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RESEARCH ARTICLE

Thermo Stability of DNA Methylation Marks in Human Sperm

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Abstract

Shipping of various tissues, including sperm, for the purposes of diagnostic testing and/or collaborative scientific pursuits is commonly performed. While many sample attributes are largely stable during the shipping process regardless of shipping environment, some physical factors such as temperature, duration of storage and other handling conditions may affect certain features of these samples including a commonly assessed epigenetic mark, DNA methylation. Since any changes to these marks may drastically alter the outcome and interpretation of a study, it is imperative that handling conditions be carefully analyzed and validated for any assay. To date few studies have addressed the stability of this important regulatory epigenetic mark under various storage conditions in general, and none have explored these effects in the storage of sperm. In this study, sperm from 18 men was assessed for potential DNA methylation alterations resultant from exposure to high heat over varying amounts of exposure time. As liquid nitrogen is among the most commonly used storage conditions and generally very stable, we compared sample aliquots stored in liquid nitrogen compared to aliquots stored at 65 °C for varying durations. We assessed differential methylation globally, at specific regions, and at the single CpG level. High temperature exposure was not associated with methylation alterations, regardless of the duration of exposure. To confirm these findings, we performed unsupervised hierarchical clustering across the CpGs tiled on the array and found no clustering based on treatment group, rather, we found a strong clustering according to the individual from which each aliquot originated. These findings suggest that high temperature exposure is unlikely to affect sperm DNA methylation signatures significantly within the exposure times studied.

Introduction

DNA methylation is an essential epigenetic mark in the gamete and is thought to play a role in many different processes in the sperm and in the embryo [1-3]. Because of the dynamic nature of this mark in mammalian sperm, it is susceptible to alterations via cellular processes based on exposure to multiple modifiers including various toxins, pollutants, diet alterations, stress exposures and even age [4-6]. While some of these alterations are likely quite benign, it has been shown in recent studies that specific patterns of epigenetic alteration are associated with various abnormalities in sperm biology and have additionally been associated with subfertility [7-9]. These associations have driven research addressing the contribution of sperm DNA methylation and, more globally, all types sperm epigenetic alterations to the pathological processes of infertility and poor pregnancy outcomes [7,8]. This work has yielded a great degree of interesting findings. While much of the data do not suggest that sperm DNA methylation is independently causative of infertility, it has been found to be significantly predictive of fertility status in men [7]. Hence, there is interest in using the DNA methylation signatures in sperm as a potential diagnostic tool.

While sperm DNA methylation appears to have potential diagnostic utility, there are major hurdles associated with the implementation of such testing. Despite continually falling costs for assays used to assess DNA methylation patterns, the expense of such an assay remains a concern. Typically, high resolution DNA methylation assessment is performed in one of two ways; via microarray, or through deep bisulfite sequencing (typically performed via reduced representation bisulfite

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sequencing or other targeted sequencing approaches). While costs of these technologies have dropped considerably in recent history, the hardware required to perform such experiments are not readily available to most clinical facilities, therefore sperm testing may shipping samples to a centralized referral laboratory. This raises important questions about the stability of methylation marks under different transport conditions. Some work has been performed in this regard in placenta and in blood [10,11]. These studies have assessed the stability of the DNA methylation mark in varying conditions including long term storage at different temperatures at or below freezing [11] and assessment of methylation signatures at repetitive elements [10]. These data are quite helpful in our understanding of what appears to be a very stable mark, however additional work is required to understand if DNA methylation patterns in the highly unique sperm DNA epigenetic landscape remain as stable as has been shown in small genomic regions from other tissue types. Further, additional genome wide information about the stability of these marks would be greatly beneficial to the scientific community as a whole for many reasons including, forensic studies assessing epigenetic signatures that can determine both identity and tissue type, archaeological studies assessing genetic and epigenetic aspects of various samples, and collaborative studies in which tissue sharing and shipping is essential.

In this study, we have assessed the impact of storage duration and temperature exposure on the sperm DNA methylome. We have specifically selected an extreme temperature (approximately the temperature that samples may reach in transport if no precautions were taken to control temperature) to test the stability of this mark. The findings of these experiments will help to define the thresholds at which a human sperm sample can be stored without alteration to the methylome.

