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Understanding the Specific Influence of Mitochondria on Cellular Health in Diabetes

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Experimental Pathology

by

Andrew Harrison Dolinko

Thesis Committee:
Professor Maria Cristina Kenney, Chair
Professor Edwin S. Monuki
Associate Professor Steven Chessler

2021

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DEDICATION

To

my parents, Laurie and Robert, and my brother Jeremy

in recognition of their unyielding love and support

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ABSTRACT OF THE DISSERTATION

Understanding the Specific Influence of Mitochondria on Cellular Health in Diabetes

By

Andrew Harrison Dolinko
Doctor of Philosophy in Experimental Pathology
University of California, Irvine 2021
Professor M. Cristina Kenney, Chair

In the past several decades, diabetes mellitus has become one of the most common chronic diseases worldwide. While the United States spends hundreds of billions of dollars on treatment for this disease, it continues to rank among the top 10 causes of death. One of its main complications, diabetic retinopathy, is the foremost cause of blindness for individuals younger than 65 years-old. In this condition, retinal blood vessels become more porous, allowing hemorrhages to form within the neural retina. The blood-retina barrier system, formed by endothelial cells lining retinal capillaries in the inner retina and retinal pigment epithelial (RPE) cells located between the outer photoreceptor layer and the choroid vasculature, loses its integrity, leading to vascular fluid leakage. This fluid accumulation within the retina, known as diabetic macular edema (DME), leads to decreased vision. Minority populations, such as people of African or Asian ancestry, are more likely to develop diabetes and diabetic retinopathy, including DME, than Caucasians.

The landmark Diabetes Control and Complications Trial (DCCT) showed that an intensive insulin regimen decreased the incidence and progression of diabetic retinopathy and other microvascular complications. Based on this work, tight, systemic control of blood glucose

through insulin and other medications, such as metformin, remains a core element of treating diabetic retinopathy. In the past, retinopathy unable to be managed by strict glycemic control could only be treated with laser to photocoagulate portions of the retina. More recently, intravitreal injections of medications that act against vascular endothelial growth factor (VEGF) have decreased the need for photocoagulation and have allowed patients to retain more of their vision and neural retina. However, at least 40% of patients with diabetic retinopathy do not respond to anti-VEGF treatments, stressing the need for further research into other therapeutic targets.

In addition to the benefits described in the DCCT, longitudinal studies of diabetic patients enrolled in this trial found that individuals who were given early intensive insulin therapy were significantly less likely than individuals initially treated with a single daily insulin injection to develop diabetic retinopathy or have worsening retinopathy for over 10 years after the initial change in treatment. Other investigations in Europe found similar results, showing that early glycemic control reduces morbidity and mortality associated with diabetes. Overall, these studies suggested that the cellular microenvironment of diabetes induces a *metabolic memory* in a patient's cells that remains long after cells are returned to a more normal, euglycemic microenvironment. By clarifying how this memory is formed, researchers and clinicians may use this knowledge to develop additional therapies to treat diabetes and its complications.

While there has been extensive research examining how the nuclear genome contributes to the development of diabetes, there has been much less focus on the role of the mitochondria and its DNA in this process. Our laboratory employs a cytoplasmic hybrid (cybrid) system, in which a parent RPE cell line is chemically-treated to remove mitochondrial DNA (mtDNA) and then fused with patient platelets containing mitochondria but no nuclear DNA. Our resulting cybrids have patient-specific mtDNA, but all cell lines have the same nuclear genome, allowing us to investigate the influence of mtDNA on cellular health and functions. Using cybrid

cell lines, we have shown that mtDNA associated with certain maternal ancestries (e.g. African versus European) or from patients with age-related macular degeneration differentially-alter levels of reactive oxygen species, pro-inflammatory genes, complement, and other factors.

The work in this dissertation uses our cybrid system to investigate how mtDNA affects RPE cell health and function. In **Chapter 1**, we examined how ancestral mtDNA background and mtDNA from diabetic versus unaffected patients may protect RPE cells against stresses seen in diabetes, such as high glucose or hypoxia. In **Chapter 2**, we investigated the mechanisms of this protective effect using RNA-seq and how it may alter cellular functions. We found that (i) mitochondria/mtDNA from African and Asian diabetic patients confers resistance against diabetic stressors compared to age-matched African and Asian non-diabetic patients; and (ii) cybrid cells with African or Asian mitochondria/mtDNA have unique differential expression of factors involved in certain gene ontology families, such as fat processing

INTRODUCTION

Diabetes – A Modern Epidemic

Within the past 30 years, diabetes has become one of the most prevalent chronic diseases affecting individuals worldwide. According to the World Health Organization (WHO), the prevalence of diabetes in the global population has drastically risen from approximately 4.7% in 1980 to 8.5% in 2014. In this time, the number of individuals affected by the disease rose from approximately 108 million in 1980 to a nearly-quadruple of 422 million in 2014. While diabetes had been associated with rich countries in the past, data from the WHO suggests this is no longer the case; the prevalence of diabetes in the lower- and upper-income group countries nearly doubled between 1980 and 2014 (~4.5-5% in 1980 to ~9% in 2014) while the prevalence in high-income countries rose more modestly (~5.5% in 1980 to ~7% in 2014).[1] Extrapolations from the International Diabetes Foundation (IDF) suggest that the prevalence of diabetes will increase even further from 8.8% in 2017 (424.9 million people) to 9.9% in 2045 (628.6 million people).[2] In the United States, a meta-analysis of the National Health Interview Surveys (NHIS) between 1980 and 2012 revealed the prevalence of diabetes had grown from 3.7% in 1980 to 8.3% in 2012, and the incidence of diabetes had risen from 3.7% in 1980 to 7.1% in 2012.[3] According to the Centers for Disease Control and Prevention (CDC), this incidence has dropped to 6.7% in 2015. Despite this, approximately 30.3 million Americans (9.4% of the population) were affected by diabetes in 2015, with an estimated 7.2 million undiagnosed individuals among them.[4]

As more individuals have become diagnosed with the disease, the economic costs of diabetes have grown. According to the IDF, the global expenditures on treating diabetes and its complications have more than tripled between within the past 15 years. While in 2007, approximately $232 billion was spent on treatment of diabetic patients between the ages of 20 and 79, nearly $727 billion was spent for individuals of this same age group in 2017. Based on current data, the IDF extrapolated that the economic burden of diabetes would rise to approximately $776 billion for all treatment costs worldwide by 2045. Additionally, the foundation's data revealed that the United States paid the most on healthcare for diabetic patients.[2] According to a report from the American Diabetes Association, the United States spent an estimated $237 billion on medical care directly related to diabetes, a remarkable rise from the $176 billion spent only 5 years prior.[5]

Despite this investment to maintain health and decrease disease burden in affected individuals, diabetes remains one of the top 10 causes of death worldwide. In 2016, the WHO found that diabetes and its complications led to the deaths of nearly 1.6 million individuals, more than all traffic deaths occurring that year. The mortality rate for this disease has nearly doubled from less than 1 million deaths in the year 2000 to that of the present day.[6] In the United States, diabetes also continues to be a primary cause of death. Despite advances in diagnosis and treatment, 2.9% of all reported deaths in both 2000 and 2016 were due to diabetes, according to the CDC.[7,8] Additionally, the percentage of all deaths in 2016 due to diabetes was found to be disproportionately higher in minority groups, such as African-Americans (4.3%) and Asians (4.0%), compared to Caucasians (2.5%).[8] Overall, these data stress that there is a significant need to effectively treat and prevent diabetes, nationally and globally, to prevent mortality.

