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# African and Asian Mitochondrial DNA Haplogroups Confer Resistance Against Diabetic Stresses On Retinal Pigment Epithelial Cybrid Cells *In Vitro*

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## Abstract

Diabetic retinopathy (DR) is the most common cause of blindness for individuals under the age of 65. This loss of vision can be due to ischemia, neovascularization and/or diabetic macular edema, which are caused by breakdown of the blood-retina barrier at the level of the retinal pigment epithelium (RPE) and inner retinal vasculature. The prevalence of diabetes and its complications differ between Caucasian-Americans and certain minority populations, such as African-Americans and Asian-Americans. Individuals can be classified by their mitochondrial haplogroups, which are collections of single nucleotide polymorphisms (SNPs) in mitochondrial DNA (mtDNA) representing ancient geographic origins of populations. In this study, we compared the responses of diabetic human RPE cybrids, cell lines containing identical nuclei but mitochondria from either European (maternal European) or maternal African or Asian individuals, to hypoxia and high glucose levels. The African and Asian diabetic ([Afr+Asi]/DM) cybrids showed (1) resistance to both hyperglycemic and hypoxic stresses; (2) downregulation of pro-apoptotic indicator *BAX;* (3) upregulation of DNA methylation genes, such as *DNMT3A* and *DNMT3B;* and (4) resistance to DNA de-methylation by the methylation inhibitor 5-Aza-2'-deoxycytidine (5-Aza-dC) compared to European diabetic (Euro/DM) cybrids. Our findings suggest that mitochondria from African and Asian diabetic subjects possess a "metabolic memory" that confers resistance against hyperglycemia, hypoxia, and demethylation, and that this "metabolic memory" can be transferred into the RPE cybrid cell lines *in vitro*.



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AUTHOR CONTRIBUTIONS

Conceived and Designed Experiments – AHD, MCK, MC, SRA; Performed the Experiments – AHD, MC, SRA; Analyzed the Data – AHD, MCK, MC, SRA; Contributed reagents/Materials/Analysis tools – MCK; Wrote the Manuscript – AHD, MCK.

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**Keywords**

Diabetic retinopathy (DR); Retinal Pigment Epithelial Cells; African and Asian Mitochondrial DNA Haplogroups; cybrid cell model; hyperglycemic and hypoxic stresses

## 1. INTRODUCTION

Diabetes has become one of the most widespread disorders affecting people worldwide. The number of individuals in the United States with this disease has more than doubled over the last decade such that 9.3% of Americans over the age of 20 (a population of at least 29 million people) as well as an estimated 8.5% of the global adult population, suffer from diabetes.[1–3] Minority populations such as African-Americans are particularly more likely to be diagnosed with diabetes and to suffer from its complications compared to Caucasians. Individuals over the age of 45 years old are much more likely to be diagnosed with diabetes as compared to those younger than 45. Obesity is a strong risk factor for developing diabetes as approximately 87.5% of all diabetic individuals have a BMI of 25 $kg/m^2$ or greater.[1] Diet and physical activity strongly influence the risk of developing diabetes, as therapy combining increased exercise with improved diet reduced the risk of developing diabetes in individuals with impaired glucose tolerance.[4] Poor health care coverage due to lack of insurance or access to health care is associated with increases in missed diabetes diagnoses and worse blood glucose, blood pressure, and overall well-being.[5,6]

Diabetes induces a shift in cellular metabolism due to decreased cellular glucose uptake. Since diabetes and its secondary complications arise from this altered metabolic environment, mitochondria may play a role in the progression of this disease. Mitochondria are unique organelles that possess their own genome. Individuals can be categorized by their mitochondrial DNA (mtDNA) into haplogroups, based on specific sets of ancestrally-derived single nucleotide polymorphisms (SNPs).[7] Some of these sets of SNPs modify the activity of protein complexes involved in mitochondrial oxidative phosphorylation (OXPHOS), influencing the risk and severity of certain diseases. For instance, mitochondria with haplogroup M mtDNA have more OXPHOS Complex I activity than those with haplogroup N mtDNA; consequently, mtDNA mutations associated with diseases such as Leber's Hereditary Optic Neuropathy (LHON) are more penetrant in haplogroup N individuals than those of haplogroup M.[8] Within European haplogroups, haplogroups H and J have more tightly-coupled OXPHOS and more mtDNA per cell than the Uk haplogroup; however, this more efficient OXPHOS is associated with increased ROS generation, which is correlated with an increased risk of neurodegenerative disease in individuals of haplogroups H and J.[9,10]

Certain mtDNA mutations and polymorphisms are also known to induce or increase the risk of developing diabetes. The A3243G mutation, an abnormal change not associated with any haplogroup, occurs within the coding sequence for mitochondrial tRNA for leucine and is associated with Mitochondrial Inherited Diabetes and Deafness (MIDD) disorder.[11,12] Patients with this condition show damaged, dysfunctional mitochondria in pancreatic cells, which may contribute to decreases in both exocrine and endocrine function.[13,14] Cells

possessing mitochondria with this mutation demonstrate a preference for glycolysis over oxidative phosphorylation and an increase in reactive oxygen species.[15]

While the A3243G mutation may induce diabetes, certain normally-occurring mtDNA variants may modify the risk of developing diabetes and its complications. For example, diabetic patients of haplogroup H, the most common European haplogroup, are more likely to develop more severe proliferative diabetic retinopathy (PDR), characterized by increased retinal neovascularization. However, patients of haplogroup Uk are at a decreased risk for PDR.[16] A large study performed by the Wallace laboratory in Taiwainese individuals showed that the N9a, F4, and D5 haplogroups are at much lower odds of having diabetes than others, suggesting that these haplogroups may be protective.[17]

Diabetic retinopathy (DR) is the leading cause of blindness in adults under the age of 65.[18] In diabetic macular edema (DME), one form of DR, fluid accumulates in the neural retina, particularly in the fovea due to breakdown of the blood-retina barrier (BRB). One component of the BRB is the retinal endothelial cells (RECs) lining the vessels of the inner retina and forming a tight barrier governing molecular flow to retinal cells. The second barrier component, between the choroid vasculature and the outer retina, is formed by tight junctions of the retinal pigment epithelial (RPE) cells. However, in DME, this collection of barriers weakens, allowing fluid, protein, and lipoprotein from blood into the layers of the retina.[19]

A major factor for DR is an increase in vascular endothelial growth factors (VEGFs). Members of the VEGF family, particularly VEGF-A, are found at increased levels in the eyes of DR patients as compared to eyes of healthy individuals.[20,21] In response to the ischemic diabetic environment, RPE cells, Müller glia, and retinal ganglion cells produce VEGF.[22] Upregulated VEGF signaling not only induces the production of new, leaky blood vessels but also increases permeability of the RPE layer.[23,24] To combat this, clinicians administer soluble (decoy) VEGF receptors or anti-VEGF antibodies into the vitreous of diabetic patients in order to significantly improve visual function and decrease neural retina thickness.[25–27] Despite this revolution in the treatment of DR, a remarkable percentage of diabetic patient eyes, between 40–50%, are resistant to anti-VEGF therapy.[28,29] It is therefore critical to characterize other cellular and molecular targets that govern blood-retina barrier (BRB) function in order to better treat DR and DME.

Although most DR and DME studies have focused on RECs and pericytes lining retinal vessels, more recently researchers have become more interested in the role of RPE cells in DME. Among other functions, the RPE transports nutrients from the choroidal vessels to the rod and cone photoreceptors and breaks down degraded outer segments of photoreceptors. While clinical and scientific data suggested that RPE function is compromised in DR, it has been difficult to determine whether DME leakage was due to an increase in RPE permeability or an inability to compensate for leakage of the inner retinal vasculature.[30,31] However, injections of small FITC-dextran molecules in a diabetic mouse model have shown both the retinal vasculature and the RPE leak, demonstrating that the RPE contributes to fluid accumulation in DR and DME.[32]

To isolate the mitochondrial influence on disease, our laboratory has developed a cybrid (cytoplasmic hybrid) model. To generate cybrids, cells from a parent line are treated with low-dose ethidium bromide to result in mtDNA disruption and degradation while keeping nuclear DNA intact, resulting in mitochondria-free $Rho_0$ cells. These cells are then fused with platelets, which contain mitochondria and mtDNA, but no nuclear DNA, to form cybrids.[33] Since the resulting cybrids have the same nuclear DNA but mitochondria from different individuals, the biochemical and molecular changes seen in culture are due to the modulation by the mitochondria. Cybrids are an excellent system to investigate retrograde signaling (from mitochondria to nuclei) and mitochondrial influences on cell behavior. Using this model, our laboratory has discovered that mitochondria of specific haplogroups modify transcription of factors involved in inflammation, complement activation, and apoptosis that confer risks of developing retinal diseases such as AMD.[34–38]

In this study, RPE cybrids with mitochondria from diabetic (DM) or non-diabetic (Non-DM) patients of European (Euro) or African and Asian ([Afr+Asi]) haplogroups were generated and analyzed to determine if the mtDNA genetic background and the diabetic status affected RPE cell homeostasis. Our results show that cybrids containing mitochondria from African and Asian diabetic subjects have increased resistance to both high glucose and hypoxia along with altered methylation patterns compared to Euro/DM cybrids. Significantly, our findings suggest that mitochondria from [Afr+Asi] subjects are modified in a manner consistent with a „molecular memory' that can be transferred to other cells *in vitro* and may confer adaptive advantages to cells. Future studies are needed to further understand the mechanisms of the „molecular memory' and its role in diabetic pathology.

## 2. MATERIALS AND METHODS

### 2.1 Human Subjects

All research involving human patients was approved by the University of California, Irvine's Institutional Review Board (#2003–3131). Written, informed consent from all enrolled patients was obtained. Clinical investigations were conducted as per the ethical principles in the Declaration of Helsinki.[39]

### 2.2 Cybrid Generation

Patient platelets were collected in tubes containing 3.2% sodium citrate, isolated by several centrifugations, and the resulting pellets were resuspended in Tris buffered saline (TBS). ARPE-19 cells, a spontaneously immortalized RPE cell line (American Type Culture Collection (ATCC), Manassas, VA), were depleted of mtDNA ($Rho_0$) through serial passages in low-dose ethidium bromide and then cultured in standard culture media (10% FBS, 100 units/mL Penicillin, 100 μg/mL Streptomycin, 25 μg/mL Fungizone (Amphotericin B: Omega Scientific, Torzana, CA), and 50 μg/mL Gentamycin in DMEM/F12) supplemented with uridine.[40] Cybrids were generated by polyethylene glycol-mediated fusion of platelets with $Rho_0$ cells according to modified procedures used by Chomyn *et al.*[41] Integration of the mitochondria into the $Rho_0$ ARPE-19 cells was verified through a combination of polymerase chain reaction (PCR), restriction enzyme digestion of these PCR products, and mtDNA sequencing to determine the haplogroups of the

mitochondria in each cybrid.[42] The cohorts of Euro and [Afr+Asi] cybrids were generated from individuals of similar ages (p = 0.61), genders, and DM versus Non-DM status (Table 1). Within the Euro and [Afr+Asi] cohorts, cybrids were paired by haplogroup and age within 12 years, but not gender matched (boxed). Clinical information on the diabetic patients from whom we acquired mitochondria show that most subjects had type 2 diabetes, and the majority had some form of diabetic retinopathy (Table 2). When we first constructed our cybrid library, we only had access to this limited clinical information.

