the

3' and 5' untranslated region (UTR) sequences (83, 129–133), and coding regions of targeted gene transcripts (134, 135). Recent evidence demonstrated that miRNAs regulate transcript abundance at the transcriptional level by binding to sequences in the promoter regions of targeted genes (136, 137). It is hypothesized that endogenously expressed miRNAs within the cells of every animal cell type at each developmental stage carry a specific miRNA profile to regulate the transcriptome (83).

It has become evident that miRNAs also have an essential role during spermatogenesis. Conditional knockdowns of the miRNA processing machinery in both the germ cells and Sertoli cells in the testis have resulted in aberrant effects on spermatogenesis. Studies in mice that have germ cells lacking Dicer, a protein involved in the maturation of miRNA from precursor miRNA, are either infertile or subfertile due to defects in both spermatogonial proliferation and differentiation, and aberrant transitions from round to elongating spermatids (138, 139). Furthermore, Sertoli cell-specific Dicer knockouts are depleted of spermatozoa and ultimately result in testicular degeneration (140). Although the molecular significance of miRNAs and their role in spermatogenesis is not clearly understood, it is evident that they are absolutely required during this process.

miRNAs have also been detected in mature spermatozoa (141–145), but their functional importance is still unknown. A recent clinical study that surveyed the sperm miRNA profiles of infertile couples identified an association between aberrant miRNA profiles and asthenozoospermia and oligoasthenozoospermia (146), suggesting that miRNAs are essential for normal sperm function. Additionally, it has been hypothesized that the miRNAs delivered to the oocyte by the sperm are involved in normal embryogenic development (144, 147–153). *In silico* miRNA target prediction and functional analysis identified miRNAs that target *IGF-2* receptor and *Dickkopf2* genes, which are involved in the regulation of growth and development (150, 154–156). Furthermore, the injection of miRNAs specific for *Kit* or *Sox9* into fertilized mouse embryos induced paramutation, a heritable mutant phenotype in the offspring (149, 157). miRNAs role in nearly all biological processes are becoming evident, and the presence of sperm-specific miRNAs in the developing zygote suggests that paternally derived miRNAs are involved in early embryogenic development (144).

Transgenerational Effects

Definition of “Transgenerational”

Inheritance of persistent biological effects following chemotherapy may result from either induced alterations in the DNA sequence *per se* (mutations) or from alterations in any of the epigenetic marks discussed in the previous sections. The appearance of disease-related mutations or altered epigenetic marks in progeny is often loosely termed a “transgenerational” effect; however, the strict definition of a transgenerational effect requires that the alteration be maintained for at least three generations following *in utero* exposures and for at least two generations following adult exposures (158). The reason for this requirement for persistence across multiple generations is obvious; in an adult (F_0 generation) exposed to chemotherapy, the germ cells of that adult, which produce the next generation (F_1 generation), are also directly exposed. The cells of the grandchildren (F_2 generation) of the treated adult are the first cells without the potential for direct exposure, making this the first generation to manifest true transgenerational effects. True transgenerational (F_2 generation) effects are of significant interest and importance because this implies a heritable consequence, either mutational or epigenetic, of the F_0 exposure.

Animal Studies of Transgenerational Effects

There is a rapidly growing literature that identifies transgenerational (F_3 generation) effects following *in utero* exposure to a variety of stressors, including nutritional deprivation (159,

160), exposures to environmental chemicals (161–163), and treatment with chemotherapeutic agents (164–166). Surprisingly, the male reproductive tract has been found to be particularly vulnerable to disruption by exposure to *in utero* stressors, including a variety of environmental chemicals that are endocrine disruptors (167–170).

With regard to exposure of adult male animals to stressors and any resulting transgenerational (F₂ generation) effects, the data are less robust but still persuasive. Adult male mice fed a high fat diet that induced obesity but not diabetes mellitus gave rise to F₁ and F₂ generation males and females with subfertility when mated to normal females (171). This effect of subfertility was paternally transmitted and transgenerational, since the dietary manipulation occurred only in the F₀ generation. In pigs, exposure of F₀ males to a diet with high amounts of methylating micronutrients resulted in paternal transmission to F₂ offspring of both phenotypic and molecular differences, including significant alterations in DNA methylation (172). Studies using radiation exposure of F₀ males have identified transgenerational (F₂ or F₃ generation) effects, including impaired cell proliferation (173–175), alterations in protein kinase signaling (176), elevated mutation rates (177, 178), and abnormalities in sperm chromatin (179).

Human Studies of Transgenerational Effects

There have been a number of human studies of progeny effects (F₁ generation) resulting from parental radiation and/or chemotherapy either during childhood or adulthood, and these studies provide contradictory evidence of generational effects (180–185). Best known is the evidence of an increased risk of paternally-mediated risk for childhood leukemia/lymphoma in the progeny of workers at the Sellafield nuclear facility (180–182), while no increase in childhood cancer was observed in progeny of survivors of the Hiroshima and Nagasaki atomic bomb explosions who were conceived more than a year after the explosions (183). The reason that the available human data is less conclusive than the animal studies regarding progeny effects may be due to technical limitations inherent to traditional human study designs focused on relatively rare endpoints; there is a reasonable expectation that the new sequencing technologies may address these technical limitations (186). There are no human studies of transgenerational (F₂ generation and beyond) effects of chemotherapy exposure.

Biomarkers of Persistent Transgenerational Risk

Studies in mice have shown genetic instability in the form of germ line mutations at ESTR (expanded simple tandem repeat) loci, and increased rates of tumors in the progeny of adult males exposed to radiation and chemotherapy (166, 178, 187). Measuring such an increase in genetic instability could be a biomarker for generational effects of paternal exposure in humans. Other potential biomarkers include alterations in epigenetic marks associated with paternal exposures, transmitted as sperm epimutations, and persisting in subsequent generations of progeny, as has been observed following *in utero* exposures in rats (167–170).

