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ORIGINAL ARTICLE

Mitochondrial DNA variants can mediate methylation status of inflammation, angiogenesis and signaling genes

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Abstract

Mitochondrial (mt) DNA can be classified into haplogroups representing different geographic and/or racial origins of populations. The H haplogroup is protective against age-related macular degeneration (AMD), while the J haplogroup is high risk for AMD. In the present study, we performed comparison analyses of human retinal cell cybrids, which possess identical nuclei, but mtDNA from subjects with either the H or J haplogroups, and demonstrate differences in total global methylation, and expression patterns for two genes related to acetylation and five genes related to methylation. Analyses revealed that untreated-H and -J cybrids have different expression levels for nuclear genes (*CFH, EFEMP1, VEGFA* and *NFkB2*). However, expression levels for these genes become equivalent after treatment with a methylation inhibitor, 5-aza-2′-deoxycytidine. Moreover, sequencing of the entire mtDNA suggests that differences in epigenetic status found in cybrids are likely due to single nucleotide polymorphisms (SNPs) within the haplogroup profiles rather than rare variants or private SNPs. In conclusion, our findings indicate that mtDNA variants can mediate methylation profiles and transcription for inflammation, angiogenesis and various signaling pathways, which are important in several common diseases.

Introduction

Mitochondria are unique organelles with their own maternally inherited DNA (mtDNA), containing a non-coding control region (MT-Dloop), important for replication and transcription. The mtDNA-coding region encodes for 37 genes, including 13 proteins, 22 tRNAs and 2 rRNAs, that are critical for oxidative phosphorylation (OXPHOS). The mtDNA can be classified into haplogroups, defined by an accumulation of single nucleotide polymorphisms (SNPs), which represent the different geographic origins of populations. Investigations report that the mtDNA haplogroups can be

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either protective or high risk for a number of diseases, including cancers, diabetes, Alzheimer's disease, Parkinson's disease and cardiomyopathies (1,2). Age-related macular degeneration (AMD) is the leading cause of vision loss in older individuals. It has been shown that the J haplogroup is associated with AMD (3–8), while the H haplogroup has a protective effect (7). Recent studies have shown that epigenetics may also play a key role in the progression of AMD (9).

Transmitochondrial cybrids (cell lines containing identical nuclei, but mtDNA from different individuals) have been used to investigate the mitochondrial–nuclear interactions for various diseases (10–22). Our previous studies have shown that cybrids containing different mtDNA haplogroups have: (i) differential expression of nuclear genes involved in cell signaling, inflammation and apoptosis pathways; and (ii) different responses to sublethal ultraviolet radiation (23–26). However, the signaling transduction mechanism(s) involved with the mitochondrial–nuclear interactions in the cybrids are not fully understood. Studies have shown that mitochondria and methylation status are interconnected. When cells were depleted of their mitochondria, the degree of DNA methylation was affected (27). Osteosarcoma cybrids with the J haplogroup mtDNA had higher levels of total methylation compared with cybrids with H haplogroup mtDNA (28). Methylation levels can be influenced by S-adenosylmethionine (SAM) formation and mitochondrial functions (29,30). Although human mtDNA has been shown to have both CpG and non-CpG methylation sites (31), the interplay between various mtDNA haplogroups associated with methylation-related nuclear gene expressions needs further elucidation.

This report compares human retinal cell cybrids possessing either the H or J haplogroup mtDNA, and demonstrates differences in: (i) total global methylation and (ii) expression patterns for two genes related to acetylation (*HAT1* and *HDAC1*) and five genes related to methylation (*MAT2B, DNMT1, DNMT3A, DNMT3B* and *MBD2*). The untreated H versus J cybrids have different expression levels for methylation-regulated nuclear genes (*CFH, EFEMP1, VEGFA* and *NFkB2*), but after treatment with 5-aza-dC, a methylation inhibitor, the expression levels become equivalent. Our results suggest that the mtDNA haplogroup background within cells can significantly influence epigenetic profiles and create an environment capable of upregulating specific major pathways critical for disease processes.

Results

Elevated levels of global DNA methylation in J versus H cybrids

All cybrid cultures were grown under identical conditions and periods of time. Results showed the 5-mC% mean value for the H cybrids was 0.007 ± 0.001 and the 5-mC% mean value for the J cybrids was 0.022 ± 0.0053 (Fig. 1). The mean difference for the H versus the J cybrids was –0.015 ± 0.005 ($P = 0.02$). This indicates that the J cybrids showed higher levels of total global methylation compared with the H cybrids.

Altered expression levels of genes associated with epigenetic pathways

The gene expression levels for 11 genes related to epigenetic modification (Fig. 2) were analyzed by Q-PCR in H cybrids versus J cybrids (Tables 1 and 2). The expression levels for *HAT1*, a histone acetyltransferase, were significantly lower in the J cybrids

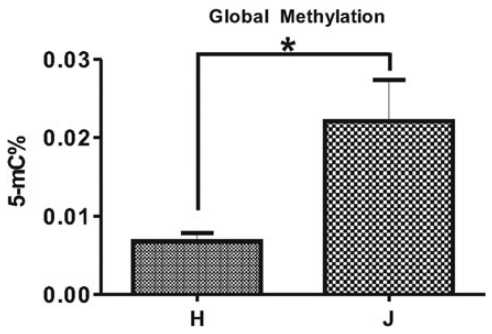

Figure 1. J cybrids show increased levels of global DNA methylation. The levels of methylated DNA (5-mC%) were quantified in J ($n = 3$) and H ($n = 3$) cybrids, cultured under identical conditions. The H cybrids had a lower 5-mC% mean value (0.007 ± 0.001) compared with the J cybrids (0.022 ± 0.0053, $P = 0.02$). Samples were run in duplicate and the experiment was repeated twice. Statistical significance is denoted by *$P < 0.05$.

compared to the H cybrids (0.63-fold, $P = 0.0001$). With respect to the deacetylase enzymes, the J cybrids showed a 0.68-fold decrease in expression for *HDAC1* gene ($P = 0.003$) compared with the H cybrids, but *HDAC6* ($P = 0.18$) and *HDAC11* ($P = 0.4$) showed similar gene expression levels for H and J cybrids. The expression levels for *SIN3A*, a transcriptional regulator protein, which promotes deacetylation, were also similar in H and J cybrids ($P = 0.19$).

The expression levels for the *MAT2B* gene, which catalyzes the biosynthesis of SAM a major methyl donor, were significantly higher in the J cybrids compared with the H cybrids (1.51-fold, $P = 0.002$). The *MAT1A* levels were minimal in both the J and H cybrids (data not shown). *DNMT1*, a DNA methyltransferase, which specifically targets the mtDNA (32), was expressed at lower levels in the J cybrids compared with the H cybrids (0.24-fold, $P = 0.0001$). The *DNMT3A* and *DNMT3B* expression levels were also significantly lower in the J cybrids compared with the H cybrids (0.3-fold, $P < 0.0001$ and 0.27-fold, $P < 0.001$). The J cybrids showed a significant decrease in the expression levels of *MBD2* compared with the H cybrids (0.4-fold, $P = 0.001$). There was no difference observed in the expression levels for *MBD4* in the H versus J cybrids (1.06-fold, $P = 0.74$).

