Incorrect DNA methylation of the DAZL promoter CpG island associates with defective human sperm†

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BACKGROUND: Successful gametogenesis requires the establishment of an appropriate epigenetic state in developing germ cells. Nevertheless, an association between abnormal spermatogenesis and epigenetic disturbances in germline-specific genes remains to be demonstrated.

METHODS: In this study, the DNA methylation pattern of the promoter CpG island (CGI) of two germline regulator genes—DAZL and DAZ, was characterized by bisulphite genomic sequencing in quality-fractioned ejaculated sperm populations from normozoospermic (NZ) and oligoasthenoteratozoospermic (OAT) men.

RESULTS: OAT patients display increased methylation defects in the DAZL promoter CGI when compared with NZ controls. Such differences are recorded when analyzing sperm fractions enriched either in normal or defective germ cells (P < 0.001 in both cases). Significant differences in DNA methylation profiles are also observable when comparing the qualitatively distinct germ cell fractions inside the NZ and OAT groups (P = 0.003 and P = 0.007, respectively). Contrastingly, the unmethylation pattern of the DAZ promoter CGI remains correctly established in all experimental groups.

CONCLUSIONS: An association between disrupted DNA methylation of a key spermatogenesis gene and abnormal human sperm is described here for the first time. These results suggest that incorrect epigenetic marks in germline genes may be correlated with male gametogenic defects.

Key words: male infertility / spermatogenesis / epigenetics / DAZ gene family / DNA methylation

Introduction

Infertility affects ~9% of all couples of reproductive age, with a male factor being detected in 30–50% of the cases (de Kretser, 1997; Boivin et al., 2007). Although these correspond mostly to abnormalities in sperm production, the number of genetic mutations unequivocally associated with human spermatogenic impairment remains low (Visser and Repping, 2010). Nevertheless, recent advances in the field of germ cell epigenetics have opened new areas for the identification of male infertility determinants.

The establishment and maintenance of the germline requires precise orchestration of epigenetic cues starting as early as in the nascent primordial germ cell (PGC) population (Sasaki and Matsui, 2008). More particularly, programmed changes in the germ cell DNA methylation state serve as developmental triggers for gametogenesis, as illustrated by the two demethylation waves in developing PGCs (Hajkova et al., 2002). Indeed, after an initial demethylation wave in migrating PGCs, a key reprogramming step occurs shortly after these cells enter a post-migratory state in the developing gonads (Maatouk et al., 2006). The latter step corresponds to the

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activation of germ cell differentiation genes via DNA demethylation of their promoter CpG islands (CGIs). Thus, the onset of the germline-specific gene expression profile is dependent on the establishment of an appropriate epigenetic signature. This signature must be perpetuated throughout fetal development, and also post-natally, for successful spermatogenesis. Accordingly, it can be hypothesized that the disruption of the epigenetic state of germ cell differentiation genes represents a causative agent of spermatogenic failure.

The vast majority of studies that have analyzed DNA methylation patterns in the context of abnormal human spermatogenesis have focused solely on imprinted genes (for selected references: Manning et al., 2001; Benchab et al., 2005; Houshdaran et al., 2007; Kobayashi et al., 2007; Marques et al., 2008; Hammoud et al., 2009; Poplinski et al., 2009; Boissonnas et al., 2010). These have demonstrated that defective methylation patterns in such genes are associated with abnormal spermatogenesis. If the latter defects were shown to be extensible to genes actually involved in the spermatogenic program this would represent a conceptual advance in the field. The fairly recent detection of DNA methylation defects outside of imprinted domains in poor quality sperm represented a tentative first step in that direction (Houshdaran et al., 2007). Nevertheless, the lack of any gametogenic function ascribable to the identified genes limited the functional relevance of the association.

