Mammalian sperm chromatin structure and assessment of DNA fragmentation

S. M. H. Andrabi

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Abstract This review article illustrates the biology of mammalian sperm chromatin structure. The possible causes of DNA (deoxyribonucleic acid) fragmentation are discussed. Also available molecular techniques for assessment of mammalian sperm DNA damage are described.

Keywords Sperm chromatin · DNA fragmentation · Mammals

Introduction

Unlike other animal species, mammalian sperm chromatin differs from somatic cells in structure and composition [1]. Sperm chromatin structure protects genetic integrity during transport of the paternal genome through the male and female reproductive tracts [2, 3]. The sperm DNA (deoxyribonucleic acid) is packed with specific small, basic proteins into a tight, almost crystalline status that is at least six times more condensed than in mitotic chromosomes [4].

Spermatids develop into spermatozoa through process of spermatogenesis. The development entails the loss of almost all of the cytoplasm and the development of a motile tail [5]. The sperm develops a highly condensed species-specific nuclear shape and replaces somatic-type histones with sperm-specific basic nuclear proteins (protamines) leading to highly packaged chromatin [6]. In some of the mammalian species like humans, rodents, boar and

ram this replacement involves a set of special proteins known as transition nuclear proteins (TP) [7–9].

There is a growing understanding about the importance of sperm DNA integrity in embryo development and afterward on health of the offspring. Therefore, the purpose of this review is to understand the structure of mammalian sperm chromatin and to know the possible causes of damage to it in the rapidly advancing postgenomic era. This essay will also evaluate the molecular techniques used for study of sperm DNA fragmentation in mammals.

Sperm chromatin structure and packaging

Histones, protamines and histone replacement

The basic unit of chromatin is the nucleosome, which consist of 146 base pairs of DNA wrapped around an octamer of core histones, including two molecules of H2A, H2B, H3 and H4 [10]. There is also a fifth histone H1 that protects additional DNA fragments linking neighboring nucleosomes [11]. More specifically, the histones H3 and H4 form a dimer, two H3–H4 dimers associate into a (H3–H4)₂ tetramer. DNA wraps around this tetramer, forming a tetrameric particle. Histones H2A and H2B heterodimerize and heterodimers associate on each side of the tetrameric particle to form a nucleosome [10, 12]. The histone modifying enzymes dictate combinations of post-translational modifications (PTM) of histones to create specific signals defining the histone code, which in turn induces localized alterations of the chromatin structure and function. A variety of PTM are lysine and arginine methylation, lysine acetylation, serine and threonine phosphorylation, and lysine ubiquitination [13]. Variants of histones H2A, H2B, H3 and H1 have been identified (refer to Tables 1 and 2),

S. M. H. Andrabi (✉)
Animal Reproduction Laboratory, Animal Sciences Institute,
National Agricultural Research Centre, Park Road,
Islamabad 45500, Pakistan
e-mail: andrabi123@yahoo.com

Table 1 Variants of histones in mammalian spermatogenic cells [13, 15]

Histone	Somatic variant	Gametogenic variant	Testis-specific variant
H1	H1b, H1c, H1d, H1e, H1°	H1a	H1t, HILS1
H2A	H2A.1, H2A.2, H2A.Z	H2A.X	TH2A
H2B	H2B.1	–	TH2B
H3	H3.1, H3.2, H3.3	–	TH3/H3t
H4	No variants	No variants	No variants

and of some of the variants have been shown to mediate specific functions such as DNA repair [14].

Protamines are highly basic proteins that are about half the size of a typical histone (5–8 kDa). From 55 to 79% of the amino acid residues of protamines are arginines, permitting a strong DNA binding. Protamines also contain a significant number of cystein residues that are very important during the final stages of sperm nuclear maturation because they participate in chromatin compaction by forming multiple inter- and intraprotamine disulfide cross-links [17].

There are two types of protamines known as the P1 protamine and the family of P2 proteins [18]. The P1 protamine is present in all of the mammalian species mentioned in Table 3. Protamine P2 is formed by the P2, P3 and P4 components, and it is only present in some of the mammalian species like man, mouse and stallion. Protamine P1 is synthesized as a mature protein, whereas the components of the P2 family are generated by proteolysis from a precursor encoded by a single gene [19–25].

Human, mouse and boar spermatozoa retain some of their original histone content which leads to the formation or the retention of less-compact nucleosome structures [37]. Importantly, the retained histones in human spermatozoa are associated with the nuclear periphery and telomeric sequences and may be among the first structures in the sperm nucleus to respond to oocyte signals for pronucleus formation [38].

The main functions that have been proposed for the protamines [18] are; (1) The generation of a condensed paternal genome with a more compact and hydrodynamic

nucleus. The spermatozoa with the more hydrodynamic nucleus have the capacity to move faster and thus the potential to fertilize the oocyte first. (2) Involvement in the imprinting of the paternal genome during spermatogenesis. Protamines may confer an epigenetic mark on some regions of the sperm genome, affecting its reactivation upon fertilization.