Methylation inhibitor studies comparing H versus J cybrids

Studies were performed to compare responses of the H and J cybrids to methylation inhibition. Briefly, H and J cybrids were treated with the methylation inhibitor 5-aza-dC and subsequently the expression levels of four nuclear genes associated with AMD were measured (Table 3). When untreated-J and -H cultures were compared with each other, the *CFH* expression levels were 0.44-fold lower in the untreated-J cybrids compared with the untreated-H cybrids ($P < 0.0001$). After 5-aza-dC treatment, the treated-J and -H cybrids expressed similar levels of *CFH* (1.07-fold, $P = 0.42$). When the untreated cybrids were compared, the *EFEMP1* gene showed 0.71-fold lower expression levels in the untreated-J cybrids compared with the untreated-H cybrids ($P = 0.015$). However, there was no difference in the expression levels for *EFEMP1* in the treated-H and -J cybrids (1.2-fold, $P = 0.2$) after treatment with 5-aza-dC. The *NFkB2* gene was expressed at lower levels in the untreated-J cybrids compared with the untreated-H cybrids (0.69-fold, $P = 0.03$), but after the 5-aza-dC treatment, the treated-H and -J cybrids expressed similar levels of *NFkB2* (1.04-fold, $P = 0.71$). *VEGFA* is a critical gene for neovascularization and is important for

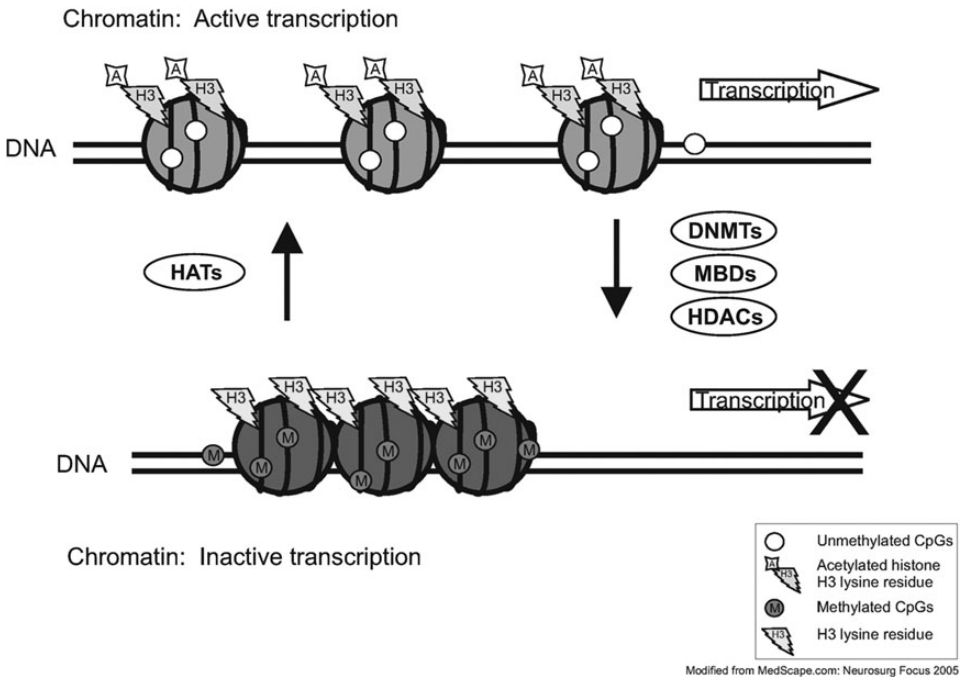

Figure 2. Schematic of the acetylation and methylation enzymes affecting transcription. Upper panel shows the active transcription state for chromatin with unmethylated CpG sites on the DNA and acetylated histone sites. Bottom panel shows the inactive transcription state for chromatin with methylated CpG sites and non-acetylated histone H3 lysine residues. HATs, histone acetyltransferase; HDACs, histone deacetylase; DNMTs, DNA (cystosine-5) methyltransferase; MBDs, methyl-CpG binding domain protein 2; H3, histone lysine residue; M, methylated CpG site.

development and disease processes. When the untreated cybrids were compared with each other, the untreated-J cybrids expressed 0.62-fold lower levels of *VEGFA* compared with the untreated-H cybrids ($P = 0.0006$). When the cybrids are demethylated with 5-aza-dC, the treated-H and -J cybrids showed similar expression levels for the *VEGFA* gene (1.1-fold, $P = 0.3$) (Fig. 3).

In the 5-aza-dC experiments, the *HPRT1* gene was used as the housekeeper for *EFEMP1, NFkB2* and *VEGFA*. The *HMBS* gene was the housekeeper for *CHF*. To determine if the changes in the demethylation experiments were due to methylation regulation of the housekeeper genes, the expression level of *HMBS* as a target gene and *HPRT1* as the housekeeper gene was measured and found to be similar before and after treatment with 5-aza-dC (0.97-fold, $P = 0.77$). As a second analysis, the *HPRT1* gene (target) was measured against *TUBB* gene (housekeeper) and similarly, there was no change in expression after treatment with 5-aza-dC (1.22-fold, $P = 0.15$). If the 5-aza-dC were causing demethylation, then the values for the genes would have varied.

Sequences comparisons of the H and J cybrids for the MT-Dloop

The entire control regions for H ($n = 7$) and J ($n = 6$) cybrids were sequenced and analyzed. Sequencing profiles for the MT-Dloops of H and J cybrids were similar to the Cambridge Reference Sequence (Table 4). The areas of greatest SNP variations were from nucleotides (np) 263–461, a region which contains the mtDNA loci for Conserved Sequence Block 2 (np 299–315), H Strand Origin (np 110–441) and Hypervariable Segment 2 (np 57–372). There was also high variability with C insertions at the np 310–321 region. The H and J cybrids had similar total numbers of CpG and non-CpG methylation sites in the MT-Dloop, as represented by ACA, CCA, TCA, GCA and ACT (33) (Table 5).

Sequence variations of the H and J cybrid mtDNA

The original three H and J cybrids have been sequenced and reported to be J1d1a, J1c1 and J1c7 along with H, H5a and H66a (24). In this investigation, additional cybrids were used and the entire mtDNA from each of the cybrids was sequenced (Tables 6 and 7) and compared with the Cambridge Reference Sequence. SNPs we define as unique are not listed in www.MitoMap.org. Private SNPs are those that do not define the haplogroup. The rs# numbers for the mtDNA SNPs were obtained from rCRS GenBank number NC_012920. The rs# numbers for 31 SNPs for the J cybrids have been listed. However, 11 of the mtDNA SNPs have not been assigned rs# numbers. For the H cybrids, there are 24 SNPs that had rs# numbers, but 17 SNPs lack the rs# identification.