In this context, the (epi) genetic regulation of the DAZ gene family is of particular relevance. This sequence family consists of Y chromosome-encoded DAZ (deleted in azoospermia), organized in four copies in the reference human Y sequence, and autosomal DAZL (deleted in azoospermia-like), mapping as a single copy to chromosome 3 (Yen, 2004). The DAZ gene family plays key roles in germline establishment and gametogenesis, with its members encoding for germ cell-specific RNA binding proteins involved in transcript transport/storage, translation initiation and protein regulation. Fittingly, the full deletion of the DAZ copies, as observed in complete AZFc deletions, results in a hypospermatogenesis phenotype (for a review: Navarro-Costa et al., 2010), whereas studies in mammalian models have demonstrated that DAZL deficiencies lead to spermatogenic arrest (Ruggiu et al., 1997; Schrans-Stassen et al., 2001; Lin and Page, 2005). Owing to their extensive similarities, DAZ and DAZL display high sequence identity levels even in non-coding regions. This is particularly relevant since the promoter CGI of both genes remains unmethylated exclusively in germ cells, the sole cell type where they are expressed (Chai et al., 1997; Yen, 2004). Experiments conducted in murine models have shown that Dazl expression is induced after the selective DNA demethylation of germ cell differentiation genes in post-migratory PGCs, and its activation is central to the establishment of the germline genetic profile (Maatouk et al., 2006; Haston et al., 2009). Taking into consideration both its relevance for male gametogenesis and regulation via DNA methylation, the DAZ gene family emerges as a suitable candidate for the characterization of the epigenetic state of germline genes in defective spermatogenesis.

In this study, we characterized the DNA methylation pattern of the DAZ and DAZL promoter CGIs in quality-fractioned sperm populations from men with normozoospermia (NZ) and oligoasthenoteratozoospermia (OAT). This represents the first time that the epigenetic state of these two key gametogenesis genes are assessed in sperm fractions from men with varying degrees of spermatogenic success.

**Materials and Methods**

This study was performed in compliance with the Helsinki Declaration and was approved by the Scientific and Ethics Committees of both Saint Mary’s Hospital and the National Institute of Health. All enrolled subjects gave their written informed consent to the analyzes.

**Inclusion criteria and clinical characteristics of the selected samples**

A total of 10 semen samples were selected from men undergoing standard spermogram analysis during clinical evaluation of infertile couples (more than two spermograms per patient). Five men were initially selected for the OAT group (average 6.7 ± 2.6 × 10^6 sperm/ml; 5 ± 4.3% normal sperm forms and 1 ± 2.2% fast progressive motility) based on a stringent interpretation of the World Health Organization criteria (1999). Since age-related changes in DNA methylation profiles have been reported in human sperm (Fanagan et al., 2006), age-matching (maximum tolerated difference: 2 years) was adopted in order to select five NZ controls (average 83.5 ± 23.1 × 10^6 sperm/ml; 36 ± 4.7% normal sperm forms and 44 ± 6.5% fast progressive motility). In accordance, the age distribution between both groups (average NZ age: 39.2 ± 7.3 y.o; OAT: 39.4 ± 7.2) did not vary significantly, as analyzed by the Mann–Whitney U-test (P = 1.000). Additionally, no evidence of ethnic differences (all men were of Portuguese ancestry), complete or partial AZF deletions [as assessed by a previously published genetic marker panel (Navarro-Costa et al., 2007)], or other confounding effects was detected between groups.

**Ejaculated sperm fractioning and purification**

Semen samples were fractioned via the use of a discontinuous 80/40 density gradient centrifugation in a silica particle matrix (PureSperm; NidaCon Laboratories AB, Sweden). Such centrifugation techniques are routinely used in ART and rely on the fractioning of cells according to their velocity of sedimentation, a reflection of morphological properties such as size, shape and compactness (Gandini et al., 1999). This results in the formation of a pellet fraction largely constituted by sperm with normal morphology, and in a supernatant interphase fraction enriched in defective germ cells (sperm with morphological abnormalities, as well as other immature cell types; Chen and Bongso, 1999; Gil-Guzman et al., 2001).

All fractions were retrieved from samples with <1 × 10^4 neutrophils/ml and were further purified by performing leukocyte depletion via immuno-magnetic cell selection using antibody complexes specific for the CD45 antigen (EasySep Human CD45 depletion kit; StemCell Technologies, France). This strategy led to highly purified sperm populations, as validated by flow cytometric analysis of all cell fractions (FACSAnia flow cytometer; Becton, Dickinson and Company, USA). The removal of this minor leukocyte population (usually increased in OAT samples), is critical because: (i) it represents the major somatic contaminant present in semen (Arata de Bellabarba et al., 2000); and (ii) it localizes to the fraction enriched in defective germ cells after the gradient centrifugation (Angelopoulos et al., 1997). Following leukocyte depletion, the microscopic evaluation of all populations was performed to assess the characteristics of the purified germ cells (Table I), and to rule out the possibility of contamination by other residual somatic cells.