Transition nuclear proteins

In some mammals (see Table 3) the histones are first replaced by a group of arginine- and lysine-rich proteins called TP which are in turn replaced by protamines. Expression of these TP is presumed to regulate changes in chromatin occurring as part of the condensation process [39]. The major TP are TP1 and TP2; TP1 is a 6.2 kDa protein with numerous basic amino acids like arginine (20%) and lysine (20%) distributed randomly throughout the molecule, but no cysteine [40]. Both TP1 and TP2 are encoded by single copy genes, Tnp1 and Tnp2, respectively. The TP1 has important DNA destabilizing properties, probably due to the presence of two tyrosine residues flanked by basic amino acids [41]. TP2 is a 13 kDa protein containing proline (13%), serine (22%), arginine (14%), lysine (9%) and cysteine (5%) basic residues [42]. By amino acid sequence analysis, Baskaran and Rao [30] identified two potential Zn finger domains in mouse and rat TP1 that may play an important role in the initiation of chromatin condensation and/or cessation of transcriptional activity during mammalian spermiogenesis [4]. Tnp2 is closely linked to the two protamine genes [43], suggesting that they arose by gene duplication and might have retained common functions [39]. In contrast, Tnp1 is on a separate chromosome [44].

Chromatin packaging

During transit through the epididymis, the protamines crosslink by forming disulphide bonds, thereby compressing sperm DNA into one sixth the volume occupied by somatic cell nuclei [4]. DNA in fixed, dehydrated mammalian sperm is packed in a way that approaches the physical limits of molecular compression [45]. To reach this degree

Table 2 Major sites of histones side-chain modification in mammalian spermatogenic cells [13, 16]

Histone	Methylation	Acetylation	Phosphorylation	Ubiquitination
H1	–	–	–	–
H2A	–	K5, K9	S1	K119
H2B	–	K5, K12, K15, K20	S14	K120
H3	R2, K4, K9, K14, R17, K23, R26, K27, K36, K79	K9, K14, K18, K23	S10, T11, S28	–
H4	No variants	No variants	No variants	No variants

Table 3 Sperm chromatin structure in different mammalian species

Specie	Histone	Protamine type	Transition protein	Reference
Boar	Present	P1	–	[26]
Bull	–	P1	–	[27, 28]
Man	Present	P1, P2	Present	[29]
Mouse	Present	P1, P2	Present	[30–32]
Rabbit	–	P1	–	[33]
Ram	–	P1	Present	[27]
Rat	–	P1	Present	[34]
Stallion	–	P1, P2	–	[27, 35, 36]

of compactness, sperm DNA–protein complexes must first be packaged differently than somatic DNA–protein complexes. For this during mid-spermiogenesis the nucleus of the round spermatid changes from spherical to a shape which is unique for each species. Evidence for the nuclear matrix being responsible for such shape changes are twofold [46]. Changes in the shape of spermatid nuclei are coincident with the production of unique nuclear matrix proteins. The second process of nuclear packaging involves replacement of the somatic cell histone with sperm specific protamine [47–49]. Chromatin in somatic cells is first packed into repeating subunits, or nucleosomes, coupled by segments of spacer DNA giving the chromatin a ‘bead son-a-string’ appearance. Each nucleosome consists of approximately 200 base pairs of DNA wrapped twice around an octamer of histone [50, 51]. A single turn of solenoid is formed by six nucleosomes twisting about one another [52–58].

Sperm DNA fragmentation

Normal sperm genetic material is required for successful fertilization, as well as for further embryo and fetal development that will result in a healthy offspring. Sperm DNA contributes half of the offspring’s genomic material and abnormal DNA can lead to derangements in the reproductive process. Damaged sperm chromatin or DNA structure is thought to arise due to environmental stresses, gene mutations or chromosomal abnormalities [59]. Sperm chromatin abnormalities may occur during, or as a result of, DNA packaging at spermiogenesis [60]. It could also be the result of free-radical (reactive oxygen species; ROS) induced damage [61]. Abortive apoptosis could also induce DNA damage [62]. The exact source/mechanism of mammalian sperm damage is yet not been precisely understood [1]. Variable sperm chromatin structure in a single human ejaculate also has been attributed to protamine content. Therefore, abnormalities in proteolytic cleavage of the processing protamine precursors may act as an additional source of human sperm chromatin

heterogeneity and potential infertility [63, 64]. Major theories related to the etiology of sperm DNA fragmentation are described below.

Packaging defects

Under normal circumstances the recombination checkpoint in the meiotic prophase does not allow meiotic division I to proceed until the DNA is fully repaired or until defective spermatocytes are ablated [65, 66]. Ligation of DNA breaks is necessary not only for preserving the integrity of the primary DNA structure but also for reassembly of the important unit of genome expression, the DNA loop-domain [67].

It has been suggested that mammalian chromatin packaging may require endogenous nuclease (topoisomerase II) activity to create and ligate nicks that facilitate protamination during spermiogenesis [68]. The DNA double strand (ds) breaks have been found in round and elongating spermatids and should be ligated until the end of meiosis I. Nicks are thought to relieve torsional stress and aid chromatin rearrangement during the displacement of histones by protamines [69]. DNAase I-hypersensitive sites were found to be localized throughout the maturing spermatid nuclei, increasing from the anterior to posterior pole of the spermatid nucleus to reflect the pattern of chromatin re-packaging and condensation. Thus, chromatin re-packaging includes a sensitive step involving endogenous nuclease activity where chromatin is loosened in a coordinated manner by histone hyper-acetylation and by the creation and ligation of breaks by topoisomerase II [69, 70]. Normally chromatin packaging around the new protamine cores is completed and DNA integrity restored during epididymal transit [71]. However, the presence of endogenous nicks in spermatozoa after epididymal transit may indicate a fault during chromatin packaging at spermiogenesis and an incomplete maturation process.