The new J cybrids were classified into J1c1a (#J13-74), J1b (#J13-107) and J1b1b1 (#J12-43). There were a total of eight private SNPs in the J cybrids: J1c1a cybrid with m.4322C>T (unique, MT-TI); J1b cybrid with m.4322C>T (unique, MT-TI), m.8200T>C (syn, MT-CO2) and m.200A>G (non-coding); and J1b1b1 cybrid with m.4664C>T (syn, MT-ND2), m.7805G>A (V-I, MT-CO2), m.14028A>G (unique, MT-ND5) and m.508A>G (non-coding). The private SNPs were found in different mitochondrial loci (MT-TI, MT-CO2, MT-ND2, and non-coding). There were seven non-synonymous, J-defining haplogroup SNPs: m.3394T>C (Y-H, MT-ND1), m.4216T>C (Y-H, MT-ND1*), m.10398A>G (T-A, MT-ND3*), m.13708G>A (A-I, MT-ND5*), m.13879T>C (S-P, MT-ND5), m.14798T>C (F-L, MT-CYB) and m.15452C>A (L-I, MT-CYB*). The four SNPs with asterisks are J-defining SNPs found in all three J cybrids. The non-coding region of the J cybrids possessed 15 SNPs, 12 of which defined the J haplogroup. It is important to note that the three J cybrids listed in this paper and the previously published J cybrid sequences (24) did not have private or unique SNPs found in all samples, supporting the idea that it is the background mtDNA haplogroup profile which causes the differences detected in the J versus H cybrids.

Table 1. Description of genes analyzed in this study

Symbol	Gene name	GenBank accession no.	Function
HAT1	Histone acetyltransferase	NM_001033085 NM_003642	Adds acetyl group; promotes transcription.
HDAC1	Histone deacetylase 1	NM_004964	Class I histone deacetylase involved in control of cell proliferation, differentiation, growth and apoptosis; represses transcription.
HDAC6	Histone deacetylase 6	NM_006044	Class II histone deacetylase.
HDAC11	Histone deacetylase 11	NM_024827	Class IV histone deacetylase localized to the nucleus; involved in regulating interleukin 10 expression.
SIN3A	Sin3 transcription regulator homolog A (yeast)	NM_001145357 NM_001145358 NM_015477	Transcriptional regulator for STAT3; Promotes deacetylation.
MAT2B	Methionine adenosyltransferase II, beta	NM_013283	Catalyzes the biosynthesis of S-adenosylmethionine, the major methyl donor; Regulatory beta subunit.
MBD2	methyl-CpG binding domain protein 2	NM_003927	Part of nuclear protein family related by the presence of a methyl-CpG binding domain (MBD). Binds specifically to methylated DNA; represses transcription from methylated gene promoters; functions as a demethylase to activate transcription.
MBD4	Methyl-CpG binding domain protein 4	NM_003925	Binds specifically to methylated DNA; Involved in protein interactions, and DNA repair.
DNMT1	DNA (cystosine-5) methyltransferase 1	NM_001130823 NM_001379	Methylates CpG residues, preferentially methylates hemimethylated DNA. Associates with DNA replication sites in S phase maintaining the methylation pattern in newly synthesized strand, essential for epigenetic inheritance.
DNMT3A	DNA (cystosine-5) methyltransferase 3A	NM_022552 NM_153759 NM_175629	Methylates *de novo* during development. Important for genomic imprinting.
DNMT3B	DNA (cystosine-5) methyltransferase 3B	NM_001207055; NM_001207056 NM_006892 NM_175848 NM_175849 NM_175850	Methylates *de novo* during development. Important for genomic imprinting.
CFH	Complement factor H	NM_000186	Essential in regulation of complement activation.
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	NM_004105 NM_018894 NM_001039348 NM_001039349	Encoded protein contains tandemly repeated EGF-like repeats followed by C-terminus fibulin-like domain.
NFκB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	NM_001077494 NM_001077493 NM_002502	Subunit of transcription factor complex nuclear factor-kappa-B complex which is central activator of inflammation and immune function genes.
VEGFA	Vascular endothelial growth factor A	NM_001025366 NM_001025367 NM_001025368 NM_001025369 NM_001025370 NM_001033756 NM_001171622 NM_001171623 NM_001171624 NM_001171625 NM_001171626 NM_001171627 NM_001171628 NM_001171629 NM_001171630 NM_001204384 NM_001204385 NM_003376	Member of PDGF/VEGF growth factor family. Acts on endothelial cells. Causes increased vascular permeability, angiogenesis, vasculogenesis, endothelial cell growth, cell migration and inhibits apoptosis. Mutations in this gene have been associated with proliferative and non-proliferative diabetic retinopathy.

Continued

Table 1. Continued

Symbol	Gene name	GenBank accession no.	Function
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_000194	Transferase, which catalyzes conversion of hypoxanthine to inosine monophosphate & guanine to guanosine monophosphate. Plays a central role in the generation of purine nucleotides through the purine salvage pathway. Endogenous control.
HMBS	Hydroxymethylbilane synthase	NM_000190 NM_001024382 NM_001258208 NM_001258209	Member of the hydroxymethylbilane synthase superfamily. Third enzyme of the heme biosynthetic pathway. Catalyzes the head to tail condensation of four porphobilinogen molecules into the linear hydroxymethylbilane. Endogenous control.
ALAS1	5′-aminolevulinate synthase 1	NM_000688 XM_005264944	This gene encodes the mitochondrial enzyme which catalyzes the rate-limiting step in heme (iron-protoporphyrin) biosynthesis. The enzyme encoded by this gene is the housekeeping enzyme; the level of the mature encoded protein is regulated by heme: high levels of heme down-regulate the mature enzyme in mitochondria while low heme levels up-regulate.
TUBB	Tubulin, beta class I	NM_178014 NM_001293213	This gene encodes a beta tubulin protein. This protein forms a dimer with alpha tubulin and acts as a structural component of microtubules.

Table 2. Differential gene expression in cybrids H versus J

Symbol	H versus J $\Delta\Delta C_T$/	H versus J Fold	H versus J *P*-value
HAT1[a]	−0.67 ± 0.16	0.63	0.0001
HDAC1[b]	−0.55 ± 0.16	0.68	0.003
HDAC6[b]	−0.43 ± 0.5	0.74	0.18
HDAC11[b]	−0.33 ± 0.38	0.8	0.4
SIN3A[b]	−0.41 ± 0.30	0.75	0.19
MAT2B[b]	0.6 ± 0.16	1.51	0.002
MBD2^^[a]	−1.3 ± 0.36	0.4	0.001
MBD4[b]	0.08 ± 0.23	1.06	0.74
DNMT1[a]	−2 ± 0.2	0.24	<0.0001
DNMT3A^[a]	−1.73 ± 0.2	0.3	<0.0001
DNMT3B^[a]	−1.88 ± 0.2	0.32	<0.0001

Fold values >1 indicate upregulation of the gene compared with H cybrids; Fold values <1 indicate downregulation of the gene compared with H cybrids; H cybrids are assigned a value of 1.
Measured versus HMBS; ^ versus ALAS1; ^^ versus TUBB as housekeeper; Fold = $2^{\Delta\Delta CT}$.
[a]$n = 7$ different H cybrids and 6 different J cybrids, with three values for each sample.
[b]$n = 3$ different H cybrids and 3 different J cybrids, with three values for each sample.