Diabetes and Its Ocular Complications

Diabetes mellitus (DM) is a complex disease that arises from improper cellular uptake and metabolism of glucose from the bloodstream. This initial insult can subsequently impair the absorption of other metabolites, such as amino acids and fatty acids. Most DM is classified into two groups, type 1 and type 2, based on the etiology of the disease.[9] In type 1 DM, young adults develop autoimmunity against insulin and factors related to its secretion, such as insulinoma antigen 2 (IA2), Zinc transporter 8 (ZnT8), and glutamic acid dehydrogenase (GAD).[10] An ensuing immune response damages pancreatic β-islet cells, leading to severe insulin deficiency. In type 2 DM, patients display a decreased tolerance for blood glucose, through a combination of impaired insulin secretion from the pancreas and a decreased response of target cells to insulin. Unlike type 1 DM, this form of diabetes is most commonly related to obesity. A study of Pima Indians, a group in which type 2 DM is highly prevalent, found that decreased insulin secretion and activity correlated with increased body weight.[11]

While type 1 and type 2 DM may progress through different mechanisms, all affected patients can develop chronic complications secondary to diabetes. One of the more concerning of these complications is diabetic retinopathy (DR), a leading cause of blindness in adults under the age of 65.[12] The pathophysiology of DR was first described by Arthur Ballantyne, who noted changes in the retinal vasculature of eyes from diabetic patients. In particular, he remarked that the cells lining the blood vessels of the inner retina, such as the pericytes and retinal endothelial cells (RECs), were more prone to cell death in the eyes of diabetic patients than those of non-diabetic

patients.[13] In support of these observations, researchers have found that retinal capillaries in canine models of diabetes show microaneurysms and decreased or lack of bloodflow, promoting the formation of basement membrane tubes devoid of RECs and pericytes.[14] In addition, REC and pericyte apoptosis was found in retinas of both human diabetic patients and diabetic rat models.[15] As a result of this destruction of the retinal vasculature, the inner retina becomes ischemic and may precipitate blindness.

This damage to the retinal vasculature can subsequently trigger more severe pathology. At first, these injured capillaries may only allow minor “dot blot” hemorrhages to leak through the endothelial cells; however, these leakages may eventually cause protein and lipids from the blood to remain in the retina after reabsorption of fluids, indicating the development of non-proliferative DR (NPDR). In this state, the neural retina becomes hypoxic, as the blood vessels cannot adequately oxygenate it.[16] From this point, the disease may progress in two forms that may coexist: proliferative DR (PDR) and diabetic macular edema (DME). In PDR, the decreased metabolite delivery to the retina and metabolic demand of the neural retina induces neovascularization from existing retinal blood vessels. However, these newly formed vessels exacerbate the problem in two ways: (1) they are more porous than the initial retinal vasculature, increasing the amount of fluid leaking into the retina, and (2) they expand into regions where capillaries are not normally found, such as the vitreous, causing mechanical strain the neural retina and increasing the chance of retinal tearing and detachment. In DME, fluid leaks from the blood vessels at a faster rate than it is removed from the retina, so it accumulates within the layers of the neural retina or between the photoreceptors and the neighboring retinal pigment epithelial (RPE) cells. In particular, fluid accumulates in the fovea, disrupting vision. In a healthy eye, fluid is prevented from entering the retina through a collection of cells that form a blood-retina barrier (BRB). The inner retina is

protected by the RECs, which are tightly connected to each other. The outer retina is nourished by the choroid capillaries, which are much more permeable than the retinal vasculature. Here, the RPE cells have a dual function: they form a barrier through tight junctions with one another, and they pump fluid out of the neural retina. As DME progresses, these barriers become more penetrable, allowing further deposition of serum proteins, lipids, and fluids into the neural retina.[16,17]

Long-Lasting Effects of Controlling Blood Glucose in Diabetes – Metabolic Memory

While hyperglycemia has been known to induce microvascular complications of diabetes for decades, the benefits of controlling blood glucose levels were not clearly demonstrated in clinical trials before the 1990s. Since then, several studies have been performed to examine the effect of rigorous control of blood glucose on the development of diabetic complications. Starting in 1982, a group of multiple clinical centers in the United States, in association with several institutes of the National Institutes of Health, recruited insulin-dependent (type 1) diabetes patients to take part in the Diabetes Control and Complications Trial (DCCT). Patients were first grouped by the absence ("Primary Prevention" group) or presence ("Secondary Prevention" group) of diabetic complications, such as NPDR. Within each group, one cohort was assigned to standard therapy, in which patients were administered 1-2 injections of insulin daily. The rest of the patients were assigned a more intensive regimen, in which patients received multiple insulin doses daily to lower glycosylated hemoglobin (HbA1C) to 6.05% as well as lower blood glucose before (70-120 mg/dL) and after meals (<180 mg/dL). Patients were closely followed until the end of the study in 1993. Their results showed that, for both

groups, average HbA1C was much lower in the intensive regimen cohort than in the standard therapy cohort. More importantly, compared to the standard therapy cohorts, the rigorous therapy cohorts showed reductions in risk of developing either progressive DR or clinical neuropathy. Based on this evidence, the study suggested that actively controlling blood glucose in diabetic patients prevents the development of further microvascular complications.[18]

In addition to decreasing the risk of complications during the DCCT, intensive therapy also decreased this risk for many years after the study. When the DCCT finished, all enrollees were shown how to perform intensive therapy. 1,375 of the DCCT patients agreed to take part in a longitudinal study, the Epidemiology of Diabetes Interventions and Complications (EDIC) study, which examined the incidence of new or worsening microvascular complications in these individuals. While average HbA1C was higher in the original standard therapy group (9.1%) than the rigorous therapy group (7.2%) at the end of the DCCT, over the next 18 years, HbA1C levels for all enrollees were similar, averaging about 8%. However, there were significant differences in the incidence and progression of diabetic retinopathy between the original intensive and standard therapy groups. The study showed, for the rigorous therapy group, there was a 71% risk reduction in developing further diabetic retinopathy 4 years after the DCCT, a 51% risk reduction 10 years after the DCCT, and a 46% risk reduction 18 years after the DCCT as compared to the standard therapy group. In addition, the incidences of PDR and DME were decreased for the intensive therapy group compared to the standard therapy group, with risk reductions of 47% and 35%, respectively.[19] These results suggest that the microenvironment of retina, influenced here by early, rigorous glucose control, imparts a lasting influence on their susceptibility to progression of DR.

While the DCCT and EDIC studies have been influential in advancing clinical

care of diabetes patients, these studies only investigated the role of glucose control on the risk of developing microvascular complications in insulin-dependent (type 1) diabetes patients. However, other clinical studies have found similar results for individuals with type 2 diabetes. For instance, Ohkubo *et al.* monitored type 2 diabetic patients in Japan over the course of 6 years. Patients were grouped based on the presence of retinopathy or nephropathy; those with at least one of these complications were placed in the secondary treatment group, and the others were placed in the primary treatment group. Within each group, patients were divided into two cohorts: one given standard therapy of 1-2 insulin injections/day with a goal of blood glucose < 140 mg/dL, and the other given 3 or more (multiple) insulin injections/day with an additional goal of having HbA1C < 7.0%. The study found that significantly fewer patients of the multiple injection cohorts in both primary and secondary treatment groups developed new or worsening retinopathy or nephropathy, compared to the conventional treatment cohorts.[20]

In addition, the STENO-2 study from Denmark demonstrated comparable results to the DCCT and EDIC studies. Type 2 diabetics were divided into groups based on the presence of nephropathy (microalbuminuria) and subdivided into standard therapy and intensive therapy cohorts as done for the DCCT. Targeted intervention and monitoring continued for 7.8 years (on average), and then all patients were taught intensive therapy. Patients were then monitored for another 5.5 years (on average). The study revealed that the intensive therapy cohorts experienced significantly lower mortality and significantly fewer cardiovascular events than the standard therapy cohorts. Furthermore, over the course of the entire study, patients given intensive therapy had a significantly lower relative risk (57%) of developing new or worsening DR as compared to patients given standard therapy.[21] As was found in the EDIC study, the beneficial effects of early glucose control persisted for years after the end of interventional study.

Similar to the STENO-2 study, the United Kingdom Prospective Diabetes Study (UKPDS) showed that rigorous control of blood glucose can decrease the risk of mortality and developing microvascular complications. In the UKPDS, type 2 diabetic patients with no history of retinopathy, cardiovascular complications, or nephropathy were placed into groups based on ideal body weight. Each group was divided into two cohorts. One cohort was treated with a control (conventional) therapy with a goal of limiting blood glucose to 15 mM. The other cohort was given a more rigorous therapy, using multiple insulin injections or insulin supplemented with sulfonylurea (chlorpropamide or glibenclamide) or metformin, with a goal of limiting blood glucose to 6 mM. The study revealed that rigorous glucose therapy significantly decreased the risk for needing photocoagulation to treat late-stage DR (relative risk = 71%).[22] After the end of the study, patients were advised to lower blood glucose (as done for the rigorous therapy group) and were monitored for 10 years. This follow-up study revealed that patients initially treated with rigorous therapy were at significantly decreased risk of diabetes-related mortality (83% relative risk for sulfonylurea cohort, 70% for metformin cohort) as compared to control cohort patients. In addition, patients treated with intensive therapy supplemented with sulfonylurea demonstrated decreased risk of developing microvascular disease, such as retinopathy (76%) as compared to control cohort patients.[23]

Taken together, the DCCT/EDIC, UKPDS, and STENO-2 studies indicate that glucose control in diabetes has a lasting impact on decreasing the risks of future complications and death. These results propose that a “Legacy Effect” is responsible for the persisting risk reduction of diabetic complications in patients treated with more intense glucose control therapy. The theory suggests that the diabetic microenvironment induces a biochemical response that instills cells with a *metabolic memory* of their

current surroundings. This metabolic memory persists when stressors in the microenvironment are removed or added.[24,25] Understanding the mechanisms behind these memories may provide researchers and clinicians with novel ways of treating diabetes.

