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INVITED REVIEW

Sperm Biology

Human sperm chromatin epigenetic potential: genomics, proteomics, and male infertility

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The classical idea about the function of the mammalian sperm chromatin is that it serves to transmit a highly protected and transcriptionally inactive paternal genome, largely condensed by protamines, to the next generation. In addition, recent sperm chromatin genome-wide dissection studies indicate the presence of a differential distribution of the genes and repetitive sequences in the protamine-condensed and histone-condensed sperm chromatin domains, which could be potentially involved in regulatory roles after fertilization. Interestingly, recent proteomic studies have shown that sperm chromatin contains many additional proteins, in addition to the abundant histones and protamines, with specific modifications and chromatin affinity features which are also delivered to the oocyte. Both gene and protein signatures seem to be altered in infertile patients and, as such, are consistent with the potential involvement of the sperm chromatin landscape in early embryo development. This present work reviews the available information on the composition of the human sperm chromatin and its epigenetic potential, with a particular focus on recent results derived from high-throughput genomic and proteomic studies. As a complement, we provide experimental evidence for the detection of phosphorylations and acetylations in human protamine 1 using a mass spectrometry approach. The available data indicate that the sperm chromatin is much more complex than what it was previously thought, raising the possibility that it could also serve to transmit crucial paternal epigenetic information to the embryo.

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INTRODUCTION

The main function of the sperm cell is to transmit to the embryo the paternal genetic message encoded in the DNA sequence together with the presence of appropriate epigenetic information.^{1–4} The most well-studied mechanism of epigenetic inheritance is the reversible methylation of cytosine residues in cytosine-guanine dinucleotides at imprinted genes, which is involved in gene expression regulation.^{5,6} However, additional potential sperm epigenetic information is also constituted by the presence of histone modifications, presence of other chromatin-associated proteins and their modifications, RNAs, a unique chromatin structure (**Figure 1**), and chromosome territories in the nucleus.^{7–15} As compared to somatic cells, not much is known so far about the potential role of these additional forms of epigenetic information in the sperm, despite that it is an emerging subject of increasing interest.^{3,10,16–19} Thus, the present review aims to cover these newer forms of epigenetic information, being focused on the chromatin structure, gene distribution and presence of chromatin proteins and their modifications in the sperm cell (**Figure 1**). Therefore, it does not aim to cover the topics of sperm DNA methylation and the presence of sperm RNAs, for which the reader is referred to other excellent reviews.^{5,7,20,21} Furthermore, the present review does not intend to cover related issues concerning

the potential origins and consequences of sperm DNA damage, for which recent reviews are also available.^{22–26}

As a source of information, PubMed articles published until submission of this review (November 10, 2014) were considered on the topics of “sperm chromatin,” “sperm chromatin packaging,” “sperm chromatin gene distribution,” “sperm histone retention,” “sperm histone modifications,” “protamine modifications,” “sperm chromatin protein composition,” and “chromatin alterations in infertile patients.” As a complement, we provide here for the first-time experimental evidence for the detection of phosphorylations and acetylations in human protamine 1 using mass spectrometry (MS).

The first part of this review starts with a discussion about the current knowledge of the sequence-specific sperm chromatin distribution, and is followed by a section on the abundant histones and protamines and the additional (less abundant) sperm chromatin proteins recently identified using MS. Finally, the review concludes with a section on the presence of genomic and proteomic alterations detected in the sperm chromatin of infertile patients, both in the gene distribution and in the presence of an altered abundance on sperm chromatin proteins.

The present review complements, expands, and updates other previously published reviews on this topic.^{3,4,8–12,17,18,21,27–36}

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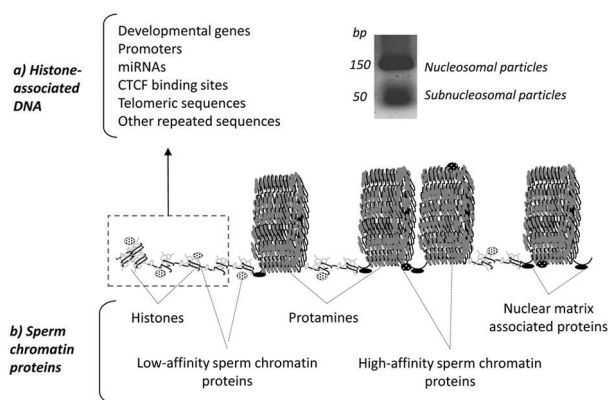


Figure 1: Gene and protein composition of the human sperm chromatin. Hypothetical model of human sperm chromatin showing the histone-associated DNA constitution (a) and protein contents (b). The model is drawn at a scale and inspired by the known nucleo-protamine toroidal structures¹²⁴ and the high-throughput sequencing data obtained from the analysis of retained human sperm nucleosomes after micrococcal nuclease digestion.^{43–45,51,52}