Table 3. Expression levels of genes before and after treatment with 5-aza-dC, a methylation inhibitor

	H Cybrids[a] versus J Cybrids (Untreated) *P*-value $\Delta\Delta C_T$/Fold	H Cybrids[a] versus J Cybrids (5-aza-dC Treated) *P*-value $\Delta\Delta C_T$/Fold
CFH[b]	<0.0001	0.42
	−1.17 ± 0.12/0.44	0.1 ± 0.13/1.07
EFEMP1[c]	0.015	0.2
	−0.48 ± 0.2/0.71	0.23 ± 0.18/1.2
NFkB2[c]	0.03	0.71
	−0.52 ± 0.22/0.69	0.06 ± 0.16/1.04
VEGFA[c]	0.0006	0.3
	−0.67 ± 0.15/0.62	0.18 ± 0.17/1.1

Fold values >1 indicate upregulation of the gene.
Fold values <1 indicate downregulation of the gene.
Fold = $2^{\Delta\Delta CT}$.
H cybrids, $n = 3$ different individuals; J cybrids, $n = 3$ different individuals. Each sample was run in triplicate. Experiment was repeated twice.
[a]H cybrids assigned a value of 1.
[b]Measured versus HMBS as housekeeper.
[c]Measured versus HPRT1 as housekeeper.

Based upon the sequences, the additional H cybrids were classified into H4a1a (#H11-10), H11a2a2 (#H11-23), H1 (#H11-35) and H1j (#H13-49). The H-defining SNPs were m.73G>A, m.2706G>A, m.7028T>C, m.11719A>G and m.14766T>C. There were a total of five private SNPs in the H cybrids. The H4a1a cybrid had no private SNPs, but four non-synonymous H-defining SNPs: m.3992T>C (M-T, MT-ND1), m.4024A>G (T-A, MT-ND1), m.13889G>A (C-Y, MT-ND5) and m.14582A>G (V-A, MT-ND6). H11a2a2 had two private SNPs: 4322C>T (unique, MT-TI and 11587C>T (unique, MT-ND4) and two non-synonymous H-defining SNPs: 8448T>C, (M-T, MT-ATP8) and 13759G>A (A-T, MT-ND5). The H1 cybrid had one private SNP, 16289A>G (non-coding, MT-Dloop). The H1j cybrid had two private SNPs: 13911A>G (syn, MT-ND5) and 14025T>C (syn, MT-ND5). The H1 and H1j cybrids did not have any non-synonymous H-defining SNPs.

Discussion

Our present studies illustrate that cybrids containing the J haplogroup mtDNA have significantly higher levels of total global methylation compared with H cybrids, a finding similar to that found in osteosarcoma cybrids (28). Since the total methylation was varied, the expression levels of methylation- and acetylation-specific genes were also examined and found to be different in H versus J cybrids. In mammalian cells, SAM is the universal methyl donor to cytosines in CpG sites in promoters. We found that the *MAT2B* gene, which catalyzes the

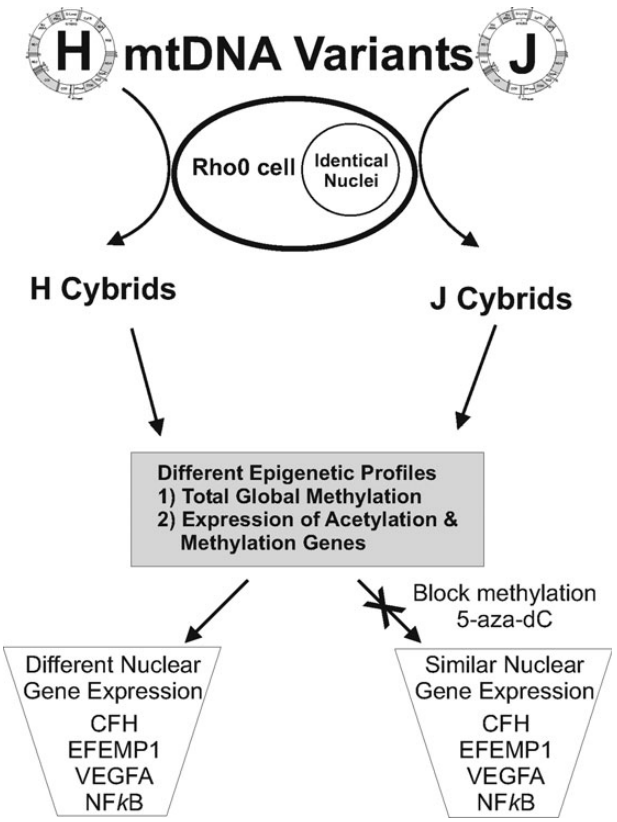

Figure 3. Schematic summarizing epigenetic profiles of H versus J cybrids. The H and J cybrids have different levels of total global methylation and expression of acetylation and methylation-related genes. Untreated H and J cybrids show significantly different transcription levels for *CFH* ($P < 0.0001$), *EFEMP1* ($P = 0.015$), *NFkB2* ($P = 0.03$) and *VEGFA* ($P = 0.0006$). After 48 h treatment with 5-aza-dC, a methylation inhibitor, then the gene expression levels are equivalent in the H and J cybrid cultures; *CFH* ($P < 0.42$, *EFEMP1* ($P = 0.2$), *NFkB2* ($P = 0.71$) and *VEGFA* ($P = 0.3$). Rho0, lacking mtDNA; 5-aza-dC, 5-aza-2′-deoxycytidine; *CFH*, Complement factor H; *EFEMP1*, EGF containing fibulin-like extracellular matrix protein1; *VEGFA*, vascular endothelial growth factor A; *NFkB2*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2.

biosynthesis of SAM from ATP and methionine, was expressed at a higher level in J cybrids compared with H cybrids. In cancer cells, increased *MAT2B* levels lead to lower SAM, which in turn facilitates the growth of hepatocellular carcinoma cells (34). The *MAT2B* levels may be influencing growth in cybrids as studies have shown that J cybrids grow more rapidly than H cybrids, which in turn grow faster than Uk cybrids (23,24,35). In J haplogroup osteosarcoma cybrids, elevated levels of *MAT1A* were expressed (28). However, our H and J RPE cybrids expressed only very low levels of *MAT1A* (the hepatic isoform) or *MAT2A* (non-hepatic isoform). This disparity may be due to differences in the underlying host cells (human RPE cells versus osteosarcoma cells). Our findings herein suggest that the *MAT2B* is the major methionine adenosyltransferase in human RPE cells *in vitro* and its expression can be modulated by the mtDNA variants within cells.