In the present study, our cybrids were cultured in a standard DMEM:F12 media similar to the recommended growth media for ARPE-19 cells according to ATCC. The recommended growth media contains 3.151 g/L, or ~17.5 mM D-Glucose.[43,44] When our cybrids were cultured in media containing 5.5 mM glucose, they depicted poor growth rates and cell viability (data not shown).

### 2.3 High Glucose and Cobalt Chloride Metabolic Activity Assays

For each experiment, cells from a diabetic cybrid line and a non-diabetic, age-matched, and general haplogroup-matched cybrid line were simultaneously grown in similar treatment conditions. Cells were plated at 20,000 cells/well in a 96-well plate and incubated overnight at 37°C with 5% $CO_2$. For each sample, cultures were run as sextuplets. Experiments were repeated three times.

**2.3.1. High Glucose ——**Cybrids(n = 3 Euro/Non-DM and Euro/DM, 4 [Afr+Asi]/Non-DM and [Afr+Asi] DM) were cultured for 48 hours in standard media (17.5 mM glucose), medium glucose media (32.5 mM glucose, ~2x higher than standard), or high glucose media (62.5 mM glucose, ~4x higher than standard) with a final volume of 100 μL per well. Since a variety of D-glucose concentrations (25 mM and greater) have been used as diabetic conditions in the literature, we chose 32.5 mM (~2x) and 62.5 mM (~4x) as our higher glucose conditions.[45]

**2.3.2. Cobalt Chloride ——**Cybrids (n = 3 Euro/Non-DM and Euro/DM, 4 [Afr+Asi]/Non-DM and [Afr+Asi] DM) were cultured for 48 hours in standard media supplemented with 0 μM, 25 μM, or 50 μM cobalt(II) chloride ($CoCl_2$, MilliporeSigma, St. Louis, MO) with a final volume of 100 μL per well.

**2.3.3. MTT Assay ——**After incubation, 10 μL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Biotium, Hayward, CA) was added to each well for 2 hours, and then the reaction was quenched by adding 200 μL DMSO. The concentration of MTT in the stock solution of Biotium's reagent is proprietary. However, the manufacturer's protocol indicates that the reagent should be diluted 1:10, as was done in the original protocol by Mosmann, suggesting that the MTT concentration in the well is approximately 0.5 mg/mL.[46] The plates were read at 570 nm (MTT) and at 630 nm (background) using an absorbance reader (Biotek Elx808 Absorbance Reader, Winooski, VT). Background was then subtracted from the MTT values. Within each cybrid line, values were normalized to the average standard glucose media value.

### 2.4 High Glucose and Hypoxia Live Cell Assays

For each experiment, cells from a diabetic cybrid line and a non-diabetic, age-matched, and general haplogroup-matched cybrid line were simultaneously grown in similar treatment conditions. Cells were plated at 500,000 cells/well in a 6-well plate and incubated overnight at 37°C, 5% $CO_2$. For each sample, cultures were run as triplicates.

**2.4.1. High Glucose ——**Cybrids (n = 3 Euro/Non-DM and Euro/DM, n = 4 [Afr+Asi]/Non-DM and [Afr+Asi]/DM) were cultured for 48 hours in standard media or high-glucose (~4x higher than standard) media with a final volume of 2 mL per well.

**2.4.2. Hypoxia ——**Cybrids (n = 6 Euro/Non-DM, n = 4 Euro/DM, n = 6 [Afr+Asi]/Non-DM, n = 4 [Afr+Asi]/DM) were cultured for 48 hours in 2 mL standard media at 37°C with 5% $CO_2$ in either room air (~21% $O_2$) or 2% $O_2$ (MCO-18M $O_2/CO_2$ Incubator, Sanyo, Osaka, Japan). A level of 2% $O_2$ was chosen because it induces sublethal cell stress, as has been shown by others.[47,48] Based on animal data and diffusion modeling, the RPE is exposed to 50–60 mm Hg $O_2$. The 2% $O_2$ used in our cybrid studies represents less than 1/3 of this level.[49] Experiments were repeated twice.

**2.4.3. Trypan Blue Assay ——**After incubation, cells from each well were trypsinized and collected separately. Cells were pelleted by centrifugation at 200 x g, subsequently resuspended in 500 μL standard media and transferred to a sample cup. Numbers of viable cells were counted using a ViCell Cell Viability Analyzer (Beckman Coultier, Brea, CA) using the trypan blue dye-exclusion method. Trypan blue is a diazo-dye that stains dead cells blue. This dye cannot permeate intact cell membranes, thus excluding live cells. An average of 50 images was acquired for each sample.

### 2.5. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

**2.5.1. *RNA Extraction & cDNA Generation* ——**Euro/Non-DM, Euro/DM, [Afr+Asi]/Non-DM, and [Afr+Asi]/DM cybrids (n = 4 per group) were grown in duplicate in 6-well plates at 500,000 cells/well and incubated overnight at 37°C with 5% $CO_2$. Cells were then cultured in in 2 mL standard media at 37°C with 5% $CO_2$ in either room air (~21% $O_2$) or 2% $O_2$ for 48 h. For each experiment, diabetic and non-diabetic cybrid cells were matched for age and haplogroup, and were simultaneously grown in similar treatment conditions. Duplicate cultures grown in identical conditions were combined and pelleted by centrifugation before isolating RNA using a PureLink RNA Mini Kit (Invitrogen, Waltham, MA) as per the manufacturer's protocol. RNA was quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and reverse-transcribed into cDNA using the Superscript IV VILO Master Mix with ezDNase Enzyme (Invitrogen) as per manufacturer's protocol.

**2.5.2. qRT-PCR Analyses to Measure Gene Expression Levels ——**qRT-PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA) to examine transcripts of angiogenesis, mitogenesis, apoptosis, and DNA methylation genes. Samples (Euro/Non-DM n = 4, Euro/Non-DM n = 4, [Afr+Asi]/Non-DM n = 3–4, [Afr+Asi]/DM n = 3–4) were analyzed in triplicate, using Power SYBR Green Master Mix

(Applied Biosystems) and primers representing specific pathways (KicqStart, MilliporeSigma and Quantitect, Qiagen, Germantown, MD) **as listed in** Supplementary Table ST1. *HPRT1* was used as the housekeeping gene for all experiments. qRT-PCR data was analyzed by the ΔΔCt method where ΔCt = [Ct (threshold value) of the target gene] – [Ct for *HPRT1*], and ΔΔCt = ΔCt of the treatment condition (hypoxia) – ΔCt of the untreated condition. Fold changes in transcript compared to the untreated condition were calculated as follows: fold change = $2^{-\Delta\Delta Ct}$.

## 2.6. Analysis of Bioenergetics

Bioenergetic profiles of Euro/Non-DM (n = 4), Euro/DM (n = 4–5), [Afr+Asi]/Non-DM (n = 4), and [Afr+Asi]/DM cybrids (n = 4–5) were determined by measuring their oxygen consumption rates (OCR) via a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). Cybrids were plated at a density of 50,000 cells/well in quintets and grown overnight at 37°C with 5% $CO_2$. Cybrids were then washed with XF Running Media (unbuffered DMEM, 17.5 mM D-Glucose (MilliporeSigma), 200 mM L-glutamine (Invitrogen) and 10 mM sodium pyruvate (Invitrogen), pH 7.4) and then grown in XF Running Media for 1 h at 37°C without added $CO_2$.

In the Seahorse XF24 Extracellular Flux Analyzer, drugs were sequentially injected into the wells as follows: First, 1 μM Oligomycin (inhibits ATP synthase), then 1 μM FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, uncouples the inner mitochondrial membrane to induce maximum respiration), and finally 1 μM Antimycin A plus 1 μM rotenone (inhibitors of Complexes III and I of the electron transport chain, respectively, that halt all mitochondrial respiration). Values were normalized to protein content per well. Total protein was harvested from each well in 40 μL of RIPA (Radioimmunoprecipitation Assay) buffer (MilliporeSigma) containing protease inhibitor (MilliporeSigma) and Phosphatase Arrest (Gbiosciences, St. Louis, MO) overnight at 4°C. After brief centrifugation, lysates were mixed with QuBit Assay Buffer (Invitrogen) and protein content was measured in a QuBit 2.0 fluorimeter (Invitrogen). Data was collected and processed using Wave 2.6 software (Seahorse Biosciences). Bioenergetic characteristics were calculated as follows: For ATP Production: 100% * ([Baseline OCR] - [OCR After Oligomycin]) / [Baseline OCR], *which measures the percent of OCR used to generate ATP through the ATP synthase via the electron transport chain*. For Spare Respiratory Capacity: 100% * ([OCR After FCCP] - [Baseline OCR]) / [Baseline OCR], *which measures the percent of additional OCR available in response to bioenergetic stresses*. For Proton Leak: 100% * ([OCR After Oligomycin] - [OCR After Antimycin A and Rotenone]) / [Baseline OCR], which measures the percent of OCR lost to protons leaking across the inner mitochondrial membrane, down the electrochemical gradient, but not moving through the ATP synthase.

## 2.7. Total Global Methylation

Total DNA methylation in Euro/Non-DM (n = 5), Euro/DM (n = 4), [Afr+Asi]/Non-DM (n = 3), and [Afr+Asi]/DM cybrids (n = 4) was measured using the MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Epigentek, Farmington, NY) according to the manufacturer's protocol.

Cybrids were cultured 48 hours in standard media supplemented with 0 μM or 250 μM 5-Aza-dC (MilliporeSigma), an inhibitor of DNA methyltransferases. Media was changed daily. Subsequently, cells were trypsinized and pelleted, and finally DNA was extracted from the cultures using the DNeasy Blood & Tissue Kit (Qiagen), as per the manufacturer's instructions.

For the ELISA assay, 100 ng of sample DNA was bound to strip wells and incubated with an anti-5-methylcytosine (anti-5-mC) antibody. After washing and adding developer reagent, enzymes on bound antibodies induce a chemiluminescent reaction. The products of these reactions were quantified by absorbance on a plate reader at 450 nm. OD values were converted to %5-mC through calculations described in the manufacturer's protocol. Samples were run in duplicate.

### 2.8. Statistical Analysis

Experimental data were compared using Unpaired Student's *t*-test or One-Way ANOVA with Bonferonni's Post-Test (GraphPad Prism, version 5.0, GraphPad Software, CA). p-values were adjusted using a Bonferonni correction. Raw p-values were multiplied by the number of comparisons in an experiment to get adjusted (reported) p-values. Data on the T16189C polymorphism was compared using the Pearson Chi-Squared test through the Simple Interactive Statistical Analysis website (SISA; http://www.quantitativeskills.com/sisa/index.htm). Results with an adjusted $p \leq 0.05$ were considered statistically significant.