Materials and Methods

Study groups

A total of 19 sperm sample were assessed for this study. All samples were received following informed consent under a University of Utah Institutional Review Board (IRB) approved protocol. Individuals who participated in this study were recruited through the University of Utah Andrology and IVF laboratories. Each sample was from patients attending this lab for a routine semen analysis. Participants were required to strictly adhere to Andrology lab collection guidelines, which include having between two and five days of abstinence prior to collection. Two studies were performed with these samples. First, we performed a discovery study with 10 participants and 10 different exposures (E1). Second, we performed a follow-up study in an independent cohort to confirm the findings from the discovery analysis (n = 9), with four of the exposures from the first study repeated (E2).

Treatment and sample processing

Samples were exposed to high heat (65 °C) for varying amounts of time to determine the stability of sperm DNA methylation marks when exposed to extremes in temperature. In the discovery study, each sample was split into 1 control group that mimicked a commonly utilized form of long-term storage slow freezing in Test Yolk Buffer (TYB; Irvine Scientific). The sample was then split into 10 additional groups: 65 °C for two hours, 65 °C for four hours, 65 °C for six hours, 65 °C for eight hours, 65 °C for 12 hours, 65 °C for one day, 65 °C for two days, 65 °C for four days, 65 °C for six days, and 65 °C 8-10 days. Each sample was stored in an Eppendorf snap cap tube in a monitored incubator during this period of time. Following incubation, samples were immediately processed through somatic cell lysis as is commonly performed in our lab [12]. We assessed the Delta Like Non-Canonical Notch Ligand 1 (DLK1) loci which is significantly differentially methylated between somatic cells and sperm to determine the purity of our samples as has been previously performed in our lab [9]. Figure 1 illustrates the lack of contamination in the samples, which we analyzed (with each treatment group from each individual represented). Following somatic cell lysis, DNA was isolated from the sperm cells in each sample via a sperm specific modification to the DNeasy column based extraction protocol commonly performed in our lab [12]. DNA was then bisulfite converted (EZ DNA Methylation Kit, Zymo) and delivered to the core lab at the University of Utah for array hybridization and processing on the illumina450k methylation array. The array results from each individual sample were processed through genome studio software to perform Quality Control (QC) to ensure that no problems in processing were present. In E1 multiple samples did not pass QC because of apparent poor conversion efficiency as well as hybridization. This sample exclusion left us with 8 samples in the liquid nitrogen treatment group, 9 in 37C group, 8 in the 65C 2 hours group, 8 in the 65C 4 hours group, 9 in the 65C 8 hours group, 9 in the 65C 12 hours group, 9 in the 65C 1 day group, 7 in the 65C 2 days group, 10 in the 65C 4 days group, 10 in the 65C 6 days group, and 9 in the 65C 8-10 days group. No issues were identified in the QC in E2.

Analysis: Upon receipt of raw array data we processed id at files in both Chip Analysis Methylation Pipeline (CHAMP) [13] and in Partek. In both approaches the raw intensity files were used to generate β-values (fraction methylation values between 0 and 1; 0 being complete absence of methylation and 1 being complete methylation) following SWAN normalization. Additionally, poorly performing probes (probes with a QC, $p < 0.05$) were filtered from downstream analysis. The remaining β-values were then log it transformed to generate m-values for differential methylation analysis of point data and for variability analysis to avoid the statis-