Our Q-PCR studies showed significantly lower expression levels for *DNMT1*, *DNMT3A* and *DNMT3B* in the J cybrids compared with the H cybrids. Shock *et al.*(32) identified *DNMT1* inside mitochondria, while others have found *DNMT3A* associated with mitochondrial fractions (36). While the degree of mtDNA methylation may provide a biomarker and diagnostic tool to identify abnormalities of mitochondrial metabolism (37), it is necessary to learn more about its significance before it might have a correlative value to diseases.

The *MBD* genes encode for a family of proteins that bind to the methylated DNA, block transcription, act as demethylases to activate transcription and are associated with various cancers (38). In our study, the J cybrids showed significantly lower levels of *MBD2* expression compared with the H cybrids. In contrast, the levels for *MBD4* gene expression were similar in the H and J RPE cybrids, which is consistent with findings in the osteosarcoma cybrids (28). Both *in vitro* and *in vivo* studies have shown that *MBD2* can bind to methylated DNA and silence IL-4, a major signaling intermediate molecule for IFN-gamma and the immune system (39,40). Differential expression of *MBD2* by the J and H cybrids could potentially lead to different methylation status and transcription levels for nuclear genes in complement and inflammation pathways, both of which are activated in AMD.

Epigenetics involves not only modification of DNA methylation, but also acetylation and deacetylation of histones. To our knowledge, we are the first group to demonstrate that the mtDNA variants can modulate the expression levels of acetylation-related genes. Recent evidence has shown that mitochondrial signaling is likely regulated through acetylation, with >1000 mitochondrial-related proteins having >4500 acetylation sites, indicating this form of regulation significantly impacts cellular homeostasis (41). Therefore, some of the acetylation-related genes in our cybrids were examined using Q-PCR. The J cybrids had significantly lower levels of *HAT1*, an acetyltransferase which adds acetyl groups to histones and promotes transcription. The inhibition of acetylation can lower inflammation and may be a possible therapy to block progression of diabetic retinopathy (42). Deregulation of acetylation and deacetylation is also associated with various cancers. Current studies are underway to identify acetylation modifiers for use as therapeutic agents (43–45).

In J cybrids, we detected significantly decreased expression levels of *HDAC1* compared with H cybrids. *HDAC1* is part of a complex responsible for deacetylation of histones, which represses transcription. When examined by GeneChip analyses, the H and J cybrids did not show differences of expression for another group of deacetylase enzymes, the sirtuins (*SIRT3, SIRT4, SIRT5*), which are actually found within mitochondria and are responsible for major protein modifications (46). In particular, the mitochondrial SIRT3 acts by deacetylation of various substrates to regulate reactive oxygen species (ROS) production and detoxification (47). This suggests that the mtDNA variants are influencing acetylation through the HDACs rather than SIRTs, but additional studies are required to understand this intricate process.

Our initial experiments showed that H and J cybrids have different levels for total global methylation and expression of acetylation and methylation genes. However, we also wanted to investigate the influences that H mtDNA versus J mtDNA might have on genes known to be regulated by methylation. *CFH* is a major susceptibility gene which confers significant risk for development of AMD (48–50). *EFEMP1* is a high-risk gene for AMD and other retinal degenerations (51,52) and misfolded *EFEMP1* causes altered RPE cell function and inflammation (53). *VEGFA* is associated with increased neovascularization found in AMD patients and is the target for anti-VEGF therapies commonly used to control the choroidal neovascularization associated with AMD. *NFkB2* is a major signaling molecule for inflammation and the activated form is associated with AMD fibrovascular membranes (54).

The H and J cybrids were cultured with and without 5-aza-dC, a DNA methyltransferase inhibitor. Although the untreated-J cybrids showed lower expression levels for *CFH* ($P < 0.0001$), *EFEMP1*

Table 4. Representative sequences showing the CpG and non-CpG sites (as represented by ACA, **CCA**, **TCA**, GCA, and *ACT*)) within the MT-Dloop from J Cybrid (J) and H Cybrid (H) compared to the revised Cambridge Reference Standard (R)

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16411R CGTGAAA TCA ATATCCC GCA CAAGAGTGCT
16411J CGTGAAA TCA ATATCCC GCA CAAGAGTGCT
16411H CGTGAAA TCA ATATCCC GCA CAAGAGTGCT

16441R ACT CTCCTCG CTCCGGGC CC A TAACA CT TG GGGGTAGCTA AAGTGA ACT G TATCCG ACAT
16441J ACT CTCCTCG CTCCGGGC CC A TAACA CT TG GGGGTAGCTA AAGTGA ACT G TATCCG ACAT
16441H ACT CTCCTCG CTCCGGGC CC A TAACA CT TG GGGGTAGCTA AAGTGA ACT G TATCCG ACAT

16501R CTGGTTCCT A CT TCA GGG TC A TAAAGCCTA AATAGC CCA C ACGTTCCCCT TAAATAAGAC
16501J CTGGTTCCT A CT TCA GGG TC A TAAAGCCTA AATAGC CCA C ACGTTCCCCT TAAATAAGAC
16501H CTGGTTCCT A CT TCA GGG CC A TAAAGCCTA AATAGC CCA C ACGTTCCCCT TAAATAAGAC

16561R A TCA CGATG
16561J A TCA CGATG
16561H A TCA CGATG

   1R GA TCACA GGT CTA TCA CCCT ATTAA CCACT CA CGGGAGCT CT CCA TGCA T TTGGTATTTT
   1J GA TCACA GGT CTA TCA CCCT ATTAA CCACT CA CGGGAGCT CT CCA TGCA T TTGGTATTTT
   1H GA TCACA GGT CTA TCA CCCT ATTAA CCACT CA CGGGAGCT CT CCA TGCA T TTGGTATTTT

  61R CGTCTGGGGG GTAT GCA CGC GATA GCA TTG CGAGACGCTG GAGCCGGA GC A CCCTATGTC
  61J CGTCTGGGGG GTGT GCA CGC GATA GCA TTG CGAGACGCTG GAGCCGGA GC A CCCTATGTC
  61H CGTCTGGGGG GTAT GCA CGC GATA GCA TTG CGAGACGCTG GAGCCGGA GC A CCCTATGTC

 121R GCA GTATCTG TCTTTGATTC CTGCC TCA TC CTATTATTTA TC GCA CCTAC GT TCA ATATT
 121J GCA GTATCTG TCTTTGATTC CTGCC TCA TC CTATTATTTA TC GCA CCTAC GT TCA ATATT
 121H GCA GTATCTG TCTTTGATTC CTGCC TCA TC CTATTATTTA TC GCA CCTAC GT TCA ATATT