**Bisulphite genomic sequencing of the DAZ and DAZL promoter CGIs**

For analysis of promoter CGI methylation levels, a bisulphite modification-based genomic sequencing strategy was adopted. Strand-specific PCR (ssPCR) was performed in bisulphite-modified DNA, with the two
genes being tested using the same treated aliquot (additional information on CGI mapping and ssPCR primer design/settings is available in the Supplementary data, methods and in Supplementary data, Table S1). Briefly, sperm DNA was extracted from 1 × 10⁶ cells in all purified fractions (DNeasy Blood & Tissue Kit; Qiagen, The Netherlands) in order to avoid a cell number bias effect, and was modified by sodium bisulphite (DNeasy Blood & Tissue Kit; Qiagen, The Netherlands) in order to confirm in all selected populations by microscopic observation. The occurrence of such somatic contaminants was minimized by the fact that these cells tend not to penetrate the cell fraction. Since early spermatids and spermatocytes are morphologically similar to leukocytes and these different cell types have been shown to co-localize to the interphase fraction in density gradients (Gandini et al., 1999), leukocyte depletion is essential for this type of analysis. Owing to their distinct morphology, the absence of contaminating Sertoli or epithelial cells was confirmed in all selected populations by microscopic observation. The occurrence of such somatic contaminants was minimized by the fact that these cells tend not to penetrate the gradient matrix due to their large size and low compactness.

The number of clones allocated to the three methylation categories was compared between the two types of experimental groups (NZ versus OAT and normal sperm-enriched versus defective germ cell-enriched fractions) using a multivariate version of Fisher’s exact test. Additionally, the distribution of clones in all experimental groups was analyzed using log linear models, with the goodness of fit of each model being tested by the lowest Akaike information criterion (AIC) value. The concept of epigenetic distance was also used to characterize inter- and intra-group DNA methylation levels (Flanagan et al., 2006). Epigenetic distance corresponds to a measure of the net dissimilarity in DNA methylation profiles of a given sequence between two samples, indicating the number of pairwise methylation differences. The higher the distance, the more dissimilar are the two individual epigenetic profiles. To characterize such distances, a programmed R subroutine was devised. Briefly, a row vector identifier representing the methylation state of all CpG positions was coded for each of the 880 clones, with ‘0’ for unmethylated cytosine and ‘1’ for methylated. The individual methylation vector of a given sample was
thus obtained by adding the 22 row vectors (corresponding to the recorded observations per sample) for each CpG position and dividing the result by the number of vectors. Euclidean distances were then calculated between the individual methylation vectors of two samples, with the final result representing the epigenetic distance between both. To increase robustness, the analysis was further expanded to include an in silico simulation algorithm based on the generation of virtual samples according to the actual recorded profiles. In this case, for each experimental group all clones from the tested individuals were pooled together, with a set of 22 randomly selected row vectors being assembled in order to establish a simulated individual. Subsequently, the epigenetic distance between this simulated individual and another intra- or inter-group assembly was calculated as above. The process was iterated 100,000 times, allowing the establishment of a density distribution curve.

**Results**

Methylation profiles were initially analyzed by ascribing the clones constituting each experimental group to one of three methylation categories as defined by the Human Epigenome Project criteria: unmethylated (<20% methylated CpGs), partially methylated