Oxidative stress

ROS are highly reactive oxidizing agents, among which are included hydrogen peroxide, superoxide and free radicals [72]. The generation of low levels of ROS is necessary for modulating gene and protein activities vital for sperm proliferation, differentiation and function. In mammalian semen, the degree of ROS generation is properly controlled by seminal antioxidants [67]. Oxidative stress is caused by an imbalance between the production of ROS and the antioxidant capacity [73]. High levels of ROS can be generated by defective spermatozoa (specifically those with retained cytoplasm), and by semen leukocytes [74]. According to Vernet et al. [75] sperm mitochondria leak electrons and are responsible for a proportion of the ROS

generated by rat spermatozoa. Also the male germ line possesses a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX5) that is potentially capable of generating ROS in the presence of calcium and NADPH [76, 77]. Other ROS-generating systems in mammalian spermatozoa may include lipoxygenase [78], tNOX [79, 80] and cytochrome P450 reductase [81].

Many studies have reported a connection between oxidative stress and DNA damage [82–85]. Inadequate oxidation of thiols during epididymal transit may result in defective spermatid protamination and disulphide bridge formation. This negatively affects the sperm chromatin in packaging, making sperm cells more vulnerable to ROS-induced DNA fragmentation [67]. The association between sperm DNA damage and sperm-derived ROS also suggests that DNA damage may be due to a defect in spermiogenesis (the period during which sperm cytoplasmic droplets are generally shed) [86]. Therefore, two factors may protect the sperm DNA from oxidative insult: the characteristic tight packaging of the DNA; and the antioxidants present in seminal plasma [83].

Abortive apoptosis

In mammalian testes, germ cells expand clonally through many rounds of mitoses before undergoing the differentiation steps that result in mature spermatozoa [59]. This clonal expansion is excessive and requires a mechanism to match the number of germ cells with the supportive capacity of Sertoli cells. Therefore, apoptosis controls the overproduction of male gametes and restricts normal proliferation levels so that they do not surpass the supportive capacity of Sertoli cells [87, 88]. The presence of spermatozoa that possess apoptotic markers, such as DNA damage, indicates that in men with abnormal semen parameters, an abortive apoptosis has taken place [89]. Failure to clear DNA damaged spermatozoa may be due to dysfunction at one or more levels. It has been postulated that men with reduced spermatogenesis may not produce enough spermatozoa to trigger apoptosis [90]. Another major component of apoptotic machinery that contributes to sperm DNA fragmentation involves members of a family of aspartic acid-directed cysteine proteases called caspases [91].

Tests for sperm DNA damage

DNA fragmentation in the sperm cell that fertilizes an oocyte can have a drastic impact on ontogeny and, later on, the health of offspring [92, 93]. Evenson et al. [94] showed a significant relationship between human and bull sperm DNA fragmentation and loss of fertility potential. Therefore, detection of damage in mammalian sperm DNA may

help to identify individuals with reduced capacity to fertilise ova or to initiate a healthy pregnancy. A number of tests are now available for the assessment of mammalian sperm DNA damage. The use of these tests has been driven largely by the emergence of assisted reproductive technologies in humans and domestic/non-domestic animals.

Both direct and indirect assay methods have been used to assess sperm DNA damage. More common direct methods for detecting DNA breaks include the Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick-end labelling assay (TUNEL) and Comet assay. While the most common indirect method for assessing DNA damage include the sperm chromatin integrity assays like sperm chromatin structure assay (SCSA). There are other tests too which are less common in use. The details of the tests for sperm DNA damage are given below;

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick-end labelling assay (TUNEL)

The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at single strand (ss)- and ds-DNA breaks in an enzymatic reaction by labelling the free 3'-OH terminal and modified nucleotides (5-bromo-2'-deoxyuridine 5'-triphosphate nucleotide) with terminal deoxynucleotidyl transferase. Sperm with normal DNA therefore have only background staining/fluorescence, while those with fragmented DNA (multiple 3'-OH ends) stain/fluoresce brightly [62] which can be quantified by flow cytometry, fluorescent microscopy or light microscopy [59].

The microscopic TUNEL assay has been modified by some researchers to include a peroxidase enzyme labeling system that catalytically generates an intense signal from chromogenic substrates. This labelling technique eliminates the problems associated with fluorescence fading in the microscopic method, thereby giving operators more time to analyze a greater number of cells for more accuracy [95–98]. Regarding use of light microscopy, some of the results reported based on this non-fluorescent technique confirm the results based on fluorescent TUNEL labeling techniques, other results seem to contradict previous findings [97–101].

Finally, TUNEL assay cannot be routinely used in experimental and clinical studies due to its application in limited mammalian species and lack of useful thresholds [102, 103].

In situ nick translation assay (ISNT)

This in situ nick translation (ISNT) assay quantifies the incorporation of biotinylated-dUTP at ssDNA breaks in a

reaction catalyzed by the template-dependant enzyme DNA polymerase I. The ISNT may detect spermatozoa that contain appreciable and variable levels of endogenous DNA damage. Currently, however, ISNT assay thresholds for post-fertilization embryo viability have not been established, which severely limits the clinical usefulness of this assay.