 181R ACA GGCGAAC ATACT ACT A AAGTGTGTTA ATTAATTAAT GCTTGTAGGA CATAATAATA
 181J ACA GGCGAAC ATACT ACT G AAGTGTGTTA ATTAATTAAT GCTTGTAGGA CATAATAATA
 181H ACA GGCGAAC ATACT ACT A AAGTGTGTTA ATTAATTAAT GCTTGTAGGA CATAATAATA

 241R ACAATTGAAT GTCT GCACA G CCACT TT CCA CACAGACA TC A TAACAAAAA ATTT CCACCA
 241J ACAATTGAAT GTCT GCACA G CCGCTTT CCA CACAGACA TC A TAACAAAAA ATTT TCACCA
 241H ACAATTGAAT GTCT GCACA G CCGCTTT CCA CACAGACA TC A TAACAAAAA ATTT CCACCA

 301R AACCCCCCCT CCCCCGCTTC TGG CCACA GC ACT AAACAC ATCTCTG CCA AACC CCA AAA
 301J AACCCCCCCTCCCCCCGCTTC TGG CCACA GC ACT AAACAC ATCTCTG CCA AACC CCA AAA
 301H AACCCCCCCCTCCCCCCGCTTCTGG CCACA GC ACT AAACAC ATCTCTG CCA AACC CCA AAA

 361R ACAAAGAACC CTAACA CCA G CCTAA CCA GA TT TCA AATTT TATCTTTTGG CGGTAT GCAC
 361J ACAAAGAACC CTAACA CCA G CCTAA CCA GA TT TCA AATTT TATCTTTTGG CGGTAT GCAC
 361H ACAAAGAACC CTAACA CCA G CCTAA CCA GA TT TCA AATTT TATCTTTTGG CGGTAT GCAC

 421R TTTTAACAG T CA CCCC CCAA CT AACACATT ATTTTCCCCT C CCACT C CCA TACTACT AAT
 421J TTTTAACAG T CA CCCC CCAA CT AACACATT ATTTTCCCCT C TCACT C CCA TACTACT AAT
 421H TTTTAACAG T CA CCCC CCAA CT AACACATT ATTTTCCCCT C CCACT C CCA TACTACT AAT

 481R C TCATCA ATA CAACCCCCGC CCA TCCTAC C CAGCA CACAC ACACCGCTGC TAACC CCA TA
 481J C TCATCA ACA CAACCCCCGC CCA TCCTAC C CAGCA CACAC ACACCGCTGC TAACC CCA TA
 481H C TCATCA ATA CAACCCCCGC CCA TCCTAC C CAGCA CACAC ACACCGCTGC TAACC CCA TA

 541R CCCCGAA CCA A CCA AACC CC A AAGACACCC C CCACA GTTT ATGTAGCTTA CCTCC TCA AA
 541J CCCCGAA CCA A CCA AACC CC A AAGACACCC C CCACA GTTT ATGTAGCTTA CCTCC TCA AA
 541H CCCCGAA CCA A CCA AACC CC A AAGACACCC C CCACA GTTT ATGTAGCTTA CCTCC TCA AA

 601R GCA ATACACT GAAAATGTTT
 601J GCA ATACACT GAAAATGTTT
 601H GCA ATACACT GAAAATGTTT
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Table 5. Potential methylation sites in the MT-Dloop for J and H Cybrids

	CpG Sites	Non-CpG Sites					Total
	CpG	ACA	CCA	TCA	GCA	ACT	
rCRS	23	26	23	15	12	15	114
J	23	26	20	17	12	14	112
H	23	26	23	14	12	14	112

rCRS, revised Cambridge Reference Standard (based upon GenBank number NC_012920).
(From http://www.mitomap.org).

($P = 0.015$), *NFkB2* ($P = 0.03$) and *VEGFA* ($P < 0.0006$) compared with the untreated-H cybrids, once the cells were demethylated, the expression levels for these were similar in J-treated and H-treated cybrids. These results suggest that methylation status may be regulated in part by mtDNA variants, because when methylation is present, the J cybrids express lower levels of these genes, but once demethylated, the levels are similar.

Methylation status of tissues is extremely important in diseases. In gastric tumor cells, demethylation with 5-aza-dC increased microRNA-195 and microRNA-378 levels, which in turn down-regulated VEGF levels (55). In hepatocellular carcinoma cells, Yang *et al.* (56) reported an inverse relationship between the degree of methylation at CpG sites and *CFH* expression levels. This is similar to what we found in the J cybrids, which also have higher total methylation, but lower expressions of *CFH* (23). Expression levels of *CFH* can correlate with metastasis, mortality risk, insulin resistance in adipose tissues and possibly tumor suppression (56–58). The degree of methylation in the *EFEMP1* promoter plays a key role in tumor metastases and disease outcomes (59–69). Therefore, the fact that mtDNA variants (J versus H haplogroups) can affect significantly, via the methylation profile, the expression levels of *CFH, EFEMP1* and *VEGFA*, suggests that targeting the methylation pathways may serve as a therapeutic target/approach in future studies.

It has been shown that 2–5% of human mtDNA has methylation at the CCGG regions (70). Normally the methylation sites for human nuclear DNA occur through the CpG sites while plants and fungi utilize non-CpG methylation sites; however, reports have shown that mtDNA actually contain both types of methylation sites, CpG and non-CpG sites (31). The MT-Dloop contains the control region possessing the Light-Strand Promoter, responsible for transcription of the *MT-ND6* gene and eight of the MT-tRNAs, and the Heavy-Strand Promoter, which controls the transcription for the other mtDNA encoded genes. Previous studies have shown that J cybrids express significantly lower MT-RNA levels than H cybrids (24,71). Based upon reported differences in MT-RNA expression levels, we speculated that mtDNA defining J haplogroups may have different numbers of CpG and non-CpG sites within the MT-Dloop. However, the numbers of CpG and non-CpG sites in the Light-Strand Promoter and Heavy-Strand Promoter for the H and J cybrids were similar, indicating that altered MT-RNA expression levels were likely not a result of SNP substitutions within those regions. Our sequencing data suggest that although the J haplogroup mtDNA have numerous SNP variants, there was no disruption of the SNP pattern within the MT-Dloop causing increased numbers of potential CpG or non-CpG methylation sites. Future studies will focus on understanding the mechanism(s) underlying differential methylation levels associated with different mtDNA variants.

Sequencing of the entire mtDNA for the cybrids showed that the majority of SNPs defined either the H or J haplogroups. There were no private or unique SNPs that were found uniformly in all of the H or J cybrids. This supports the concept that the differences in gene expression are not due to a single mutation, private or unique SNP, but rather: (i) the accumulation of multiple SNPs in the J or H haplogroups or (ii) a particular, as of yet, unidentified SNP, which defines the haplogroup, but has properties to modulate nuclear gene expression via a retrograde signaling mechanism. In addition, all of the J cybrids belonged in the haplogroup J1 family, with none being part of the J2 haplogroup, the other main branch of the J haplogroup. This may be significant because J1 and J2 branches are quite different, especially when mutations in complex III are considered. The SNP m.195T>C found in H11a2a2 cybrid has been found to be associated with bipolar disorder (72) (reported in www.MitoMap.org). None of the other SNPs in either the H or J cybrids were associated with diseases when compared with the '*Reported Mitochondria DNA Base Substitution in Diseases*' or the '*Coding and Control Region Point Mutations*' sections. Research studies need to be completed before we fully comprehend how the mtDNA variants: (i) mediate total global methylation levels; (ii) affect expression levels of epigenetic-related genes or (iii) influence the transcription of non-energy-related nuclear genes. Further investigations aimed at elucidating the mechanisms of these mtDNA variants will help in paving the way to personalized therapeutic advancements and interventions.