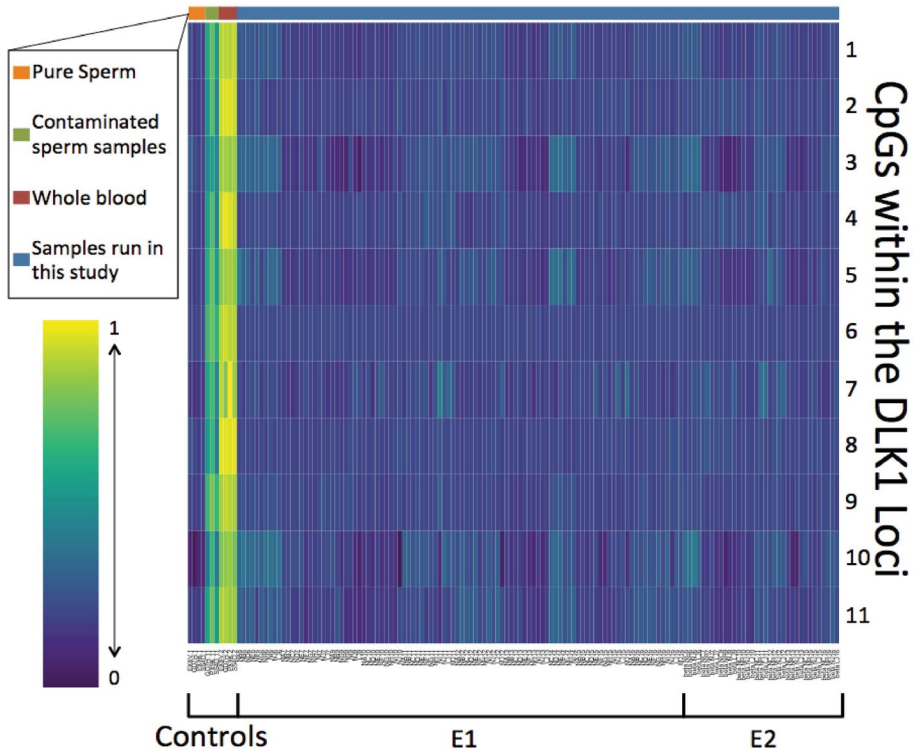

Figure 1: Heat map demonstrating the purity of samples (absence of somatic cell DNA) analyzed in the study by screening methylation at the Delta Like Non-Canonical Notch Ligand 1 (DLK1) differentially methylated region.

tic issues associated with heteroscedasticity. M-values were used to calculate differential methylation and variation in downstream analyses.

Differential methylation analysis

Differential methylation analysis was performed on m-values at three different levels. Specifically, we assessed methylation alterations at the Global, regional, and CpG level. Global methylation analysis was conducted by averaging swan normalized beta values across all probes in the array for each test group. Beta values were also averaged across all groups considering gene features (Promoter, body, etc.) as well as CpG density association (*CpG island; south shelf, 2kb flanking shore; north shelf, 2kb flanking shore; south shore, 2kb flanking island; north shore, 2kb flanking island).* Point data analysis was performed using the ChAMP pipeline specifically to identify differentially methylated CpGs in the genome. This analysis was repeated for every comparison (each test group compared to storage in liquid nitrogen). Differential methylation was considered significant at any given CpG using a Bonferroni corrected p-value of 0.05 as a threshold of significance. Differential methylation analysis was also performed at the regional level by the sliding window approach using the application "Methylation Array Scanner", which is part of the publically available USeq package of bioinformatics tools. Two thresholds were applied to identify windows with significant differential methylation. A Benjamini Hochberg corrected t-test FDR (false discovery rate) of ≤ 0.0001 and an absolute log 2 ratio ≥ 0.2 were considered significant findings. This approach has been a reliable and informative approach previously used in methylation studies [4,12].

Unsupervised Hierarchical Clustering (HC)

HC was performed in R to determine if treatment groups aggregate together. The purpose of utilizing this analysis in the context of our experiment is to confirm our genome wide differential methylation analysis. In brief, if samples cluster based on treatment type, we can assume there is an effect of treatment on the samples. However, if they cluster based on the individual from whom the sample originated (the expected outcome of any methylation study analyzing multiple samples from the same individuals) this would suggest that the various treatments have no strong and/or consistent impacts on sperm DNA methylation signatures.

