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Repeated Assessment by High-Throughput Assay Demonstrates that Sperm DNA Methylation Levels Are Highly Reproducible

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Abstract

Objective—To assess reliability of high-throughput assay of sperm DNA methylation.

Design—Observational study comparing DNA methylation of sperm isolated from three divided and twelve longitudinally collected semen samples.

Setting—Academic Medical Center

Patients—One man undergoing screening semen analysis during evaluation of the infertile couple and two healthy fertile male volunteers.

Interventions—Spermatozoa were separated from seminal plasma and somatic cells using gradient separation. DNA was extracted from spermatozoa, and DNA methylation was assessed at 1,505 DNA-sequence specific sites.

Main Outcome Measures—Repeatability of sperm DNA methylation measures, estimated by correlation coefficients.

Results—DNA methylation levels were highly correlated within matched sets of divided samples (all $r = 0.97$) and longitudinal samples (average $r = 0.97$).

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High-throughput DNA methylation analysis of gradient-separated human sperm demonstrated high correlation between divided samples and among multiple samples collected over time.

Conclusions—The described methodology reliably assesses methylation of sperm DNA at large numbers of sites. Methylation profiles were consistent over time. High-throughput assessment of sperm DNA methylation is a promising tool for studying the role of epigenetic state in male fertility.

Introduction

A promising new area of reproduction research relates epigenetic state of spermatozoa to male factor infertility (1). Epigenetic processes are central to establishing and maintaining identity and function of specialized cell types. Gametogenesis, fertilization, and early development of the zygote and embryo are accompanied by dramatic and nearly genome-wide changes in epigenetic state, termed epigenetic reprogramming (2). In the mammalian male germ cell lineage, epigenetic changes begin around the time of lineage specification and continue throughout spermatogenesis (3-5). These include extensive demethylation and remethylation of DNA (6), covalent modification of histones and exchange of histones for protamines (7), and may additionally involve action of multiple classes of small RNA molecules (8).

The most extensively characterized epigenetic mark is DNA methylation – presence of a methyl group covalently bound to the cytosine residue in a CpG sequence of DNA. DNA methylation profiles characteristic of numerous specific cell and tissue types have now been described. DNA of spermatozoa is hypomethylated compared with somatic cell lineages(9), and methylation of sperm DNA varies somewhat within and between spermatozoa of individual healthy men (10).

Altered DNA methylation profiles have been identified in numerous diseases (11). The promise of research characterizing epigenetic state of normal and diseased tissue is illustrated by epigenetic studies of neoplasms, which have identified etiologically and clinically distinct subtypes of cancer (12) and opened avenues of translational research aimed at using DNA methylation markers to develop novel approaches to treatment and prevention (13, 14), and to predict response to therapy (15).

Preliminary studies suggest that abnormalities of DNA methylation may also impact male fertility. In murine models, DNA methyltransferase enzymes, which mediate methylation of DNA, are expressed in a highly regulated fashion in the male germline, and disturbance of such expression has been shown to compromise fertility (6); moreover, significant preimplantation loss is observed following experimental exposure of male animals and germ cells to the DNA methylation inhibiting drug 5-azacytidine (16). In humans, clinic-based studies indicate that levels of DNA methylation are abnormal in spermatozoa of men with compromised fertility or abnormal semen parameters (17-22), and that additional epigenetic marks measured in sperm -- histone modification, histone variants, and protamine ratios -- may also be related to subfertile phenotypes (1). Thus proper epigenetic programming and maintenance of the germ line may be of central importance to male fertility, and epigenetic marks in the male germ cell lineage may prove to be useful in prevention and decision making in clinical diagnostic, treatment, and in vitro settings.