DNA breakage detection–fluorescence in situ hybridization test (DBD-FISH)

DNA breakage detection–fluorescence in situ hybridization (DBD-FISH) is a procedure that allows in situ detection and quantification of DNA breaks in the whole genome or within specific DNA sequence areas, cell by cell [104]. Sperm cells embedded in an inert agarose matrix on a slide are treated with an alkaline unwinding solution that transforms DNA ss breaks and ds breaks, as well as alkali-labile sites, into ssDNA. After deproteinization and dehydration, the microgels are incubated with DNA probes, as in standard FISH procedures. As DNA breaks increase within a specific DNA sequence area, more ssDNA is produced by the alkali in the DNA region and more of the specific probe hybridizes, producing a stronger FISH signal. The level of DNA breakage in different DNA sequence areas of the nucleus is analyzed by measuring the hybridization of different probes [105, 106]. The procedure being new is not common in use in experimental protocols or clinics and needs to be extensively evaluated for different mammalian sperm.

Sperm chromatin structure assay (SCSA)

The sperm chromatin structure assay (SCSA) that depends on the fact that abnormal sperm chromatin has a greater susceptibility to the physical induction of partial DNA denaturation in situ [6]. The SCSA exploits the metachromatic properties of acridine orange (AO) to monitor the susceptibility of sperm chromatin to heat or acid-induced denaturation [6, 103]. SCSA used for the first time showed that sperm nuclear DNA was more resistant to heat induced denaturation in fertile compared with sub-fertile or infertile men and bulls [94]. Heat induced denaturation has since been replaced with acid induced denaturation due to the similarity of results and the greater ease of the later technique [107].

This flow cytometric based technique measures the ratio of denatured ssDNA (red fluorescence) to native dsDNA (green fluorescence). The SCSA accurately estimates the percentage of sperm chromatin damage expressed as DFI (DNA fragmentation index) with a cut-off point of 30% to differentiate between fertile and infertile samples [108]. The 30% threshold is described as chromatin damage identified

in 30% of the spermatozoa indicated an abnormality in the entire population causing in vivo infertility [3].

Acridine orange assay (AOA)

The acridine orange assay (AOA) was introduced by Tejada et al. [109] as a simplified microscopic method, which relies in visual interpretation of fluorescing spermatozoa rather than expensive flow cytometry equipment involved in SCSA [103]. The AOA measures the susceptibility of sperm nuclear DNA to acid-induced denaturation in situ by quantifying the metachromatic shift of AO fluorescence from green (native or dsDNA) to red (denatured or ssDNA). Some research groups have used AOA in attempt to improve male fertility evaluations, however, the predictive values of the test for fertility remains controversial [110–113] as observer subjectivity may hinder the results [6]. In recent studies by Martin et al. [114, 115] and Chohan et al. [116] it is found that AOA is not that much sensitive as TUNEL and SCSA for evaluating sperm DNA fragmentation.

Comet assay

In a single-cell gel electrophoresis, the amount of low-molecular weight DNA assessed by measuring the length and area of the comet formed during electrophoresis of spermatozoa [117, 118]. In this assay, spermatozoa are stained with a fluorescent DNA-binding dye. One of the principles of the comet assay is that nicked double stranded DNA tends to remain in the comet head, whereas short fragments of nicked double and single stranded DNA migrate into the tail area [119]. Therefore, spermatozoa with high levels of DNA strand breaks would show increased comet tail fluorescent intensity [120] and comet tail length [121]. The results are interpreted by comparing the number of cells with comet tails with total number of spermatozoa [117, 118]. The assay is based on fluorescent microscopy, the assay requires an experienced observer to analyze the slides and interpret the results [6]. Also the assay is classified as alkaline and neutral COMET assay depending on the type of damage being investigated [119, 120].

Regarding the alkaline COMET assay (pH>10), it denatures sperm DNA and therefore, identifies both ss and dsDNA breaks. Whereas, the neutral COMET assay may be more sensitive to dsDNA breaks and therefore, better able to identify DNA damage related to infertility as the conditions of the assay (pH 9) do not denature DNA [3]. Moreover, the neutral COMET assay only measures ds breaks and closely associated ss breaks, but does not measure strand breaks associated with alkali labile sites [121–123]. It is documented that these alkali labile DNA

fragments, which are abundant in spermatozoa, could decrease the sensitivity of the assay to DNA fragments resulting from dsDNA breaks that are indicative of DNA damage and infertility [85, 119]. Hence, neutral COMET assay is better able to identify DNA damage related to infertility [103]. The major problem with COMET assay is that it is a labour intensive test.

Sperm chromatin dispersion assay (SCDA)

More recently, sperm chromatin dispersion assay (SCDA) has been described as a simple and inexpensive method for analysis of mammalian sperm DNA fragmentation [124]. The SCDA is based on the principal that spermatozoa with non-fragmented DNA are immersed in an agarose matrix and exposed to lysing solution; the resulting deproteinized nuclei show extended halos of DNA dispersion as monitored by fluorescent or light microscopy. For some species such as bovine the technique is modified and the spermatozoa with fragmented DNA on exposure to lysing solution exhibit extended halos of DNA dispersion which can also be monitored by fluorescent or light microscopy. The major advantage of the SCDA is that it does not require the determination of fluorescence intensity. On other hand, the SCDA does not give much information about the extent of spermatozoal DNA damage, since its endpoints consist of subjectively quantifying the percentage of spermatozoa with dispersed or non-dispersed nucleoids.