Conclusion and Outlook

Men undergoing chemotherapy treatment for cancer are vulnerable to the toxic and long-lasting reproductive effects of these compounds. These concerns are especially important with regards to couples concerned with conceiving children of their own because these gonadotoxic effects may not only impact their offspring, but also subsequent generations as well. Current clinical assessments rely heavily on traditional fertility endpoints, such as semen parameters, and do not leverage the quantitative information acquired from more advanced genetic and molecular assays for measuring sperm integrity. These advanced approaches are proven to be as sensitive, if not more so as testicular histopathology, and

could act as important clinical biomarkers for assessing damage and recovery following chemotherapy treatment.

References

1. Trasler JM, Hales BF, Robaire B. Paternal cyclophosphamide treatment of rats causes fetal loss and malformations without affecting male fertility. *Nature*. 1985; 316:144–6. [PubMed: 4040213]
2. Trasler JM, Hales BF, Robaire B. Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny. *Biol Reprod*. 1986; 34:275–83. [PubMed: 3082378]
3. Trasler JM, Hales BF, Robaire B. A time-course study of chronic paternal cyclophosphamide treatment in rats: effects on pregnancy outcome and the male reproductive and hematologic systems. *Biol Reprod*. 1987; 37:317–26. [PubMed: 3676391]
4. Bieber AM, Marcon L, Hales BF, Robaire B. Effects of chemotherapeutic agents for testicular cancer on the male rat reproductive system, spermatozoa, and fertility. *J Androl*. 2006; 27:189–200. [PubMed: 16278370]
5. Marcon L, Hales BF, Robaire B. Reversibility of the effects of subchronic exposure to the cancer chemotherapeutics bleomycin, etoposide, and cisplatin on spermatogenesis, fertility, and progeny outcome in the male rat. *J Androl*. 2008; 29:408–17. [PubMed: 18296477]
6. Cai L, Hales BF, Robaire B. Induction of apoptosis in the germ cells of adult male rats after exposure to cyclophosphamide. *Biol Reprod*. 1997; 56:1490–7. [PubMed: 9166702]
7. Sjoblom T, West A, Lahdetie J. Apoptotic response of spermatogenic cells to the germ cell mutagens etoposide, adriamycin, and diepoxybutane. *Environ Mol Mutagen*. 1998; 31:133–48. [PubMed: 9544191]
8. Bergada I, Milani C, Bedecarras P, Andreone L, Ropelato MG, Gottlieb S, et al. Time course of the serum gonadotropin surge, inhibins, and anti-Mullerian hormone in normal newborn males during the first month of life. *J Clin Endocrinol Metab*. 2006; 91:4092–8. [PubMed: 16849404]
9. Grinspon RP, Loreti N, Braslavsky D, Bedecarras P, Ambao V, Gottlieb S, et al. Sertoli cell markers in the diagnosis of paediatric male hypogonadism. *J Pediatr Endocrinol Metab*. 2012; 25:3–11. [PubMed: 22570945]
10. Young J, Chanson P, Salenave S, Noel M, Brailly S, O'Flaherty M, et al. Testicular anti-mullerian hormone secretion is stimulated by recombinant human FSH in patients with congenital hypogonadotropic hypogonadism. *J Clin Endocrinol Metab*. 2005; 90:724–8. [PubMed: 15536161]
11. Grinspon RP, Ropelato MG, Bedecarras P, Loreti N, Ballerini MG, Gottlieb S, et al. Gonadotrophin secretion pattern in anorchid boys from birth to pubertal age: pathophysiological aspects and diagnostic usefulness. *Clin Endocrinol (Oxf)*. 2012; 76:698–705. [PubMed: 22098623]
12. Grinspon RP, Ropelato MG, Gottlieb S, Keselman A, Martinez A, Ballerini MG, et al. Basal follicle-stimulating hormone and peak gonadotropin levels after gonadotropin-releasing hormone infusion show high diagnostic accuracy in boys with suspicion of hypogonadotropic hypogonadism. *J Clin Endocrinol Metab*. 2010; 95:2811–8. [PubMed: 20371659]
13. Grinspon RP, Rey RA. Anti-mullerian hormone and sertoli cell function in paediatric male hypogonadism. *Horm Res Paediatr*. 2010; 73:81–92. [PubMed: 20190544]
14. Kenney LB, Laufer MR, Grant FD, Grier H, Diller L. High risk of infertility and long term gonadal damage in males treated with high dose cyclophosphamide for sarcoma during childhood. *Cancer*. 2001; 91:613–21. [PubMed: 11169946]
15. Page ST. Physiologic role and regulation of intratesticular sex steroids. *Curr Opin Endocrinol Diabetes Obes*. 2011; 18:217–23. [PubMed: 21478750]
16. Islam N, Trainer PJ. The hormonal assessment of the infertile male. *Br J Urol*. 1998; 82:69–75. [PubMed: 9698664]
17. Pierik FH, Burdorf A, de Jong FH, Weber RF. Inhibin B: a novel marker of spermatogenesis. *Ann Med*. 2003; 35:12–20. [PubMed: 12693608]
18. Bonde JP, Giwercman A, Ernst E. Identifying environmental risk to male reproductive function by occupational sperm studies: logistics and design options. *Occupational and environmental medicine*. 1996; 53:511–9. [PubMed: 8983461]