The Role of Anti-VEGF Therapy in Treating DR

While intensive maintenance of glucose control decreases the risk of developing more severe DR, it does not necessarily stop any existing DR from progressing further. For instance, in a canine model of diabetes, properly-controlled glucose (good control) through insulin injections and a monitored diet after induction of DM prevented the development of DR. Canines that were not treated with insulin (poor control) showed DR. However, canines that became diabetic, were left for 2.5 years with poor control, then treated with good control for 2.5 years continued to develop DR despite having normal glucose levels.[26] These data suggest that glucose control is more effective when administered at earlier stages of DM. However, other therapy is required to prevent the progression of any DR that exists at the time of starting glucose control.

From the 1970s to the mid-2000s, laser photocoagulation of the retina was used to treat DR. Since the initial impairment of nutrient flow to the retina induces neovascularization, the goal of laser therapy was to prevent the further expansion of retinal vasculature. However, such photocoagulation also ablated the laser-treated regions of the retina. In pan-retinal therapy, the entire periphery of the retina was photocoagulated in order to spare the macula and, thus, central vision. Such drastic treatments were eventually reserved for only the most severe cases. A clinical study by

the Early Treatment Diabetic Retinopathy Study Research Group found that laser treatment of distinguishable foci in early DR prevented the development of more severe complications and improved visual acuity compared to patients who were not given laser therapy at that time.[27]

Since then, advances in molecular biology inspired the development of more targeted therapies to treat DR-induced neovascularization. Since PDR arises from the proliferation of blood vessels in a hypoxic retina, researchers sought to determine which molecular cues induce this pathology. These investigations found that protein levels and RNA transcripts of vascular endothelial growth factors (VEGFs) were elevated in the eyes of diabetic patients compared to those of non-diabetic subjects.[28] In particular, VEGF-A transcript was significantly increased in hypoxic RPE cells compared to levels found in untreated controls.[29] Consistent with this evidence, other studies found that in addition to increasing the permeability of RECs, VEGFs also decrease the barrier function of RPE cells *in vitro* and *in vivo*.[30,31] Supporting these results, cells that maintain the neural retinal microenvironment, the RPE and the Müller glia, were found to increase production of VEGFs in a diabetic mouse model compared to non-diabetic controls. Furthermore, Müller glia-specific VEGF-A knockout mice showed decreased inflammation and decreased vascular leakage in their retinas compared to those of wild type diabetic littermates, further implicating VEGF in the pathology of DR.[32]

Based on these results, researchers investigated if small molecules that bind VEGFs could prevent the induction of ocular neovascularization. For instance, researchers found that injecting monoclonal antibodies against VEGF in a macaque model of ocular ischemia significantly prevented neovascularization of the iris in treated animals as compared to controls.[33] In addition, another group generated chimeras of the ligand-binding domains of either human or mouse VEGF-receptor fused to Fc

fragments to determine how well these “decoy” receptors could inhibit VEGF signaling. The researchers found that the chimeras significantly decreased REC proliferation *in vitro* when cells were cultured in media supplemented with VEGF or taken from pericytes grown in hypoxia. Furthermore, these chimeric receptors decreased retinal neovascularization in a mouse model of neonatal hypoxia.[34] Overall, these results suggested that inhibition of VEGFs could prevent retinal angiogenesis in conditions such as DR.

Since VEGF inhibitors effectively prevented pathologic angiogenesis and its complications in cell and animal models of diabetes, clinicians and investigators administered similar compounds to patients in order to determine if these molecules could constitute a minimally invasive, drug-based therapy. One of the first of these compounds to be clinically tested in patients with ocular disease was pegaptanib, a 28-base RNA oligomer that specifically binds VEGF-165. Age-related macular degeneration (AMD) patients treated with pegaptinib exhibited milder decreases in visual acuity than patients who were not given the drug. More importantly, significantly more patients treated with pegaptinib showed improved visual acuity than untreated patients. In addition, there were significantly fewer patients in the pegaptinib-treated group who experienced severe losses in visual acuity than in the untreated group.[35] These results suggested that pegaptanib would be an effective therapy for preventing vision loss in AMD and other microvascular retinal diseases.

Since then, three other anti-VEGF drugs, which are most commonly used in treating DR and AMD, have shown efficacy in preventing neovascularization. The first of these, bevacizumab, is a monoclonal anti-VEGF antibody used to treat metastatic colon cancer and commonly administered off-label to patients to prevent microvascular disease in the eye. In a Phase II clinical trial, DME patients treated with bevacizumab

demonstrated decreases in retinal thickness from baseline more rapidly than patients treated with focal laser therapy. In addition, bevacizumab treatment led to improved visual acuity in treated patients as compared to those given laser therapy.[36] The second of these drugs, ranibizumab, is a monoclonal Fab portion of an antibody that specifically targets VEGF. Unlike bevacizumab, ranibizumab was specifically produced as a treatment for microvascular disease of the eye. In two clinical trials, RISE and RIDE, clinicians administered ranibizumab to patients with DME. In both trials, patients treated with ranibizumab showed significant increases in visual acuity and decreases in retinal thickness compared to untreated subjects.[37] The most recent anti-VEGF drug is aflibercept, a chimeric molecule with the ligand-binding portions of the VEGFR-1 and -2 receptors fused to an Fc antibody fragment. In the DA VINCI clinical trial, clinicians compared the efficacy of aflibercept to laser therapy in DME patients. Similar to the clinical trials for bevacizumab and ranibizumab, this study showed that patients treated with aflibercept showed significantly higher increases in visual acuity and significantly greater decreases in central retinal thickness as compared to patients treated with photocoagulation.[38] Based on these results, anti-VEGF therapy has become the primary treatment for treating DR and other retinal microvascular diseases.

While anti-VEGF therapy has proven to be a remarkable, minimally-invasive method for treating DR, its effectiveness is limited to only a portion of DR patients.

In a clinical trial by the Diabetic Retinopathy Clinical Research (DRCR) Network, patients treated with ranibizumab were monitored over the course of 2 years for changes in visual function and retinal thickness. While visual acuity was improved for the majority of patients compared to baseline, between 40-50% of subjects had central retinal thickness > 250 μm at the 2-year timepoint, signifying unresolved DME.[39] Similar results were shown in another study by the DRCR Network where DME patients were treated with

either bevacizumab, ranibizumab, or aflibercept and monitored for 1 year. As was the case in the study with ranibizumab alone, this study revealed that 46.6% of all subjects showed unresolved DME (macula thickness > 250 μm) after 1 year.[40] Overall, these studies suggest that while anti-VEGF therapy improves visual acuity for most DME patients, other treatments that target molecular pathways and retinal cellular functions that influence the BRB are necessary to treat the underlying pathology of DME.