Conclusions

In mammals the spermiogenesis can be divided into two phases. In the first phase, the nucleus is round, contains histones as the major basic nuclear proteins and second phase involves dramatic changes in chromatin structure, nuclear shaping and condensation. In many mammalian species except of a few, the histones are removed and replaced by other proteins, resulting in sperm containing one or more protamines as the major nuclear protein. The replacement of histones and deposition of protamines involves major remodelling of the chromatin and a high level of DNA strand breaks is formed which are then repaired by ligation. Moreover, intermediate proteins appear in this histone to protamine transition known as TP. Sperm chromatin abnormalities may occur during, or as a result of, DNA packaging at spermiogenesis. It could also be the result of free-radical (ROS) induced damage. Abortive apoptosis could also induce DNA damage. The exact source/mechanism of mammalian sperm damage is yet not been precisely understood. In recent years, the rapid advance of molecular biology has resulted in numerous techniques to assess sperm DNA fragmentation. Of these,

the TUNEL, COMET, and SCSA have been shown to have wider application because of strong prognostic power in assessing male infertility or sub-fertility.

References

1. Lewis SE, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell Tissue Res* 2005;322:33–41.
2. Zini A, Bielcki R, Phang D, Zenzes MT. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation in fertile and infertile men. *Fertil Steril* 2001;75:674–7.
3. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male fertility and comparisons with other techniques. *J Androl* 2002;23:25–43.
4. Fuentes-Mascorro G, Serrano H, Rosado A. Sperm chromatin. *Arch Androl* 2000;45:215–25.
5. Holstein A-F, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. *Reprod Biol Endocrinol* 2003;1:107.
6. Agarwal A, Said TM. Sperm chromatin assessment. In: Gardner DK, Weissman A, Howles CM, Shoham A, editors. *Textbook of assisted reproductive technology and clinical perspectives*. London: Taylor & Francis; 2004. p. 93–106.
7. Akama K, Sato H, Furihata-Yamauchi M, Komatsu Y, Tobita T, Nakano M. Interaction of nucleosome core DNA with transition proteins 1 and 3 from boar late spermatid nuclei. *J Biochem* 1996;119:448–5.
8. Chevallier P, Chirat F, Sautiere P. The amino acid sequence of the ram spermatid protein 3—a transition protein TP3 or TP4? *Eur J Biochem* 1998;258:460–4.
9. Steger K, Klonisch T, Gavenis K, Drabent B, Doenecke D, Bergmann M. Expression of mRNA and protein of nucleoproteins during human spermiogenesis. *Mol Hum Reprod* 1998;4:939–45.
10. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 1997;389:251–60.
11. Khochbin S. Histone H1 diversity: bridging regulatory signals to linker histone function. *Gene* 2001;271:1–12.
12. Arents G, Moudrianakis EN. Topography of the histone octamer surface: repeating structural motifs utilized in the docking of nucleosomal DNA. *Proc Natl Acad Sci USA* 1993;90:10489–93.
13. Govin J, Caron C, Lestrat C, Rousseaux S, Khochbin S. The role of histones in chromatin remodeling during mammalian spermiogenesis. *Eur J Biochem* 2004;271:3459–69.
14. Malik HS, Henikoff S. Phylogenomics of the nucleosome. *Nat Struct Biol* 2003;10:882–91.
15. D'Occhio MJ, Hengstberger KJ, Johnston SD. Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. *Anim Reprod Sci* 2007;101:1–17.
16. Marzluff WF, Gongidi P, Woods KR, Jin J, Maltais LJ. The human and mouse replication-dependent histone genes. *Genomics* 2002;80:487–98.
17. Loir M, Lanneau M. Structural function of the basic nuclear proteins in ram spermatids. *J Ultrastruct Res* 1984;86:262–72.
18. Oliva R. Protamines and male infertility. *Hum Reprod* 2006;12:417–35.
19. McKay DJ, Renaux BS, Dixon GH. Human sperm protamines amino-acid sequences of two forms of protamine P2. *Eur J Biochem* 1986;156:5–8.
20. Yelick PC, Balhorn R, Johnson PA, Corzett M, Mazrimas JA, Kleene KC, Hecht NB. Mouse protamine 2 is synthesized as a