## 3. RESULTS

### 3.1. [Afr+Asi]/DM cybrids are resistant to hyperglycemic stress

Since diabetes is characterized by hyperglycemia, cybrids were first challenged with either 2x (medium glucose) or 4x (high glucose) glucose compared to the standard culture media (standard glucose; normalized to 1 for each group). There were significant decreases in metabolic activity (mean ± SEM) in medium and high glucose for Euro/Non-DM cybrids (medium glucose: 0.76 ± 0.02 relative metabolic activity of standard glucose, $p < 0.0006$; high glucose: 0.65 ± 0.02, $p < 0.0006$), and Euro/DM cybrids (medium glucose: 0.82 ± 0.02, $p = 0.0048$; high glucose: 0.72 ± 0.02, $p < 0.0006$) compared to the standard glucose-treated samples (Fig. 1A). The [Afr+Asi]/Non-DM cybrids also had decreased metabolic activity compared to the standard glucose-treated samples (medium glucose: 0.82 ± 0.02, $p < 0.0006$; high glucose: 0.72 ± 0.02, $p < 0.0006$). In contrast, the metabolic activity of [Afr+Asi]/DM cybrids did not significantly change in medium glucose (0.93 ± 0.02, $p = 0.086$) or high glucose (0.93 ± 0.02, $p = 0.070$) as compared to the standard glucose-treated samples (Fig. 1B). [Afr+Asi]/Non-DM cybrids had significantly lower viability than similarly-treated [Afr+Asi]/DM cybrids at both medium glucose ($p < 0.0006$) and high glucose ($p < 0.0006$). Euro/Non-DM cybrids treated with medium glucose had no significant difference in metabolic activity compared to similarly-treated Euro/DM cybrids ($p = 0.17$) while Euro/Non-DM cybrids treated with high glucose had a statistically significant increase in metabolic activity compared to similarly-treated Euro/DM cybrids ($p = 0.018$) (Figs. 1A, 1B).

Next, we compared these cybrids using the trypan blue dye exclusion assay. The ratio of live cells in a treatment condition to live cells in a standard condition reflects cell viability. We discovered that high glucose did not significantly decrease live cell numbers in [Afr +Asi]/DM cybrids compared to cells grown in standard glucose (0.90 ± 0.04 relative ratio of live cells of untreated, $p = 0.74$) while there were significant decreases in live cell numbers for [Afr+Asi]/Non-DM cybrids (0.77 ± 0.03, $p < 0.0006$), Euro/Non-DM cybrids (0.84 ± 0.04, $p = 0.036$), and Euro/DM cybrids (0.86 ± 0.02, $p = 0.0024$) compared to cells grown in standard glucose. The relative viable cell ratio for Non-DM cybrids treated with high glucose was not significantly different from than that of DM cybrids in high glucose for both Euro ($p \approx 1$) and Afri+Asi groups ($p = 0.29$) (Fig. 1C).

Experiments comparing treatment of ARPE-19 cells with high mannitol (60 mM, 17.5 mM glucose) versus high glucose for 48 hours reveal that mannitol induces a significant decrease in metabolic activity measured by MTT (Mannitol: 0.79 ± 0.06, $p = 0.019$; Glucose: 0.89 ± 0.04, $p = 0.10$), suggesting that high osmolarity affects metabolic activity (Fig. S7A). However, mannitol did not induce a decrease in live cell numbers measured by trypan blue dye-exclusion assay while high glucose did (Mannitol: 1.00 ± 0.03, $p = 0.89$; Glucose: 0.91 ± 0.02, $p = 0.0025$) (Fig. S7B). Overall, these findings suggest that the [Afr+Asi]/DM cybrids uniquely resist hyperglycemic stresses.

### 3.2. [Afr+Asi]/DM cybrids are resistant to hypoxic stress

While the [Afr+Asi]/DM cybrids show resistance to decreases in metabolic activity after exposure to high glucose, we speculated that the cybrids may be more vulnerable to other types of stressors in the process, such as hypoxic stress that is often associated with diabetes. To test this, cybrids were grown in the presence or absence of cobalt chloride ($CoCl_2$), a chemical used to mimic the hypoxic state. MTT assay data revealed that at the highest (50 μM) concentrations of $CoCl_2$, metabolic activity did not significantly decrease for Euro/DM cybrids (0.98 ± 0.01, $p = 0.85$) but did significantly decrease for [Afr+Asi]/DM cybrids (0.93 ± 0.01, $p < 0.0006$), Euro/Non-DM cybrids (0.91 ± 0.02, $p < 0.0006$) and [Afr+Asi]/Non-DM cybrids (0.79 ± 0.01, $p < 0.0006$). Interestingly, at 50 μM $CoCl_2$, metabolic activity was significantly increased in DM cybrids compared to Non-DM cybrids for both European ($p < 0.0006$) and African and Asian groups ($p < 0.0006$). Lower levels (25 μM) of $CoCl_2$ significantly decreased metabolic activity for all cybrids (Euro/Non-DM: 0.88 ± 0.01, $p < 0.0006$; Euro/DM: 0.91 ± 0.02, $p < 0.0006$; [Afr+Asi]/Non-DM: 0.89 ± 0.01, $p < 0.0006$; [Afr+Asi]/DM: 0.88 ± 0.01, $p < 0.0006$). These levels were not significantly different from those of similarly-treated European ($p = 0.63$) or African and Asian groups ($p \approx 1$) (Fig. 2A,B).

To further investigate the response to hypoxia, cybrids were cultured in either room air or 2% oxygen and numbers of live cells were measured using the trypan blue dye exclusion assay. The [Afr+Asi] cybrids grown in 2% oxygen significantly increased in live cell counts compared to similar cultures grown in room air (Non-DM: 1.20 ± 0.04, $p < 0.0004$; DM: 1.14 ± 0.04, $p = 0.0024$) (Fig. 2D). There were no significant changes for Euro cybrids (Non-DM: 0.99 ± 0.03, $p \approx 1$; DM: 0.99 ± 0.02, $p \approx 1$) (Fig. 2C). In both experiments, only

the [Afr+Asi]/DM cybrids thrived in hypoxic conditions, suggesting that the [Afr+Asi]/DM cybrids resist hypoxic stress in addition to hyperglycemic stress.

### 3.3. [Afr+Asi]/DM cybrids have significant differential expression of *BAX* but not mitogenic markers

In response to the ischemic diabetic environment, RPE cells, Müller glia, and retinal ganglion cells produce VEGF.[22] To determine if our cybrids behave similarly, we performed qRT-PCR for *VEGFA*, finding that all cybrids significantly increased expression of *VEGFA* in hypoxic conditions compared to cybrids grown in room air. Gene expression in the room air cultures was assigned a value of 1. The VEGF transcript levels were increased in Euro/Non-DM cybrids (4.42 ± 0.24-fold, $p < 0.0004$), Euro/DM cybrids (4.41 ± 0.65 -fold, $p = 0.0076$), [Afr+Asi]/Non-DM cybrids (5.50 ± 1.23 -fold, $p = 0.043$), and [Afr +Asi]/DM cybrids (7.19 ± 1.50 -fold, $p = 0.023$) (Fig. 3A). However, *HIF1A* was downregulated for the majority of cybrids grown in hypoxia (Euro/Non-DM: 0.95 ± 0.14 -fold, $p \approx 1$; Euro/DM: 0.53 ± 0.06 -fold, $p = 0.0008$; [Afr+Asi]/Non-DM: 0.52 ± 0.12 -fold, $p = 0.028$; [Afr+Asi]/DM: 0.48 ± 0.10, -fold $p = 0.0056$) (Fig. S8A).

Wild-type ARPE-19 cells grown in 2% oxygen over a 36 hour time-course showed a decrease in *HIF1A* compared to untreated/baseline (6 hours: 0.79 ± 0.03 –fold, $p = 0.022$; 12 hours: 0.56 ± 0.01 – fold, $p < 0.0004$; 24 hours: 0.48 ± 0.01 –fold, $p < 0.0004$; 36 hours: 0.58 ± 0.02, -fold, $p < 0.0004$) (Fig. S8B). In contrast, after 12 hours of incubation in 2% oxygen, *VEGFA* levels increased compared to untreated/baseline (6 hours: 0.91 ± 0.08 – fold, $p \approx 1$; 12 hours: 1.74 ± 0.05 -fold, $p = 0.0020$; 24 hours: 3.92 ± 0.27 -fold, $p = 0.0020$; 36 hours: 4.55 ± 0.10, -fold $p < 0.0004$) (Fig. S8C).

“Next, we performed qRT-PCR with representative markers of molecular pathways influenced by mitochondria. Only the [Afr+Asi]/DM cybrids showed a significant decrease in expression of pro-apoptotic indicator *BAX* in hypoxic conditions (0.84 ± 0.02 -fold, $p = 0.002$) while the [Afr+Asi]/Non-DM cybrids showed a non-significant lower level in *BAX* (0.84 ± 0.10 -fold, $p = 0.073$). Remarkably, the European cybrids showed no significant change in *BAX* expression levels (Euro/Non-DM: 0.96 ± 0.10 -fold, $p \approx 1$; Euro/DM: 1.01 ± 0.18 -fold, $p \approx 1$) (Fig. 3B). However, all cybrids grown in hypoxia demonstrated a decrease in expression of *PPARGC1A*, a master mitochondrial biogenesis regulator, compared with cultures grown in room air (Euro/Non-DM: 0.12 ± 0.01 -fold, $p < 0.0004$; Euro/DM: 0.31 ± 0.11 -fold, $p = 0.0024$; [Afr+Asi]/Non-DM: 0.09 ± 0.02 -fold, $p < 0.0004$; [Afr+Asi]/DM: 0.16 ± 0.04, fold $p < 0.0004$) (Fig. 3C). *TFAM*, a transcription factor downstream of PGC-1α that regulates mtDNA copy number and transcription, was not significantly altered in any of the cybrids grown in 2% hypoxic conditions (Euro/Non-DM: 1.10 ± 0.08 –fold; Euro/DM: 1.13 ± 0.19 -fold; [Afr+Asi]/Non-DM: 0.98 ± 0.06 -fold; [Afr+Asi]/DM: 0.97 ± 0.11 -fold; all $p \approx 1$) (Fig. 3D). Moreover, the mitochondrial copy numbers of Non-DM and DM cybrids were not significantly different for both groups of cybrids (Euro/DM: 0.84 ± 0.19 – fold of Euro/Non-DM, $p = 0.38$; [Afr+Asi]/DM: 0.98 ± 0.13 – fold of [Afr+Asi]/DM, $p = 0.89$) (Fig. S9). Overall, these data suggest that Non/Euro DM mitochondria are unique in that they induce downregulation of *BAX* but not mitogenic genes in response to hypoxic stress.

### 3.4. [Afr+Asi] and Euro cybrids have similar mitochondrial bioenergetics profiles.

The XF24 Extracellular Flux Analyzer was employed to determine differences in mitochondrial OXPHOS between Euro and [Afr+Asi] cybrids. In general, the bioenergetic profiles of DM and Non-DM cybrids were similar between groups. The amount of OCR used for ATP production was lower in DM cybrids as compared to their Non-DM cybrid counterparts (Euro/Non-DM: 41.55 ± 3.74 % of baseline OCR vs. Euro/DM: 25.65 ± 5.19, p = 0.15; [Afr+Asi]/Non-DM: 44.87 ± 7.75 vs. [Afr+Asi]/DM: 29.56 ± 7.61, p = 0.62) (Fig. 4A). In addition, the DM cybrids showed substantially less spare respiratory capacity compared to the Non-DM (Euro/Non-DM: 39.56 ± 6.02 % vs. Euro/DM: 10.31 ± 20.09, p = 0.75; [Afr+Asi]/Non-DM: 45.19 ± 12.13 vs. [Afr+Asi]/DM: 9.40 ± 16.96, p = 0.46) (Fig. 4B). Moreover, DM cybrids showed increased levels of proton leak compared to the Non-DM cybrids. (Euro/Non-DM: 17.58 ± 3.84 % vs. Euro/DM: 26.69 ± 4.41, p = 0.51; [Afr +Asi]/Non-DM: 18.99 ± 4.18 vs. [Afr+Asi]/DM: 24.17 ± 4.54, p ≈ 1) (Fig. 4C). In summary, the changes in bioenergetic measurements between DM and Non-DM cybrids are similar for European and [African plus Asian] cybrids.