The Role of the RPE in DR

Since the early pathology seen in DR arises from changes to the RECs and pericytes that form the inner BRB, clinical treatments have focused on preventing proliferation of these cells while maintaining their survival. In contrast, researchers have given far less consideration to the RPE, which forms the outer BRB, and its role in the progression of DR. While RPE cells are derived from the same embryonic tissue as the neural retina, the RPE is highly specialized to maintain the microenvironment of the outer neural retina. RPE cells sit on the Bruch's membrane, separating the photoreceptors of the outer neural retina from the choroid capillaries. In order to supply nutrients from the choroid to the neural retina and replenish ion concentrations around the photoreceptors, RPE cells employ an array of transmembrane proteins.[41] The RPE cells also play an integral role in the recycling of photopigments. Light that enters the outer segments of photoreceptors converts these pigments into a form that inhibits neurotransmitter release; however, light also damages photoreceptor proteins and lipids through formation of reactive oxygen species (ROS). Photoreceptors regularly slough off portions of these outer segments, and RPE cells undergo phagocytosis to absorb and

eventually break down these cellular fragments.[42] Aside from proteins and lipids, these fragments also contain all-trans-retinols, Vitamin A derivatives that are produced during phototransduction. RPE cells possess a critical enzyme, RPE65, which converts all-trans-retinols to 11-cis-retinol. Patients who lack functional RPE65 develop Leber congenital amaurosis, a condition marked by loss of photoreceptor activity, visual loss, and potential blindness at an early age.[43]

In addition to these functions, RPE cells form the outer BRB. The choroid vessels, which supply nutrients to the RPE and the outer neural retina, are more fenestrated than the retinal vasculature. To prevent fluid from seeping into the neural retina from the choroid vessels, the RPE forms tight junctions between RPE cells. While researchers have studied the role of REC barrier function in the development of DR and DME for many years, investigators have more recently sought to determine how permeability of the RPE affects the development of these conditions. Studies in diabetic patients and animal models suggested that the RPE barrier becomes impaired in DR. In one study, mice rendered diabetic through treatment with streptozotocin, a pancreatic islet-specific toxin had horseradish-peroxidase (HRP) injected into their blood as a tracer. The study showed that more HRP accumulated in the retinas of diabetic mice as compared to untreated controls.[44] However, this finding was contentious because HRP can increase permeability of blood vessels and, potentially, the RPE itself. Despite this, clinical researchers such as Vinores *et al* used serum albumin as a tracer to evaluate BRB leakage in DM patients. These researchers found that while a remarkable amount of albumin accumulated in the inner retinas of diabetic patients, there was also a moderate amount of albumin near the RPE in these diabetic patients as compared to albumin in non-diabetic subjects.[45] Consistent with these results, other clinicians found that in a subset of patients with NPDR, fluorescein injected into the bloodstream did not

leak into the retina, which would be characteristic of retinal vascular leakage. These results suggested that the RPE could become leaky in the absence of underlying inner BRB deterioration.[46] While these results suggested that RPE function is compromised in diabetic retinopathy, they could not determine whether the leakage seen was truly an increase in RPE permeability or an inability to compensate for leakage of the inner retinal vasculature. To resolve this, in a recent study, researchers injected small FITC-dextran molecules into the bloodstream of streptozotocin-treated mice. The study found that, after 1 minute of circulation, the injected FITC-dextran molecules leaked through both the retinal vasculature and the RPE without overlap, supporting the idea that the RPE actually becomes more porous in DR and DME.[47]

Contributions of the Nuclear Genome to the Development of DM and DR

In order to further understand the molecular mechanisms underlying diabetes and its complications, researchers have investigated the nuclear genome, searching for particular alleles that may confer risks of developing or resistance to developing these conditions. Diabetes is known to be a complex culmination of environmental and genetic factors. However, several decades ago, twin studies and other observations of familial inheritance suggested that type 1 DM has a genetic component.[48] In 1973, Drs. Singal and Blajchman used antisera to show that certain antigens of the major histocompatibility complex (MHC), also known as the Human Leukocyte Antigen HLA) complex, were significantly increased in individuals with type 1 DM, as compared to non-diabetic or type 2 DM subjects.[49] A subsequent study investigated the influence of HLA alleles on the risk of acquiring type 1 DM in patients and their families. The study

found that certain alleles, such as the MHC class I alleles *HLA-B8* and *-B15* as well as the MHC class II alleles *HLA-DR3* and *-DR4*, were more significantly associated with type 1 DM subjects than non-diabetic controls. In contrast, other MHC class II alleles, such as *HLA-DR2*, *-DR5*, and *–DR7* were less significantly associated with type 1 DM subjects than non-diabetic controls.[50] Overall, these early studies supported the idea that the development of diabetes was in part due to the presence of certain risk alleles.

Years later, advancements in genetic sequencing and knowledge of the human genome inspired researchers to look for genes outside of the HLA complex that influence the risk of developing diabetes. In linkage studies of type 1 DM patients, subjects from thousands of different families had their genomes sequenced. These studies found that the odds of finding certain loci, such as chromosome 6p21.3 (containing *HLA-DQβ1*) and chromosome 11p15.5 (containing the insulin-encoding *INS* gene), within pairs of siblings with type 1 diabetes were significant, but other genes found in individual studies could not be reproduced.[51,52] In contrast, genetic linkage analyses of type 2 diabetic families could not reproducibly find any loci that were significantly shared by sibling pairs. To resolve this, researchers sequenced genomes of type 2 diabetic subjects and unaffected controls to examine particular genes of interest. These case-control studies revealed certain genes were significantly associated or unassociated with type 2 diabetic patients: *PPARG* (in which the Pro22Ala allele decreases risk),[53] the β-islet cell K+ channel protein-encoding gene *KCNJ11* (in which the Glu23Lys allele increases risk),[54] and the Wnt-pathway transcription factor-encoding gene *TCF7L2* (in which an allele with a specific intronic sequence increases risk).[55] While these linkage and association analyses revealed genes whose variants influence the risk of acquiring DM, the analyses were limited as they could only focus on a small number of factors at a time. To overcome this, researchers then employed single

nucleotide polymorphism (SNP) microarrays to categorize nuclear DNA. Over the past decade, genome-wide association scan (GWAS) studies have identified tens more genes that are positively associated with type 1 or type 2 DM, though most genes only show modest increases in risk.[56,57]

In particular, a number of GWAS studies have been performed on subjects with DR to identify genes that increase risk of developing this complication. Unfortunately, most of these studies have not generated reproducible results. One study from the Candidate Gene Association Resource (CARe) found that the *SELP* gene, which encodes a cell-surface receptor on endothelial cells involved in neutrophil invasion of inflamed tissue, was significantly associated with DR in one Caucasian DR cohort. However, this gene was not significant for other DR cohorts in the study, including a second, separate Caucasian cohort. Overall, the study could not find a gene whose expression was unique in all DR subjects as compared to unaffected individuals.[58] On the other hand, a more recent GWAS study was able to show significant and reproducible association of a candidate gene with multiple cohorts of DR patients. In this study, DNA from a group of type 2 DM Caucasian subjects (336 DR, 508 non-DR) was sequenced, revealing several candidate SNPs. When the same analysis was performed for a separate cohort of Caucasians and for a cohort of Indian descent, most of the genes found in the initial group were not significant. However, the *GRB2* gene, whose gene product activates the MAPK pathway with Insulin Receptor Substrate 1 (IRS1) in response to insulin signaling, remained significantly associated with DR in all cohorts. In addition, the researchers speculated that other candidate genes were not reproducibly significant in previous studies because those studies were done in smaller cohorts of particular ethnicities.[59] This implies that while no one gene may be associated with DR

in all patients, certain genes of interest found in GWAS studies may be significant for patients in certain ethnic groups.

The Influence of the Mitochondria in Diabetes and Diabetic Retinopathy

Since diabetes alters cellular metabolism, arising from a lack of cellular glucose uptake, it is reasonable to investigate the role of mitochondria in this disease. Mitochondria are well known for their ability to generate ATP from pyruvate through oxidative phosphorylation. In addition, mitochondria are unique organelles that possess their own genome, separate from nuclear DNA. Mitochondrial DNA (mtDNA) exists as a circular 16,569 base pair structure without introns. It encodes 2 rRNAs – the 12S and 16S subunits, 22 tRNA with a genetic code that differs slightly from the nuclear tRNA code, and 13 protein encoding genes. All of the major gene products from mtDNA are involved in the electron transport chain.[60] Using restriction endonuclease-treated samples of mtDNA, researchers have found that mtDNA is inherited maternally.[61,62] Although a recent study claims that paternal mtDNA may be inherited along with maternal mtDNA in rare cases,[63] the data presented do not clearly support the study's hypothesis, as the reported paternal mtDNA may actually have been nuclear DNA.[64]

While the general structure of mtDNA is the same for all individuals, mtDNA sequences vary from person to person. One source of mtDNA variance arises from naturally occurring, ancestrally-derived collections of SNPs in mtDNA, also known as haplogroups. In a 1991 study, Douglas Wallace's laboratory investigated differences in mtDNA sequence patterns in individuals from 62 different ethnic backgrounds. Using restriction enzyme digests of mtDNA, the researchers found that similarities in the

fragment patterns of individuals' mtDNA could be seen in subjects with common ancestral origins. Based on the data, the researchers constructed a mtDNA dendrogram. It showed that the cohort of African descent had the largest amount of heterogeneity with a large genetic distance from all other groups; this implies that the African mtDNA has the earliest origin. In contrast, mtDNA of the indigenous people of the Americas had the least amount of variation, suggesting that these populations had the most recent origin.[65] These findings strongly paralleled the migrations of human beings, based on archaeological data. Using similar restriction enzyme fragment length polymorphism (RFLP) studies, other groups refined this dendrogram. Over time, the branches became known as *haplogroups*. mtDNA of earliest African origin are all part of the L haplogroup; European haplogroups, such as H, U, and K, arose from a more recent origin; and haplogroups of indigenous Americans and Pacific Asians, such as the D haplogroup, have the most recent origin.[66]