For human research, ejaculated sperm are the germ cell lineage members of greatest practical interest. Differing methods have been used to assess methylation of DNA isolated from sperm. Reliability of such measures has yet to be demonstrated, however. Immunostain of methylated cytosine has the advantage of globally assaying DNA, but lacks sequence specificity. Early studies using this technique found little association between sperm DNA methylation and semen parameters (23), or fertilization rate and embryo quality following IVF (18). Subsequent research queried specific sequence elements by single-strand

conformation analysis (24), MethyLight (19), combined bisulfite PCR restriction analysis (21), pyrosequencing (22), PCR Luminex (25) or bisulfite sequencing (1, 17, 20). Each reported abnormal methylation of at least one imprinted gene in DNA from poor quality sperm, consistent with the hypothesis that defects of sperm production may be related to deregulated imprinting (26). Three studies also measured consensus sites for repetitive elements, reporting no notable association between semen quality and DNA methylation at abundantly repeated *Alu* (21) and *LINE1* elements (19-21); however, hypermethylation of lower abundance SAT2CHROM1 elements was associated with poor semen quality (19). The single study interrogating numerous individual CpG islands not associated with recognized imprinted regions found several sites to be hypermethylated in DNA of poor quality sperm, suggesting widespread disruption of DNA methylation in abnormal sperm (19).

Progress in this area of fertility research would be accelerated by use of high-throughput methodology for efficiently assessing methylation marks in sperm DNA. Validation of such measures would be a significant step toward characterizing epigenetic states that may distinguish poor quality human sperm from fertile counterparts, and thence identifying epigenetic marks with biological, clinical and predictive significance to male fertility. Issues to examine in order to soundly design such studies include whether sperm DNA methylation can be reliably measured using high-throughput commercial panels that interrogate thousands of sites, and whether levels of sperm DNA methylation vary appreciably over time or in relation to length of abstinence. We used a high-throughput panel of DNA methylation assays to address these questions in a carefully selected set of samples of ejaculated sperm.

Materials and Methods

Patients

A single semen sample collected by one man undergoing screening semen analysis as part of the evaluation of the infertile couple was studied, as were longitudinal series of six semen samples provided by each of two fertile male volunteers.

Study Design

To determine intra-assay variability in measures of sperm DNA methylation, three individual semen samples, one collected as a screening semen analysis and two samples collected by a single fertile volunteer, were each divided into three aliquots. Each aliquot was processed separately for analysis of sperm DNA methylation by sperm separation, DNA isolation and sodium bisulfite conversion.

To determine whether site-specific measures of DNA methylation change over time, we analyzed multiple sperm samples donated by each of two fertile men on the following schedules (designating as 1 the day each man donated the first sample). Volunteer A provided samples on days 1, 333, 340, 347, 354 and 361 (after the first sample was donated, nearly a year passed, then five additional samples were collected at one week intervals). Volunteer B provided samples on days 1, 8, 15, 22, 29 and 36.

Sample Donation and Semen Analysis

Ejaculated semen samples were collected by each man into a sterile container, from which a small aliquot was removed for semen analysis. The remainder of each sample was transferred to a screw-top tube and stored at -20°C for a period ranging from 27 to 388 days, when all samples were thawed and processed for DNA methylation analysis by separation of sperm from seminal plasma, DNA isolation, and bisulfite conversion.

Standard semen analysis was performed as previously described using WHO criteria and Strict Morphology (27). Semen volume, sperm concentration, sperm motility, and leukocyte count were measured using the MicroCell chamber (Conception Technologies, San Diego, CA). Sperm morphology was assessed using prestained slides (TestSimplets, Spectrum Technologies, Healdsburg, CA), and percentages of normal and morphologically abnormal sperm were documented. Presence of white blood cells, round cells, or epithelial cells was determined by phase microscopy using the MicroCell and by light microscopy on TestSimplet slides.

Sperm Separation

Semen samples were allowed to thaw at 37°C. Sperm were separated from seminal plasma using Isolate® Sperm Separation Medium (Irvine Scientific, Santa Ana, CA), a density gradient centrifugation column designed to separate cellular contaminants (including leukocytes, round cells, and miscellaneous debris) from spermatozoa (28). Separation was performed according to the manufacturer's protocol (29). The purity of separated sperm from leukocytes and other cells was documented for each separated sample by phase microscopy using the MicroCell chamber and by light microscopy using TestSimplet slides.