### 3.5. [Afr+Asi]/DM Cybrids resist decreases in global DNA methylation from DNA methyltransferase inhibition

The total global methylation levels were compared by culturing cybrids at room air conditions in the presence or absence of a DNMT1 inhibitor, 5-aza-dC (Figure 5). Compared to untreated controls (assigned value at 100%), we found that the DNA was demethylated to a similar degree in 5-Aza-dC-treated Euro/Non-DM and Euro/DM cultures (Euro/Non-DM: 70.8 ± 11.9 % versus Euro/DM: 68.6 ± 11.0%, p ≈ 1). The 5-aza-dC treated [Afr+Asi]/Non-DM cultures showed a large decrease in total global DNA methylation (31.0 ± 18.3 %) compared to untreated. In contrast, [Afr+Asi]/DM cultures were not significantly affected by 5-aza-dC treatment (98.4 ± 11.5%) compared to untreated, indicating unique resistance to DNA demethylation in the presence of 5-Aza-dC, a known DNA methyltransferase inhibitor. The total global methylation levels in [Afr+Asi]/Non-DM cybrids were significantly lower after 5-aza-dC treatment compared to [Afr+Asi]/DM cybrids (p = 0.044). These data suggest that [Afr+Asi]/DM cybrids resist demethylation from 5-Aza-dC.

To determine if our cybrids differentially express DNA methylation factors, we performed qRT-PCR for several methyltransferases and methylation co-factors. All hypoxia-treated cybrids showed a decrease in the DNA methyltransferase *DNMT1* (Euro/Non-DM: 0.41 ± 0.06 -fold, p < 0.0001; Euro/DM: 0.49 ± 0.05 -fold, p < 0.0001; [Afr+Asi]/Non-DM: 0.46 ± 0.04 -fold, p < 0.0001; [Afr+Asi]/DM: 0.54 ± 0.10 -fold, p = 0.0025) (Fig. 6A). Only the Euro/Non-DM cybrids had a decrease in the S-adenylmethionine synthase *MAT2B* (Euro/Non-DM: 0.73 ± 0.07 -fold, p = 0.0091; Euro/DM: 1.02 ± 0.10 -fold, p = 0.85; [Afr+Asi]/Non-DM: 0.95 ± 0.07 -fold, p = 0.48; [Afr+Asi]/DM: 1.21 ± 0.12 -fold, p = 0.12) (Fig. 6D). Interestingly, the hypoxia-treated [Afr+Asi]/DM cybrids showed a consistently significant increase in DNA methylation-related genes compared to untreated cultures: the DNA methyltransferases *DNMT3A* (Euro/Non-DM: 1.24 ± 0.16 -fold, p = 0.18; Euro/DM: 1.54 ± 0.20 -fold, p = 0.037; [Afr+Asi]/Non-DM:

1.43 ± 0.28 -fold, p = 0.18; [Afr+Asi]/DM: 2.10 ± 0.37 -fold, p = 0.025) (Fig. 6B) and *DNMT3B* (Euro/Non-DM: 1.44 ± 0.29 -fold, p = 0.18; Euro/DM: 2.11 ± 0.48 -fold, p = 0.060; [Afr+Asi]/Non-DM: 1.09 ± 0.02 -fold, p = 0.0011; [Afr+Asi]/DM: 2.67 ± 0.56 -fold, p = 0.025) (Fig. 6C). In addition, hypoxia-treated [Afr+Asi]/DM cybrids had increased transcript levels of the methyltransferase-recruiting scaffold protein *MBD2* (Euro/Non-DM: 1.34 ± 0.18 -fold, p = 0.097; Euro/DM: 1.49 ± 0.17 -fold, p = 0.028; [Afr+Asi]/Non-DM: 1.54 ± 0.24 -fold, p = 0.068; [Afr+Asi]/DM: 1.71 ± 0.11 -fold, p = 0.0006) (Fig. 6E) and the RNA methyltransferase *TRDMTs1* (Euro/Non-DM: 1.09 ± 0.07 -fold, p = 0.23; Euro/DM: 1.38 ± 0.05 -fold, p < 0.0001; [Afr+Asi]/Non-DM: 1.24 ± 0.07 -fold, p = 0.016; [Afr +Asi]/DM: 1.82 ± 0.26 -fold, p = 0.021) (Fig. 6F), suggesting that the distinct responses of these cybrids to stress are due to differential regulation of DNA methylation.

### 3.6. The behavior of our cybrids is not likely due to the presence of the diabetes-associated T16189C polymorphism.

Certain mtDNA polymorphisms that do not affect the structure of RNA and protein products may predispose an individual to disease. In particular, the T16189C mutation has been highly associated with type 2 diabetes in Asian and Caucasian subjects.[50,51] A meta-analysis suggested that this polymorphism may not be associated with type 2 diabetes in African populations.[52] We examined the mtDNA sequences of our cybrids to determine if this polymorphism is more prevalent in our diabetic cybrids than our non-diabetic cybrids. We found that, of the DM cybrids for which we had information, 3 out of 8 had 16189C while the others were 16189T (Table 3). Of those, 1 out of 4 Euro/DM cybrids and 2 out of 4 [Afr+Asi]/DM cybrids had this polymorphism. Within our cybrids, the association of this polymorphism and diabetic status was not statistically significant (p = 0.84), suggesting that the T16189C mutation is unlikely to account for the distinct behavior of our cybrids.

## 4. DISCUSSION

In this study, we investigated the effects on cell viability of diabetic mitochondria from subjects of European or African and Asian ancestry using RPE cybrid cells. Our results showed that mitochondria isolated from African and Asian diabetic patients conferred resistance against hyperglycemic and hypoxic stresses and could induce unique transcriptional changes. Cybrid models have been used to compare mitochondria from individuals with the major diabetes-susceptible versus diabetes-resistant haplogroups[53,54] and from individuals with known mtDNA mutations associated with diabetes (A3243G).[55,56] However, to our knowledge, this is the first study to use cybrids to determine the effect of these mitochondria on how the cell lines respond to stress.

Two of the major stresses promoting retinal pathology in diabetes are hyperglycemia and hypoxia. Earlier studies revealed a decrease in ARPE-19 cell viability in response to high glucose conditions.[57–59] In this study, we found that Euro/Non-DM, Euro/DM, and [Afr +Asi]/Non-DM cybrids significantly decreased in metabolic activity when treated for 48 hours with high glucose. In contrast, the [Afr+Asi]/DM cybrids did not have a significant decline in viability in high glucose. This property may arise from differences in metabolic processing of glucose between individuals with [[Afr+Asi]] mtDNA versus Euro mtDNA.

For instance, one study found that non-obese African-American women had lower insulin sensitivity and mitochondrial content in skeletal muscle compared to Caucasian women. These measures were also correlated to mitochondrial oxidative capacity, which was lower in the African-American cohort.[60] Previous cybrid studies have shown that mitochondria with L haplogroup mtDNA (of African descent) have lower oxidative capacity and express mtDNA transcripts more efficiently than H haplogroup cybrids.[61] These data, along with our findings, suggest that cybrids containing [Afr+Asi] mtDNA may have greater metabolic flexibility. Additionally, our cybrid studies suggest that the mtDNA variants and adaptations distinguishing Euro versus [Afr+Asi] subjects are incorporated into the mitochondria and transferable into recipient $Rho_0$ cells *in vitro*, implying a “metabolic memory” of previous conditions.

In addition to high blood glucose, diabetes induces a hypoxic microenvironment for RPE and neural retinal cells. Previous studies demonstrated that ARPE-19 cells decrease in cell viability in response to hypoxia and chemicals that mimic hypoxia.[62,63,47] In this study, we found that Non-DM cybrids (both Euro and [Afr+Asi]) and [Afr+Asi]/DM cybrids showed significant decreases in cell viability from high $CoCl_2$ while Euro/DM cybrids did not. However, when analyzed by trypan blue assay, [Afr+Asi] cybrids (both DM and Non-DM) showed significant increases in viable cells grown in 2% $O_2$ while Euro cybrids (both DM and Non-DM) showed no change. These results may differ from each other for technical reasons. The MTT assay measures NAD(P)H-oxidoreductase activity, which measures metabolic activity and is thought to represent the number of viable cells. In contrast, the trypan blue assay measures dye exclusion and accurately represents live versus dead cells[64] $CoCl_2$ induces Hif1α and Hif2α activity, which is only a part of the cellular processes that occur in physical hypoxia. [65] In contrast, incubation in 2% oxygen achieves the multiple aspects of hypoxia and is a more accurate stressor. Overall, only the [Afr+Asi]/DM cybrids showed resistance to high $CoCl_2$ compared to their Non-DM counterparts and thrived in low oxygen conditions. Since the nuclear DNA in all of our cybrids is the same, these data suggest that [Afr+Asi]/DM mitochondria uniquely confer resistance to hyperglycemia and hypoxia.

One explanation of this property could be that the [Afr+Asi]/DM mitochondria originated from hypoxia-adapted populations. For instance, the Sherpa have adapted to higher altitudes with lower oxygen through improved efficiency in coupling mitochondrial oxygen consumption to energy production through OXPHOS. However, they had a lower capacity for generating energy from fatty acids.[66] Alternatively, it may be that the [Afr+Asi]/DM mitochondria are more capable of shifting their threshold for hypoxia tolerance than [Afr+Asi] mitochondria. To illustrate, a Chinese population study showed that D4 haplogroup individuals were more resistant to developing pulmonary edema at high altitudes while the B haplogroup subjects had a much greater risk of pulmonary edema.[67] These data support the concept that the mtDNA plays a role in how well individuals can adjust to sustained decreases in oxygen levels.

To determine possible mechanisms for this property, qRT-PCR on RNA from cybrids cultured in 2% $O_2$ for 48 hours was performed. Stressors commonly present in the diabetic retinal microenvironment are known to induce apoptosis,[68,69] decrease mitogenesis and

mitochondrial replication,[70] and increase VEGFs to promote neovascularization.[71–73] Consistent with these findings, all groups of cybrids cultured in hypoxia significantly increased expression of VEGF-A transcript. Remarkably, we found that the [Afr+Asi]/DM cybrids were the only group to show a significant decrease in transcript of the pro-apoptotic indicator *BAX* in response to hypoxia, suggesting that [Afr+Asi]/DM mitochondria alter the transcriptome to prevent apoptosis. Interestingly, the [Afr+Asi]/Non-DM cybrids showed a decreased, though not statistically significant, trend in *BAX* expression in hypoxia. These decreases in *BAX* are supported by our findings that numbers of viable cells in [Afr+Asi] cybrids in hypoxic conditions are increased compared to controls. However, in all hypoxia-treated cybrids, the *PPARGC1A* transcript was significantly decreased while levels of *TFAM*, a downstream effector, were unchanged, suggesting that the resistance to hypoxia conferred by [Afr+Asi]/DM mitochondria does not act through mitogenic pathways. Our findings suggest that [Afr+Asi]/DM mitochondria uniquely alter the transcriptome to resist decreases in cell viability from hypoxia.