While the polymorphisms between haplogroups do not cause disease on their own, other mtDNA mutations can precipitate debilitating diseases. One such mutation, A3243G, was first detected in patients affected by a multisystem disorder termed mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). A study from the Horai lab found that 26/31 MELAS patients harbored this mtDNA mutation, but it was not present in any of the study's control subjects' mtDNA. This mutation was found to occur in the coding region of tRNA for Leucine at a conserved site in mammalian, other vertebrate, and invertebrate mtDNA.[67] Remarkably, this mutation was also detected in individuals suffering from a multisystem condition termed maternally inherited diabetes and deafness (MIDD). When mtDNA from affected members of 3 different families was screened for mutations, only the A3243G mtDNA mutation was found to be common to all subjects, though only 10-30% of mtDNA in any

individual had this mutation.[68] In a subsequent study, the Maassen group generated cellular hybrid (cybrid) lines by combining a parent cell line devoid of mtDNA with enucleated fibroblasts containing either wild-type mtDNA or A3243G-mtDNA. Using these cell lines, this group found that the function of oxidative phosphorylation (OXPHOS) complexes I and IV were remarkably decreased in A3243G cybrids compared to wild-type cybrids. These results suggested that the A3243G mtDNA mutation not only impacts energy production but also is transferable to other cells *in vitro*.[69]

Though diabetes may arise from abnormal mutations such as A3243G, certain naturally-occurring mtDNA haplogroups may increase or decrease the risk of developing diabetes. In one study, the mtDNA of Japanese and Korean individuals with or without DM were collected and analyzed, and the results indicated that individuals of the N9a haplogroup were less likely to have diabetes than to be unaffected. In addition, for a majority of the sample groups, individuals of the D and F haplogroups were more likely to be diabetic than unaffected.[70] A separate, smaller study sequenced and compared the mtDNA of diabetic and obese Japanese individuals, revealing that certain SNPs the M8a haplogroup were associated with diabetic patients but not non-diabetic, obese subjects.[71] In contrast to these findings, a study of Chinese individuals found that N9a haplogroup subjects were not significantly associated with either having diabetes or being unaffected.[72] These conflicting results suggested that interactions between the nuclear genome and the mitochondria have a profound effect on the relative risk of developing diabetes. To clarify the role of the N9a and other haplogroups in developing diabetes, Dr. Doug Wallace and others performed a large study of Taiwanese individuals, both diabetic and non-diabetic. The study showed that the individuals of the N9a, F4, and D5 haplogroups were significantly less likely to be diabetic than unaffected.

The differences between these results and those of previous studies were likely due to the frequency of these haplogroups in each study's cohort. The F and D haplogroups were found to be at least twice as prevalent in the Taiwanese population than the Korean and Japanese populations, so stronger inferences about relative risk for diabetes could be drawn from the Taiwanese population. In addition, there were many fewer N9a subjects overall in the Chinese study than there were in the Taiwanese study.[73] Overall, these studies suggest that certain haplogroups, such as N9a may be protective against developing diabetes, but the effect of the mtDNA background may be influenced by nuclear genes.

Since mitochondria have been implicated in the development of diabetes, researchers have also investigated the influence of certain haplogroups on the progression and prevalence of DR. In an initial study by the Samuels lab, DR patients were categorized by haplogroup in order to determine if the presence of PDR, NPDR, and DME were associated with mtDNA background. The study found that, for patients with any DR, subjects of haplogroup H were more likely to have PDR than NPDR while individuals of haplogroup Uk were less likely to have PDR than NPDR. This study also showed that there were no significant differences in the presence of DME between the H, Uk, and non-H/non-Uk cohorts.[74] To expand upon their findings, the Samuels lab recruited diabetic patients, with or without DR, to investigate if the H or Uk haplogroups are more likely to be associated with DR. As was the case in the previous study, individuals of the H haplogroup with DR were significantly more likely to have PDR than NPDR, and subjects of the Uk haplogroup were significantly less likely to have PDR than NPDR, even after adjustment for age, sex, or duration of DM. However, when all subjects of the H or Uk haplogroups were considered and the data was adjusted for age, sex, or duration of DM, neither haplogroup was significantly associated with an

increased or decreased risk of having DR.[75] Based on the results of these two studies, mtDNA of European haplogroups may differentially influence the extent of DR but do not affect the risk of developing DR. Yet, a more recent study of Australian subjects, with or without DR, has challenged these previous findings. On one hand, the Australian study found that the presence of either H or Uk haplogroup was not significantly associated with an increase or decrease in DR risk, supporting the previous data. On the other hand, the Australian study determined that DR patients of the H or Uk haplogroups were not significantly associated with PDR or NPDR. Since this study enrolled at least 4 times as many subjects compared to the previous studies, the authors concluded that their results were more accurate, concluding that European haplogroups do not differentially affect the presence or severity of DR.[76]

Using Cybrid Models to Examine the Influence of the Mitochondria on Cell Health and Function

Though most studies on the role of haplogroups in diseases such as DM and DR examine large groups of patients, the work in this dissertation employs a cybrid model to examine the influence of mitochondria on retinal cell function. Specifically, our laboratory has generated cybrids from ARPE-19 cells, a spontaneously immortalized RPE line. We chemically-treat of these parent cells to render them devoid of mtDNA and subsequently fuse them with mitochondria-rich platelets from clinically well-characterized patients to generate our cybrid lines. One weakness of this system is that it focuses on one cell type in isolation, so communication between the various retinal neurons and the RPE is not present. Furthermore, our cell lines have only one nuclear genetic background – that of

a 19-year-old male – so we cannot examine differences in mitochondrial influence on male and female nuclear genomes. Nonetheless, the cybrid system has several advantages over other cell models:

1. By keeping the nuclear genome constant, any differences in cell health and function between the various cybrid lines are due to the presence of particular mitochondria and mtDNA. Such differences also demonstrate that the mitochondrial influence is transferable in an *in vitro* system.

2. ARPE19 cells are spontaneously immortalized,[77] so our cybrid lines may be grown for multiple passages while maintaining genomic stability and proliferative ability.

3. The cybrid model permits us to examine how particular mtDNA backgrounds render cells more or less susceptible to various stressors they may encounter in disease. In addition, we can treat our cells with various therapeutic agents, such as anti-VEGF drugs, to determine if certain haplogroups allow cells to receive a greater benefit from treatment.

Our laboratory has used this system to demonstrate significant differences in cellular health and activity between cells of diverse haplogroups. In two of our laboratory's studies, we compared cybrids of the J haplogroup, another European haplogroup, with H haplogroup cybrids. We found that while J cybrids produce less ROS than H cybrids, J cybrids express fewer transcripts for mtDNA-encoded OXPHOS genes

and are more glycolytic than H cybrids. In addition, J cybrids expressed lower transcript levels of complement genes (e.g. C1R, C1S, C3), the complement pathway inhibitor CFH, pro-inflammatory genes such as IL33, and the apoptosis inhibitor BCL2L13 than H cybrids.[78,79] However, these previous measurements were performed in the absence of a cellular stressor. When these cybrids were treated with ultraviolet radiation (UV), J cybrids expressed significantly less transcript of complement pathway inhibitors (e.g. CFH, CD55) but significantly more transcript of inflammatory factors (e.g. IL33, EFEMP1) than H cybrids.[80] Together, these data suggest that the J haplogroup is more susceptible to UV stress than the H haplogroup. While the H haplogroup appeared to be protective in these studies, we found that the K haplogroup may be more protective against a stressor found in AMD. When we examined H and K cybrids at baseline, the K cybrids were significantly more enriched for mtDNA OXPHOS transcripts than H cybrids. Additionally, the K cybrids produced more Apolipoprotein E, a protein that can bind to amyloid-β (Aβ) in hard retinal deposits (drusen) to improve their clearance from the retina. Consistent with this finding, the K cybrids were resistant to decreases in cell viability from active Aβ while the H cybrids showed a significant decrease in viability.[81]

We have also compared H and L cybrids to determine how these backgrounds may influence susceptibility to conditions such as Alzheimer's disease and DM. We found that while L cybrids consumed less oxygen for ATP turnover through OXPHOS than H cybrids, the L cybrids also expressed higher levels of mtDNA-encoded OXPHOS transcripts and produced less ROS than H cybrids. These data suggest that L cybrids produce energy through OXPHOS more efficiently than H cybrids. However, L cybrids also express significantly lower levels of complement pathway transcripts (e.g. C1R, C3) and higher levels of pro-inflammatory factors such as IL33, suggesting that the mtDNA

background may induce these cells to have different responses to disease-related stressors.[82]

To further understand the influence of mtDNA in diseases such as DM, this dissertation uses our cybrid system to examine two aspects of our cells. In **Chapter 1**, we asked how mitochondria of different haplogroups (European versus African+Asian) and diabetic statuses influence cell health. In **Chapter 2**, we investigated how these mitochondria affect cellular functions of our RPE cybrids. Our results suggest that certain mitochondrial backgrounds protect against decreases in cell health and function. The differentially-expressed pathways and genes we identified may prove valuable as targets for future DM and DR therapy.