19. Meistrich ML, Wilson G, Brown BW, da Cunha MF, Lipshultz LI. Impact of cyclophosphamide on long-term reduction in sperm count in men treated with combination chemotherapy for Ewing and soft tissue sarcomas. *Cancer*. 1992; 70:2703–12. [PubMed: 1423201]
20. Chatterjee R, Mills W, Katz M, McGarrigle HH, Goldstone AH. Germ cell failure and Leydig cell insufficiency in post-pubertal males after autologous bone marrow transplantation with BEAM for lymphoma. *Bone Marrow Transplant*. 1994; 13:519–22. [PubMed: 8054905]
21. Charak BS, Gupta R, Mandrekar P, Sheth NA, Banavali SD, Saikia TK, et al. Testicular dysfunction after cyclophosphamide-vincristine-procarbazine-prednisolone chemotherapy for advanced Hodgkin's disease. A long-term follow-up study. *Cancer*. 1990; 65:1903–6. [PubMed: 2115396]
22. Chapman RM, Sutcliffe SB, Rees LH, Edwards CR, Malpas JS. Cyclical combination chemotherapy and gonadal function. Retrospective study in males. *Lancet*. 1979; 1:285–9. [PubMed: 84944]
23. Whitehead E, Shalet SM, Blackledge G, Todd I, Crowther D, Beardwell CG. The effects of Hodgkin's disease and combination chemotherapy on gonadal function in the adult male. *Cancer*. 1982; 49:418–22. [PubMed: 7059904]
24. Gerl A, Mühlbayer D, Hansmann G, Mraz W, Hiddemann W. The impact of chemotherapy on Leydig cell function in long term survivors of germ cell tumors. *Cancer*. 2001; 91:1297–303. [PubMed: 11283930]
25. Howell SJ, Radford JA, Ryder WD, Shalet SM. Testicular function after cytotoxic chemotherapy: evidence of Leydig cell insufficiency. *J Clin Oncol*. 1999; 17:1493–8. [PubMed: 10334536]
26. Jahnukainen K, Heikkinen R, Henriksson M, Cooper TG, Puukko-Viertomies L-R, Mäkitie O. Semen quality and fertility in adult long-term survivors of childhood acute lymphoblastic leukemia. *Fertil Steril*. 2011; 96:837–42. [PubMed: 21868007]
27. Howell SJ, Radford JA, Smets EM, Shalet SM. Fatigue, sexual function and mood following treatment for haematological malignancy: the impact of mild Leydig cell dysfunction. *Br J Cancer*. 2000; 82:789–93. [PubMed: 10732747]
28. Goldberg RB, Rabin D, Alexander AN, Doelle GC, Getz GS. Suppression of plasma testosterone leads to an increase in serum total and high density lipoprotein cholesterol and apoproteins A-I and B. *J Clin Endocrinol Metab*. 1985; 60:203–7. [PubMed: 3917267]
29. Gerl A. Vascular toxicity associated with chemotherapy for testicular cancer. *Anticancer Drugs*. 1994; 5:607–14. [PubMed: 7534146]
30. Meinardi MT, Gietema JA, van der Graaf WT, van Veldhuisen DJ, Runne MA, Sluiter WJ, et al. Cardiovascular morbidity in long-term survivors of metastatic testicular cancer. *J Clin Oncol*. 2000; 18:1725–32. [PubMed: 10764433]
31. Holmes SJ, Whitehouse RW, Clark ST, Crowther DC, Adams JE, Shalet SM. Reduced bone mineral density in men following chemotherapy for Hodgkin's disease. *Br J Cancer*. 1994; 70:371–5. [PubMed: 8054287]
32. Burrello N, Vicari E, Calogero AE. Chromosome abnormalities in spermatozoa of patients with azoospermia and normal somatic karyotype. *Cytogenet Genome Res*. 2005; 111:363–5. [PubMed: 16192717]
33. Egozcue J, Blanco J, Anton E, Egozcue S, Sarrate Z, Vidal F. Genetic analysis of sperm and implications of severe male infertility--a review. *Placenta*. 2003; 24 (Suppl B):S62–5. [PubMed: 14559032]
34. Ryu HM, Lin WW, Lamb DJ, Chuang W, Lipshultz LI, Bischoff FZ. Increased chromosome X, Y, and 18 nondisjunction in sperm from infertile patients that were identified as normal by strict morphology: implication for intracytoplasmic sperm injection. *Fertil Steril*. 2001; 76:879–83. [PubMed: 11704105]
35. Sarrate Z, Blanco J, Anton E, Egozcue S, Egozcue J, Vidal F. FISH studies of chromosome abnormalities in germ cells and its relevance in reproductive counseling. *Asian J Androl*. 2005; 7:227–36. [PubMed: 16110350]
36. Attia SM, Schmid TE, Badary OA, Hamada FM, Adler ID. Molecular cytogenetic analysis in mouse sperm of chemically induced aneuploidy: studies with topoisomerase II inhibitors. *Mutat Res*. 2002; 520:1–13. [PubMed: 12297139]

