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ORIGINAL ARTICLE *Reproductive genetics*

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; Benchaib *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 spermiogram analysis during clinical evaluation of infertile couples (more than two spermiograms per patient). Five men were initially selected for the OAT group (average $6.7 \pm 2.6 \times 10^6$ sperm/ml $5 \pm 4.3\%$ normal sperm forms and $1 \pm 2.2\%$ 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 (Flanagan *et al.*, 2006), age-matching (maximum tolerated difference: 2 years) was adopted in order to select five NZ controls (average $83.5 \pm 23.1 \times 10^6$ sperm/ml; $36 \pm 4.7\%$ normal sperm forms and $44 \pm 6.5\%$ 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 $<1 \times 10^4$ neutrophils/ml and were further purified by performing leukocyte depletion via immunomagnetic 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 (FACSAria 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

Table I Individual spermiogram parameters and characterization of the analyzed sperm populations.

		Spermiogram parameters (major)			Post-gradient purification			
					Normal sperm-enriched fraction[a]		Defective germ cell-enriched fraction[b]	
Sample code[c]	Age (y.o)	Sperm concentration ($\times 10^6$/ml)	Normal morphology[d] (%)	Fast progressive motility (%)	Normal morphology (%)	Immature germ cells[e] (%)	Normal morphology (%)	Immature germ cells (%)
NZ_1	38	84.0	31	35	75	0	11	3
NZ_2	31	50.0	42	50	64	0	21	6
NZ_3	51	91.2	40	45	71	0	12	3
NZ_4	37	78.4	33	50	69	0	17	3
NZ_5	39	114.0	35	40	72	0	5	5
OAT_1	36	7.5	5	0	52	2	0	11
OAT_2	51	3.0	5	5	65	2	9	7
OAT_3	37	10.0	2	0	33	4	3	13
OAT_4	32	5.6	1	0	27	6	0	14
OAT_5	41	7.2	12	0	50	3	10	9

[a]Figures correspond to cells recovered from the pellet fraction of a density gradient centrifugation using a discontinuous silane-coated silica particle matrix (PureSperm; NidaCon Laboratories AB, Sweden), according to the manufacturer's instructions. Prior to manual scoring, cells were subjected to immunomagnetic leukocyte depletion using tetrameric antibody complexes specific both for the CD45 antigen and dextran-coated nanoparticles (EasySep Human CD45 depletion kit; StemCell Technologies, France).
[b]Figures correspond to cells recovered from the gradient supernatant interphase. This cell fraction is enriched in abnormal human sperm and in some immature germ cells, as previously reported (Gil-Guzman *et al.*, 2001). Leukocyte depletion was performed, as above, prior to manual scoring.
[c]NZ, normozoospermia; OAT, oligoasthenoteratozoospermia; both according to the World Health Organization criteria (1999). Sperm morphology was evaluated according to the strict Tygerberg's criteria (Kruger *et al.*, 1988).
[d]Normal sperm morphology, as defined by strict criteria: oval head with appropriate contour, dimensions and width/length ratio; fully developed and defined acrosome encompassing most of the distal part of the head; linear neck with no associated cellular debris; normal sized midpiece with no evident thickening; single uncoiled tail with no abnormalities.
[e]Immature germ cells were identified by post-staining cellular morphology and corresponded mainly to elongating spermatids, as expected from the use of samples with low levels of round cells. 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.

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 SI). Briefly, sperm DNA was extracted from 1×10^5 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 conversion (Imprint DNA modification kit; Sigma-Aldrich, Germany). Following ssPCR amplification, fragments were cloned into a pCR2.1 TOPO vector (Invitrogen, USA), and random positive colonies were screened for the correct amplicon via colony PCR. Twenty-two selected clones per individual cell fraction were sequenced (BigDye Terminator Cycle Sequencing v2.0; Applied Biosystems, CA, USA), with the resulting electropherograms being subjected to quality control for partial cytosine conversion (minimum conversion rate: 95%) using the BiQ Analyzer software (Bock *et al.*, 2005). Although the *DAZ* gene is organized in two, four or six copies in the Y chromosome population (Repping *et al.*, 2006), the amplified region in this assay is conserved between copies, as confirmed both by *in silico* sequence analysis and by the absence of nucleotide variants in the amplified products. Thus, each sequenced ssPCR amplicon reflects the epigenetic state of one of the *DAZ* copies present in a given Y chromosome, with amplification kinetics allowing the identification of disturbances irrespective of the identity of the altered copy. The methylation status of the CpG residues was assessed both by *in silico* analysis with the CpGviewer algorithm (Carr *et al.*, 2007), and operator inspection of the electropherograms. In net numbers, a total of 880 clones were sequenced and analyzed between the two genes: 110 clones per cell fraction (normal sperm versus defective germ cell-enriched) in both the NZ and OAT groups. Each cloned *DAZL* amplicon contained 31 CpGs, as opposed to 12 CpGs for *DAZ* (Fig. 1). Of the 18.920 analyzed CpGs, the methylation state of 4 could not be assigned due to uninformative results (for full clone profiling see Supplementary data, Fig. S1).