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CHAPTER 1

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*.

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.

MATERIALS AND METHODS

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]

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 1.1)**. 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 1.2)**. 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).

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.

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]

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.

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.

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.

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.

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.

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.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

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.

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 Supplemental Table S1.1**. *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 = $2^{-\Delta\Delta Ct}$.

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*.

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.

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.

RESULTS

[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 (**Figure 1.1A**). 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 (**Figure 1.1B**). [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 ($p = 0.018$) (**Figures 1.1A, 1.1B**).

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 ($p = 0.29$) (**Figure 1.1C**).

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 (**Figure S1.1A**). 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, $p = 0.0025$) (**Figure S1.1B**). Overall, these findings suggest that the [Afr+Asi]/DM cybrids uniquely resist hyperglycemic stresses.

[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 ($p \approx 1$) (**Figure 1.2A,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, $p = 0.0024$) (**Figure 1.2D**). There were no significant changes for Euro cybrids (Non-DM: 0.99 ± 0.03, $p \approx 1$; DM: 0.99 ± 0.02, $p \approx 1$) (**Figure 1.2C**). 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.

[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, $p = 0.023$) (**Figure 1.3A**). 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 $p = 0.0056$) (**Figure S1.2A**).

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, $p < 0.0004$) (**Figure S1.2B**). 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 $p < 0.0004$) (**Figure S1.2C**).

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 ≈ 1; Euro/DM: 1.01 ± 0.18 -fold, p ≈ 1) (**Figure 1.3B**). 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) (**Figure 1.3C**). *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 ≈ 1) (**Figure 1.3D**). 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) (**Figure S1.3**). 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.

[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) (**Figure 1.4A**). 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) (**Figure 1.4B**). 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) (**Figure 1.4C**). In summary, the changes in bioenergetic measurements between DM and Non-DM cybrids are similar for European and [African plus Asian] cybrids.

[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 1.5**). 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) (**Figure 1.6A**). 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) (**Figure 1.6D**). 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) (**Figure 1.6B**) 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) (**Figure 1.6C**). 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) (**Figure 1.6E**) 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) (**Figure 1.6F**), suggesting that the distinct responses of these cybrids to stress are due to differential regulation of DNA methylation.

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 1.3**). 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.

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 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 1.7**). 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.

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FIGURES

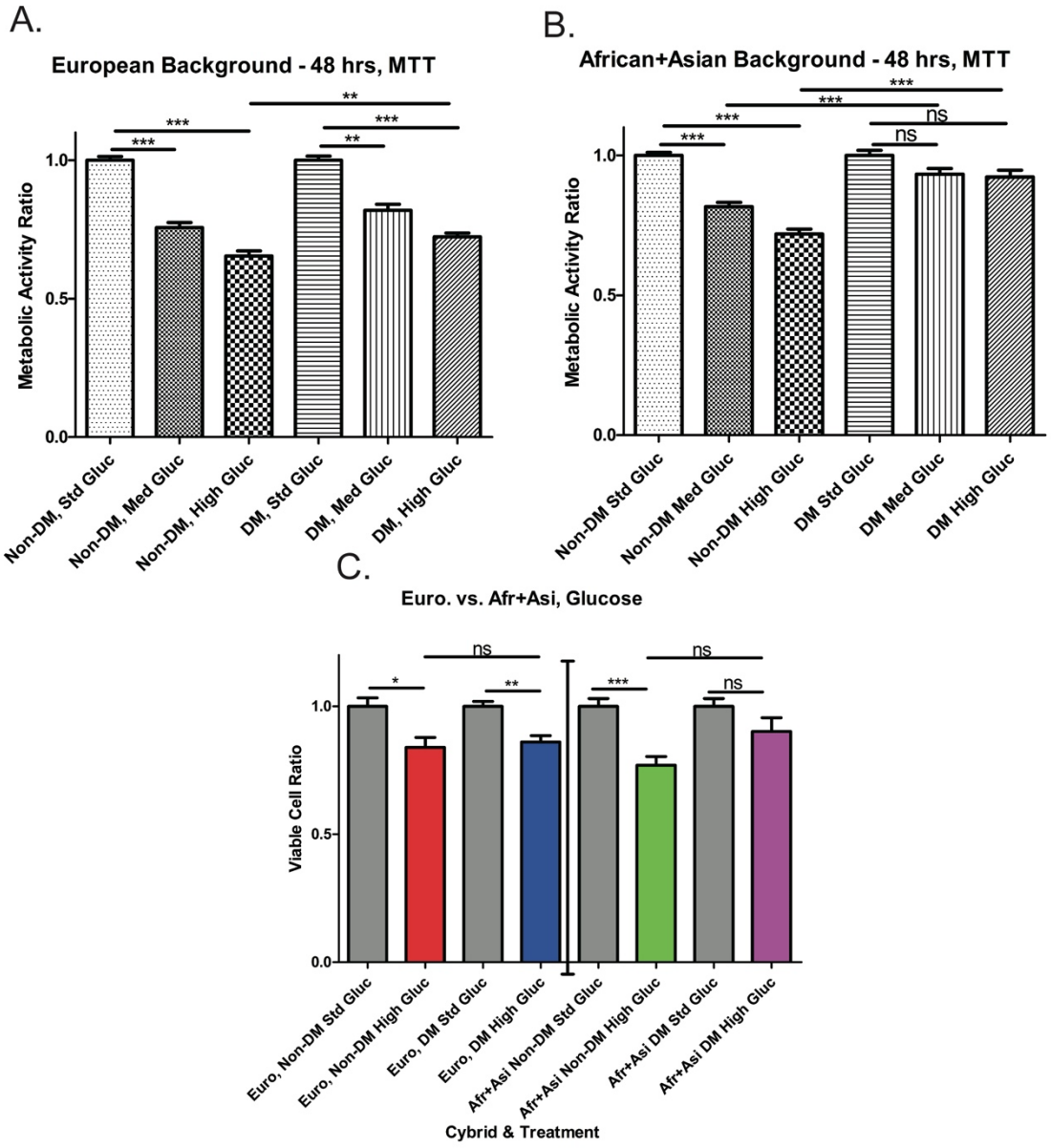


Figure 1.1. [Afr+Asi] DM cybrids resist decreases in viability from high glucose.
(A and B) Changes in viability after 48 hours normalized to Standard glucose (Std) controls, measured by MTT assay. (n = 3 Euro/Non-DM and Euro/DM, 4 [Afr+Asi]/Non-DM and [Afr+Asi] DM; 20 independent experiments total) (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 1.2. [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 1.2.