One way that [Afr+Asi]/DM mitochondria could confer resistance to stress is through improved OXPHOS. Mouse models of diabetes and cells from diabetic patient donors express significantly less mitochondrial transcripts for OXPHOS factors compared to control animals and non-diabetic patient cells, respectively.[74] Mitochondria from retinal cells exposed to high glucose exhibit decreased ATP production and decreased spare respiratory capacity compared to untreated cultures.[75,76] Consistent with these results, we found that both Euro/and [Afr+Asi]/DM cybrids showed decreased trends in oxygen consumption used for ATP production in spare respiratory capacity compared to non-diabetic controls. Additionally, these cybrids showed increased trends in oxygen consumption lost to proton leak, suggesting that all DM mitochondria have decreased efficiency in forming ATP through OXPHOS. Since the DM cybrids demonstrated similar impairments in bioenergetics compared to their respective Non-DM cybrids, these data suggest that the unique response to stress in [Afr+Asi]/DM cybrids (compared to Euro/DM cybrids) does not arise from changes in OXPHOS.

One other possible mechanism for the resistance imbued by [Afr+Asi]/DM mitochondria is through modulation of DNA methylation. One way such information may be encoded in mitochondria is through methylation of mtDNA. The role of mtDNA in mammalian cells is a subject of much controversy. Studies in mouse and human cells suggest that mtDNA can be methylated in certain regions, such as the D-loop, a region that regulates mitochondrial transcription and DNA replication.[77,78] In particular, a study by Almeida and Cheng in primary cells from human patients showed that increases in the HOMA-IR insulin resistance measurement are significantly correlated with increases in DNA methylation of the ND6 and D-loop regions.[79] However, other work indicates that mtDNA methylation in mtDNA may be quite rare, at a level of 2% or less. Such work also suggests that increases in mtDNA methylation may reflect changes in mtDNA secondary structures.[80–82]

One reason why mtDNA methylation may not have been seen in those studies is that the cells were cultured in the absence of stressors. Kowluru and coworkers report that a REC cell line exposed to high glucose showed increased methylation of the D-loop. Chromatin immunoprecipitation also showed elevated DNMT1 association with the D-loop in RECs

cultured in high glucose; if the elevated mtDNA methylation in high glucose were an artifact of DNA supercoiling, DNMT1-binding would have been lower than in the untreated condition. As a result of increased mtDNA methylation, these cells expressed lower transcript levels of OXPHOS-related factors. RECs from patients affected by diabetic retinopathy showed similar decreases in OXPHOS-related transcripts, increases in mtDNA methylation, and increases in DNMT1-binding in the D-loop compared to cells from unaffected patients.[83] Further investigation in RPE cells showed D-loop methylation persisted when these cells were removed from the glycemic challenge.[84] More recent data suggests methylated cytosines in mtDNA of RECs cultured in high glucose can undergo deamination to thymines. This production of base mismatches was absent when cells were cultured in high glucose with 5-aza-2′-deoxycytidine (5-Aza-dC), a DNMT1 inhibitor, suggesting that the process is dependent on DNMTs.[85] These findings suggest that mitochondria have a “metabolic memory” of the retinal diabetic microenvironment.

In our study, the cybrids were cultured in the presence of 5-Aza-dC and total global DNA methylation levels were measured. The Euro/Non-DM, Euro/DM, and [Afr+Asi]/Non-DM cybrids showed decreased DNA methylation (29.2%, 37.8% and 69%, respectively) compared to untreated controls. Surprisingly, [Afr+Asi]/DM cybrids resisted demethylation from 5-Aza-dC, showing only 1.6% decline. These results suggested that [Afr+Asi]/DM mitochondria uniquely promote the preservation of DNA methylation.

To determine if molecular pathways involved in DNA methylation are differentially-expressed in [Afr+Asi]/DM cybrids, the levels of DNMTs and related factors were analyzed. Results showed that *DNMT1* levels were decreased for all cybrids grown in hypoxia, but only the [Afr+Asi]/DM cybrids had significantly elevated expression levels of *DNMT3A*, *DNMT3B*, *MBD2,* and *TRDMT1*. In summary, the presence of [Afr+Asi]/DM mitochondria prevents DNA demethylation and increases transcription of *DNMT3A*, *DNMT3B*, *MBD2*, and *TRDMT1,* which may lead to altered epigenetic regulation of downstream genes in response to hypoxia. Future studies will be necessary to understand the mechanisms involved.

Other studies have also shown that hypoxia upregulates genes promoting DNA methylation and changes in epigenetics. For example, Watson *et al*. found that incubating cardiac fibroblasts in hypoxic conditions resulted in upregulation of *DNMT1* and *DNMT3B*, which were correlated with increased expression of genes associated with fibrosis, such as α-smooth actin and Collagen-1. Treatment with either 5-Aza-dC or small RNA inhibitor of DNMTs prevented the upregulation in these pro-fibrotic genes, suggesting that the increase in pro-fibrotic genes was a consequence of higher DNA methylation levels.[86] DNA methylation has also been associated with ocular diseases such as glaucoma.[87] These data are consistent with our finding that transcripts of certain DNA methylation factors, such as *DNMT3B*, are elevated in hypoxic [Afr+Asi]/DM cultures.

Our methylation results lead us to speculate that the modification(s) of the mitochondria in the [Afr+Asi]/DM patients are retained as a “memory” that can then be transferred into other cells *in vitro*, modulate DNA methylation characteristics and result in altered regulation of downstream genes. The concept of “metabolic memory” in diabetes has been described by

the Kowluru group and others as the collection of beneficial or deleterious adaptations to a previous metabolic environment that persist for an extended period of time after the metabolic environment is changed. The influence of high glucose in diabetes can modify epigenetics for nuclear genes, such as that of insulin, and mtDNA regions.[88,89,85] In our study, we found differences in total global DNA methylation and expression levels of methylation genes. It is reasonable to hypothesize that chronically high glucose could lead to alterations in epigenetic status that can be passed on through mitochondrial influence.

However, our findings could be explained by other mechanisms inherent to the mtDNA. For instance, certain haplogroups may produce more ROS, which can act as signaling molecules, than others, particularly in stress conditions.[9] In addition, different haplogroups may express mitochondrially-derived OXPHOS factors, such as cytochrome b and the NADH dehydrogenase subunit 4, at higher levels than others. While increases in these factors may bolster energy production, such changes may also increase the amount of ROS produced and thus alter the downstream signaling patterns.[34,61] One other way haplogroups may influence function is through biochemical signals from the mitochondria to the rest of the cell, known as retrograde signaling. For instance, we and others have found that small, mitochondrial-derived peptides (MDPs), such as humanin, can protect cells against apoptosis and DNA damage.[90,91] In particular, the Cohen laboratory has shown that protein levels of SHLP2, another MDP, are higher in Caucasians than African-Americans, but low SHLP2 is correlated with increase prostate cancer risk only in Caucasians and not in African-Americans.[92] Another example of mitochondrial modulation of nuclear genomes is that cybrids with normal versus AMD mitochondria express different microRNA levels that modulate expression of angiogenesis, apoptosis and autophagy pathways.[93] While our findings suggest that epigenetic changes are involved, the mechanisms are unclear and will require future investigations.

Given the clinical data that certain minorities are more likely to develop diabetes and diabetic retinopathies than Caucasians, the data from our study are unexpected and paradoxical. However, a similar phenomenon has been shown with the T mtDNA haplogroup, in which diabetic haplogroup T subjects were more likely to have diabetic retinopathy and coronary artery disease compared to diabetic individuals of the H haplogroup.[94] These data implied that T haplogroup mitochondria might be more susceptible to stress. Yet surprisingly, a study using HEK293 cybrids showed that T cybrids had greater cell viability than H cybrids when treated with hydrogen peroxide, a stressor that promotes reactive oxygen species (ROS) formation.[95]

We recognize that there are limitations to this study. First, we performed our experiments *in vitro* using cybrids derived from the ARPE-19 cell line. These cells are widely used as RPE cell models as they express tissue-specific markers, such as CRALBP, BEST1, and RPE65, though the levels of protein products are decreased compared to primary cultures.[96,97] Our lab has shown that our parent cell line also expresses these markers.[98] Functionally, ARPE-19 cells resemble primary RPE cells as they both demonstrate polarized secretion of cytokines in response to IL-1β administration.[99] However, ARPE-19 cells lack certain surface markers, such as claudin-19, resulting in substantially lower barrier strength than that of primary cell cultures.[100] Despite this, we use our cybrid system as an elegant

model to study the specific influence of mitochondria on cellular health and transcription while keeping the nuclear DNA sequence the same between different cybrid lines. Since the same parent, mitochondria-free $Rho_0$ ARPE-19 cell line can be fused with platelets from many different patients to form unique cybrid lines, we have generated a total of 25 cybrid lines from diabetic and non-diabetic patients for this study. It is not feasible to use primary cultures for these experiments due to the cells' limited lifetime *in vitro* and the fact that they have different nuclear DNA, which would confound our ability to examine the specific influence of mitochondria.

The second drawback is that we had limited information on the clinical phenotype. However, there were similar numbers of diabetic individuals with either background retinopathy or no retinopathy (Euro n = 2, [Afr+Asi] n = 3) and non-proliferative diabetic retinopathy (Euro n = 3, [Afr+Asi] n = 2). Unfortunately, the study lacked diabetic subjects with proliferative diabetic retinopathy in either group. While our findings showed significant differences between Euro and [Afr+Asi] cybrids *in vitro*, further studies will be necessary to discern if the differences are due to the mtDNA haplogroups or other clinical parameters that were not taken into account in this study. In future studies, we will expand our cybrid library to include larger numbers of diabetics. In addition, we will gather information about the clinical severity of their retinopathy, the extent of any nephropathy or neuropathy they may have, the presence of any other diabetic complications, and the status of their blood sugar control.