37. Schmid TE, Xu W, Adler ID. Detection of aneuploidy by multicolor FISH in mouse sperm after in vivo treatment with acrylamide, colchicine, diazepam or thiabendazole. *Mutagenesis*. 1999; 14:173–9. [PubMed: 10229918]
38. Attia SM, Badary OA, Hamada FM, Hrabe de Angelis M, Adler ID. The chemotherapeutic agents nocodazole and amsacrine cause meiotic delay and non-disjunction in spermatocytes of mice. *Mutat Res*. 2008; 651:105–13. [PubMed: 18069049]
39. Barton TS, Wyrobek AJ, Hill FS, Robaire B, Hales BF. Numerical chromosomal abnormalities in rat epididymal spermatozoa following chronic cyclophosphamide exposure. *Biol Reprod*. 2003; 69:1150–7. [PubMed: 12773405]
40. De Mas P, Daudin M, Vincent MC, Bourrouillou G, Calvas P, Mieusset R, et al. Increased aneuploidy in spermatozoa from testicular tumour patients after chemotherapy with cisplatin, etoposide and bleomycin. *Hum Reprod*. 2001; 16:1204–8. [PubMed: 11387293]
41. Martin RH, Ernst S, Rademaker A, Barclay L, Ko E, Summers N. Analysis of sperm chromosome complements before, during, and after chemotherapy. *Cancer Genet Cytogenet*. 1999; 108:133–6. [PubMed: 9973940]
42. Tempest HG, Ko E, Chan P, Robaire B, Rademaker A, Martin RH. Sperm aneuploidy frequencies analysed before and after chemotherapy in testicular cancer and Hodgkin's lymphoma patients. *Hum Reprod*. 2008; 23:251–8. [PubMed: 18083744]
43. Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study. *Team Fertil Steril*. 2000; 73:43–50.
44. Tesarik J, Mendoza C, Greco E. Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. *Hum Reprod*. 2002; 17:184–9. [PubMed: 11756385]
45. Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online*. 2003; 7:65–70. [PubMed: 12930576]
46. Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl*. 2005; 26:349–53. [PubMed: 15867002]
47. Smit M, Romijn JC, Wildhagen MF, Veldhoven JL, Weber RF, Dohle GR. Decreased sperm DNA fragmentation after surgical varicocelectomy is associated with increased pregnancy rate. *J Urol*. 2010; 183:270–4. [PubMed: 19913801]
48. Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod*. 2004; 19:1401–8. [PubMed: 15117894]
49. Larson KL, DeJonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod*. 2000; 15:1717–22. [PubMed: 10920092]
50. Matzuk MM, Lamb DJ. Genetic dissection of mammalian fertility pathways. *Nat Cell Biol*. 2002; 4 (Suppl):s41–9. [PubMed: 12479614]
51. Matzuk MM, Lamb DJ. The biology of infertility: research advances and clinical challenges. *Nature medicine*. 2008; 14:1197–213.
52. Aoki VW, Christensen GL, Atkins JF, Carrell DT. Identification of novel polymorphisms in the nuclear protein genes and their relationship with human sperm protamine deficiency and severe male infertility. *Fertil Steril*. 2006; 86:1416–22. [PubMed: 16989827]
53. Aoki VW, Moskovtsev SI, Willis J, Liu L, Mullen JB, Carrell DT. DNA integrity is compromised in protamine-deficient human sperm. *J Androl*. 2005; 26:741–8. [PubMed: 16291969]
54. Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod*. 2002; 66:1061–7. [PubMed: 11906926]
55. Bujan L, Walschaerts M, Moinard N, Hennebicq S, Saias J, Brignon F, et al. Impact of chemotherapy and radiotherapy for testicular germ cell tumors on spermatogenesis and sperm DNA: a multicenter prospective study from the CECOS network. *Fertil Steril*. 2013

56. O'Flaherty C, Hales BF, Chan P, Robaire B. Impact of chemotherapeutics and advanced testicular cancer or Hodgkin lymphoma on sperm deoxyribonucleic acid integrity. *Fertil Steril.* 2010; 94:1374–9. [PubMed: 19591994]
57. Spermon JR, Ramos L, Wetzels AM, Sweep CG, Braat DD, Kiemeny LA, et al. Sperm integrity pre- and post-chemotherapy in men with testicular germ cell cancer. *Hum Reprod.* 2006; 21:1781–6. [PubMed: 16601009]
58. Heller CG, Clermont Y. Spermatogenesis in man: an estimate of its duration. *Science.* 1963; 140:184–6. [PubMed: 13953583]
59. Pacheco SE, Anderson LM, Sandrof MA, Vantangoli MM, Hall SJ, Boekelheide K. Sperm mRNA transcripts are indicators of sub-chronic low dose testicular injury in the Fischer 344 rat. *PLoS ONE.* 2012; 7:e44280. [PubMed: 22952946]
60. Delbes G, Chan D, Hales BF, Trasler JM, Robaire B. Selective induction of glutathione S-transferases in round spermatids from the Brown-Norway rat by the chemotherapeutic regimen for testicular cancer. *Reprod Toxicol.* 2013; 36:24–32. [PubMed: 23200778]
61. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. *Lancet.* 2002; 360:772–7. [PubMed: 12241836]
62. Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. Reproductive biology: delivering spermatozoan RNA to the oocyte. *Nature.* 2004; 429:154. [PubMed: 15141202]
63. Ostermeier GC, Goodrich RJ, Diamond MP, Dix DJ, Krawetz SA. Toward using stable spermatozoal RNAs for prognostic assessment of male factor fertility. *Fertil Steril.* 2005; 83:1687–94. [PubMed: 15950637]
64. Sandler E, Johnson GD, Mao S, Goodrich RJ, Diamond MP, Hauser R, et al. Stability, delivery and functions of human sperm RNAs at fertilization. *Nucleic Acids Res.* 2013; 41:4104–17. [PubMed: 23471003]
65. Lambard S, Galeraud-Denis I, Martin G, Levy R, Chocat A, Carreau S. Analysis and significance of mRNA in human ejaculated sperm from normozoospermic donors: relationship to sperm motility and capacitation. *Mol Hum Reprod.* 2004; 10:535–41. [PubMed: 15100385]
66. Brahem S, Mehdi M, Landolsi H, Mougou S, Elghezal H, Saad A. Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. *Urology.* 2011; 78:792–6. [PubMed: 21813165]
67. Wang G, Guo Y, Zhou T, Shi X, Yu J, Yang Y, et al. In-depth proteomic analysis of the human sperm reveals complex protein compositions. *J Proteomics.* 2013; 79:114–22. [PubMed: 23268119]
68. Baker MA, Naumovski N, Hetherington L, Weinberg A, Velkov T, Aitken RJ. Head and flagella subcompartmental proteomic analysis of human spermatozoa. *Proteomics.* 2013; 13:61–74. [PubMed: 23161668]
69. Johnston DS, Wooters J, Kopf GS, Qiu Y, Roberts KP. Analysis of the human sperm proteome. *Ann N Y Acad Sci.* 2005; 1061:190–202. [PubMed: 16467268]
70. Baker MA, Reeves G, Hetherington L, Müller J, Baur I, Aitken RJ. Identification of gene products present in Triton X-100 soluble and insoluble fractions of human spermatozoa lysates using LC-MS/MS analysis. *Proteomics Clin Appl.* 2007; 1:524–32. [PubMed: 21136703]
71. Petricoin EF, Belluco C, Araujo RP, Liotta LA. The blood peptidome: a higher dimension of information content for cancer biomarker discovery. *Nat Rev Cancer.* 2006; 6:961–7. [PubMed: 17093504]
72. Siva AB, Kameshwari DB, Singh V, Pavani K, Sundaram CS, Rangaraj N, et al. Proteomics-based study on asthenozoospermia: differential expression of proteasome alpha complex. *Molecular Human Reproduction.* 2010; 16:452–62. [PubMed: 20304782]
73. Martínez-Heredia J, de Mateo S, Vidal-Taboada JM, Ballescà JL, Oliva R. Identification of proteomic differences in asthenozoospermic sperm samples. *Hum Reprod.* 2008; 23:783–91. [PubMed: 18281682]
74. Lefièvre L, Chen Y, Conner SJ, Scott JL, Publicover SJ, Ford WCL, et al. Human spermatozoa contain multiple targets for protein S-nitrosylation: an alternative mechanism of the modulation of sperm function by nitric oxide? *Proteomics.* 2007; 7:3066–84. [PubMed: 17683036]