SEQUENCE-SPECIFIC SPERM CHROMATIN DNA DISTRIBUTION

The structure and composition of the inert mammalian sperm chromatin have been extensively studied during the past three decades^{27,37–52} (Table 1). Classical sperm chromatin dissection experiments were based on two main approaches: (1) DNA digestion by endonucleases (typically DNase I and micrococcal nuclease; MNase),^{39,40,42–46,48–52} and (2) disruption of histone-DNA associations by 0.65 M NaCl followed by the digestion of histone-free DNA (typically by EcoRI and BamHI).^{37,38,41,43,51} Subsequent DNA analyses using probes, polymerase chain reaction, chromatin immunoprecipitation, microarrays or high-throughput sequencing techniques have provided exciting results (Table 1).

A growing body of evidence suggests the existence of a sequence-specific packaging by histones and protamines involved in potential epigenetic inheritance.^{9,12,17,27,37,42,53,54} The first studies in this field were focussed on the analysis of specific gene families or clusters, showing a potential involvement of DNA distribution in postfertilization events (Table 1). Interestingly, members of β -globin gene family active in embryonic yolk sac (ϵ - and γ -globin) contained histone-associated regions, while no presence of β - and δ -globin (inactive in embryonic yolk sac) was found.³⁸ Furthermore, Wykes and Krawetz reported a nonrandom distribution of PRM1-PRM2-TNP2 *loci*, repetitive sequences and selected genes throughout the chromatin.⁴¹ This programmatic sperm DNA distribution has been confirmed recently by the genome-wide sperm nucleosome profiles generated by other groups (Table 1). Particularly, an enrichment of human and mouse retained sperm nucleosomes at developmental *loci* was reported, which included imprinted genes, microRNAs, HOX genes, promoters of developmental transcriptional signaling factors, GC-rich sequences, and transcription start sites of most housekeeping genes^{43,44,47,51} (Figure 1).

Of relevance, we have recently contributed to the knowledge of sperm chromatin reporting the existence in human sperm histone-associated chromatin of not only classical nucleosomes, but also of smaller MNase-sensitive regions.⁵¹ Although an association with specific histone variants is still unexplored, these so-called subnucleosomal particles were seen to be enriched in alternative and not overlapping developmental *loci*.⁵¹ These results allowed speculation

that additional levels of sperm chromatin packaging might be involved in postfertilization gene regulation. A recent study using mouse sperm has also suggested the presence of two types of footprints obtained after MNase digestion, one corresponding to nucleosomes, and a shorter one (<80 bp) associated with other DNA-binding proteins such as transcription factors.⁵⁰ Similarly, zebrafish (which do not employ protamines for sperm DNA packaging) have a multivalent chromatin constituted by gene sets implicated in embryo development processes and associated with distinctive types of nucleosome packaging.⁵⁵ All these data are therefore suggesting an exciting dynamic behavior of the sperm chromatin.

The role of sperm histone-retention in male epigenetic inheritance becomes even more significant considering that regulatory *loci* are marked by specific histone methylation patterns.^{3,9,12,17,44,45,56} In fact, human and mice genes related to spermatogenesis and cellular homeostasis seem to be associated with activating modifications (H3K4me2), while developmental gene promoters may be related not only with activating (H3K4me2 and H3K4me3) but also with repressive histone marks (H3K27me3). Interestingly, this bivalent gene marking is showing an overlap with embryonic stem cells, suggesting a role in the establishment of embryonic totipotency.^{44,45}

However, histone-retention constitutes just 5% to 15% of sperm chromatin while the major part is indeed tightly packaged by protamines (Figure 1). This higher level of compaction is required not only to avoid any transcriptional and translational activity, but also to reduce the accessibility of external and internal nucleases^{28,57,58} (Figure 1). In fact, although protamine-packaging is not necessary for proper embryo development,^{59,60} it appears to be important for DNA integrity maintenance.^{61–65} Therefore, if the nucleo-protamine structure is ensuring the transmission of intact male genome to the next generation, an important question remaining to be answered is why crucial male developmental *loci* are vulnerable by virtue of being associated with nucleosomes.