- precursor whereas mouse protamine 1 is not. *Mol Cell Biol* 1987;7:2173–9.
21. Sautière P, Martinage A, Bélaïche D, Arkhis A, Chevaillier P. Comparison of the amino acid sequences of human protamines HP2 and HP3 and of intermediate basic nuclear proteins HPS1 and HPS2. Structural evidence that HPS1 and HPS2 are pro-protamines. *J Biol Chem* 1988;263:11059–62.
  22. Chauvière M, Martinage A, Debarle M, Sautière P, Chevaillier P. Molecular characterization of six intermediate proteins in the processing of mouse protamine P2 precursor. *Eur J Biochem* 1992;204:759–65.
  23. Green GR, Balhorn R, Poccia DL, Hecht NB. Synthesis and processing of mammalian protamines and transition proteins. *Mol Reprod Dev* 1994;37:255–63.
  24. Queralt R, Oliva R. Demonstration of trans-acting factors binding to the promoter region of the testis-specific rat protamine P1 gene. *Biochem Biophys Res Commun* 1995;208:802–12.
  25. Wouters-Tyrou D, Martinage A, Chevaillier P, Sautière P. Nuclear basic proteins in spermiogenesis. *Biochimie* 1998;80:117–28.
  26. Banerjee S, Smallwood A. Chromatin modification of imprinted H19 gene in mammalian spermatozoa. *Mol Reprod Dev* 1998;50:474–84.
  27. Gusse M, Chevaillier PH. Electron microscope evidence for the presence of globular structure in different sperm chromatin. *J Cell Biol* 1980;87:280–84.
  28. Oliva R, Dixon GH. Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Prog Nucleic Acid Res Mol Biol* 1991;40:25–94.
  29. Gatewood JM, Cook GR, Balhorn R, Bradbury EM, Schmid CV. Sequence-specific packaging of DNA in human sperm chromatin. *Science* 1987;236:962–4.
  30. Baskaran R, Rao MRS. Mammalian spermatid specific protein, TP2, is a zinc metalloprotein with two finger motifs. *Biochem Biophys Res Commun* 1991;179:1491–9.
  31. Pogany GC, Corzett M, Weston S, Balhorn R. DNA and protein content of mouse sperm implication regarding sperm chromatin structure. *Exp Cell Res* 1981;136:127–36.
  32. Rhim JA, Connor W, Dixon GH, Harendza CJ, Evenson DP, Palmiter RD, Brinster RL. Expression of an avian protamine in transgenic mice disrupts chromatin structure in spermatozoa. *Biol Reprod* 1995;52:20–32.
  33. Koheler JK. A freeze-etching study of rabbit spermatozoa with particular reference to head structure. *J Ultrastruct Res* 1970;33:598–614.
  34. Koheler JK, Würschmidt U, Larsen MP. Nuclear and chromatin structure in rat spermatozoa. *Gamet Res* 1983;8:357–70.
  35. Livolant F. Cholesteric organization of DNA in the stallion sperm head. *Tiss Cell* 1983;16:535–55.
  36. Sipski ML, Wagner TE. The total structure and organization of chromosomal fibers in eutherian sperm nuclei. *Biol Reprod* 1977;16:428–40.
  37. 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.
  38. Gineitis AA, Zalenskaya IA, Yau PM, Morton BE, Zalensky AO. Human sperm telomere-binding complex involves histone H2B and secures telomere membrane attachment. *J Cell Biol* 2000;151:1591–8.
  39. Meistrich ML, Mohapatra B, Shirley CR, Zhao M. Roles of transition nuclear proteins in spermiogenesis. *Chromosoma* 2003;111:483–8.
  40. Kistler WS, Noyes C, Hsu R, Henrikson RL. The amino acid sequence of a testis-specific basic protein that is associated with spermatogenesis. *J Biol Chem* 1975;250:1847–53.
  41. Schumacher JM, Artzt K, Braun RE. Spermatid perinuclear ribonucleic acid-binding protein binds microtubules in vitro and associates with abnormal manchettes in vivo in mice. *Biol Reprod* 1998;59:69–76.
  42. Grimes SR, Platz RD, Meistrich ML, Hnilica LS. Partial characterization of a new basic nuclear protein from rat testis elongated spermatids. *Biochem Biophys Res Commun* 1975;67:182–9.
  43. Engel W, Keime S, Kremling H, Hameister H, Schluter G. The genes for protamine 1 and 2 (PRM1 and PRM2) and transition protein 2 (TNP2) are closely linked in the mammalian genome. *Cytogenet Cell Genet* 1992;61:158–9.
  44. Heidaran MA, Kozak CA, Kistler WS. Nucleotide sequence of the Stp-1 gene coding for rat spermatid nuclear transition protein 1 (TP1): homology with protamine P1 and assignment of the mouse Stp-1 gene to chromosome 1. *Gene* 1989;75:39–46.
  45. Illison L. Spermatozoal head shape in two inbred strains of mice and their F1 and F2 progenic. *Aust J Biol Sci* 1969;22:947–63.
  46. Ostermeier GC, Sartor-Bergfelt R, Susko-Parrish JL, Parrish JJ. Bull fertility and sperm nuclear shape. *AgBiotechNet* 2000;2:1–6.
  47. Meistrich ML, Brock WA, Grimes SR, Platz RD, Hnilica LS. Nuclear protein transitions during spermatogenesis. *Fed Proc* 1978;37:2522–5.
  48. Warrant RW, Kim SH. Helix-double helix interaction shown in the structure of a protamine transfer RNA complex and a nucleoprotamine model. *Nature* 1978;271:130–5.
  49. Balhorn RA. model for the structure of chromatin in mammalian sperm. *J Cell Biol* 1982;93:298–305.
  50. Kornberg RD. Chromatin structure: repeating unit of histones and DNA. *Science* 1974;184:868–71.
  51. Thoma F, Koller T, Klug A. Involvement of histone H1 in the organization of the nucleosome and the salt-dependent superstructures of chromatin. *J Cell Biol* 1979;83:402–27.
  52. Ciejek EM, Tsai MJ, O'Malley BW. Actively transcribed genes are associated with the nuclear matrix. *Nature* 1983;306:607–9.
  53. Robinson SI, Small D, Idzerda R, McKnight GS, Vogelstein B. The association of transcriptionally active genes with the nuclear matrix of the chicken oviduct. *Nucleic Acids Res* 1983;11:5113–30.
  54. Jarman AP, Higgs DR. Nuclear scaffold attachment sites in the human globin gene complexes. *EMBO J* 1998;7:3337–44.
  55. Boulukas T. Nature of DNA sequences at the attachment regions of genes to the nuclear matrix. *J Cell Biochem* 1998;52:14–22.
  56. Bode J, Stengert-Iber M, Kay V, Schlake T, Dietz-Pfeilstetter A. Scaffold/matrix-attached regions: topological switches with multiple regulatory functions. *Crit Rev Eukaryot Gene Expr* 1996;6:115–38.
  57. Singh GB, Kramer JA, Krawetz SA. Mathematical model to predict regions of chromatin attachment to the nuclear matrix. *Nucleic Acids Res* 1997;25:1419–25.
  58. Walter WR, Singh GB, Krawetz SA. MARs mission update. *Biochem Biophys Res Commun* 1998;242:419–22.
  59. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod* 2003;9:331–45.
  60. Sailer B, Jost L, Evenson D. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J Androl* 1995;16:80–7.
  61. Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998;59:1037–46.
  62. Gorczyza W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res* 1993;53:1945–51.
  63. de Yebra L, Ballescà JL, Vanrell JA, Corzett M, Balhorn R, Oliva R. Detection of P2 precursors in the sperm cells of infertile