In conclusion, the RPE cybrids containing [Afr+Asi]/DM mitochondria (1) resist decreases in cell viability after exposed to either high glucose or hypoxia; (2) express higher levels of DNA methylation genes; and (3) are resistant to de-methylation by 5-Aza-dC compared to Euro/DM cybrids (Figure 7). These findings support the hypothesis that [Afr+Asi]/DM mitochondria have a "molecular memory" of a prior microenvironment that is transferrable *in vitro* and can modulate DNA methylation mechanisms in [Afr+Asi]/DM cybrids. Future studies will be necessary to characterize the molecular mechanism(s) for these properties and determine how it affects cellular functions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

| | |
|---|---|
| **DR** | Diabetic retinopathy |
| **RPE** | retinal pigment epithelial |
| **mtDNA** | mitochondrial DNA |
| **[Afr+Asi]/DM** | African and Asian diabetic patients |

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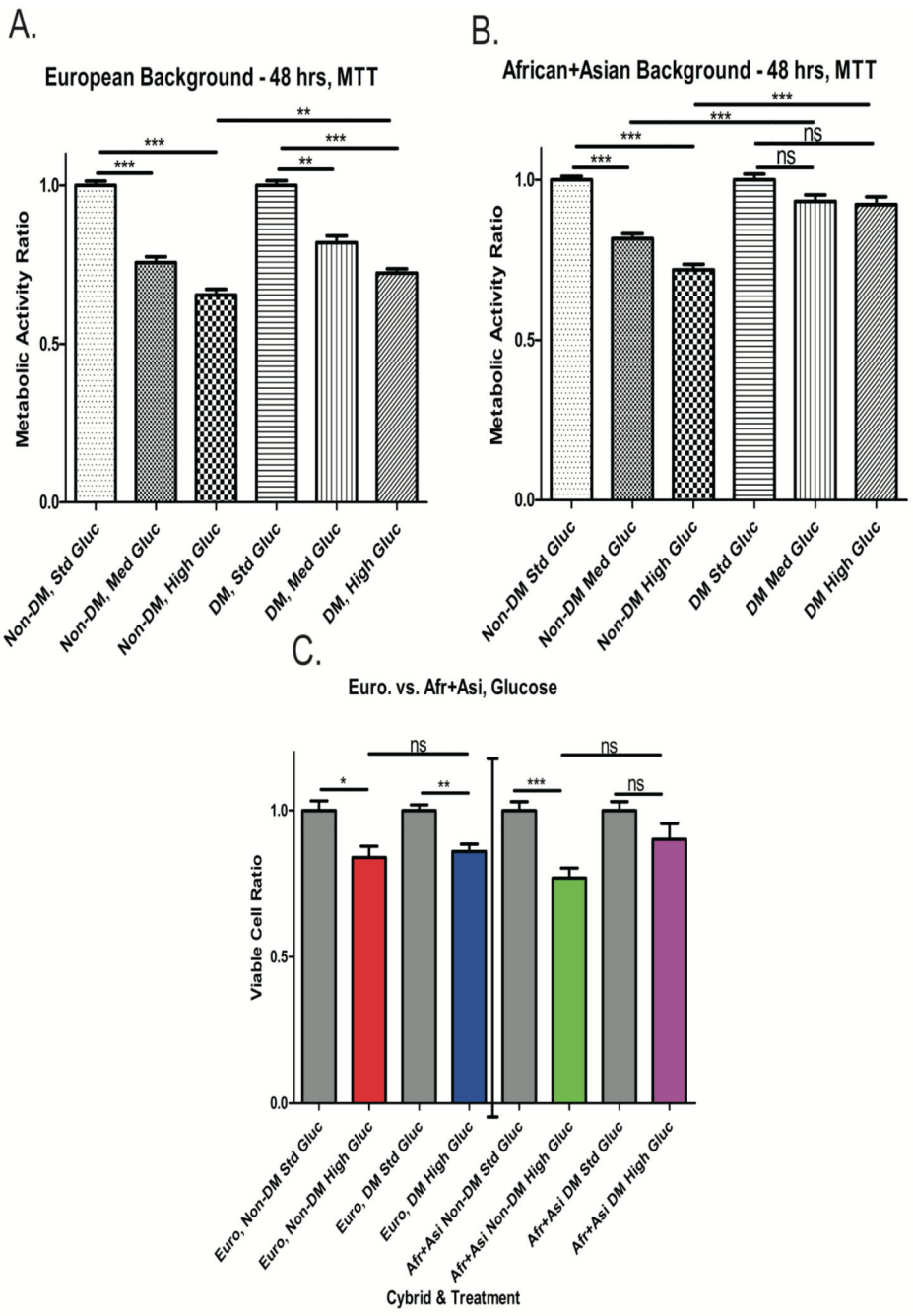



**Figure 1. [Afr+Asi] DM cybrids resist decreases in viability from high glucose.**

(A and B) Changes in viability after 48 hours normalized to Standard glucose (Std) controls, measured by MTT assay. (n = 3 Euro/Non-DM and Euro/DM, 4 [Afr+Asi]/Non-DM and [Afr+Asi] DM; 20 independent experiments total) (C) Changes in viability after 48 hours normalized to untreated (Std glucose) controls, measured by trypan blue dye-exclusion assay. (n = 3 Euro/Non-DM and Euro/DM, n = 4 [Afr+Asi]/Non-DM and [Afr+Asi]/DM; 7 independent experiments total) * = $p < 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

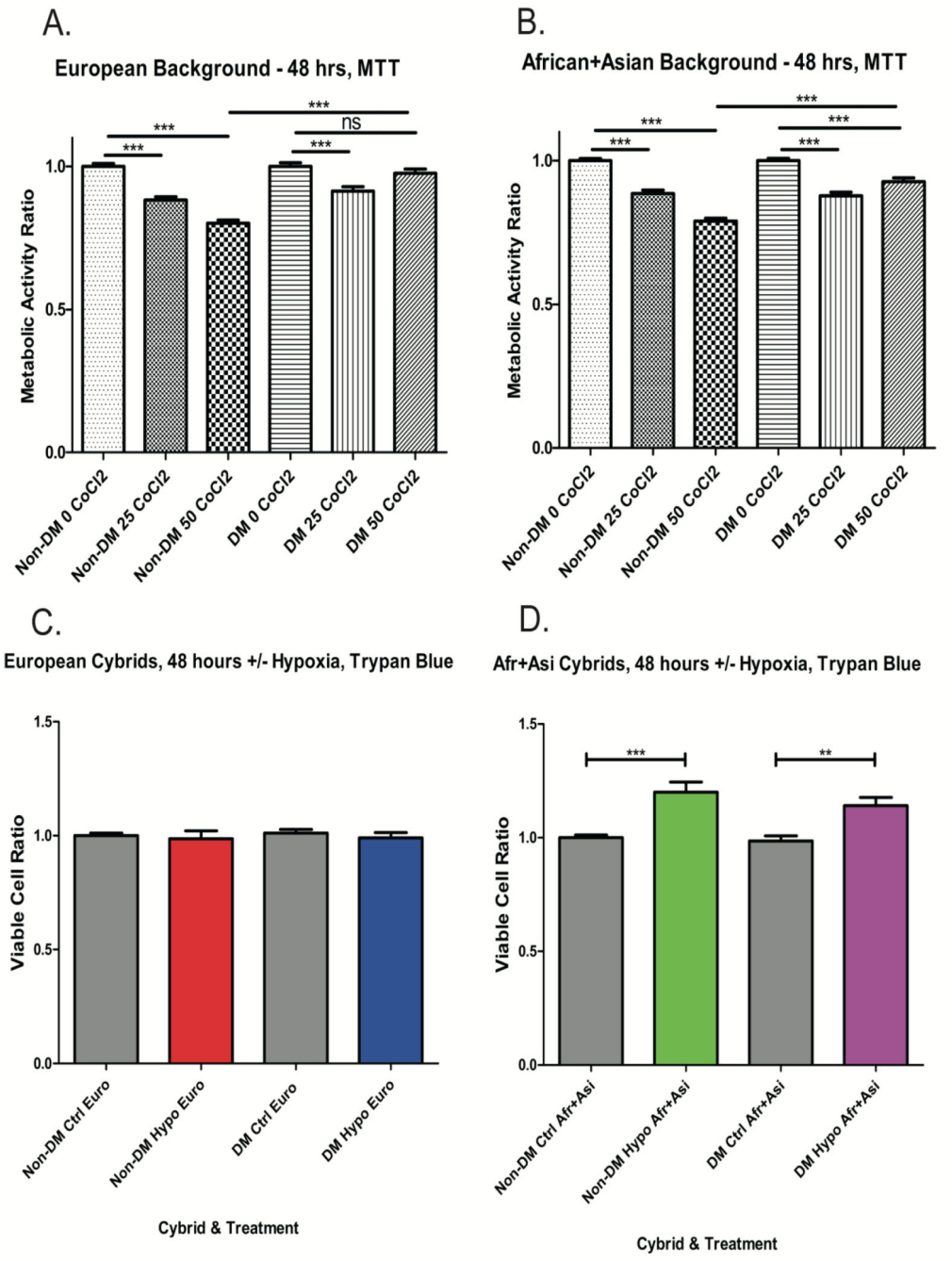



**Figure 2. [Afr+Asi] DM cybrids resist decreases in viability from hypoxic stress.**

(A and B) Changes in viability after 48 hours ±$CoCl_2$, measured by MTT assay and normalized to untreated controls. (n = 3 Euro/Non-DM and Euro/DM, 4 [Afr+Asi]/Non-DM and [Afr+Asi] DM) (C and D) Changes in viability after 48 hours. Cybrids were cultured ±2% O2, and metabolic activity was measured by trypan blue dye exclusion assay, normalized to untreated controls. (n = 6 Euro/Non-DM, n = 4 Euro/DM, n = 6 [Afr+Asi]/Non-DM, n = 4 [Afr+Asi]/DM; 18 independent experiments total) * = $p < 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