In this regard, contrasting results with those already mentioned regarding sequence-specific sperm DNA distribution, have been reported by several groups during the past years (Table 1). Interestingly, by using similar strategies for chromatin dissection and sequence analysis in human, mouse and bovine sperm, it has been shown that nucleosomes might be moderately retained at unique DNA sequences and regulatory regions (Table 1). In contrast, a majority of sperm histones seemed to be localized to the nuclear periphery, within distal intergenic regions and introns, and associated with centromere and telomere repeats and retrotransposons (LINE and SINE; Figure 1).^{39,40,45,48–50,52} Obtaining such different results following the same strategies could be due to a technical issue. Carone *et al.* suggest in their study that promoter nucleosomes, although being less abundant in sperm, are more stable to MNase digestion. In this regard, an extensive nuclear digestion of chromatin would degrade more abundant nucleosomes in gene deserts and thus reveal only those associated with regulatory regions.⁵² This hypothesis seems to be consistent with the identification of distal DNase I-hypersensitive regions characterized by an enrichment at CTCF-binding sites, depletion in H3K4me3 and presence of H3K9ac and H4K12ac in human and mouse spermatozoa^{43,46} (Table 1).

Whatever the case may be, the sperm nucleosome association with repetitive sequences would be also in agreement with a potential function of the sequence-specific sperm chromatin DNA distribution in postfertilization processes. For instance, it is known that telomeres are involved in microtubule-guided movement during male pronucleus development.^{40,66} Furthermore, retrotransposons

could be conducting regulatory functions for host genes, by serving as a scaffold for the transcription factor binding repertoire

in preimplantational processes.^{20,52} Interestingly, CTCF has been suggested to be a key mediator of epigenetic chromatin remodeling

Table 1: DNA distribution analyses through sperm chromatin in healthy or normozoospermic men and in model species

Study	Sample	Chromatin dissection approach	DNA analysis approach	Main results
Human sperm				
Gatewood <i>et al.</i> 1987 ³⁷	Human (not specified)	0.65 M NaCl+BamHI digestion	DNA probes and Southern hybridization	Existence of sequence-specific nucleo-histone and nucleo-protamine components
Gardiner-Garden <i>et al.</i> 1998 ³⁸	Healthy donors	0.65 M NaCl+BamHI/DraI digestion	DNA probes and Southern hybridization	β -globin gene family members active in embryonic yolk sac (ϵ - and γ -globin) contains histone-associated regions
Zalenskaya <i>et al.</i> 2000 ⁴⁰	Healthy donors	Micrococcal nuclease digestion (30 U mg ⁻¹ DNA)	DNA probes and Southern hybridization	Soluble chromatin fraction enriched in telomeric DNA
Wykes and Krawetz, 2003 ⁴¹	Healthy donors	0.65 M NaCl+BamHI/EcoRI or PvuII digestion	PCR amplification and Southern hybridization	Nonrandom distribution of PRM1-PRM2-TNP2 locus, repetitive sequences and selected genes throughout sperm chromatin
Nazarov <i>et al.</i> 2008 ⁴²	Healthy donors	Endogenous nucleases and micrococcal nuclease (20 U mg ⁻¹ DNA) digestion	Two-dimensional electrophoresis in agarose gels and atomic force microscopy	Existence of sequence-specific nucleo-histone and nucleo-protamine components. Two distinct chromatin domains associated with histones
Arpanahi <i>et al.</i> 2009 ⁴³	Normozoospermic men and mouse sperm	0.65 M NaCl+BamHI/EcoRI and micrococcal nuclease digestion (5 U/100 \times 10 ⁶ sperm)	ChIP and microarray analysis	Endonuclease-sensitive DNA sequences associated with promoter sequences and sequences recognized by CTCF
Hammoud <i>et al.</i> 2009 ⁴⁴	Healthy donors	Micrococcal nuclease digestion (10–160 U)	ChIP or DNA gel purification combined with high-throughput sequencing or array analysis. DNA methylation analysis	Retained sperm nucleosomes enriched at imprinted gene clusters, microRNAs clusters, HOX gene clusters and promoters of developmental transcriptional signalling factors. Histones modifications localize to particular developmental <i>loci</i>
Brykczynska <i>et al.</i> 2010 ⁴⁵	Normozoospermic men and mouse sperm	Micrococcal nuclease digestion	ChIP combined with microarray analysis or high-throughput sequencing	H3K4me2 and H3K27me3 are retained in regulatory sequences and mark promoters of genes important in spermatogenesis and early embryo development. Modest enrichment of TSS in nucleosomes
Vavouri and Lehner, 2011 ⁴⁷	Arpanahi <i>et al.</i> 2009 ⁴³ ; Hammoud <i>et al.</i> 2009 ⁴⁴ ; Brykczynska <i>et al.</i> 2010 ⁴⁵	-	<i>In silico</i> analysis of data from Arpanahi <i>et al.</i> 2009 ⁴⁴ ; Hammoud <i>et al.</i> 2009 ⁴⁴ ; Brykczynska <i>et al.</i> 2010 ⁴⁵	Nucleosome retention in genic and nongenic regions of the genome, GC-rich sequences with high intrinsic nucleosome affinity and TSS of most housekeeping genes. Link of nucleosome retention and DNA-methylation-free regions in the early embryo
Samans <i>et al.</i> 2014 ⁵²	Healthy donors and bovine sperm	Micrococcal nuclease digestion (20 U/10 \times 10 ⁶ sperm)	DNA purification and high-throughput sequencing	Retained sperm nucleosomes enriched at centromere repeats and retrotransposons (LINE1 and SINE1). Nucleosomes-associated genes relevant for preimplantation development
Castillo <i>et al.</i> 2014 ⁵¹	Normozoospermic men	0.65 M NaCl+BamHI/EcoRI and micrococcal nuclease digestion (0.07 U/100 \times 10 ⁶ sperm)	DNA purification and high-throughput sequencing	Retained sperm nucleosomes and subnucleosomal particles enriched at developmental genes, gene promoters and CpG promoters. Two sets of nuclear proteins differing in chromatin affinity
Mouse sperm				
Pittoggi <i>et al.</i> 1999 ³⁹	Epididymal sperm	Endonuclease digestion	Southern blot, en-labelling, cloning and sequencing, FISH	Hypersensitive chromatin domains retained nucleosomes and are enriched at retroposon DNA with a peripheral distribution
Saida <i>et al.</i> 2011 ⁴⁶	Caudal sperm	Micrococcal nuclease digestion (5 U/100 \times 10 ⁶ sperm)	Array analysis (CGH profiling) and FISH	Nuclease sensitive chromatin regions strongly enriched at chromosomal regions with an excess of promoters and sequences recognized by CTCF
Meyer-Ficca <i>et al.</i> 2013 ⁴⁹	Sperm WT and impaired PAR metabolism	Micrococcal nuclease digestion (0.25 U)	FISH	Preferential localization of nucleosomes to the chromocenter, retained in repetitive sequences. Impaired PAR metabolism increase histone retention
Erkek <i>et al.</i> 2013 ⁴⁸	Caudal sperm and round spermatids	Micrococcal nuclease digestion (15 U/2 \times 10 ⁶ sperm)	ChIP and high-throughput sequencing	Retained sperm nucleosomes enriched at hypomethylated CpG-rich sequences, histone H3.3 and H3K4me3
Carone <i>et al.</i> 2014 ⁵⁰	Sperm and mouse ESCs	Micrococcal nuclease digestion (1 U 10 ⁻⁸ sperm) of formaldehyde-crosslinked sperm	ChIP and high-throughput sequencing or paired-end deep sequencing, FISH	Retained sperm nucleosomes enriched at gene-poor genomic regions, with a small subset of histones retained over promoters of developmental regulators