Methylation variability analysis

Analysis of variability was performed using the R software package. The overarching goal of this approach was to identify intra-group (all study groups) variability for every CpG assessed on the array and to compare these findings between the control and test groups. First, M-values for all samples analyzed in our study undergo "CpG-wise" mean centralization in R resulting in Center Scaled (CS) values descriptive of the absolute distance from average for each CpG in every individual. CS values were then utilized to describe differences in variation between the various treatment groups with increased distance from the mean equating

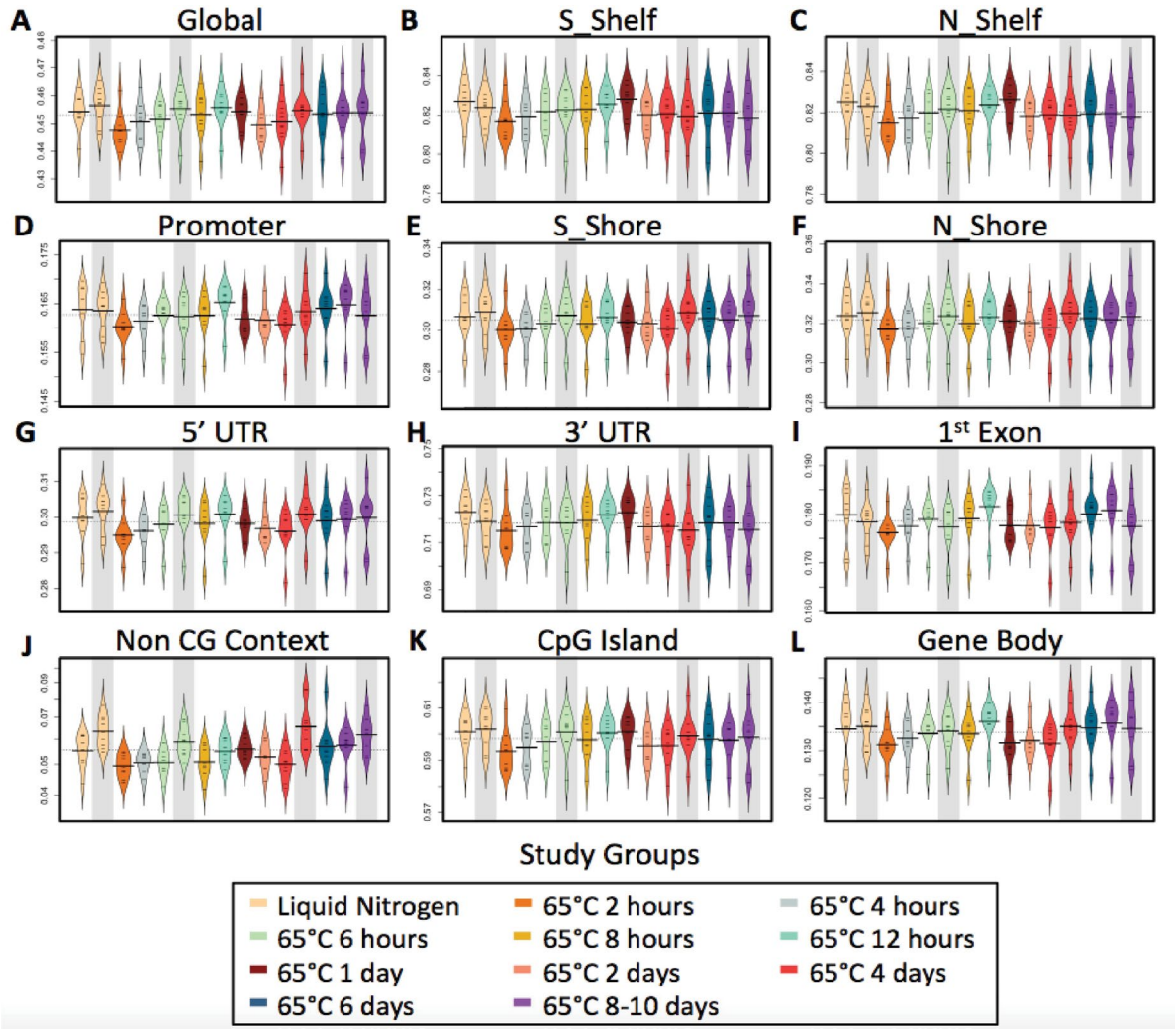

Figure 2: Bean plot of average fraction methylation values of the samples in our studies (E1 with white background, E2 with grey background). Multiple regions were screened to observe potential differences in methylation including (A) Global (entire covered genome); (B) South shelf (2 kb flanking shore); (C) North shelf (2 kb flanking shore); (D) Promoter; (E) South shore (2 kb flanking island); (F) North shore (2 kb flanking island); (G) 5' UTR (average ~3-5 kb in length based on UCSC annotation); (H) 3' UTR (average ~3-5 kb in length based on UCSC annotation); (I) 1st Exon; (J) Non-CG context (methylation on cytosine residues not in a cytosine-phosphate-guanine dinucleotide); (K) CpG island; (L) Gene body methylation.

to increased variability. With these data, we compared average variability across all probes in our treatment groups.

Results

Global methylation