DNA Isolation

DNA was isolated from purified sperm as previously described (30, 31). In brief, following separation from seminal plasma sperm were lysed by addition of 0.5 ml lysis buffer (30) and 0.1X saline sodium citrate and incubated at 55°C overnight or longer to complete the lysis procedure. DNA was precipitated by addition of one volume isopropanol to the lysate and recovered by either lifting the precipitate using a pipette tip or by centrifugation at 13,000 rpm for 20 minutes, depending on the amount of DNA. DNA was dissolved in TE buffer, and stored at -20°C until use.

Bisulfite Conversion of DNA

Sodium bisulfite conversion was performed as previously described (12). The amount of DNA in each aliquot was normalized, and a bisulfite-dependent, DNA methylation-independent control reaction was performed to confirm relative amounts of DNA in each sample.

Assessment of DNA Methylation

DNA methylation of each sample was assessed using the Illumina GoldenGate Cancer Panel I (31), which scores DNA methylation levels as β -values (32) at 1,505 CpG sites in 808 genes. This method employs two probe pairs for each CpG site to be interrogated, one pair corresponding to the methylated state and the other to the unmethylated state. Each pair includes a locus-specific oligonucleotide and an allele-specific oligonucleotide. All oligonucleotides for all CpG sites are pooled together and assayed simultaneously. PCR templates generated by extension and ligation of these oligonucleotides are subsequently amplified by fluorescently labeled primers and hybridized to a bead array (31). β -values are calculated as the ratio of the methylated signal over the total fluorescent signal and take on values between 0 and 1, with 0 representing no detectable DNA methylation and ~0.99 representing very high levels. Individual values that did not pass Illumina's quality-control, described below, were deleted.

Statistical Analysis

Density Plots—Density plots of sperm DNA methylation profiles were created using a biweight kernel. We removed probes with single nucleotide polymorphisms (SNPs) that are likely to capture genotypes at adjacent sites of nucleotide sequence variation, rather than

true DNA methylation, and probes that did not register fluorescence signals above background according to the manufacturer's criteria. Detection p-value for each locus is calculated based on signal intensities of methylated and unmethylated probes in comparison to average signal intensities of Cy3 and Cy5 negative controls. P-values less than 0.05 represent significant detection above background for individual β -value measurement, while p-values above 0.05 are considered to have non-significant detection and were scored as missing values (32). Any probe that had one or more missing value on any sample was excluded from further analysis.

Estimating correlations—We estimated both Pearson and Spearman correlation in β -values from replicate samples. Average within-sample correlation was computed by averaging correlation coefficients over all pairs of matched samples. This is compared to the average between-sample correlation, the average over all pairs of unmatched samples, in order to assess whether the similarity of the DNA methylation profile between matched samples is higher than the similarity between unmatched samples of sperm. We used bootstrap sampling to compute a 95% confidence interval (CI) for the difference in within- and between-sample correlation. Many loci typically showed consistently high or consistently low β -values across all samples. Analyses were repeated using subsets of the more variable probes by removing those for which β -values were either <0.1 or >0.9 for all samples.

Results

Semen Analysis

Results of semen analysis for all studied samples appear in Table 1.

Reproducibility of DNA Methylation Levels within Samples

Figure 1 Triplicate panels show density plots of the DNA methylation profiles for the three samples each divided into triplicate aliquots before processing and analysis (probes with SNPs ($n=125$) and missing data ($n=48$) are omitted). Pearson correlations between triplicate aliquots of individual samples were very high (all $r>0.976$; Table 2). Two samples, each analyzed in triplicate, were provided by a single individual on separate occasions seven days apart and following different times of abstinence (6 versus 48 hours); these samples had differing sperm concentrations (4.3×10^6 versus 56×10^6 per mL). For the comparison of correlations within and between samples, the correlations between samples processed and analyzed in triplicate (6 correlations) did not differ from the correlations between pairs of aliquots from samples taken seven days apart (difference = 3×10^{-5} , 95% bootstrap CI = -0.0006 - 0.0006). If we make the same comparison, but considering pairs of samples from different sperm donors, the average within sample correlation, 0.983, exceeded the average between sample correlation of 0.978 (difference = 0.005, 95% bootstrap CI= 0.003 - 0.007).