Contd...



Table 1: Contd...

Study	Sample	Chromatin dissection approach	DNA analysis approach	Main results
Zebrafish sperm				
Wu <i>et al.</i> 2011 ⁵⁵	Sperm	-	ChIP and sequential ChIP and microarray analysis	Although all the genome is packaged by nucleosomes, gene sets with a particular function in embryo development are packaged by distinctive types of chromatin

PCR: polymerase chain reaction; FISH: fluorescence *in situ* hybridization; CGH: comparative genomic hybridization; PRM1: protamine 1; PRM2: protamine 2; ChIP: chromatin immunoprecipitation; TNP2: transition protein 2; TSS: transcription start site; PAR: poly (ADP-ribose); WT: wild-type; ESCs: embryonic stem cells; CpG: cytosine-guanine dinucleotides; LINE1: long interspersed elements-1; SINE1: short interspersed element-1

not only during male germ cell development, but also in embryonic genome activation.^{43,46,50}

Taking all these results together, one could hypothesize a model of sperm chromatin structure with a selected subset of relevant regulatory *loci* packaged in dynamic nucleosomal structures, which together with repeated sequences would be closely involved in the regulation of early postfertilization processes (e.g., sperm chromatin remodeling in male pronucleus; **Figure 1**). These epigenetic signatures may be potentially important in preimplantational stages, in order to initiate key processes, and preserve the integrity of other male developmental genes required for later stages of embryogenesis.

ABUNDANT SPERM CHROMATIN-ASSOCIATED PROTEINS – HISTONES AND PROTAMINES

Similarly to the sperm DNA distribution analyses, the study of the protein component of sperm chromatin is also providing information supporting the possibility of a potential sperm epigenetic inheritance.