Global methylation was analyzed by assessing the average of all probes on the array. No significant differences between storage in liquid nitrogen or by any other storage methods were identified (Figure 2A). Similarly, we analyzed any differences in methylation at specific genomic contexts between the control and test groups. We found no significant alterations between samples stored in liquid nitrogen and those exposed to heat for various amounts of time at CpG island contexts (CpG Islands, Shores, Shelves, and in the "Open Sea"), in regions associated with genes (promoter, 5'UTR, 3'UTR, 1st exon, and gene body), or at cytosine methylation not in the CpG context (Figure 2B, Figure 2C, Figure 2D, Figure 2E, Figure 2F, Figure 2G, Figure 2H, Figure 2I, Figure 2J, Figure 2K and Figure 2L). Our second, conformation analysis revealed the same lack of significance when considering only the test groups and one liquid nitrogen control group (Figure 2).

Single CpG analysis

We assessed potential methylation differences between each group at the single CpG level. We assessed these potential differences with two analysis pipelines, ChAMP and Partek. We were unable to identify any single CpG with significantly altered methylation between any one group by either analysis. This lack of significance was also identified in our second confirmation analysis of an independent cohort. While some groups were slightly lower or higher than others, these differences failed to reach significance largely due to inconsistent changes.

Unsupervised Hierarchical Clustering

HC was performed in R to determine if treatment groups aggregate together, thus suggesting that the various treatments have real and consistent impacts