75. Waddington, CH. The Strategy of the Genes: A Discussion of some Aspects of theoretical Biology. 1957.
76. Bird A. Perceptions of epigenetics. *Nature*. 2007; 447:396–8. [PubMed: 17522671]
77. Holliday R. The inheritance of epigenetic defects. *Science*. 1987; 238:163–70. [PubMed: 3310230]
78. Csankovszki G, Nagy A, Jaenisch R. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. *J Cell Biol*. 2001; 153:773–84. [PubMed: 11352938]
79. Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, et al. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*. 2004; 429:900–3. [PubMed: 15215868]
80. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. *Science*. 2001; 293:1068–70. [PubMed: 11498573]
81. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*. 2003; 33 (Suppl):245–54. [PubMed: 12610534]
82. Manikkam M, Guerrero-Bosagna C, Tracey R, Haque MM, Skinner MK. Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PLoS ONE*. 2012; 7:e31901. [PubMed: 22389676]
83. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116:281–97. [PubMed: 14744438]
84. Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*. 1997; 389:251–60. [PubMed: 9305837]
85. Felsenfeld G, Groudine M. Controlling the double helix. *Nature*. 2003; 421:448–53. [PubMed: 12540921]
86. Strahl BD, Allis CD. The language of covalent histone modifications. *Nature*. 2000; 403:41–5. [PubMed: 10638745]
87. Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod*. 1991; 44:569–74. [PubMed: 2043729]
88. Wykes SM, Krawetz SA. The structural organization of sperm chromatin. *J Biol Chem*. 2003; 278:29471–7. [PubMed: 12775710]
89. Meistrich ML, Trostle-Weige PK, Lin R, Bhatnagar YM, Allis CD. Highly acetylated H4 is associated with histone displacement in rat spermatids. *Mol Reprod Dev*. 1992; 31:170–81. [PubMed: 1372808]
90. Nair M, Nagamori I, Sun P, Mishra DP, Rhéaume C, Li B, et al. Nuclear regulator Pygo2 controls spermiogenesis and histone H3 acetylation. *Dev Biol*. 2008; 320:446–55. [PubMed: 18614164]
91. Hazzouri M, Pivot-Pajot C, Faure AK, Usson Y, Pelletier R, Sèle B, et al. Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone deacetylases. *Eur J Cell Biol*. 2000; 79:950–60. [PubMed: 11152286]
92. Bench GS, Friz AM, Corzett MH, Morse DH, Balhorn R. DNA and total protamine masses in individual sperm from fertile mammalian subjects. *Cytometry*. 1996; 23:263–71. [PubMed: 8900468]
93. Gatewood JM, Cook GR, Balhorn R, Schmid CW, Bradbury EM. Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones. *J Biol Chem*. 1990; 265:20662–6. [PubMed: 2243112]
94. Tanphaichitr N, Sobhon P, Taluppeth N, Chalermisarachai P. Basic nuclear proteins in testicular cells and ejaculated spermatozoa in man. *Exp Cell Res*. 1978; 117:347–56. [PubMed: 720415]
95. Zalensky AO, Breneman JW, Zalenskaya IA, Brinkley BR, Bradbury EM. Organization of centromeres in the decondensed nuclei of mature human sperm. *Chromosoma*. 1993; 102:509–18. [PubMed: 8243163]
96. Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. *Nature*. 2009; 460:473–8. [PubMed: 19525931]
97. Ooi SL, Henikoff S. Germline histone dynamics and epigenetics. *Curr Opin Cell Biol*. 2007; 19:257–65. [PubMed: 17467256]