Although the sperm nucleo-histone domain constitutes a minor part of the total sperm chromatin (**Figure 1**), up to 46 different histones and histone variants have been detected so far as part of the human sperm nuclear proteome.¹⁷ Nucleosomes are dynamic, rather than static, structures, and this aspect may be especially relevant in the male germ line, where different testis-specific histones variants (and their modifications) are expressed throughout mammalian spermatogenesis.^{67,68} The role of noncanonical histones during sperm differentiation is well-documented and becomes essential for different stages, principally after meiosis. In particular, testicular H1 histone, histone H2A.Bdb, and histone H2B type 1-A (TH2B in mouse) are known to be involved in histone hyperacetylation and nucleosome destabilization prior the incorporation of transition proteins and protamines.⁶⁹⁻⁷² However, histone roles should not be restricted to spermatogenic processes, as an analogous hyperacetylation-based paternal chromatin remodeling occurs after fertilization. Therefore, it would be logical to think that the sperm-derived histone variants that remain in the zygotic nucleus could be also participating at that stage.^{17,56,70,71}

Histone participation during spermatogenic and postfertilization processes becomes fundamental due to the ability to carry modifications (mainly methylations, acetylations, and ubiquitinations), defining the so-called histone code.^{4,11,12,17,35,73,74} Besides the specific sperm histone-methylation patterns, already pointed out in the above section, histone acetylations are also noteworthy. In fact, in addition to the acetylation wave that takes place prior to the nucleo-histone-to-nucleo-protamine transition in spermatids,⁷⁵⁻⁸⁰ histone H4K8ac, and H4K12ac have been also observed preceding full decondensation in the zygotic nucleus.⁸¹ Also, interesting was the discovery of a new histone modification, crotonylation, which seems to mark postmeiotically activated genes on autosomes, as well as specific X/Y-linked genes, enabling their postmeiotic activation despite the general repression of the haploid sex chromosomes in the sex body.⁸²

Similar to histones, it is also worth-considering in detail the protamine amino-acid sequences, as protamines are considered critical in the maintenance of sperm chromatin status. Protamines are the most abundant proteins in the mammalian sperm nucleus (**Figure 1**) and their distinctive physicochemical characteristics (such as extreme basicity and high proportion of arginines and cysteines; **Figure 2**) confer elevated protein stability,^{27,28,57,58} at least in Eutherian mammals. For this reason, the identification of potential posttranslational modification (PTM) sites in the sequence of protamine 1 (P1) or protamine 2 (P2) is an intriguing field of study which could increase our knowledge of the sperm chromatin epigenetic landscape.

Reported PTM include the detection of human P1 mono- and di-phosphorylated sites (N-terminal region) and P2 mono-phosphorylated sites (middle region), which seem to be implicated in sperm chromatin remodeling during spermatogenesis.^{27,83-85} The different location of phosphorylated serines between human protamines was suggested to indicate distinct roles for P1 and P2 during sperm maturation.⁸⁵ Protamines also experience further processing during epididymal maturation, when disulfide bonds and zinc bridges are formed between cysteine residues to stabilize the toroidal structure.^{25,28,58,86,87}

An additional important contribution to our knowledge of protamine PTMs has recently been forthcoming in a study of murine protamine sequences using a novel proteomic workflow based in MS.⁷³ Thus, Brunner *et al.* were able to detect phosphorylation sites using MS in mouse P1 and P2 corresponding to those previously reported in human sperm using conventional procedures. In addition, although P2 family members were not analyzed separately, P2 residues carrying acetylations and methylations were also identified.⁷³ It is interesting to highlight that while acetylations and methylations were detected in the same sequence, acetylations and phosphorylations seemed to be exclusive, suggesting nonrandom protamine processing in mouse spermatozoa.⁷³

Consistent with the above observations in mice, we report here for the first time the analysis of the intact (not digested) human protamine 1 amino acid sequence through MS (**Figure 2**). Following a similar strategy to that used in mouse sperm, we were able to detect in human P1 mono-, di- and tri-phosphorylations by MS (**Figure 2**). In addition, P1 seemed to carry combinations of different PTMs, which included di-acetylations with mono-methylation and, in contrast to mouse P1, di-phosphorylations with mono-acetylation (**Figure 1**). Concerning human P2 family, the potential presence of PTMs (methylations and acetylations) was only detected in the component P3 (data not shown). Despite this evidence for the presence of phosphorylations and acetylations in mouse (Brunner *et al.* 2014) and human (**Figure 2**) protamines, we believe that the existence of a protamine code with a similar function to the histone code (where PTMs are involved in gene expression) is unlikely. The main reasons are the presence of the semicrystalline chromatin status conferred by the nucleo-protamine toroidal packaging and the fact that the sperm chromatin is