- patients who have reduced protamine P2 levels. *Fertil Steril* 1998;69:755–9.
64. Evenson DP, Jost LK, Corzett M, Balhorn R. Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. *J Androl* 2000;21:739–46.
  65. Page AW, Orr-Weaver TL. Stopping and starting the meiotic cell cycle. *Curr Opin Genet Dev* 1997;7:23–31.
  66. Bannister LA, Schimenti JC. Homologous recombinational repair proteins in mouse meiosis. *Cytogenet Genome Res* 2004;107:191–200.
  67. Erenpreiss J, Spano M, Erenpreisa J, Bungum M, Giwercman A. Sperm chromatin structure and male fertility: biological and clinical aspects. *Asian J Androl* 2006;8:11–29.
  68. McPherson SM, Longo FJ. Chromatin structure–function alterations during mammalian spermatogenesis—DNA nicking and repair in elongating spermatids. *Eur J Histochem* 1993;37:109–28.
  69. McPherson SM, Longo FJ. Endogenous nicks in elongating spermatid DNA—involvement of DNA topoisomerase-ii and protamine. *Mol Biol Cell* 1992;3:A102.
  70. Laberge RM, Boissonneault G. On the nature and origin of DNA strand breaks in elongating spermatids. *Biol Reprod* 2005;73:289–96.
  71. Erenpreiss J, Bars J, Lipatnikova V, Erenpreisa J, Zalkalns J. Comparative study of cytochemical tests for sperm chromatin integrity. *J Androl* 2001;22:45–53.
  72. Warren JS, Johnson KJ, Ward PA. Oxygen radicals in cell injury and cell death. *Pathol Immunopathol Res* 1987;6:301–15.
  73. Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. *Urology* 1996;48:835–50.
  74. Zini A, Libman J. Sperm DNA damage: importance in the era of assisted reproduction. *Curr Opin Urol* 2006;16:428–34.
  75. Vernet P, Fulton N, Wallace C, Aitken RJ. Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. *Biol Reprod* 2001;65:1102–13.
  76. Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, Irvine S. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev* 1997;47:468–82.
  77. Banfi B, Molnar G, Maturana A, Steger K, Hegedus B, Demareux N, Krause KH. A Ca(2)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem* 2001;276:37594–601.
  78. Oliu EH, Sprecher H. Metabolism of polyunsaturated fatty acids by an (n-6)-lipoxygenase associated with human ejaculates. *Biochim Biophys Acta* 1989;1002:283–91.
  79. Aitken RJ, Ryan AL, Curry BJ, Baker MA. Multiple forms of redox activity in populations of human spermatozoa. *Mol Hum Reprod* 2003;9:645–61.
  80. Morré DJ, Morré DM. Cell surface NADH oxidases (ECTO-NOX proteins) with roles in cancer, cellular time-keeping, growth, aging and neurodegenerative diseases. *Free Radic Res* 2003;37:795–808.
  81. Baker MA, Krutskikh A, Curry BJ, McLaughlin EA, Aitken RJ. Identification of cytochrome P450-reductase as the enzyme responsible for NADPH-dependent lucigenin and tetrazolium salt reduction in rat epididymal sperm preparations. *Biol Reprod* 2004;71:307–18.
  82. Cummins JM, Jequier AM, Kann R. Molecular biology of human male infertility: links with ageing, mitochondrial genetics and oxidative stress? *Mol Reprod Dev* 1994;37:345–62.
  83. Twigg JP, Irvine DS, Aitken RJ. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum Reprod* 1998;13:1864–71.
  84. Donnelly ET, McClure N, Lewis SE. The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis* 1999;14:505–12.
  85. Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ. DNA integrity in human spermatozoa: relationships with semen quality. *J Androl* 2000;21:33–44.
  86. Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl* 1996;17:276–87.
  87. Sinha Hikim AP, Rajavashisth TB, Sinha Hikim I, Lue Y, Bonavera JJ, Leung A, Wang C, Swerdloff RS. Significance of apoptosis in the temporal and stage-specific loss of germ cells in the adult rat after gonadotropin deprivation. *Biol Reprod* 1997;57:1193–201.
  88. Sinha Hikim AP, Swerdloff RS. Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod* 1999;4:38–47.
  89. Huszar G, Sbracia M, Vigue L, Miller DJ, Shur BD. Sperm plasma membrane remodelling during spermiogenetic maturation in men: relationship among plasma membrane beta 1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. *Biol Reprod* 1997;56:1020–4.
  90. Francavilla S, D'Abrizio P, Rucci N, Silvano G, Properzi G, Straface E, Cordeschi G, Necozione S, Gnassi L, Arizzi M, Ulisse S. Fas and Fas ligand expression in fetal and adult human testis with normal or deranged spermatogenesis. *J Clin Endocrinol Metab* 2000;85:2692–700.
  91. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998;281:1312–6.
  92. Haaf T, Ward DC. Higher order nuclear structure in mammalian sperm revealed by in situ hybridisation and extended chromatin fibres. *Exp Cell Res* 1995;219:604–11.
  93. Pérez-Llano B, Enciso M, García-Casado P, Sala R, Gosálvez J. Sperm DNA fragmentation in boars is delayed or abolished by using sperm extenders. *Theriogenology* 2006;66:2137–43.
  94. Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 1980;240:1131–3.
  95. Host E, Lindenberg S, Ernst E, Christensen F. DNA strand breaks in human spermatozoa: a possible factor to be considered in couples suffering from unexplained fertility. *Acta Obstet Gynec Scand* 1999;78:622–5.
  96. Host E, Lindenberg S, Kahn JA, Christensen F. DNA strand breaks in human sperm cells: a comparison between men with normal and oligozoospermic sperm samples. *Acta Obstet Gynec Scand* 1999;78:336–9.
  97. Host E, Lindenberg S, Smidt-Jensen S. DNA strand breaks in human spermatozoa: correlation with fertilisation in vitro in oligozoospermic men and in men with unexplained fertility. *Acta Obstet Gynec Scand* 2000;79:189–93.
  98. Host E, Lindenberg S, Smidt-Jensen S. The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. *Acta Obstet Gynec Scand* 2000;79:559–63.
  99. Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol Reprod* 1997;56:602–7.
  100. Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor quality sperm samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril* 1998;69:528–32.
  101. Ahmadi A, Ng S-C. Fertilising ability of DNA damaged spermatozoa. *J Exp Zool* 1999;284:696–704.