Statistical analysis and characterization of epigenetic distances

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

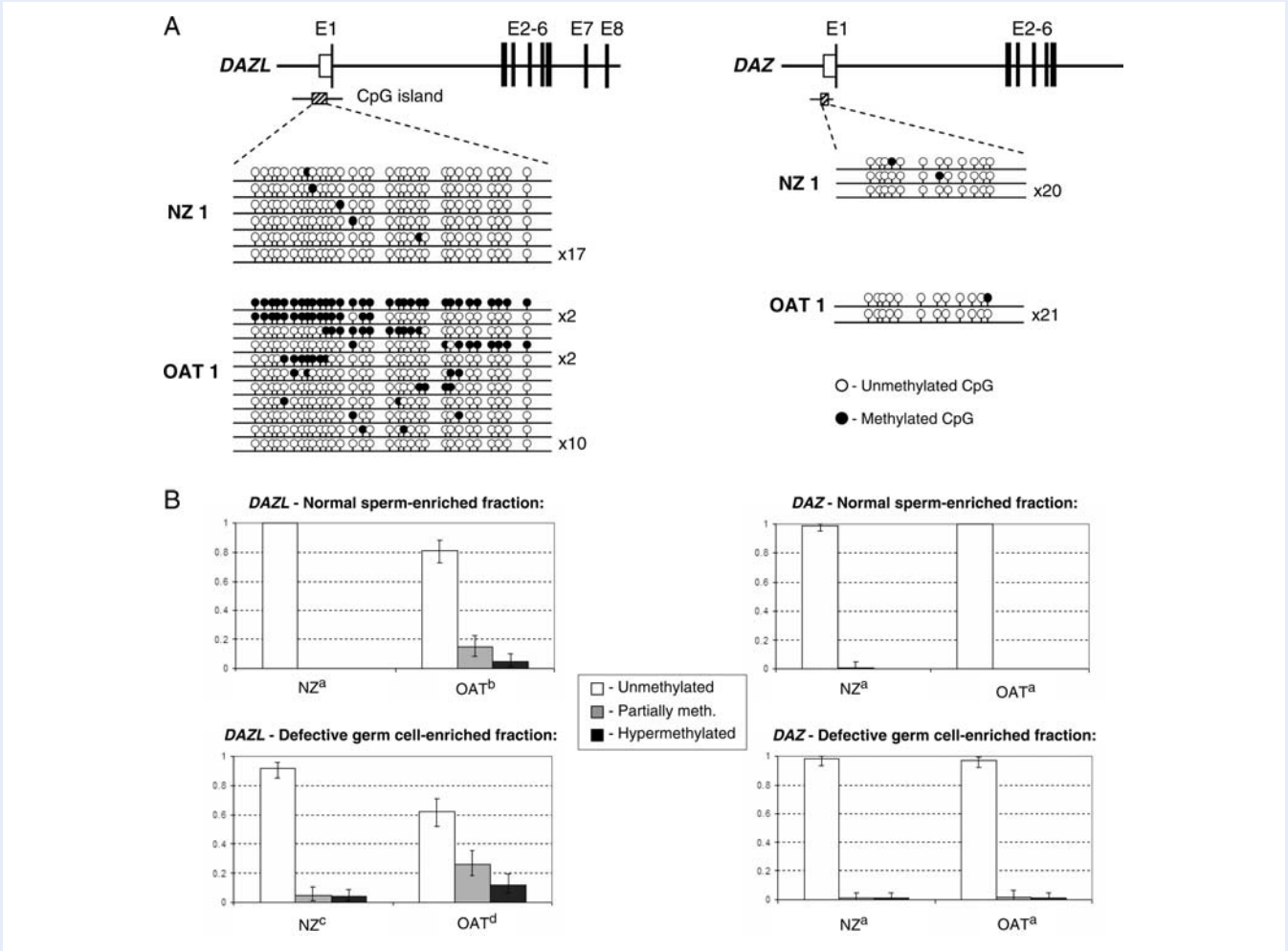

Figure 1 Sperm DNA methylation state of the *DAZL* and *DAZ* promoter CGIs. (**A**) Schematics represent the structure of the 5′ region of both genes, with exons (up to exon 8) depicted by black vertical lines and the 5′ UTR by a white box. The horizontal line under each cartoon represents the promoter 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 unmethlyated 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.