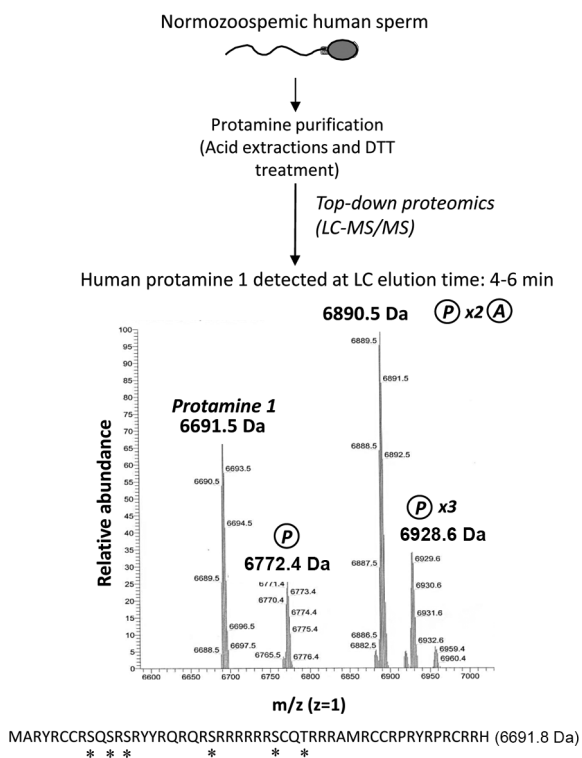


Figure 2: Analysis of the intact human sperm protamine sequences by MS. After following the indicated workflow and LC-MS/MS analysis, the registers obtained demonstrate the presence of the intact unmodified Protamine 1 mass and masses of the protamine 1 containing different combinations of phosphorylation and acetylation. Methods: human sperm cells from normozoospermic sperm donors were collected and processed as described⁹² following World Health Organization guidelines¹²⁵ and in accordance with the ethical and internal review board guidelines. Seminal plasma was discarded and sperm were treated with acid and reducing agents,⁹² in order to obtain a protein extract constituted mostly by a mixture of purified protamine 1 (P1) and protamine 2 (P2) (top). The correct protamine purification was confirmed through acid-urea polyacrylamide gel electrophoresis, as previously described,⁶¹ prior to the analysis by LC-MS/MS. Spectra corresponding to the liquid chromatography elution time 4–6 min obtained after MS analysis of nondigested proteins (top-down proteomics) is shown in the middle of the image. Mass peaks corresponding to nonmodified P1, mono-phosphorylated P1, di-phosphorylated and mono-acetylated P1, and tri-phosphorylated P1 were observed (note that each peak is showing a mass/charge (m/z) value corresponding to a specific “m,” since “z” is set as 1). A maximum error of 200 ppm between theoretical and experimental masses was considered to give a match as valid. At the bottom of the figure, the human P1 amino-acid sequence is indicated together with its theoretical “m.” Potential phosphorylation and acetylation sites are highlighted with an asterisk. DTT: dithiothreitol; MS: mass spectrometry; LC-MS/MS: liquid chromatography followed by tandem mass spectrometry.

transcriptionally silent.^{17,27,28,57,58,88–90} Instead, there is substantial evidence for the involvement of protamine phosphorylation in the deposition of recently synthesized protamines to DNA.^{27,83–85,91} What remains still unknown is whether other modifications, such as acetylations of protamines, could also have a role during spermatogenesis. The possibility is thus open to study a potential involvement of protamine PTMs in the mature chromatin structure and in chromatin structural transitions taking place after fertilization (such as the chromatin destabilization in the male pronucleus prior maternal histones incorporation), and how this could be related to gene expression in the early embryo.

These novel protamine PTMs reported here (Figure 2) were obtained following a protamine purification procedure using sperm

samples from normozoospermic men as previously described in our lab⁹² coupled to MS analysis of the nondigested protein extracts. The small size of the protamines (Figure 2) allows the detection of the intact amino acid sequence using an elegant high-throughput technique known as top-down proteomics.^{73,93} Following this strategy, spectra showing peaks with mass/charge values (m/z) were obtained and used to determine the mass of every protein component of the extract (Figure 2). Because “z” was set as 1, each MS peak provided a unique “m” (Daltons), which was subsequently compared with that corresponding to human P1, or mature and immature individual human P2 family members. Incorporations of “m” to protamines (corresponding to PTMs) were evaluated using information from the Unimod database (http://www.unimod.org/modifications_list.php?), setting 200 ppm as the maximum error acceptable to consider valid an m match.