Materials and Methods

Cybrid cultures and culture conditions

Institutional review board (#2003-3131) approval was obtained from the University of California-Irvine. For DNA analyses, 20 ml of peripheral blood was collected by venipuncture in tubes containing sodium citrate buffer. DNA was isolated with a DNA extraction kit (PUREGENE, Qiagen, Valencia, CA). Platelets were isolated by a series of centrifugation steps and final pellets were suspended in Tris-buffered saline (TBS). The ARPE-19 cells deficient in mtDNA (Rho0) were created by serial passage in low-dose ethidium bromide as previously described (73). Cybrids were created by polyethylene glycol fusion of platelets with Rho0 ARPE-19 cells according to modified procedures of Chomyn (74). Cybrids were cultured until confluent in DMEM-F12 containing 10% dialyzed fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 50 μg/ml gentamycin and 17.5 mM glucose as described previously (23). The H and J cybrids used in this set of experiments were previously shown to have similar mtDNA copy numbers (23). To eliminate potential technical variability, all experiments were performed under exactly the same controlled conditions. For each experiment, all H and J cybrids were at passage 5 and cultured side-by-side in an incubator using identical media conditions.

Inhibition of methylation in cybrid cultures

These experiments were designed to determine if the different mtDNA variants (H versus J haplogroups) influence the RNA expression for nuclear genes with known methylation sites in their promoter regions (*EFEMP1* and *VEGFA*) or key genes associated with signaling transduction or complement pathways (*NFkB2* and *CFH*). H and J cybrids ($n = 3$ for each cybrid, each from different individuals) were plated for 24 h, media were removed and replaced with the same media containing a final concentration of 250 nM 5-aza-2′-deoxycytidine (5-aza-dC, Sigma-Alrich, St Louis, MO, USA). Cells were treated with 5-aza-dC for 48 h, the culture media being replaced after 24 h

Table 6. Sequencing results for the entire mtDNA in J Cybrids

Loci: MT-	AA Change	rs#	J 13-74	J1c1a	J 13-107	J1b	J 12-43	J1b1b1
RNR1		2 853 518	750A>G	[a]	750A>G	[a]	750A>G	[a]
RNR1		2 001 030	1438A>G	[a]	1438A>G	[a]	1438A>G	[a]
RNR2		3 928 306	3010G>A	J1	3010G>A	J1	3010G>A	J1
RNR2	Unique	na					3847T>C	J1c17
ND1	Y-H	41 460 449	3394T>C	J1c1				
ND1	Y-H	1 599 988	4216T>C	JT	4216T>C	JT	4216T>C	JT
TI	Unique	na	4322C>T	[b]	4322C>T	[b]		
ND2	SYN	na					4664C>T	[b]
ND2	SYN	3 021 086	4769A>G	[a]	4769A>G	[a]	4769A>G	[a]
ND2	A-T	3 021 088					5460G>A	J1b1
CO2	V-I	na					7805G>A	[b]
CO2	SYN	na			8200T>C	[b]		
CO2	Non-Coding	8896			8269G>A	J1b	8269G>A	J1b
ATP6	T-A	2 001 031	8860A>G	[a]	8860A>G	[a]	8860A>G	[a]
CO3	SYN	na	9635A>C	J1c1a				
ND3	T-A	2 853 826	10398A>G	J	10398A>G	J	10398A>G	J
TR		2 00 478 835					10410T>A	J1b1b1
ND4	SYN	3 915 952	11251A>G	JT	11251A>G	JT	11251A>G	JT
ND4	SYN	na	11623C>T	J1c1a				
ND5	SYN	28 359 172	12612A>G	J	12612A>G	J	12612A>G	J
ND5	A-I	28 359 178	13708G>A	J	13708G>A	J	13708G>A	J
ND5	S-P	2 00 380 057					13879T>C	J1b1
ND5	SYN	3 70 031 192	13899T>C	J1c1a				
ND5	unique	na					14028A>G	[b]
CYB	F-L	28 357 681	14798T>C	J1c				
CYB	T-A	2 853 508	15326A>G	[a]	15326A>G	[a]	15326A>G	[a]
CYB	L-I	5 27 236 209	15452C>A	JT	15452C>A	JT	15452C>A	JT
Dloop	Non-Coding	1 47 903 261	16069C>T	J	16069C>T	J	16069C>T	J
Dloop	Non-Coding	1 47 029 798	16126T>C	JT	16126T>C	JT	16126T>C	JT
Dloop	Non-Coding	41 419 246			16145G>A	J1b	16145G>A	J1b
Dloop	Non-Coding	na			16222C>T	J1b		
Dloop	Non-Coding	1 38 126 107			16261C>T	J1b	16261C>T	J1b
Dloop	Non-Coding	62 581 312					150C>T	J1b17
Dloop	Non-Coding	3 72 099 630			200A>G	[b]		
Dloop	Non-Coding	41 323 649	228G>A	J1c				
Dloop	Non-Coding	2 853 515	263A>G	[a]			263A>G	[a]
Dloop	Non-Coding	na					271C>T	J1b1b
Dloop	Non-Coding	41 528 348	295C>T	J	295C>T	J	295C>T	J
Dloop	Non-Coding	41 402 146	462C>T	J1	462C>T	J1	462C>T	J1
Dloop	Non-Coding	na	482T>	J1c1				
Dloop	Non-Coding	28 625 645	489T>C	J	489T>C	J	489T>C	J
Dloop	Non-Coding	1 13 683 159					508A>G	[b]

[a]Private: non-haplogroup defining SNP found in both H and J cybrids.
[b]Private: non-haplogroup defining SNP found in single H or J cybrid.
na, rs# not assigned. Unique: SNP not listed on www.mitomap.org.

with fresh media containing the compound. Cells were pelleted, RNA isolated and cDNA synthesized as described in what follows. Q-PCR was performed with primers for *CFH*, *EFEMP1*, *NFkB2* and *VEGFA*.