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

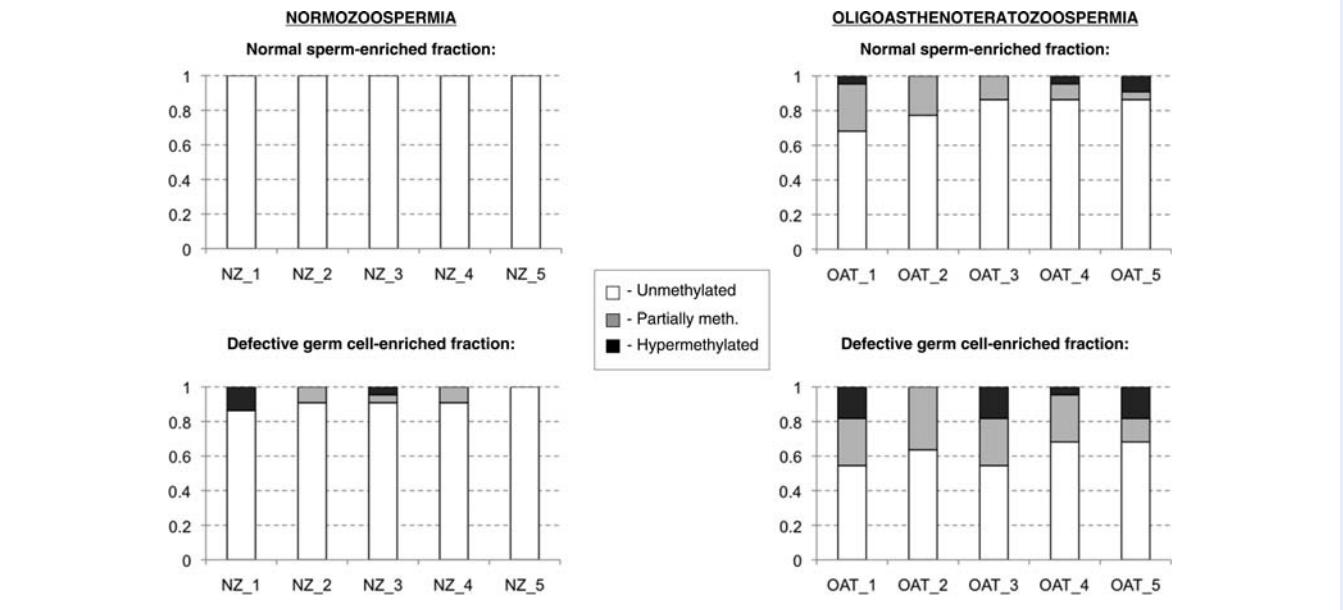

Figure 2 Allocation of the *DAZL* promoter CGI clones to the three methylation categories in all tested samples. NZ and OAT men are numbered 1–5, as plotted in the *x*-axis. Please note that two different individual cell populations (normal sperm-enriched and defective germ cell-enriched) were isolated in all men, for a total of 20 samples. Twenty-two clones were analyzed per sample. The use of log linear models provided no evidence supporting the existence of intra-group sample heterogeneities.

(20–80% methylation) and hypermethylated (>80% methylation). 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 traduced in 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 ± 0.01 and 0.20 ± 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.