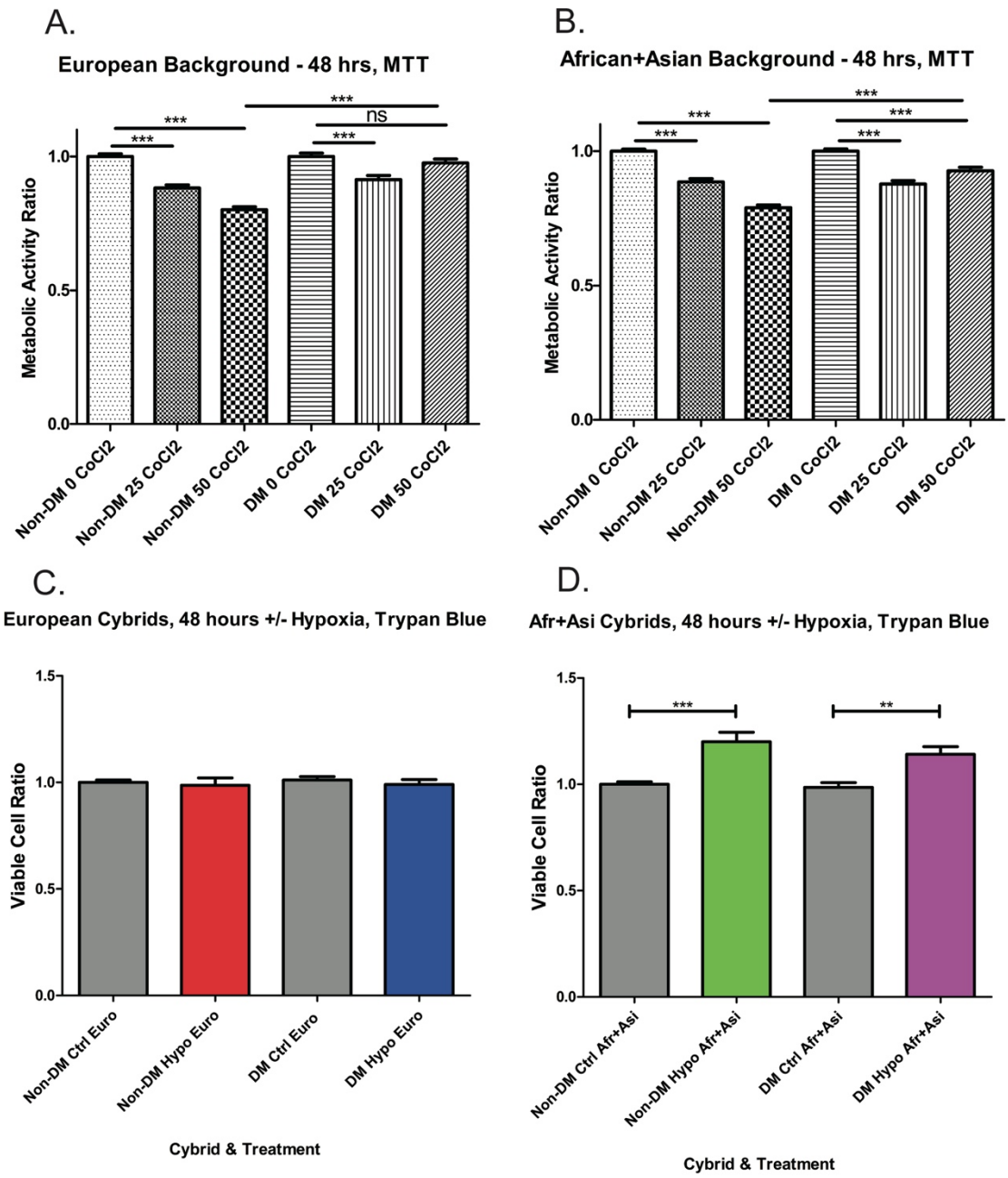
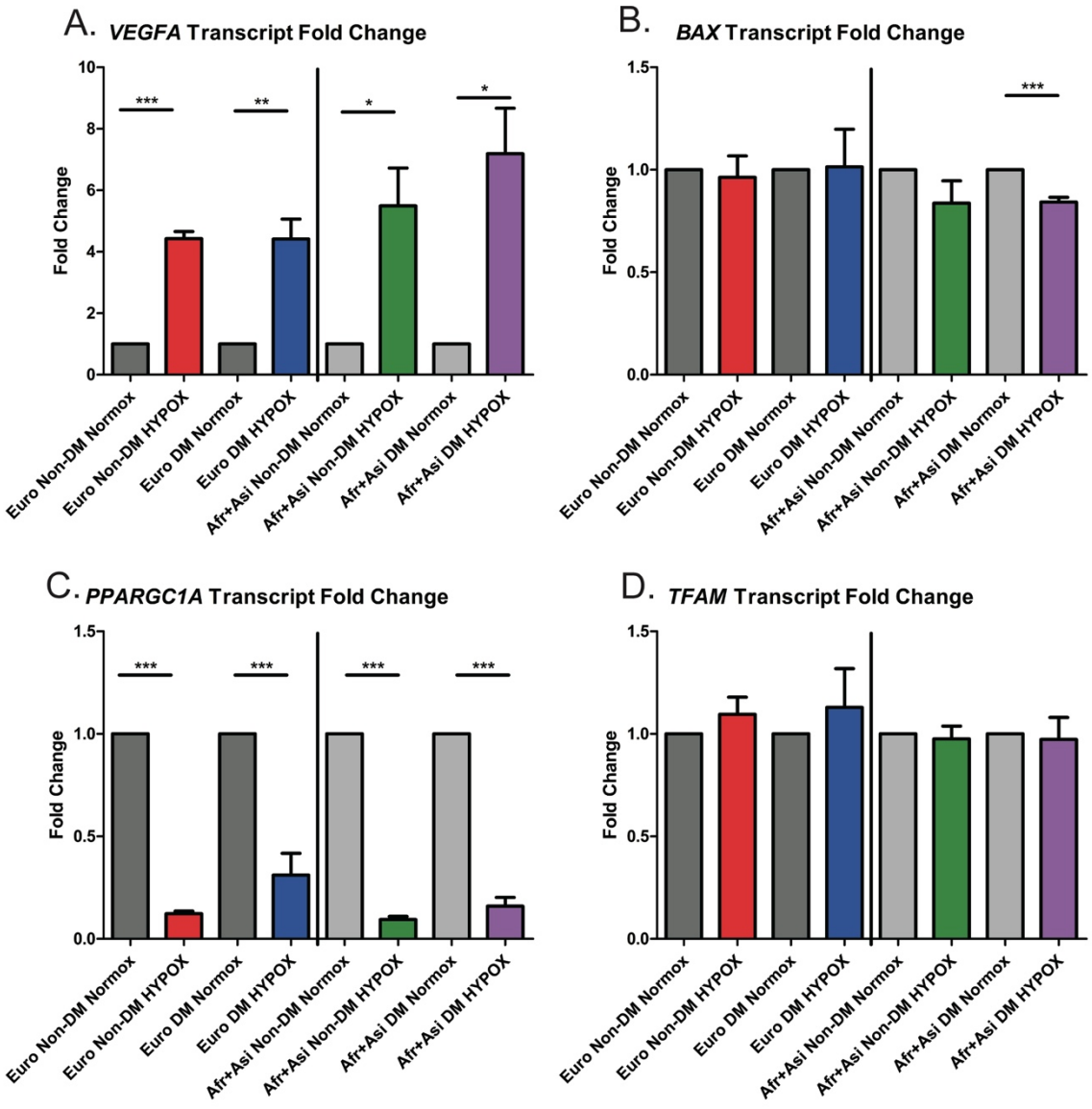

A.
European Background - 48 hrs, MTT
Metabolic Activity Ratio
1.0
0.5
0.0
***
***
***
***
ns
Non-DM 0 CoCl2
Non-DM 25 CoCl2
Non-DM 50 CoCl2
DM 0 CoCl2
DM 25 CoCl2
DM 50 CoCl2
B.
African+Asian Background - 48 hrs, MTT
Metabolic Activity Ratio
1.0
0.5
0.0
***
***
***
***
Non-DM 0 CoCl2
Non-DM 25 CoCl2
Non-DM 50 CoCl2
DM 0 CoCl2
DM 25 CoCl2
DM 50 CoCl2
C.
European Cybrids, 48 hours +/- Hypoxia, Trypan Blue
Viable Cell Ratio
1.5
1.0
0.5
0.0
Non-DM Ctrl Euro
Non-DM Hypo Euro
DM Ctrl Euro
DM Hypo Euro
Cybrid & Treatment
D.
Afr+Asi Cybrids, 48 hours +/- Hypoxia, Trypan Blue
Viable Cell Ratio
1.5
1.0
0.5
0.0
***
**
Non-DM Ctrl Afr+Asi
Non-DM Hypo Afr+Asi
DM Ctrl Afr+Asi
DM Hypo Afr+Asi
Cybrid & Treatment

Figure 1.3. 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 1.3.