Identification of cybrid haplogroups

Cybrid DNA was extracted from cell pellets using a spin column kit (DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA, USA) as described previously (24). The mitochondrial haplogroups were identified by PCR along with restriction enzyme digestions, allelic discrimination and sequencing (5,24). The major defining SNP for the H haplogroup is T7028C. The major SNP defining the J haplogroup is G13708A. Allelic discrimination was performed at the GenoSeq UCLA Genotyping and Sequencing Core. Data were analyzed with Sequence Detection Systems software (ABI7900HT). The mtDNA sequences were compared with the classification from www.phylotree.org and www.MitoMap.com. In an earlier publication, the entire mtDNA sequences for H cybrids ($n = 3$) were categorized as H, H5a and H66a, and the J haplogroup cybrids ($n = 3$) were classified into J1d1a, J1c1 and J1c7 (24). These cybrids were used for the global methylation and inhibitor studies. Additional H (H4a1a, H11a2a2, H1 and H1j; total of $n = 7$) and J (J1c1a, J1b, J1b1b1, total of $n = 6$) cybrids were also sequenced and used for the Q-PCR analyses.

Global DNA methylation assay

The global DNA methylation status was detected using the MethylFlash Methylated DNA Quantification Kit (Colormetric)

Table 7. Sequencing results for the entire mtDNA in H Cybrids

Loci: MT-	AA Change	rs#	H 11-10	H4a1a	H 11-23	H11a2a2	H 11-35	H1	H 13-49	H1j
RNR1		2 853 518	750A>G	[a]	750A>G	[a]	750A>G	[a]	750A>G	[a]
RNR1		3 888 511			961T>G	H11a				
RNR1		2 001 030	1438A>G	[a]	1438A>G	[a]	1438A>G	[a]	1438A>G	[a]
RNR2		2 854 128	2706A>G	H	2706A>G	H	2706A>G	H	2706A>G	H
RNR2		3 928 306					3010G>A	H1	3010G>A	H1
ND1	T-M	41 402 945	3992C>T	H4						
ND1	T-A	41 504 646	4024A>G	H4a						
TI	Unique	na			4322C>T	[b]				
ND2	SYN	na							4733T>C	H1j
ND2	SYN	3 021 086	4769A>G	H2a1d[c]	4769A>G	H2a1d[c]	4769A>G	H2a1d[c]	4769A>G	H2a1d[c]
ND2	SYN	41 419 549	5004T>C	H4						
NC3	Non-Coding	na			5585G>A	H11a2a2				
CO1	SYN	na	6951G>A	H4a1a4b2						
CO1	SYN	2 015 062	7028T>C	H	7028T>C	H	7028T>C	H	7028T>C	H
CO2	SYN	8896	8269G>A	H4a1a						
ATP8	M-T	na			8448T>C	H11				
ATP6	T-A	2 001 031	8860A>G	[a]	8860A>G	[a]				
ATP6	SYN	28 358 270	9123G>A	H4						
ND4	Unique	na			11587C>T	[b]				
ND4	SYN	2 853 495	11719G>A	H	11719G>A	H	11719G>A	H	11719G>A	H
ND4	SYN	na					12130T>C	H45b		
ND5	A-T	na			13759G>A	H11				
ND5	C-Y	na	13889G>A	H4a1a3						
ND5	SYN	na							13911A>G	[b]
ND5	SYN	na							14025T>C	[b]
ND6	SYN	2 853 815	14365C>T	H4a1						
ND6	V-A	41 354 845	14582A>G	H4a						
ND6	SYN	na			14587A>G	H11a2				
CYB	T-I	na	14766T>C	H	14766T>C	H	14766T>C	H	14766T>C	H
CYB	T-A	2 853 508	15326A>G	[a]	15326A>G	[a]	15326A>G	[a]	15326A>G	[a]
CYB	SYN	na			15670T>C	H11a2a2				
Dloop	Non-Coding	3 134 562			16140T>C	H11a2a				
Dloop	Non-Coding	55 749 223					16189T>C	[c]		
Dloop	Non-Coding	na			16265A>G	H11a2a2				
Dloop	Non-Coding	na					16289A>G	[b]		
Dloop	Non-Coding	na			16293A>G	H11a				
Dloop	Non-Coding	34 799 580			16311T>C	H11[c]				
Dloop	Non-Coding	3 087 742	73 G>A	H	73 G>A	H	73 G>A	H	73 G>A	H
Dloop	Non-Coding	62 581 312	150C>T							
Dloop	Non-Coding	2 857 291			195T>C	[c]				
Dloop	Non-Coding	2 853 515	263A>G	[a]	263A>G	[a]	263A>G	[a]	263A>G	[a]

[a]Private: non-haplogroup defining SNP found in both H and J cybrids.
[b]Private: non-haplogroup defining SNP found in single H or J cybrid.
[c]Back mutation to ancestral state; na, rs# not assigned.
Unique: SNP not listed on www.mitomap.org.

(EpiGenTek, Farmingdale, NY, USA) according to the manufacturer's protocol. The amount of DNA used in the assay was 100 ng. Briefly, the H ($n = 3$) and J ($n = 3$) cybrids were cultured until confluent and DNA isolated as described earlier. The DNA was bound to strip wells that were specifically treated by the manufacturer to have a high DNA affinity. The methylated fraction of DNA was detected using capture and detection antibodies and then quantified through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at 450 nm. The amount of methylated DNA (5-mC%) was proportional to the OD intensity measured with an absorbance plate reader (Bio-Tek, Winooski, VT, USA), calculated according to the kit's formulas for relative methylation status of two different DNAs. Samples were run in duplicate and the experiment was repeated twice.

RNA extraction, amplification of cDNA and quantitative PCR (Q-PCR) analyses

Cells from cybrid cultures were pelleted and RNA isolated using the RNeasy Mini-Extraction kit (Qiagen, Inc., Valencia, CA, USA) as described previously (24). This study compares relative differences in gene expression levels between the H and J cybrids. For Q-PCR analyses, 100 ng of individual RNA samples were reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Inc.). Q-PCR was performed in triplicate using 10 different primers (QuantiTect Primer Assay, Qiagen, Inc.) for genes associated with various aspects of acetylation (*HAT1, HDAC1, HDAC6, HDAC11, SIN3A)* or methylation *(MAT2B, MBD4, DNMT1, DNMT3A* and *DNMT3B*). The Q-PCR was performed on

individual samples using a QuantiFast SYBR Green PCR Kit (Qiagen) on a Bio-Rad iCycler iQ 500 detection system. For the various target genes, housekeeping genes that had comparable amplification efficiencies to the genes of interest were chosen in order to maximize the accuracy of our $\Delta\Delta C_T$ values. The housekeeper genes were either *HPRT1* (hypoxanthine phosphorbosyltransferase 1), *HMBS* (hydroxymethylbilane synthase), *ALAS1* (aminolevulinate, delta-synthase) or *TUBB* (Tubulin, beta class 1).

Statistical analyses

Statistical analyses of the data were performed by ANOVA (GraphPad Prism, version 5.0). Newman–Keuls multiple-comparison or the two-tailed *t*-tests were used to compare the data within each experiment. $P < 0.05$ was considered statistically significant. Error bars in the graphs represent standard error of the mean (SEM).

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Conflict of Interest statement. None declared.

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