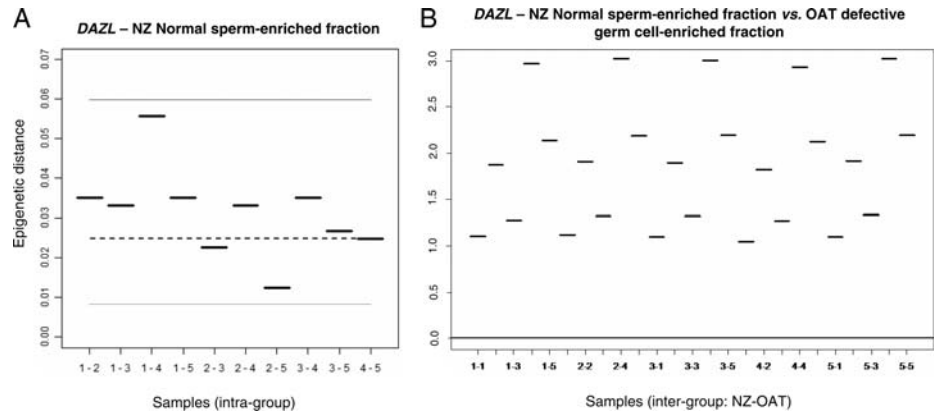

Figure 3 Intra- and inter-group epigenetic distances for the *DAZL* promoter CGI. This distance represents the net dissimilarity in DNA methylation profiles between two sequences: the higher the distance, the more dissimilar are the compared samples. (**A**) Intra-group epigenetic distances in the normal sperm-enriched fraction of NZ men. Numbers in the *x*-axis indicate the identity of the pair-wise comparisons (individuals 1 to 5) inside the experimental group. Please note the minimal distance scores (*y*-axis range: 0–0.07), illustrative of intra-group epigenetic homogeneity. Horizontal guidelines represent the expected range of epigenetic distances if the number of pair-wise observations was increased to 100 000 via *in silico* simulation. The dashed guideline represents the median distance value and the top and bottom guidelines the distance scores for the 97.5 and 2.5% quantiles of the simulation curve, respectively. (**B**) Inter-group epigenetic distances when comparing between the normal sperm-enriched fraction of NZ men and the defective germ cell-enriched fraction of OAT patients. First and second sample number identifiers in the *x*-axis refer to NZ and OAT samples, respectively. Please note the significantly increased distance values (*y*-axis range: 0–3.0) when compared with that of intra-group comparisons. Collapsed guidelines indicate the distance scores for the 97.5, 50.0 and 2.5% quantiles of the simulation curve obtained for the normal sperm-enriched fraction of NZ men (see above).

Indeed, the extremely reduced average percentage of normal sperm in the defective germ cell-enriched fraction of NZ men (13.2 $\pm$ 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 $\pm$ 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.,

F.L., I.C. and C.C.-J. were responsible for patient selection. J.G. and C.E.P. participated in the design of the study, coordinated its development and helped to draft the manuscript.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Conflict of interest: none declared.

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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
GenBank: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/omim/
RepeatMasker: http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker

References

Angelopoulos T, Krey L, McCullough A, Adler A, Grifo JA. A simple and objective approach to identifying human round spermatids. *Hum Reprod* 1997;**12**:2208–2216.

Arata de Bellabarba G, Tortolero I, Villarroel V, Molina CZ, Bellabarba C, Velazquez E. Nonsperm cells in human semen and their relationship with semen parameters. *Arch Androl* 2000;**45**:131–136.

Benchaib M, Braun V, Ressnikof D, Lornage J, Durand P, Niveleau A, Guerin JF. Influence of global sperm DNA methylation on IVF results. *Hum Reprod* 2005;**20**:768–773.

Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. *Bioinformatics* 2005;**21**:4067–4068.

Boissonnas CC, Abdalaoui HE, Haelewyn V, Fauque P, Dupont JM, Gut I, Vaiman D, Jouannet P, Tost J, Jammes H. Specific epigenetic alterations of IGF2-H19 locus in spermatozoa from infertile men. *Eur J Hum Genet* 2010;**18**:73–80.

Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod* 2007;**22**:1506–1512.

Carr IM, Valleley EM, Cordery SF, Markham AF, Bonthron DT. Sequence analysis and editing for bisulphite genomic sequencing projects. *Nucleic Acids Res* 2007;**35**:e79.

Chai NN, Phillips A, Fernandez A, Yen PH. A putative human male infertility gene DAZLA: genomic structure and methylation status. *Mol Hum Reprod* 1997;**3**:705–708.

Chen MJ, Bongso A. Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. *Hum Reprod* 1999;**14**:759–764.

de Kretser DM. Male infertility. *Lancet* 1997;**349**:787–790.

Flanagan JM, Popendikyte V, Pozdniakovaite N, Sobolev M, Assadzadeh A, Schumacher A, Zangeneh M, Lau L, Virtanen C, Wang SC *et al*. Intra- and inter-individual epigenetic variation in human germ cells. *Am J Hum Genet* 2006;**79**:67–84.