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**Figure 1** Sperm DNA methylation state of the DAZL and DAZ promoter CGIs. (A) Schematics represent the structure of the 5’ region of both genes, with exons (up to exon 8) depicted by black vertical lines and the 5’ UTR by a white box. The horizontal line under each cartoon represents the promoter CGIs, as identified by the CpGProD algorithm (for more information please consult the Supplementary data, methods). The amplified region is indicated by a diagonal pattern box. ‘Lollipop’ diagrams for the methylation state of the analyzed CpGs serve as illustrative examples of the variation between NZ and OAT men in the two genes (for full methylation profiles please consult Supplementary data, Fig. S1). Each line represents an independent bisulphite-sequenced clone with the multiplier on the right indicating the number of identical observations in the depicted sample (for each sample a total of 22 clones were sequenced). Open and closed circles indicate unmethylated and methylated CpGs, respectively. (B) Mean distribution of unmethylated (white bars), partially methylated (grey bars) and hypermethylated (black bars) clones between the NZ and OAT groups for the two qualitatively distinct germ cell fractions (normal sperm or defective germ cell-enriched). Charts on the left refer to DAZL and those on the right to DAZ. Data are represented as mean ± SEM, with 110 observations recorded per experimental group (n = 880). Twenty-two clones were individually sequenced in each cell fraction isolated from all NZ and OAT men (five men in each group). Statistical difference in the number of DAZL clones allocated to each methylation category was detected when comparing NZ to OAT men (P < 0.001 for either germ cell fraction), and also between the qualitatively different germ cell populations inside the NZ and OAT groups (P = 0.003 and P = 0.007, respectively). Different superscripts within each gene denote statistical difference.
Results displayed considerable differences between the two genes in terms of epigenetic signatures in ejaculated sperm (Fig. 1). Contrary to DAZL, DAZ unmethylation was properly established in all experimental groups, with unmethylation frequencies exceeding 0.97 (107/110) for all the tested cell fractions. The consistent DAZ unmethylation levels are of relevance in two aspects. Firstly, they demonstrate that ejaculated male germ cells can maintain the epigenetic signature of previous spermatogenic stages. Secondly, the virtual absence of somatic-like DAZ methylation patterns (fully methylated clones: 1/440) indicates the lack of somatic contaminants in the purified germ cell populations. Both observations validate the selected experimental strategy and illustrate the feasibility of using ejaculated sperm to assess the germline DNA methylation profile of spermatogenesis genes.

Unlike what was observed for DAZ, clear methylation differences between the tested groups were recorded for DAZL. More specifically, the number of clones allocated to each methylation category varied significantly when comparing between the NZ and OAT groups using a multivariate version of Fisher’s exact test ($P$, 0.001 for fractions enriched either in normal or defective germ cells; Fig. 1). Appropriately, the frequency of unmethylated DAZL clones in the normal sperm-enriched fraction decreased from 1 in NZ men to 0.81 (89/110) in OAT patients. This reduction was even more evident in the fraction enriched in defective germ cells: from 0.92 (101/110) to 0.62 (68/110), for NZ and OAT men, respectively. Furthermore, the significant differences were extensible to the comparison between normal and defective germ cell fractions inside the NZ and OAT sample groups ($P = 0.003$ and $P = 0.007$, respectively). In these cases, an increased frequency of partially and hypermethylated clones were recorded in the defective germ cell-enriched fractions.

The use of log linear models to analyze methylation frequencies in the four experimental groups indicated that the goodness of fit of models testing for intra-group differences was significantly reduced when compared with those testing at the inter-group level (AIC: 110.9–210.4 versus 30.68–69.5, respectively). These results revealed no evidence for intra-group heterogeneities, arguing against any outlier bias effect in the DAZL inter-group methylation differences (Fig. 2).

The analysis of methylation levels by measuring epigenetic distances supports the assertion that normal sperm from NZ men display a unique DAZL epigenetic signature. Indeed, when testing samples for inter-group distances the highest scores were observed when comparing normal sperm-enriched fractions of NZ men with any other group. Most significantly, the comparison of such population with either the normal sperm or defective germ cell-enriched fractions of OAT men produced a 2.6- and 9.5-fold increase of epigenetic distances when compared with intra-group measures (Fig. 3). Reassuringly, minimal intra-group distances were recorded for both DAZ and DAZL (average distances of $0.02 \pm 0.01$ and $0.20 \pm 0.14$, respectively), indicative of extensive epigenetic similarities between the samples constituting each group (Fig. 3).

**Discussion**

In this study, we provide evidence for an association between the OAT phenotype and epigenetic imbalances in DAZL. Since morphologically abnormal sperm also display increased levels of DAZL methylation defects and OAT samples are enriched in such cells, it can be argued that this quantitative variation accounts for the majority of the recorded methylation differences between the NZ and OAT groups. Nevertheless, data indicate that this effect cannot be considered sufficient to fully account for the extensive differences.
DAZL phenotype would be less evident in cells with oous tubule lumen as the result of developmental arrest. This arrest of specialization of functional similarities. Given that DAZ and DAZL are an intriguing result when taking into account their extensive germ cell development in the latter patients.