Consistency of DNA Methylation Levels between Serial Samples

Figure 1 Time course panels show density plots of the DNA methylation profiles for 12 serial samples (two subjects, six samples each; $N=1341$ probes). Time course 1 showed slight non-overlap of the density curves. After computing the mean and standard deviation of the β -values for each of the 12 samples, we found that the first sample in this series, taken one year prior to the remaining five samples, appeared to be an outlier with mean DNA methylation (0.263) and standard deviation (0.386) falling slightly below the lower limit of the 95% confidence interval (CI) for the estimates (95% CI for the mean for the six samples in Time course 1: 0.264-0.283; 95% CI for the standard deviation: 0.387-0.400).

We find high reproducibility among the samples provided at serial visits (Table 3), with an average within-subject correlation of 0.974, which is higher than the average between-subject correlation of 0.972 (difference = 0.002, 95% bootstrap CI=0.001-0.003).

Sensitivity Analyses

Conclusions regarding both reproducibility within divided samples and consistency over serial samples were unchanged in further analyses estimating Spearman correlations and using filtered sets of probes excluding those with little variation (all <0.1 or all >0.9) (results not shown).

Discussion

Increased interest in epigenetic regulation of reproduction -- including a possible role of improper epigenetic state in male factor infertility and IVF outcomes -- necessitates demonstration of reliability of methods for assessing sperm DNA methylation. A purely technical concern is whether high-throughput assays of DNA methylation reliably assess methylation of DNA isolated from human sperm. Additional questions of biological interest must also be answered in order to soundly design human studies of sperm DNA methylation. These include whether DNA methylation profiles differ amongst multiple ejaculates collected by an individual, or in response to abstinence. We addressed these issues in the present study by evaluating reproducibility of intra-assay measures of DNA methylation, and whether sperm DNA methylation patterns change over time or depend on duration of abstinence.

Our data indicate high reproducibility of DNA methylation levels within samples. This finding is consistent with published data establishing that GoldenGate and related high-throughput assays of DNA methylation are highly reliable (32, 33). Our data also indicate that there is high reproducibility among serial samples, as within-subject correlation of DNA methylation measures exceeds between-subject correlation. Slightly higher values were observed for the single sample collected a year earlier than others in one series; whether this observation is spurious or represents an effect of sample storage, aging, or some other process remains to be determined. The observed reproducibility of DNA methylation levels in sperm of fertile volunteers at numerous time points suggests that sperm samples can be provided for DNA methylation research in the clinical setting. We also report that a short period of abstinence does not change the profile of DNA methylation, evidenced by the observation that DNA methylation profile following six hours of abstinence did not differ significantly from profiles reported following 36-120 hours of abstinence. Therefore, even though semen parameters are related to abstinence time, reported associations between sperm DNA methylation levels and semen parameters likely represent true biological phenomena, rather than sporadic results arising through confounding by abstinence time.

Our present results indicate that sperm DNA methylation is stable in serial semen samples collected by healthy fertile volunteers. This is to our knowledge the first report of reliability of high-throughput assays of DNA methylation in human sperm, and as such has several limitations. The sample size was modest, as is appropriate for initial assessment of costly assays. Observed agreement between repeated split samples indicates that research questions requiring larger numbers of participants -- such as characterization of DNA methylation profiles distinguishing infertile or subfertile sperm from fertile samples -- can reasonably be conducted using a single assay for each sample, thus rendering such studies economically feasible. Serial sampling was also limited to follow-up periods of several months in length, so agreement among repeated samples implies that future research can reasonably schedule collection of specimens without concern that short term phenomena such as seasonal influences would greatly influence sperm DNA methylation. However, additional agreement

studies are needed to assure that research questions requiring several years of follow-up would not be subject to longer term changes in sperm DNA methylation which could potentially be related to factors such as aging (34), changes in diet (35), toxicant exposure (36), and oxidative stress (37). In conclusion, results presented here support the premise that high-throughput assessment of DNA methylation of ejaculated sperm is a promising tool for studying the role of the epigenetic state of sperm DNA in male factor infertility.