Gandini L, Lenzi A, Lombardo F, Pacifici R, Dondero F. Immature germ cell separation using a modified discontinuous Percoll gradient technique in human semen. *Hum Reprod* 1999;**14**:1022–1027.

Gil-Guzman E, Ollero M, Lopez MC, Sharma RK, Alvarez JG, Thomas AJ Jr, Agarwal A. Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. *Hum Reprod* 2001;**16**:1922–1930.

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA. Epigenetic reprogramming in mouse primordial germ cells. *Mech Dev* 2002;**117**:15–23.

Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT. Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. *Fertil Steril* 2009 (in press).

Haston KM, Tung JY, Reijo Pera RA. Dazl functions in maintenance of pluripotency and genetic and epigenetic programs of differentiation in mouse primordial germ cells in vivo and in vitro. *PLoS ONE* 2009;**4**:e5654.

Houshdaran S, Cortessis VK, Siegmund K, Yang A, Laird PW, Sokol RZ. Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. *PLoS ONE* 2007;**2**:e1289.

Kee K, Angeles VT, Flores M, Nguyen HN, Reijo Pera RA. Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. *Nature* 2009;**462**:222–225.

Kobayashi H, Sato A, Otsu E, Hiura H, Tomatsu C, Utsunomiya T, Sasaki H, Yaegashi N, Arima T. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. *Hum Mol Genet* 2007;**16**:2542–2551.

Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril* 1988;**49**:112–117.

Lin Y, Page DC. Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. *Dev Biol* 2005;**288**:309–316.

Maatouk DM, Kellam LD, Mann MR, Lei H, Li E, Bartolomei MS, Resnick JL. DNA methylation is a primary mechanism for silencing postmigratory primordial germ cell genes in both germ cell and somatic cell lineages. *Development* 2006;**133**:3411–3418.

Manning M, Lissens W, Liebaers I, Van Steirteghem A, Weidner W. Imprinting analysis in spermatozoa prepared for intracytoplasmic sperm injection (ICSI). *Int J Androl* 2001;**24**:87–94.

Marques CJ, Costa P, Vaz B, Carvalho F, Fernandes S, Barros A, Sousa M. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. *Mol Hum Reprod* 2008;**14**:67–74.

Navarro-Costa P, Pereira L, Alves C, Gusmao L, Proenca C, Marques-Vidal P, Rocha T, Correia SC, Jorge S, Neves A *et al.* Characterizing partial AZFc deletions of the Y chromosome with amplicon-specific sequence markers. *BMC Genomics* 2007;**8**:342.

Navarro-Costa P, Goncalves J, Plancha CE. The AZFc region of the Y chromosome: at the crossroads between genetic diversity and male infertility. *Hum Reprod Update* 2010 (in press).

Poplinski A, Tuttelmann F, Kanber D, Horsthemke B, Gromoll J. Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *Int J Androl* 2009.

Repping S, van Daalen SK, Brown LG, Korver CM, Lange J, Marszalek JD, Pyntikova T, van der Veen F, Skaletsky H, Page DC *et al.* High mutation rates have driven extensive structural polymorphism among human Y chromosomes. *Nat Genet* 2006;**38**:463–467.

Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P, Dorin J, Cooke HJ. The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis. *Nature* 1997;**389**:73–77.

Sasaki H, Matsui Y. Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nat Rev Genet* 2008;**9**:129–140.

Schrans-Stassen BH, Saunders PT, Cooke HJ, de Rooij DG. Nature of the spermatogenic arrest in Dazl -/- mice. *Biol Reprod* 2001;**65**:771–776.

Vangompel MJ, Xu EY. A novel requirement in mammalian spermatid differentiation for the DAZ-family protein Boule. *Hum Mol Genet* 2010;**19**:2360–2369.

Visser L, Repping S. Unravelling the genetics of spermatogenic failure. *Reproduction* 2010;**139**:303–307.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*, 4th edn. Cambridge, UK: Cambridge University Press, 1999.

Wu W, Lu C, Xia Y, Shen O, Ji G, Gu A, Song L, Wang X. Lack of association between DAZ gene methylation patterns and spermatogenic failure. *Clin Chem Lab Med* 2010;**48**:355–360.

Yen PH. Putative biological functions of the DAZ family. *Int J Androl* 2004; **27**:125–129.