102. Haines GA, Hendry JH, Daniel CP, Morris ID. Increased levels of comet-detected spermatozoa DNA damage following in vivo isotopic- or X-irradiation of spermatogonia. *Mutat Res* 2001;495:21–32.
103. Fraser L. Structural damage to nuclear DNA in mammalian spermatozoa: its evaluation techniques and relationship with male infertility. *Pol J Vet Sci* 2004;7:311–21.
104. Fernandez JL, Goyanes VJ, Ramiro-Diaz J, Gosalvez J. Application of FISH for in situ detection and quantification of DNA breakage. *Cytogenet Cell Genet* 1998;82:251–6.
105. Fernandez JL, Vazquez-Gundin F, Delgado A, Goyanes VJ, Ramiro-Diaz J, de la Torre J, Gosalvez J. DNA breakage detection-FISH (DBD-FISH) in human spermatozoa: technical variants evidence different structural features. *Mutat Res* 2000;453:77–82.
106. Fernandez JL, Gosalvez J. Application of FISH to detect DNA damage: DNA breakage detection-FISH (DBD-FISH). *Methods Mol Biol* 2002;203:203–16.
107. Evenson DP, Higgins PJ, Grueneberg D, Ballachey BE. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. *Cytometry* 1985;6:238–53.
108. Potts RJ, Newbury CJ, Smith G, Notarianni LJ, Jefferies TM. Sperm chromatin changes associated with male smoking. *Mutat Res* 1999;423:103–11.
109. Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil Steril* 1984;42:87–91.
110. Foresta C, De Carlo E, Mioni R, Zorzi M. Sperm nuclear chromatin heterogeneity in infertile subjects. *Andrologia* 1989;21:384–90.
111. Peluso JP, Luciano AA, Nulsen JC. The relationship between alterations in spermatozoal deoxyribonucleic acid, heparin binding sites, and semen quality. *Fertil Steril* 1992;57:665–70.
112. Hoshi K, Katayose H, Yanagida K, et al. The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil Steril* 1996;66:634–9.
113. Duran EH, Gurgan T, Gunalp S, Enginsu ME, Yarali H, Ayhan A. A logistic regression model including DNA status and morphology of spermatozoa for prediction of fertilization in vitro. *Hum Reprod* 1998;13:1235–9.
114. Martins CF, Dode MN, Bao SN, Rumpf R. The use of the acridine orange test and the TUNEL assay to assess the integrity of freeze-dried bovine spermatozoa DNA. *Genet Mol Res* 2007;6:94–104.
115. Martins CF, Bao SN, Dode MN, Correa GA, Rumpf R. Effects of freeze-drying on cytology, ultrastructure, DNA fragmentation, and fertilizing ability of bovine sperm. *Theriogenology* 2007;67:1307–15.
116. Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *J Androl* 2006;27:53–9.
117. Anderson D, Dobrzynska MM, Basaran N. Effect of various genotoxins and reproductive toxins in lymphocytes and sperm samples in the Comet assay. *Teratog Carcinog Mutagen* 1997;17:29–43.
118. Anderson D, Basaran N, Dobrzynska MM, Basaran AA, Yu TW. Modulating effects of flavonoids on food mutagens in human blood and sperm samples in the Comet assay. *Teratog Carcinog Mutagen* 1997;17:45–58.
119. Klaude M, Eriksson S, Nygren J, Ahnstrom G. The comet assay: mechanisms and technical considerations. *Mutat Res* 1996;363:89–96.
120. Hughes CM, Lewis SEM, McKelvey-Martin VJ, Thompson W. The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. *Hum Reprod* 1998;13:1240–7.
121. Singh N, Stephens R. X-ray induced DNA double-strand breaks in human sperm. *Mutagenesis* 1998;13:75–9.
122. Haines G, Marples B, Daniel P, Morris I. DNA damage in human and mouse spermatozoa after in vitro-irradiation assessed by the COMET assay. *Adv Exp Med Biol* 1998;444:79–91.
123. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988;175:184–91.
124. Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *J Androl* 2003;24:59–66.