The gold-standard strategies to detect and quantify protamines have been so far based on protein separation using acid-urea polyacrylamide gel electrophoresis and visualization by Coomassie blue gel staining,^{21,61,65,92,94–98} the use of specific antibodies for Western blot detection,^{97,99,100} and determination of the protamine amino-acid sequences using EDMAN cycle protein sequencing.^{83,85} However, these strategies were quite time-consuming implying a relative limitation. Thus, emerging approaches to identify protamines based in MS strategies⁷³ (Figure 2) seem to have the potential to be more efficient and accurate, in addition to allowing a higher-throughput analysis of patients and model systems. All these reported data are therefore supporting the prospective use of top-down proteomics for protamine PTMs patterns analyses in fertile men, the identification of alterations in infertile patients, and the potential identification of prognostic markers in assisted reproduction.

LOW ABUNDANCE SPERM CHROMATIN-ASSOCIATED PROTEINS

The MS-based study of the male gamete has also largely demonstrated that the sperm chromatin protein composition is more complex than initially considered.^{17,101,102} In fact, besides histones and protamines, a large number of zinc finger- and bromodomain-containing proteins, transcription factors, histone modifiers, and other DNA-related proteins have been identified as part of the human sperm nucleus^{17,51,101,103–107} (Figure 1). Interestingly, similar trends were also found in other mammalian species which suggests a conservation of the rich sperm chromatin protein profile.¹⁷

Thus, it should be accepted that the sperm nucleus contains a subset of sperm nuclear proteins mainly involved in chromatin organization and gene expression which are delivered to the oocyte upon fertilization.^{11,17} This fact consequently leads to speculation concerning the purpose of keeping these proteins in the transcriptionally inert male gamete. In this regard, sperm chromatin proteins could be considered at three levels: (1) spermatogenesis residual with no function at all in the mature sperm cell or in the zygote, (2) proteins with a structural or functional role in the mature sperm chromatin, and (3) proteins implicated in future events such as male pronucleus chromatin remodeling or transcriptional regulation of histone-bound paternal genes after fertilization.^{17,30,51,104,107,108} Of relevance, for many proteins, these three facets would not be necessarily mutually exclusive. For example, this could be the case for the well-studied bromodomain testis-specific protein, the histone-chaperone protein SET, transcription factors involved in differentiation and developmental processes, PHD Finger proteins, effectors of the histone code, or some proteins involved in the regulation of the DNA methylation, replication, repair and transcription.^{17,51,69,109–111}

Also interesting is the fact that sperm nuclear proteins can be differentiated according to their chromatin affinity into two subsets with different functional profiles⁵¹ (**Figure 1**). To this extent, high-affinity proteins might be mainly involved in structural roles, which could be related with the nucleo-protamine structural organization and function. In contrast, the low-affinity subset of proteins could conceivably be involved in a higher variety of roles, including protein metabolism, DNA packaging, DNA/RNA binding, and transcription, among others. Of relevance, all those proteins with potential regulatory roles in the sperm nucleus appear to be weakly attached to the chromatin.⁵¹ These data suggest that in addition to the sequence-specific distribution of the genes, the spermatozoon is organized such that many different layers of potential epigenetic information are distributed through the chromatin landscape for delivery to the oocyte at fertilization and the subsequent regulation of early embryogenesis.

In order to unravel the sperm chromatin epigenetic potential, it is also necessary to take into account the role of the nuclear matrix and the proteins associated with this structure. Similarly to the somatic cells, sperm chromatin is organized into nuclease sensitive DNA segments attached to the nuclear matrix at 50 kb intervals (**Figure 1**).^{8,112} The nuclear matrix itself is thought to incorporate Topoisomerase II-B (TOP2B). TOP2B is a matrix-associated protein reported to have a role in spermiogenesis, reducing nucleosome supercoiling by double-strand DNA breaks and promoting chromatin remodeling.¹¹³ The paternal matrix associated regions (MARs), including DNA and protein composition, seem to be inherited by the fertilized embryo and are thought to be essential for paternal pronuclear DNA replication.^{8,34,59,60} In fact, proper initiation pronuclear formation and DNA synthesis in fertilized mouse oocytes has been demonstrated using spermatozoa exhibiting a high degree of DNA degradation, as long as *in situ* DNA loop attachments to the nuclear matrix were preserved.⁵⁹ Therefore, as it happens in somatic cells, the sperm replication machinery is also assembled into the nuclear matrix, which is serving as a scaffold.³⁴ Several lines of evidence suggest that the same MAR sites could also be representing points of DNA cleavage, with a potential role in DNA integrity maintenance, in which TOP2B might also be involved.^{22,34,114}

GENOMIC AND PROTEOMIC ALTERATIONS IN SPERM CHROMATIN OF INFERTILE PATIENTS

As has been discussed in the previous sections, sperm chromatin is characterized by specific genomic and proteomic features, involved in the correct formation of the male gamete as well as the embryo. Therefore, an important question that still remains to be considered is whether alterations in sperm epigenetic signature can lead to male infertility.