98. Gardiner-Garden M, Ballesteros M, Gordon M, Tam PP. Histone- and protamine-DNA association: conservation of different patterns within the beta-globin domain in human sperm. *Mol Cell Biol.* 1998; 18:3350–6. [PubMed: 9584175]
99. Gatewood JM, Cook GR, Balhorn R, Bradbury EM, Schmid CW. Sequence-specific packaging of DNA in human sperm chromatin. *Science.* 1987; 236:962–4. [PubMed: 3576213]
100. Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley EJ, Roloff TC, et al. Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nat Struct Mol Biol.* 2010; 17:679–87. [PubMed: 20473313]
101. Carrell DT, Hammoud SS. The human sperm epigenome and its potential role in embryonic development. *Mol Hum Reprod.* 2010; 16:37–47. [PubMed: 19906823]
102. Faure AK, Pivot-Pajot C, Kerjean A, Hazzouri M, Pelletier R, Péoc'h M, et al. Misregulation of histone acetylation in Sertoli cell-only syndrome and testicular cancer. *Mol Hum Reprod.* 2003; 9:757–63. [PubMed: 14614037]
103. Sonnack V, Failing K, Bergmann M, Steger K. Expression of hyperacetylated histone H4 during normal and impaired human spermatogenesis. *Andrologia.* 2002; 34:384–90. [PubMed: 12472623]
104. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev.* 2002; 16:6–21. [PubMed: 11782440]
105. Bird AP. CpG-rich islands and the function of DNA methylation. *Nature.* 1986; 321:209–13. [PubMed: 2423876]
106. Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet.* 1998; 19:219–20. [PubMed: 9662389]
107. Bestor T, Laudano A, Mattaliano R, Ingram V. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J Mol Biol.* 1988; 203:971–83. [PubMed: 3210246]
108. Bourc'his D, Bestor TH. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature.* 2004; 431:96–9. [PubMed: 15318244]
109. Hata K, Kusumi M, Yokomine T, Li E, Sasaki H. Meiotic and epigenetic aberrations in Dnmt3L-deficient male germ cells. *Mol Reprod Dev.* 2006; 73:116–22. [PubMed: 16211598]
110. Trasler JM. Gamete imprinting: setting epigenetic patterns for the next generation. *Reprod Fertil Dev.* 2006; 18:63–9. [PubMed: 16478603]
111. Webster KE, O'Bryan MK, Fletcher S, Crewther PE, Aapola U, Craig J, et al. Meiotic and epigenetic defects in Dnmt3L-knockout mouse spermatogenesis. *Proc Natl Acad Sci USA.* 2005; 102:4068–73. [PubMed: 15753313]
112. Boissonnas CC, Abdalaoui HE, Haelewyn V, Fauque P, Dupont JM, Gut I, et al. Specific epigenetic alterations of IGF2-H19 locus in spermatozoa from infertile men. *Eur J Hum Genet.* 2010; 18:73–80. [PubMed: 19584898]
113. Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT. Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertil Steril.* 2010; 94:1728–33. [PubMed: 19880108]
114. Houshdaran S, Cortessis VK, Siegmund K, Yang A, Laird PW, Sokol RZ. Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. *PLoS ONE.* 2007; 2:e1289. [PubMed: 18074014]
115. Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsunomiya T, et al. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. *Hum Mol Genet.* 2007; 16:2542–51. [PubMed: 17636251]
116. Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A, et al. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. *Mol Hum Reprod.* 2008; 14:67–74. [PubMed: 18178607]
117. Marques CJ, Carvalho F, Sousa M, Barros A. Genomic imprinting in disruptive spermatogenesis. *Lancet.* 2004; 363:1700–2. [PubMed: 15158633]
118. Pathak S, Kedia-Mokashi N, Saxena M, D'Souza R, Maitra A, Parte P, et al. Effect of tamoxifen treatment on global and insulin-like growth factor 2-H19 locus-specific DNA methylation in rat

- spermatozoa and its association with embryo loss. *Fertil Steril.* 2009; 91:2253–63. [PubMed: 18778817]
119. Poplinski A, Tüttelmann F, Kanber D, Horsthemke B, Gromoll J. Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *Int J Androl.* 2010; 33:642–9. [PubMed: 19878521]
 120. Sato A, Hiura H, Okae H, Miyauchi N, Abe Y, Utsunomiya T, et al. Assessing loss of imprint methylation in sperm from subfertile men using novel methylation polymerase chain reaction Luminex analysis. *Fertil Steril.* 2011; 95:129–34. 34, e1–4. [PubMed: 20655520]
 121. Navarro-Costa P, Nogueira P, Carvalho M, Leal F, Cordeiro I, Calhaz-Jorge C, et al. Incorrect DNA methylation of the DAZL promoter CpG island associates with defective human sperm. *Hum Reprod.* 2010; 25:2647–54. [PubMed: 20685756]
 122. Wu W, Shen O, Qin Y, Niu X, Lu C, Xia Y, et al. Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (MTHFR). *PLoS ONE.* 2010; 5:e13884. [PubMed: 21085488]
 123. Aston KI, Punj V, Liu L, Carrell DT. Genome-wide sperm deoxyribonucleic acid methylation is altered in some men with abnormal chromatin packaging or poor in vitro fertilization embryogenesis. *Fertil Steril.* 2012; 97:285–92. [PubMed: 22154369]
 124. Pacheco SE, Houseman EA, Christensen BC, Marsit CJ, Kelsey KT, Sigman M, et al. Integrative DNA methylation and gene expression analyses identify DNA packaging and epigenetic regulatory genes associated with low motility sperm. *PLoS ONE.* 2011; 6:e20280. [PubMed: 21674046]
 125. Geekiyanage H, Chan C. MicroRNA-137/181c regulates serine palmitoyltransferase and in turn amyloid β , novel targets in sporadic Alzheimer's disease. *J Neurosci.* 2011; 31:14820–30. [PubMed: 21994399]
 126. Hudson RS, Yi M, Esposito D, Watkins SK, Hurwitz AA, Yfantis HG, et al. MicroRNA-1 is a candidate tumor suppressor and prognostic marker in human prostate cancer. *Nucleic Acids Res.* 2011
 127. Kalis M, Bolmeson C, Esguerra JLS, Gupta S, Edlund A, Tormo-Badia N, et al. Beta-cell specific deletion of Dicer1 leads to defective insulin secretion and diabetes mellitus. *PLoS ONE.* 2011; 6:e29166. [PubMed: 22216196]
 128. Siegel SR, Mackenzie J, Chaplin G, Jablonski NG, Griffiths L. Circulating microRNAs involved in multiple sclerosis. *Mol Biol Rep.* 2012
 129. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell.* 1993; 75:843–54. [PubMed: 8252621]
 130. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell.* 1993; 75:855–62. [PubMed: 8252622]
 131. Lee I, Ajay SS, Yook JI, Kim HS, Hong SH, Kim NH, et al. New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites. *Genome Res.* 2009; 19:1175–83. [PubMed: 19336450]
 132. Lytle JR, Yario TA, Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci USA.* 2007; 104:9667–72. [PubMed: 17535905]
 133. Moretti F, Thermann R, Hentze MW. Mechanism of translational regulation by miR-2 from sites in the 5' untranslated region or the open reading frame. *RNA.* 2010; 16:2493–502. [PubMed: 20966199]
 134. Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature.* 2008; 455:1124–8. [PubMed: 18806776]
 135. Zhou X, Duan X, Qian J, Li F. Abundant conserved microRNA target sites in the 5'-untranslated region and coding sequence. *Genetica.* 2009; 137:159–64. [PubMed: 19578934]
 136. Kim DH, Saetrom P, Snøve O, Rossi JJ. MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci USA.* 2008; 105:16230–5. [PubMed: 18852463]