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Figure 1.

Reproducibility of Sperm DNA Methylation, each panel graphically displaying DNA Methylation results for marker panels assessed within matched sets of sperm samples. Triplicate panels: Sperm DNA methylation based on 1332 filtered probes assessed in each of three semen samples after each was divided into three aliquots then both processed and assessed for DNA methylation in triplicate. Time course panels: DNA methylation based on 1341 filtered probes for twelve serial samples provided over time by two fertile volunteers (six samples per man; Time course 1, volunteer A; Time course 2, Volunteer B).

Table 1

Standard Semen Analysis Results and Donation Schedule for Semen Samples Used in Analyses of Sperm DNA Methylation

Comparison for Experiment	Within Divided Samples	Between Longitudinal Samples														
		Fertile Volunteer A							Fertile Volunteer B							
Day of Semen Collection ^a	NA ^a	340	347	1	333	340	347	354	361	1	8	15	22	29	36	
Abstinence time(hours)	48	48	6	72	120	48	6	36	120	48	24	48	48	48	48	
Volume (mL)	4.0	4.5	2.0	7.5	6.5	4.5	2.0	4.0	5.5	2.0	2.0	2.5	1.8	1.8	2.0	
Concentration (10 ⁶ sperm/mL)	16	56	4.3	53	72	56	4.3	21	69	62	31	63	44	30	80	
Motility (%)	41	73	56	88	75	73	56	76	74	40	40	40	50	50	50	
Morphology (%)	5	23	NA ^b	NA ^b	20	23	NA ^b	14	33	18	21	15	NA ^b	30	40	
Leukocyte count (× 10 ⁶)	0.8	0.4	0.2	0.0	0.2	0.4	0.2	0.0	0.6	NA ^b	5.6	1.8	0.8	0.0	0.0	

^aDesignating as 1 the day each volunteer provided first sample; NA indicates a single semen sample provided by corresponding individual

^bMorphology not recorded due to paucity of sperm

Table 2

Between-aliquot correlation coefficients of β values for three sperm samples with DNA methylation assessed by Illumina GoldenGate technology; for each sample each of the three aliquots was separately processed and subjected to DNA methylation analysis; correlation coefficients for N=1332 reactions filtered to remove probes at SNP adjacent sites and with missing data.

Sample 1				Sample 2				Sample 3			
Aliquot				Aliquot				Aliquot			
1	2	3		1	2	3		1	2	3	
1	1.000	0.988	0.986	1.000	0.985	0.987	1.000	0.976	0.983		
2	0.988	1.000	0.985	0.985	1.000	0.984	0.976	1.000	0.976		
3	0.986	0.985	1.000	0.987	0.984	1.000	0.983	0.976	1.000		

Table 3

Between-time point correlation coefficients of β values for successive sperm samples provided by each of two fertile donors over specified days, with DNA methylation assessed by Illumina Golden Gate technology; correlation coefficients for N=1341 reactions filtered to remove probes at SNP adjacent sites and missing data

Fertile Volunteer A										Fertile Volunteer B									
Day of Specimen Collection										Day of Specimen Collection									
Day	1	333	340	347	354	361	Day	1	8	15	22	29	36						
1	1.000	0.961	0.969	0.968	0.967	0.969	1	1.000	0.986	0.982	0.981	0.983	0.978						
333	0.961	1.000	0.962	0.964	0.963	0.966	8	0.986	1.000	0.981	0.980	0.981	0.978						
340	0.969	0.962	1.000	0.977	0.977	0.972	15	0.982	0.981	1.000	0.975	0.976	0.974						
347	0.968	0.964	0.977	1.000	0.977	0.977	22	0.981	0.980	0.975	1.000	0.974	0.973						
354	0.967	0.963	0.977	0.977	1.000	0.973	29	0.983	0.981	0.976	0.974	1.000	0.973						
361	0.969	0.966	0.972	0.977	0.973	1.000	36	0.978	0.978	0.974	0.973	0.973	1.000						