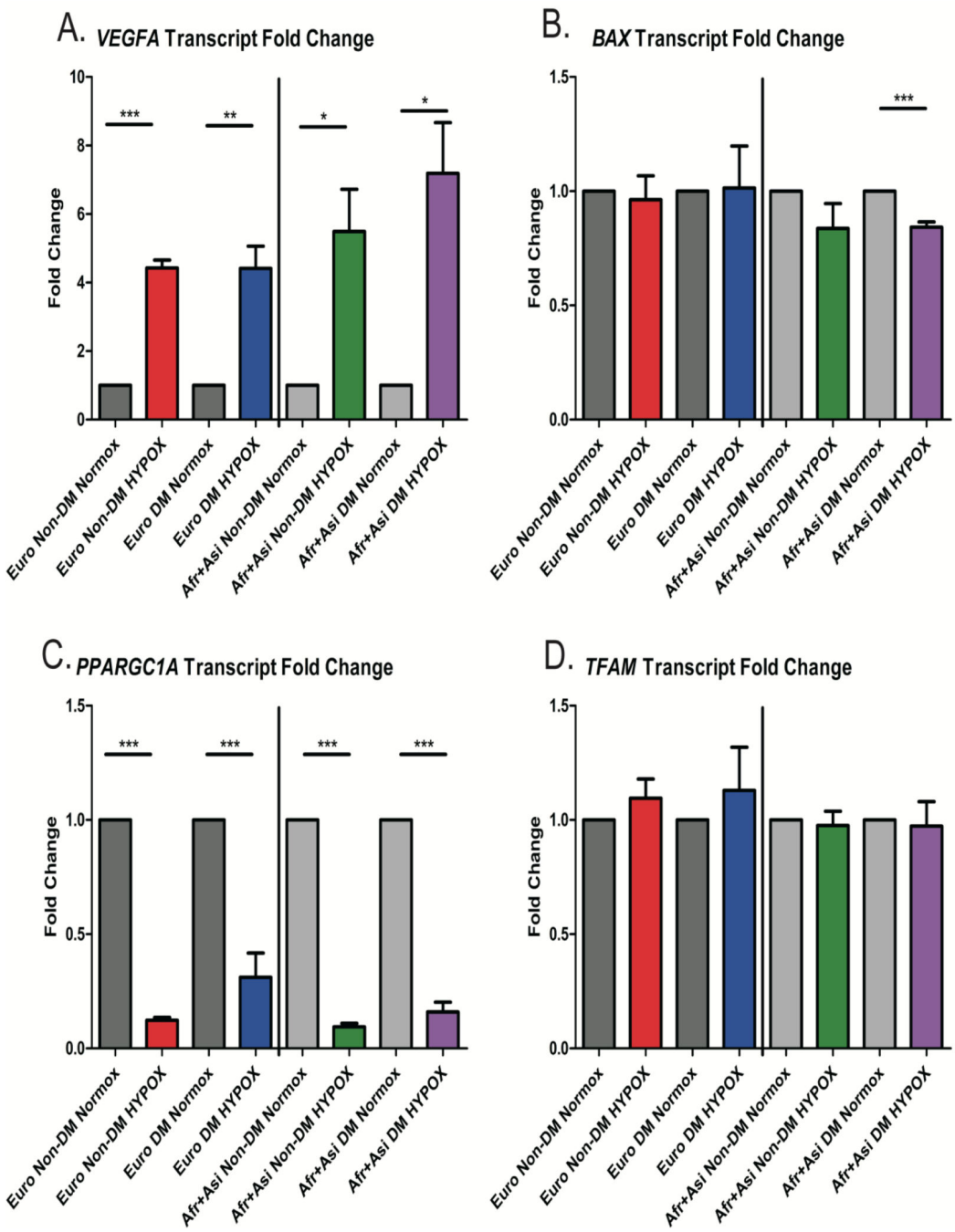



**Figure 3. qRT-PCR of Selected Targets in cybrids grown ± hypoxic conditions.**

RNA from cybrids grown 48 hours ±2% $O_2$ was measured by qRT-PCR against (A) VEGF-A, a pro-angiogenesis factor, (B) *BAX*, a marker of apoptosis, (C) *PPARCG1A*, a master regulatory factor for mitogenesis, and (D) *TFAM*, a transcription factor involved in mtDNA transcription and replication. (Euro/Non-DM n = 4, Euro/Non-DM n = 4, [Afr+Asi]/Non-DM n = 3–4, [Afr+Asi]/DM n = 3–4; 11 independent experiments total) * = $p < 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

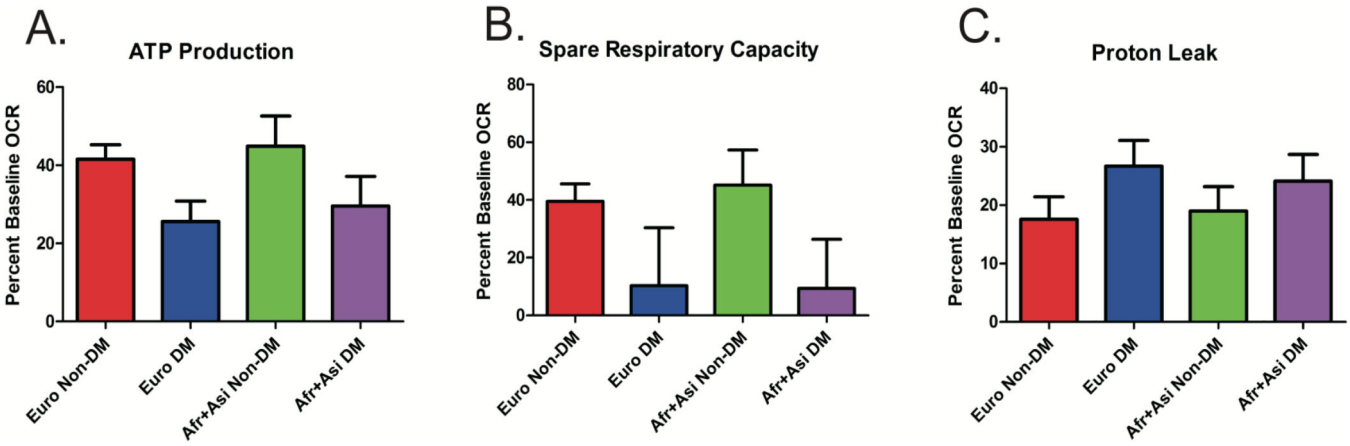



**Figure 4. Euro and [Afr+Asi] cybrids have similar bioenergetic profiles in baseline conditions.**

Measurements of the amount of Oxygen Consumption Rate (OCR) relative to untreated conditions used for (A) ATP production, (B) Spare Respiratory Capacity, and (C) Proton Leak using the Seahorse XF24 flux bioanalyzer. (Euro/Non-DM n = 4, Euro/DM n = 4–5, [Afr+Asi]/Non-DM n = 4, and [Afr+Asi]/DM cybrids n = 4–5; 10 independent experiments total)

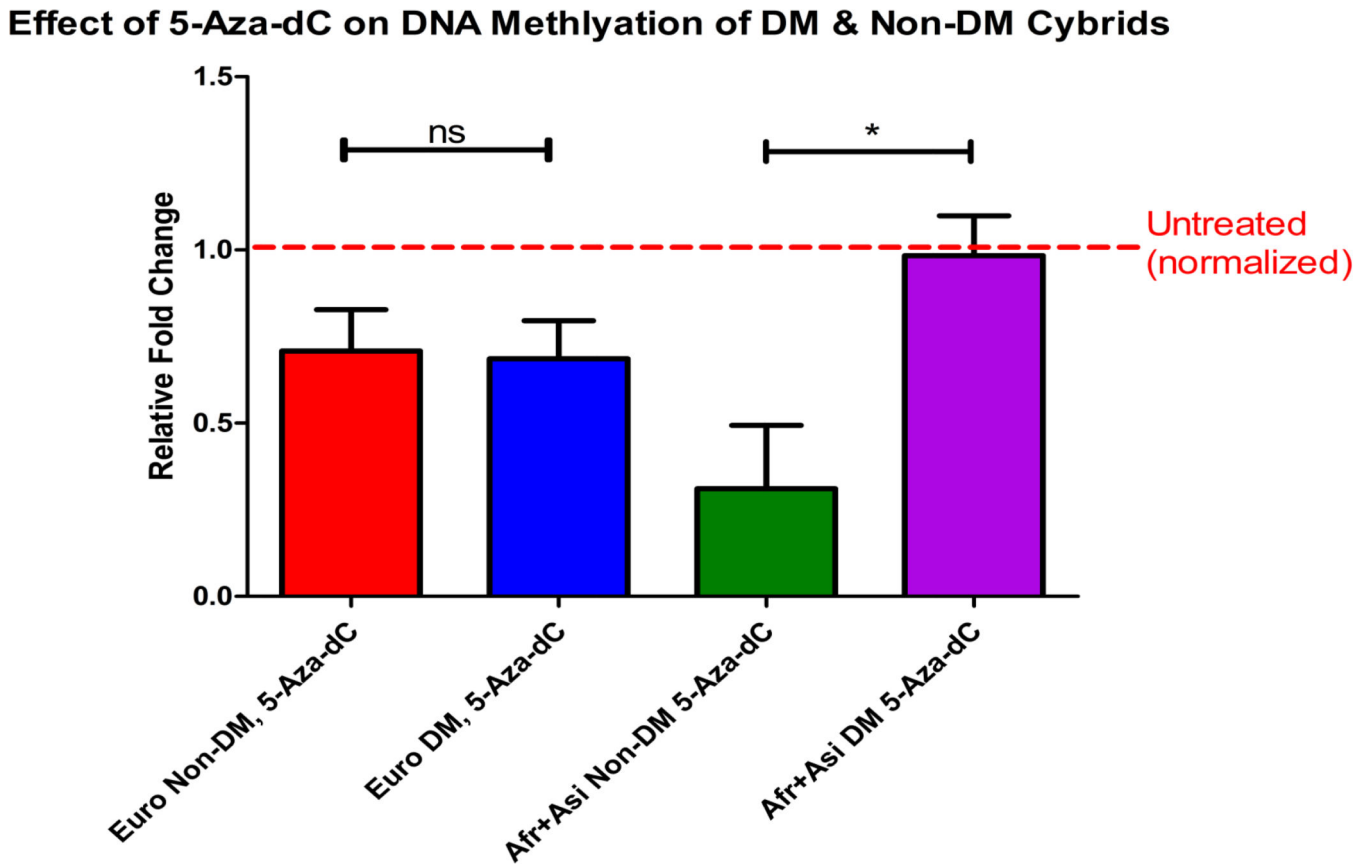



**Figure 5. Euro and [Afr+Asi] cybrids have different levels of DNA methylation after treatment with 5-Aza-dC.**

Amount of DNA methylation of Euro and [Afr+Asi] cybrids grown ± 5-Aza-2-deoxycytidine (5-Aza-dC), a DNA methyltransferase inhibitor, as measured by an ELISA against 5-methylcytosine. (Euro/Non-DM n = 5, Euro/DM n = 4, [Afr+Asi]/Non-DM n = 3, [Afr+Asi]/DM cybrids n = 4; 8 independent experiments total) * = $p < 0.05$