137. Place RF, Li L-C, Pookot D, Noonan EJ, Dahiya R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci USA*. 2008; 105:1608–13. [PubMed: 18227514]
138. Hayashi K, Chuva de Sousa Lopes SM, Kaneda M, Tang F, Hajkova P, Lao K, et al. MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. *PLoS ONE*. 2008; 3:e1738. [PubMed: 18320056]
139. Maatouk DM, Loveland KL, Mcmanus MT, Moore K, Harfe BD. Dicer1 is required for differentiation of the mouse male germline. *Biol Reprod*. 2008; 79:696–703. [PubMed: 18633141]
140. Papaioannou MD, Pitetti J-L, Ro S, Park C, Aubry F, Schaad O, et al. Sertoli cell Dicer is essential for spermatogenesis in mice. *Dev Biol*. 2009; 326:250–9. [PubMed: 19071104]
141. Amanai M, Brahmajosyula M, Perry ACF. A restricted role for sperm-borne microRNAs in mammalian fertilization. *Biol Reprod*. 2006; 75:877–84. [PubMed: 16943360]
142. Curry E, Ellis SE, Pratt SL. Detection of porcine sperm microRNAs using a heterologous microRNA microarray and reverse transcriptase polymerase chain reaction. *Mol Reprod Dev*. 2009; 76:218–9. [PubMed: 19012322]
143. Curry E, Safranski TJ, Pratt SL. Differential expression of porcine sperm microRNAs and their association with sperm morphology and motility. *Theriogenology*. 2011; 76:1532–9. [PubMed: 21872314]
144. Krawetz SA, Kruger A, Lalancette C, Tagett R, Anton E, Draghici S, et al. A survey of small RNAs in human sperm. *Hum Reprod*. 2011; 26:3401–12. [PubMed: 21989093]
145. Liu W-M, Pang RTK, Chiu PCN, Wong BPC, Lao K, Lee K-F, et al. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc Natl Acad Sci USA*. 2012; 109:490–4. [PubMed: 22203953]
146. Abu-Halima M, Hammadeh M, Schmitt J, Leidinger P, Keller A, Meese E, et al. Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments. *Fertil Steril*. 2013; 99:1249–55. e16. [PubMed: 23312218]
147. Castanotto D, Tommasi S, Li M, Li H, Yanow S, Pfeifer GP, et al. Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. *Mol Ther*. 2005; 12:179–83. [PubMed: 15963934]
148. Dadoune J-P. Spermatozoal RNAs: what about their functions? *Microsc Res Tech*. 2009; 72:536–51. [PubMed: 19283828]
149. Grandjean V, Gounon P, Wagner N, Martin L, Wagner KD, Bernex F, et al. The miR-124-Sox9 paramutation: RNA-mediated epigenetic control of embryonic and adult growth. *Development*. 2009; 136:3647–55. [PubMed: 19820183]
150. Krawetz SA. Paternal contribution: new insights and future challenges. *Nat Rev Genet*. 2005; 6:633–42. [PubMed: 16136654]
151. Matzke MA, Birchler JA. RNAi-mediated pathways in the nucleus. *Nat Rev Genet*. 2005; 6:24–35. [PubMed: 15630419]
152. Morris KV, Chan SW-L, Jacobsen SE, Looney DJ. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science*. 2004; 305:1289–92. [PubMed: 15297624]
153. Weinberg MS, Villeneuve LM, Ehsani A, Amarzguioui M, Aagaard L, Chen Z-X, et al. The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *RNA*. 2006; 12:256–62. [PubMed: 16373483]
154. Mao B, Wu W, Li Y, Hoppe D, Stanek P, Glinka A, et al. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature*. 2001; 411:321–5. [PubMed: 11357136]
155. Mao B, Niehrs C. Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. *Gene*. 2003; 302:179–83. [PubMed: 12527209]
156. Ostermeier GC, Goodrich RJ, Moldenhauer JS, Diamond MP, Krawetz SA. A suite of novel human spermatozoal RNAs. *J Androl*. 2005; 26:70–4. [PubMed: 15611569]
157. Rassoulzadegan M, Grandjean V, Gounon P, Vincent S, Gillot I, Cuzin F. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature*. 2006; 441:469–74. [PubMed: 16724059]

158. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Genet.* 2007; 8:253–62. [PubMed: 17363974]
159. Ponzio BF, Carvalho MH, Fortes ZB, do Carmo Franco M. Implications of maternal nutrient restriction in transgenerational programming of hypertension and endothelial dysfunction across F1–F3 offspring. *Life sciences.* 2012; 90:571–7. [PubMed: 22365957]
160. Burdge GC, Hoile SP, Uller T, Thomas NA, Gluckman PD, Hanson MA, et al. Progressive, transgenerational changes in offspring phenotype and epigenotype following nutritional transition. *PLoS ONE.* 2011; 6:e28282. [PubMed: 22140567]
161. Guerrero-Bosagna C, Skinner MK. Environmentally induced epigenetic transgenerational inheritance of phenotype and disease. *Mol Cell Endocrinol.* 2012; 354:3–8. [PubMed: 22020198]
162. Manikkam M, Guerrero-Bosagna C, Tracey R, Haque MM, Skinner MK. Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PLoS ONE.* 2012; 7:e31901. [PubMed: 22389676]
163. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PLoS ONE.* 2013; 8:e55387. [PubMed: 23359474]
164. Baulch JE, Raabe OG, Wiley LM. Heritable effects of paternal irradiation in mice on signaling protein kinase activities in F3 offspring. *Mutagenesis.* 2001; 16:17–23. [PubMed: 11139595]
165. Kujjo LL, Chang EA, Pereira RJ, Dhar S, Marrero-Rosado B, Sengupta S, et al. Chemotherapy-induced late transgenerational effects in mice. *PLoS ONE.* 2011; 6:e17877. [PubMed: 21437292]
166. Glen CD, Dubrova YE. Exposure to anticancer drugs can result in transgenerational genomic instability in mice. *Proceedings of the National Academy of Sciences of the United States of America.* 2012; 109:2984–8. [PubMed: 22308437]
167. Guerrero-Bosagna C, Savenkova M, Haque MM, Nilsson E, Skinner MK. Environmentally induced epigenetic transgenerational inheritance of altered Sertoli cell transcriptome and epigenome: molecular etiology of male infertility. *PLoS ONE.* 2013; 8:e59922. [PubMed: 23555832]
168. Guerrero-Bosagna C, Covert TR, Haque MM, Settles M, Nilsson EE, Anway MD, et al. Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reprod Toxicol.* 2012; 34:694–707. [PubMed: 23041264]
169. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PLoS ONE.* 2013; 8:e55387. [PubMed: 23359474]
170. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations. *PLoS ONE.* 2012; 7:e46249. [PubMed: 23049995]
171. Fullston T, Palmer NO, Owens JA, Mitchell M, Bakos HW, Lane M. Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod.* 2012; 27:1391–400. [PubMed: 22357767]
172. Braunschweig M, Jagannathan V, Gutzwiller A, Bee G. Investigations on transgenerational epigenetic response down the male line in F2 pigs. *PLoS ONE.* 2012; 7:e30583. [PubMed: 22359544]
173. Baulch JE, Raabe OG, Wiley LM, Overstreet JW. Germline drift in chimeric male mice possessing an F2 component with a paternal F0 radiation history. *Mutagenesis.* 2002; 17:9–13. [PubMed: 11752228]
174. Slovinská L, Elbertová A, Misúrová E. Transmission of genome damage from irradiated male rats to their progeny. *Mutat Res.* 2004; 559:29–37. [PubMed: 15066571]
175. Wiley LM, Baulch JE, Raabe OG, Straume T. Impaired cell proliferation in mice that persists across at least two generations after paternal irradiation. *Radiat Res.* 1997; 148:145–51. [PubMed: 9254733]
176. Baulch JE, Raabe OG, Wiley LM. Heritable effects of paternal irradiation in mice on signaling protein kinase activities in F3 offspring. *Mutagenesis.* 2001; 16:17–23. [PubMed: 11139595]

177. Barber R, Plumb MA, Boulton E, Roux I, Dubrova YE. Elevated mutation rates in the germ line of first- and second-generation offspring of irradiated male mice. *Proc Natl Acad Sci USA*. 2002; 99:6877–82. [PubMed: 11997464]
178. Dubrova YE, Plumb M, Gutierrez B, Boulton E, Jeffreys AJ. Transgenerational mutation by radiation. *Nature*. 2000; 405:37. [PubMed: 10811208]
179. Baulch JE, Li M-W, Raabe OG. Effect of ATM heterozygosity on heritable DNA damage in mice following paternal F0 germline irradiation. *Mutat Res*. 2007; 616:34–45. [PubMed: 17161850]
180. Dickinson HO, Parker L. Leukaemia and non-Hodgkin's lymphoma in children of male Sellafield radiation workers. *Int J Cancer*. 2002; 99:437–44. [PubMed: 11992415]
181. Gardner MJ, Hall AJ, Snee MP, Downes S, Powell CA, Terrell JD. Methods and basic data of case-control study of leukaemia and lymphoma among young people near Sellafield nuclear plant in West Cumbria. *BMJ*. 1990; 300:429–34. [PubMed: 2107893]
182. Gardner MJ, Snee MP, Hall AJ, Powell CA, Downes S, Terrell JD. Results of case-control study of leukaemia and lymphoma among young people near Sellafield nuclear plant in West Cumbria. *BMJ*. 1990; 300:423–9. [PubMed: 2107892]
183. Yoshimoto Y, Mabuchi K. Mortality and cancer risk among the offspring (F1) of atomic bomb survivors. *J Radiat Res*. 1991; 32 (Suppl):294–300. [PubMed: 1762118]
184. Little MP, Goodhead DT, Bridges BA, Bouffler SD. Evidence relevant to untargeted and transgenerational effects in the offspring of irradiated parents. *Mutat Res*. 2013
185. Signorello LB, Mulvihill JJ, Green DM, Munro HM, Stovall M, Weathers RE, et al. Congenital anomalies in the children of cancer survivors: a report from the childhood cancer survivor study. *J Clin Oncol*. 2012; 30:239–45. [PubMed: 22162566]
186. Demarini DM. Declaring the existence of human germ-cell mutagens. *Environ Mol Mutagen*. 2012; 53:166–72. [PubMed: 22351488]
187. Nomura T. Transgenerational effects of radiation and chemicals in mice and humans. *J Radiat Res*. 2006; 47 (Suppl B):B83–97. [PubMed: 17019056]

Figure 1.
Example of sperm FISH depicting normal X and Y bearing sperm (A), and sperm with sex chromosome disomy (B).