OAT-intrinsic epigenetic disturbances that may undermine correct normal sperm). This observation points to the existence of the defective germ cell-enriched fraction of NZ men (13.2 ± 6.1%) is associated with a far lower degree of epigenetic disruption than that of the normal sperm-enriched fraction of OAT men (45.4 ± 15.3% of normal sperm). This observation points to the existence of OAT-intrinsic epigenetic disturbances that may undermine correct germ cell development in the latter patients.

The clearly distinct epigenetic profiles recorded between DAZ and DAZL are an intriguing result when taking into account their extensive functional similarities. Given that DAZ represents a male-specific specialization of DAZL functions for meiotic entry, it can be envisaged that cells with DAZ methylation defects might not reach the seminiferous tubule lumen as the result of developmental arrest. This arrest phenotype would be less evident in cells with DAZL defects, owing both to the functional rescue warranted by DAZ in pre-meiotic stages and to the fairly lax post-meiotic checkpoint mechanisms. Alternatively, the distinct nuclear contexts occupied by DAZ and DAZL during male meiosis (XY body versus fully paired bivalents, respectively) may underlie a different propensity for the acquisition of epigenetic defects between both determinants. Recently, a lack of association between DAZ DNA methylation defects and spermatogenic failure has also been reported, yet the sequencing of a single clone per tested sample dramatically reduced the analytical power to identify more subtle disruptions (Wu et al., 2010).

The present findings encourage the development of subsequent studies aimed at demonstrating a causal link between the recorded epigenetic imbalances and abnormal spermatogenesis. Analyzing gene expression levels in patients with DAZ and/or DAZL epigenetic imbalances will represent a first step in that direction. Nevertheless, due to the precise expression windows of these two genes and to the transcriptional and cytoplasmic clean up that occurs prior to the completion of spermatogenesis, such analyzes will require the use of highly purified immature germ cells from distinct spermatogenic stages. Since the isolation of the latter from testicular biopsies is a technically complex task, this research line represents a considerable challenge. In addition, the present results also justify the effort to identify and characterize new germline regulators under DNA methylation control. Recent evidence suggesting that murine BOLL (the ancestral member of the DAZ family) plays a role in sperm cytodifferentiation makes this gene a particularly attractive candidate, even more so since its epigenetic regulation remains unknown (Vangompel and Xu, 2010). It should equally be acknowledged that DAZL is a master regulator of the gametogenic program in females (Kee et al., 2009). Accordingly, it can be envisaged that the epigenetic disruption of DAZL may also negatively impact the oocyte pool, leading to decreased ovarian reserve. Therefore, bridging the results obtained in this report to female gametogenesis represents an interesting additional prospect.

In conclusion, the results presented in this report provide novel descriptive insight into epigenetic factors that may underlie abnormal sperm production. By demonstrating for the first time an association between defective DNA methylation of a key germline gene and gametogenic abnormalities, these data will hopefully serve as catalyst for future developments in the field.

**Authors’ roles**

P.N.-C. designed the study, performed the bioinformatics analysis and the bisulphite genomic sequencing, and drafted the manuscript. P.N. was involved in data analysis and *in silico* simulations and participated in the drafting of the respective sections of the manuscript. M.C.,
Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

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Web Resources
The URLs for data and tools presented herein are as follows:

BiQ Analyzer: http://biq-analyzer.bioinf.mpi-inf.mpg.de/
BiSearch: http://bisearch.enzim.hu/
ClustalW2: http://www.ebi.ac.uk/Tools/clustalw2/index.html
CpGProD: http://pbil.univ-lyon1.fr/software/cpgprod.html
CpGviewer: http://dna.leeds.ac.uk/cpgviewer/
Ensembl: http://www.ensembl.org/Homo_sapiens/Info/Index
RepeatMasker: http://www.repeatmask.org/cgi-bin/WEBRepeatMasker

BiQ Analyzer: http://biq-analyzer.bioinf.mpi-inf.mpg.de/
BiSearch: http://bisearch.enzim.hu/
ClustalW2: http://www.ebi.ac.uk/Tools/clustalw2/index.html
CpGProD: http://pbil.univ-lyon1.fr/software/cpgprod.html
CpGviewer: http://dna.leeds.ac.uk/cpgviewer/
Ensembl: http://www.ensembl.org/Homo_sapiens/Info/Index
RepeatMasker: http://www.repeatmask.org/cgi-bin/WEBRepeatMasker

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