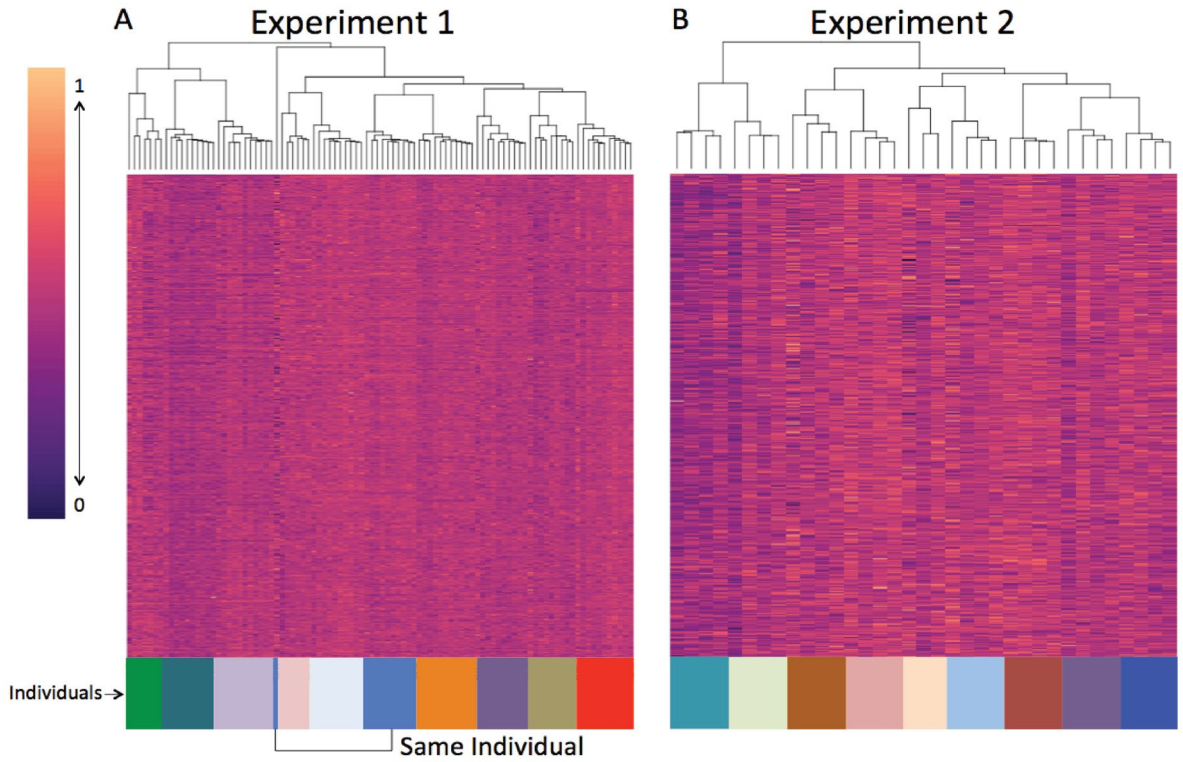

Figure 3: Heat map of unsupervised HC of all 450 k array probes in E1 and E2. Samples cluster based on the individual and not by treatment. All samples that passed initial screening included in this analysis (thus the reduced number of data points from some individuals).

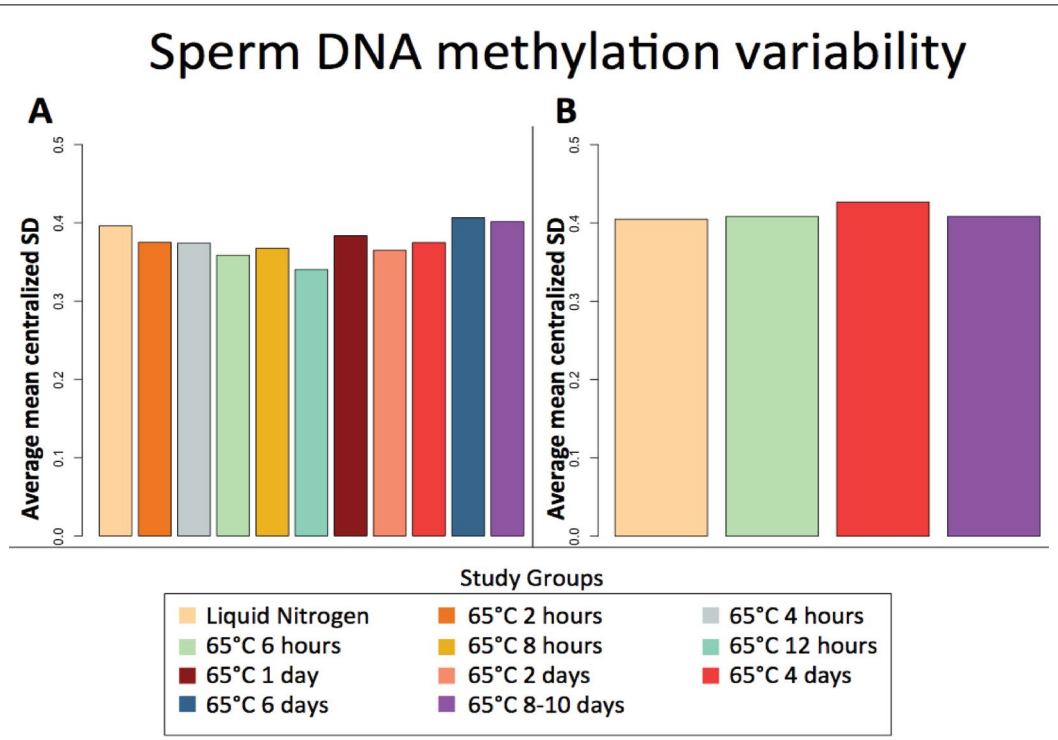

Figure 4: Histograms illustrating the variability in average DNA methylation across the genome between test groups. (A) 11 treatments were performed in E1; (B) 4 of those conditions were repeated in E2. No significant differences were identified between any of the treatment groups.

on sperm DNA methylation signatures. In performing unsupervised HC, we found that the aliquots from each sample cluster in very specific patterns. With only a single exception, all aliquots (treatments) from the same patient cluster together (Figure 3). Such clustering by individual is expected if no other outside factors (in this case, heat exposures) generate consistent alterations in the methylome.