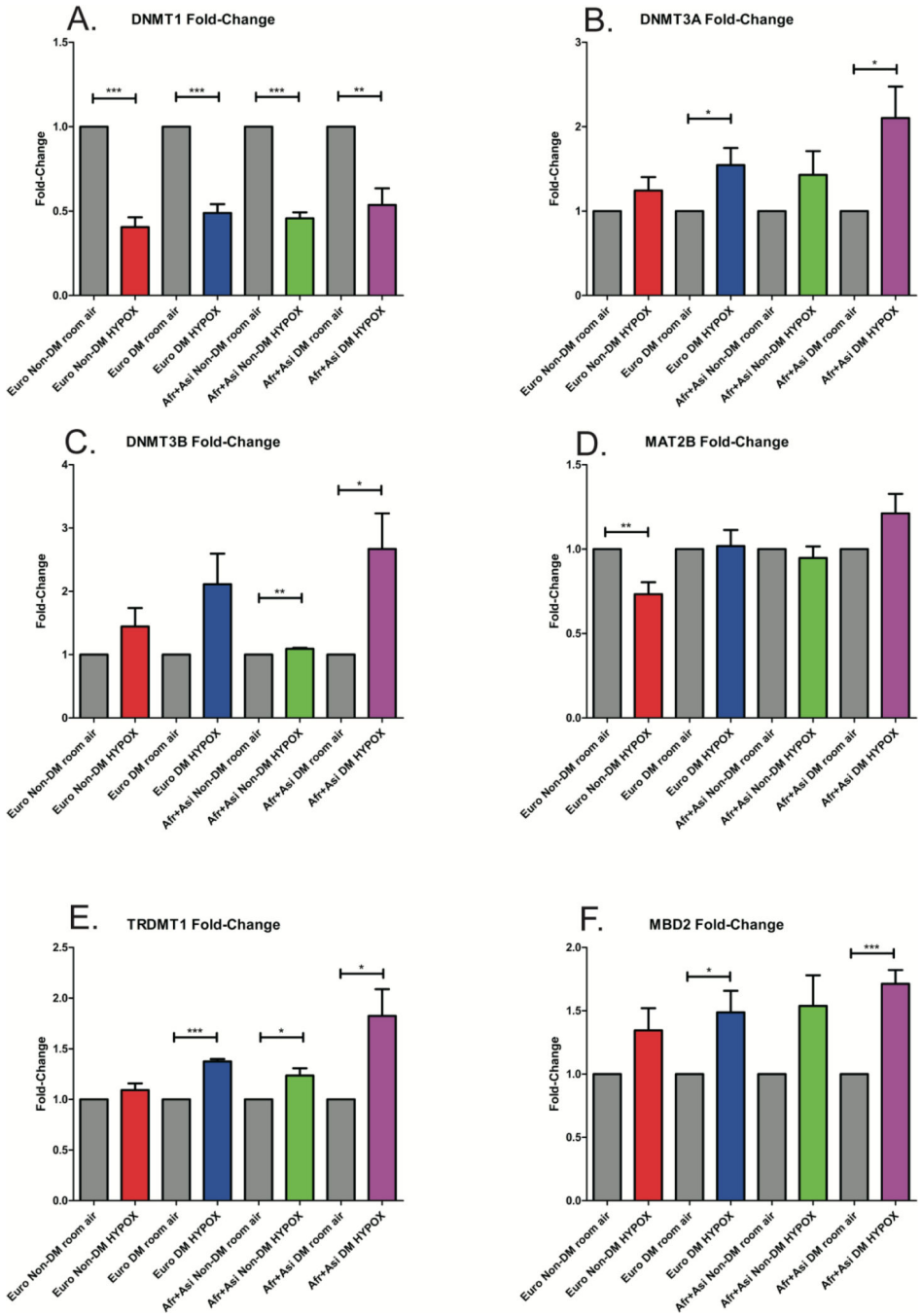
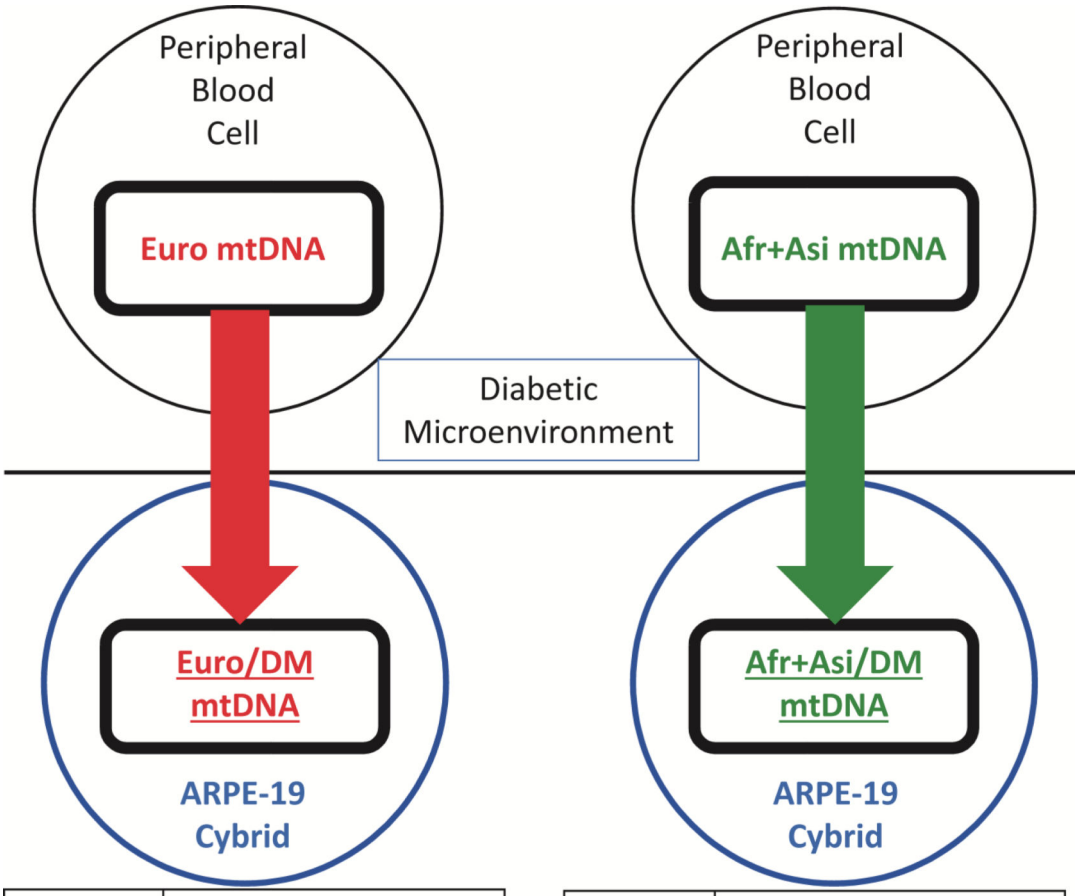



**Figure 6. qRT-PCR of DNA Methylation Factors in cybrids grown ±hypoxic conditions.**

RNA from cybrids cultured 48 h ±2% O2 was analyzed by qRT-PCR to measure transcript levels of DNA methylation genes: (A) *DNMT1*, (B) *DNMT3A*, and (C) *DNMT3B* (DNA methyltransferases); (D) *MAT2B*, an S-adenylmethionine synthase, (E) *MBD2*, a methyltransferase-recruiting scaffold protein, and (F) *TRDMT1*, an RNA methyltransferase. (Euro/Non-DM n = 4, Euro/Non-DM n = 4, [Afr+Asi]/Non-DM n = 3–4, [Afr+Asi]/DM n = 3–4; 8 independent experiments total) * = $p < 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$




| Stressor | Euro/DM Result |
|---|---|
| High Glucose | • ⬇ Metabolic Activity<br>• ⬇ Live cells |
| Hypoxia | • Metabolic Activity – Resist ⬇<br>• Live cells – No Change<br>• Transcripts - Similar to Non-DM |
| 5-Aza-dC | • ⬇ DNA methylation |

| Stressor | Afr+Asi/DM Result |
|---|---|
| High Glucose | • **Resist** ⬇ Metabolic Activity<br>• **Resist** ⬇ Live cells |
| Hypoxia | • Metabolic Activity – Resist ⬇<br>• Live cells – ⬆<br>• Transcripts – **Unique, Different From** Non-DM |
| 5-Aza-dC | • DNA methylation **persists** |

**Figure 7. [Afr+Asi]/DM mitochondria confer transferable resistance to both high glucose and hypoxia.**

Summary of data from this study.

**Table 1.**

**Euro and [Afr+Asi] cybrids used in these experiments have a similar average age, p = 0.61.**

Bold Box, Upper Panel: Cybrids listed in pairs of Non-DM and DM cybrids of similar mtDNA haplogroup and age were used for high glucose and hypoxia experiments. The average ages of Euro and [Afr+Asi] cybrids are not significantly different from each other. **(2 column image)**

| | **European** | | | | | | **African and Asian** | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cybrid** | **Haplogroup** | **Age** | **Sex** | **DM/Non-DM** | | **Cybrid** | **Haplogroup** | **Age** | **Sex** | **DM/Non-DM** |
| 11.08 | K1c1c | 24 | M | Non-DM | | 11.30 | L1b2a | 54 | F | DM |
| 13.103 | K1a3 | 25 | F | DM | | 11.31 | L2b2 | 42 | M | Non-DM |
| 13.45 | H1e1a | 47 | F | Non-DM | | 11.38 | L0a1a1 | 38 | F | Non-DM |
| 16.186 | H1 | 48 | M | DM | | 13.124 | L1b2a | 31 | M | DM |
| 10.06 | U5 | 53 | M | Non-DM | | 13.126 | L1b1a7 | 40 | F | Non-DM |
| 16.184 | U | 60 | M | DM | | 15.176 | L0a1a | 32 | M | DM |
| 14.135 | H1c3 | 75 | F | Non-DM | | 11.18 | D4a6 | 39 | M | Non-DM |
| 16.190 | H | 80 | F | DM | | 13.55 | D4a2b | 48 | F | DM |
| 13.65 | H4a1a4b | 52 | F | Non-DM | | 11.17 | L3e1a1a | 64 | F | Non-DM |
| 15.161 | H5a1 | 67 | M | Non-DM | | 13.125 | L1c2a1 | 52 | F | Non-DM |
| 16.185 | U5a1a1 | 68 | M | DM | | 16.182 | L | 78 | M | DM |
| 10.07 | H66a | 49 | M | Non-DM | | | | | | |
| 11.10 | H4a1a4b2 | 30 | M | Non-DM | | | | | | |
| 11.35 | H1b5 | 30 | F | Non-DM | | | | | | |
| | | | | | | | | | | |
| | Avg Age (yrs) | 50.57 | | **p = 0.61** | | | Avg Age (yrs) | 47.27 | | |
| | # Male | 8 | | | | | # Male | 5 | | |
| | # Female | 6 | | | | | # Female | 6 | | |
| | | | | | | | | | | |
| Non-DM | Avg Age (yrs) | 47.44 | | | | Non-DM | Avg Age (yrs) | 45.83 | | |
| | # Male | 5 | | | | | # Male | 2 | | |
| | # Female | 4 | | | | | # Female | 4 | | |
| | | | | | | | | | | |
| DM | Avg Age (yrs) | 56.20 | | | | DM | Avg Age (yrs) | 48.60 | | |
| | # Male | 3 | | | | | # Male | 3 | | |
| | # Female | 2 | | | | | # Female | 2 | | |

**Table 2.**

Diabetes and diabetic retinopathy status of patients whose mitochondria were used in our cybrids. (2 column image)

| Cybrid | Haplogroup | Age | Diabetes Type | DR Status |
|---|---|---|---|---|
| 13.55 | D4a2b | 45 | Type 2 | Background DR |
| 13.124 | L1b2a | 31 | Type 1 | No DR |
| 15.176 | L0a1a | 32 | Type 2 | No DR |
| | | | | |
| 13.103 | K1a3 | 25 | Type 2 | Background DR |
| 16.190 | H | 80 | Type 2 | Background DR |
| | | | | |
| 11.30 | L1b2a | 54 | Type 2 | NPDR |
| 16.182 | L | 78 | Type 2 | NPDR |
| | | | | |
| 16.184 | U | 60 | Type 2 | NPDR |
| 16.185 | U5a1a1 | 68 | Type 2 | NPDR |
| 16.186 | H1 | 48 | Type 2 | NPDR |

**Table 3.**

DNA Base Identity at Position 16189 in our cybrids.

| Cybrid # | Haplogroup | DM Status | mtDNA residue 16189 |
|---|---|---|---|
| 10.06 | U5 | No | C |
| 10.07 | H66a | No | C |
| 11.08 | K1c1c | No | T |
| 11.10 | H4a1a4b2 | No | T |
| 11.17 | L3e1a1a | No | T |
| 11.35 | H1b5 | No | C |
| 13.45 | H1e1a | No | T |
| 13.65 | H4a1a4b | No | T |
| 14.135 | H1c3 | No | T |
| 15.161 | H5a1 | No | T |
| 13.103 | K1a3 | **Yes** | T |
| 16.184 | U | **Yes** | T |
| 16.185 | U5a1a1 | **Yes** | T |
| 16.186 | H1 | **Yes** | C |
| 16.190 | H | **Yes** | N/A |
| | | | |
| 11.18 | D4a6 | No | T |
| 11.31 | L2b2 | No | T |
| 11.38 | L0a1a1 | No | C |
| 13.125 | L1c2a1 | No | T |
| 13.126 | L1b1a7 | No | C |
| 11.30 | L1b1a | **Yes** | C |
| 13.55 | D4a2b | **Yes** | T |
| 13.124 | L1b2a | **Yes** | C |
| 15.176 | L0a1a | **Yes** | T |
| 16.182 | L | **Yes** | N/A |