Several studies have demonstrated alterations in sperm DNA methylation patterns,^{115–117} RNA content,^{20,118} and histone retention^{54,119,120} in infertile/subfertile patients. Alterations in histone retention could affect sperm function at two levels. First, the altered histone retention would directly imply an alteration in protamine content and in the tightly regulated P1/P2 ratio, which is indicative of poor semen quality, increased DNA damage and decreased fertility.^{9,21,28,31,57,58,61,62,64,65,90,92,95,97,99,100,121} Second, it would lead to rearrangements in chromatin organization of developmental *loci* and genes, which may have an impact on normal embryo development. In fact, the histone-specific sequence packaging found in healthy men (**Figure 1**) has been shown to be lost in patients with subfertility, resulting in random sperm DNA chromatin distribution.¹¹⁹ In this

study, Hammoud *et al.* analyzed MNase-sensitive sperm chromatin regions from three patients with poor embryogenesis after *in vitro* fertilization (IVF) and four men with altered protamination. After genome-wide analysis based on high-throughput sequencing, five of the seven infertile men showed nonprogrammatic retention of histones. Histone modifications were also evaluated, showing a reduction of developmental promoters enriched with H3K4me3 and H3K27me3 in most infertile men, although the localization of modified histones was unaltered.¹¹⁹

Our group has also recently demonstrated the presence of altered histone content in infertile patients using a high-throughput quantitative proteomic approach based on protein isobaric labelling of proteins.¹²⁰ The significance of sperm nuclear proteomic profiles was recently highlighted when cells from normozoospermic men that were able to perform IVF but whose female partners did not achieve a pregnancy, were compared with those from men with similar semen parameters but with successful pregnancy outcomes (excluding female factor). Interestingly, altered levels of proteins specifically involved in chromatin assembly and metabolism were detected in the so-called “no pregnancy group.” In particular, six histones variants (H2A type 1-A, H2A type 1-C, H2A type 2-C, H2B type 1A, H3 and H4) and a protein involved in protamine 1 phosphorylation (SRSF protein kinase 1; SRPK1) were identified deregulated with abnormally high abundance.¹²⁰ These results were thus consistent with the hypothesis that an alteration in the sperm chromatin proteome may result in epigenetic errors contributing to failed embryo development.

Additional complementary data have also been obtained in mouse models with impaired poly (ADP-ribose) (PAR) metabolism, which is involved in the nucleo-histone-to-nucleo-protamine transition during spermiogenesis.^{49,122} Affected PAR mice sperm was shown to carry compromised chromatin with abnormally increased histone retention, although being still associated with repetitive sequences.^{49,122}

Of relevance, Ihara's study went a step further and analyzed for the first time the genome of embryos generated with chromatin affected (by altered PAR metabolism) spermatozoa. Interestingly, it was shown that a statistically significant portion of the differentially expressed genes in mouse embryos corresponded to paternal gene *loci* showing altered histone retention caused by PAR impairment.¹²² Despite this and in contrast to the data obtained in human spermatozoa, fertility was not compromised in this model, as embryos developed to term.¹²²

Infertility can be considered a multifactorial disease in which alterations in the epigenetic constitution of the sperm chromatin could be involved. The application of genomic and proteomic high-throughput strategies is thus helping to unravel the potential contribution of sperm chromatin organization to male infertility. Therefore, all these data have the potential in the future to be useful in the identification of putative biomarkers for the diagnosis and prognosis of idiopathic male infertility.^{107,123}

CONCLUSION

About 92% of the human sperm chromatin is formed by highly compact toroidal nucleo-protamine complexes while the remaining 8% is organized with histones. Of importance, there is a nonrandom distribution of the genes, gene sequences, and repetitive elements that has the potential to be involved in the sperm chromatin reorganization in the oocyte and perhaps in the selective activation of key paternal genes in the early embryo. In addition to the protamines and histones, the sperm chromatin also contains many additional chromatin-associated proteins with the potential to provide different layers of epigenetic information or serve in the reorganization of the

paternal chromatin after fertilization. Alterations in the distribution of histones and the additional sperm chromatin-associated proteins are also being detected in infertile patients. Altogether the information so far available indicates that sperm chromatin is much more complex than it was previously thought and that it contributes in the transmission of information to the zygote that may be crucial for the paternal pronuclear chromatin remodeling and embryo development.

AUTHOR'S CONTRIBUTIONS

JC and RO analyzed MS data of protamines, drafted the manuscript and approved the final manuscript. JME analyzed MS data of protamines, critically reviewed the manuscript and approved the final version. JLB characterized the clinical samples used for MS detection of protamines, critically reviewed the manuscript and approved the final version.

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COMPETING FINANCIAL INTERESTS

All authors declare no competing interests.

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