Variability analysis

Average variability (as defined by the average absolute distance from the mean) was analyzed as averages

within each group to identify any differences in variability between the groups we assessed. We found no significant difference in the interindividual variance between groups assessed in either the initial discovery study or in the follow-up confirmation analysis (Figure 4). Differences were assessed by two tailed t-tests of averages of each individual within each test group.

Discussion

Our data demonstrate that heat exposure does not appear to significantly or consistently impact sperm DNA methylation patterns. We have shown that no consistent measurable global alterations occur as a result of heat exposure for any duration (from a few hours to 10 days). Even when assessing DNA methylation at the regional or single CpG level, we fail to find any specific sites where DNA methylation alterations occur. To ensure accuracy, this was analyzed with multiple software packages and approaches.

To further establish the lack of effect, we capitalized on the unique nature of the samples in our two cohorts. Specifically, we used the multiple repeated assessments of samples from the same individual (each of which were treated differently) and assessed all available aliquots from each sample via unsupervised HC to determine the nature of clustering among these samples. In effect, we sought to identify (considering all CpGs tiled on the array that passed initial quality thresholds) the attributes that created consistent similarity between samples. With such an approach, we expect to find the majority of clustering to coincide with the individual from which the sample originated unless a strong outside influence resulted in consistent alterations to the sperm methylome. Indeed, we identified virtually perfect fit to this null model, with only a single aliquot from a one individual not clustering with the rest of the aliquots from which the sample originated. In every other case from both experiments, there appeared to be no effect from the treatment, since all aliquots from the same individual clustered together within discrete groups. Together, these data strongly suggest that the effect of high temperature (65 °C) on the sperm DNA methylome is extremely low in magnitude or non-existent, at least in the relatively short term. It should be noted however that the fact alone that HC analysis demonstrated that the individuals cluster together more tightly than do the treatments definitively prove that no smaller alterations may be occurring in a subset of the samples. However, when taking together our data regarding point data alterations, variability analysis, and global assessment of changes, there are no clear indications that temperature exposure alters DNA methylation signatures in sperm consistently among the samples that we assessed.

It is clear from these findings that there should be very little concern in shipping samples between labs with transport of only a few days. Further, the storage conditions appear to have a low likelihood of impacting the findings in DNA methylation studies. Thus, it is reasonable to find alternatives to more expensive shipping methods in the case of sperm DNA methylation studies.

In many ways, these data are bit surprising, as a great deal can occur on the molecular level over the period of 10 days at 65 °C. There are many potential explanations as to why this lack of effect occurs. Among the most plausible is the fact that DNA methylation is quite stable under many conditions. If this is true, we would expect that the nature of these findings is not unique to sperm and that any cell type that undergoes prolonged extreme temperature exposure would have similar findings. While this may be accurate, an alternative explanation is also plausible. Because of the unique nature of the mammalian sperm cell, a great deal of molecular effort is expended in ensuring the protection of the sperm DNA though protamination. In fact, the sperm nucleus is between 6 and 20 times more compact than any other cell as a result of the massive chromatin remodeling events that occur throughout spermiogenesis [14]. This reprograming contributes to the quiescent nature of the mature sperm, but also facilitates a tremendous amount of protection from the environment. The unique nature of the sperm epigenetic landscape, may lend itself to a more robust environment that provides enhanced protection from outside influences. In this way, we may expect the sperm to be uniquely resistant to such modifiers.

While these data represent only a portion of the potential environments a sample in transit may encounter, the data do suggest that even in extreme temperatures sperm DNA methylation patterns appear to remain stable. Future studies may be required to further understand the stability of many types of epigenetic marks under various conditions, but if labs are utilizing sperm samples strictly for DNA methylation analysis, investigators may consider using more economical approaches to sample transit.

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