A. VEGFA Transcript Fold Change
B. BAX Transcript Fold Change
C. PPARGC1A Transcript Fold Change
D. TFAM Transcript Fold Change
Fold Change
Euro Non-DM Normox
Euro Non-DM HYPOX
Euro DM Normox
Euro DM HYPOX
Afr+Asi Non-DM Normox
Afr+Asi Non-DM HYPOX
Afr+Asi DM Normox
Afr+Asi DM HYPOX

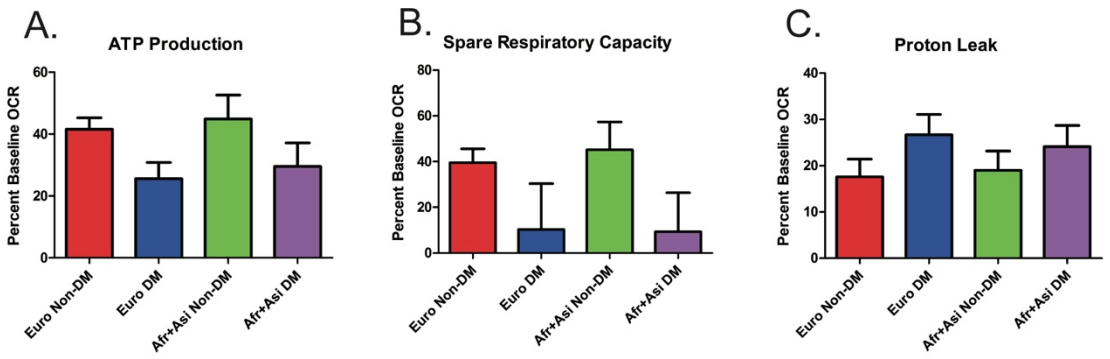


Figure 1.4. Euro and [Afr+Asi] cybrids have similar bioenergetic profiles in baseline conditions. Measurements of the amount of Oxygen Consumption Rate (OCR) relative to untreated conditions used for (A) ATP production, (B) Spare Respiratory Capacity, and (C) Proton Leak using the Seahorse XF24 flux 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)

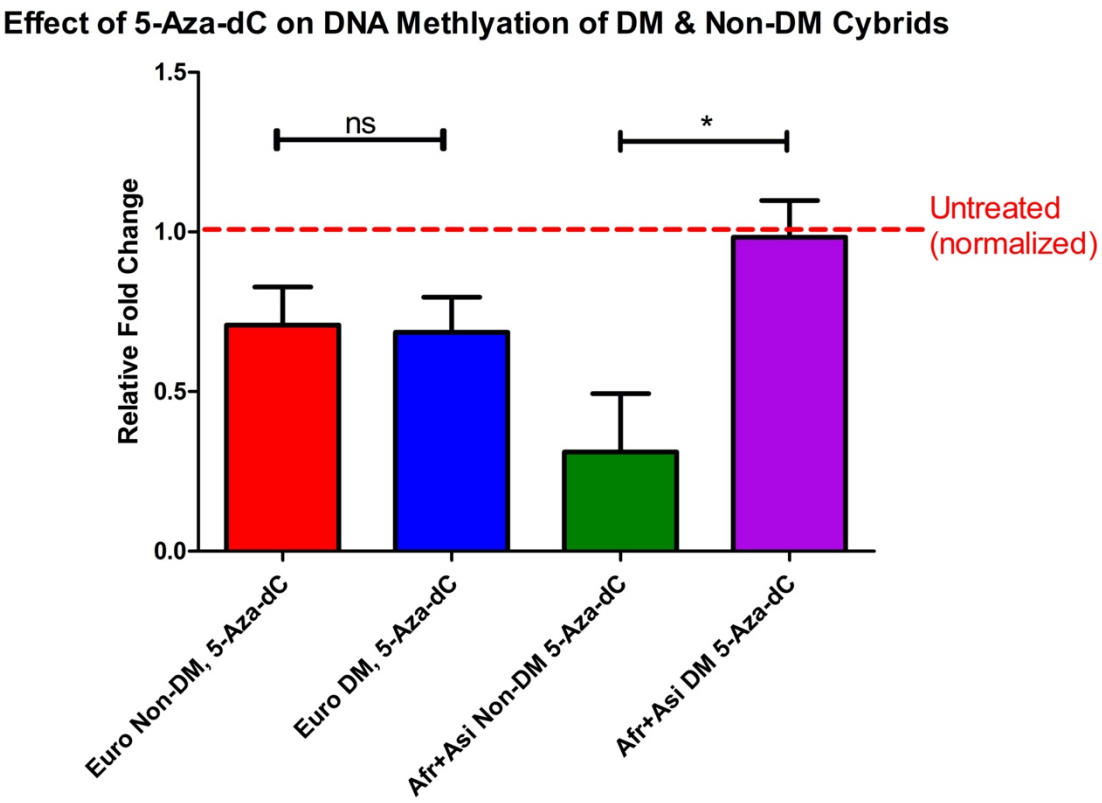
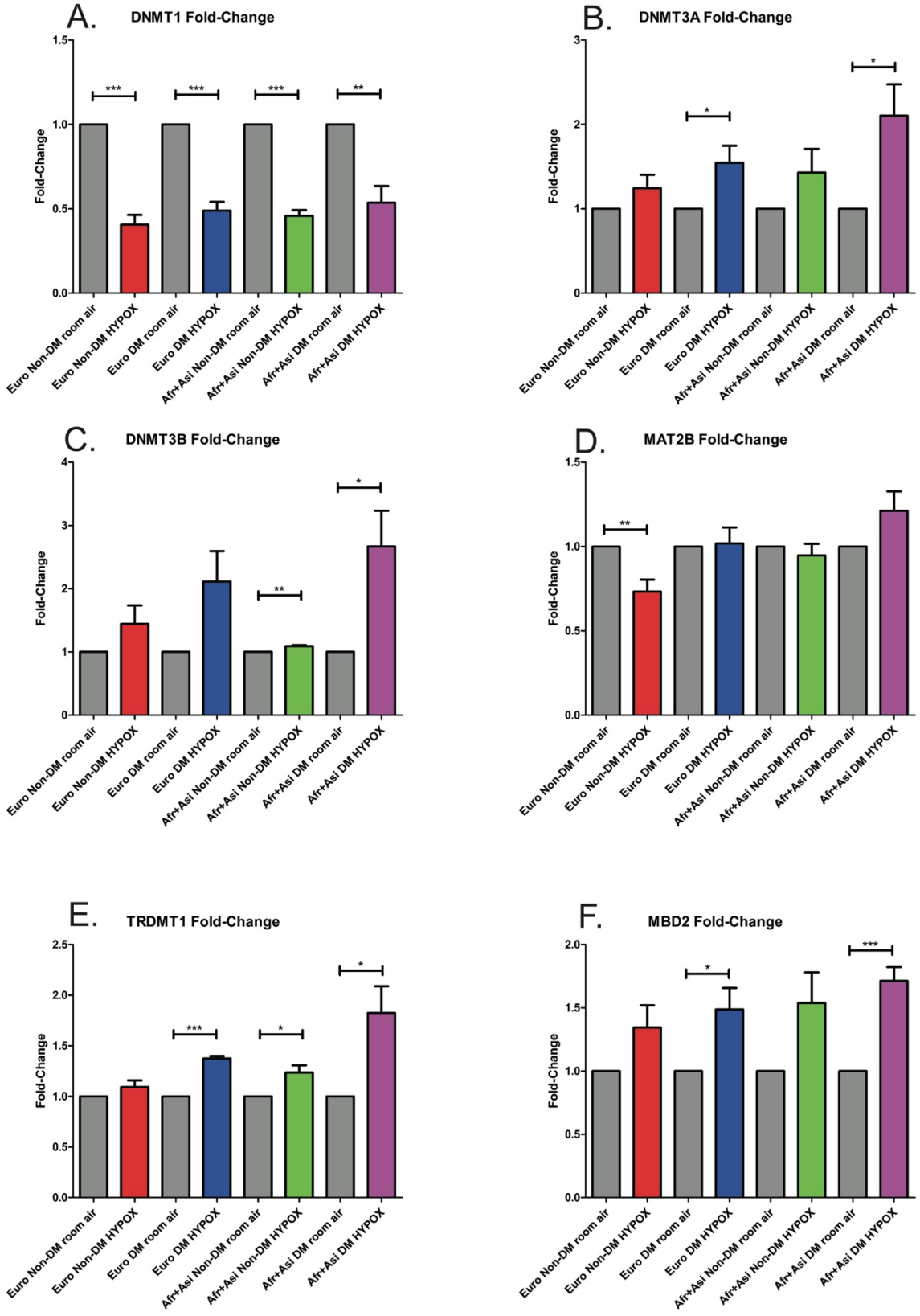
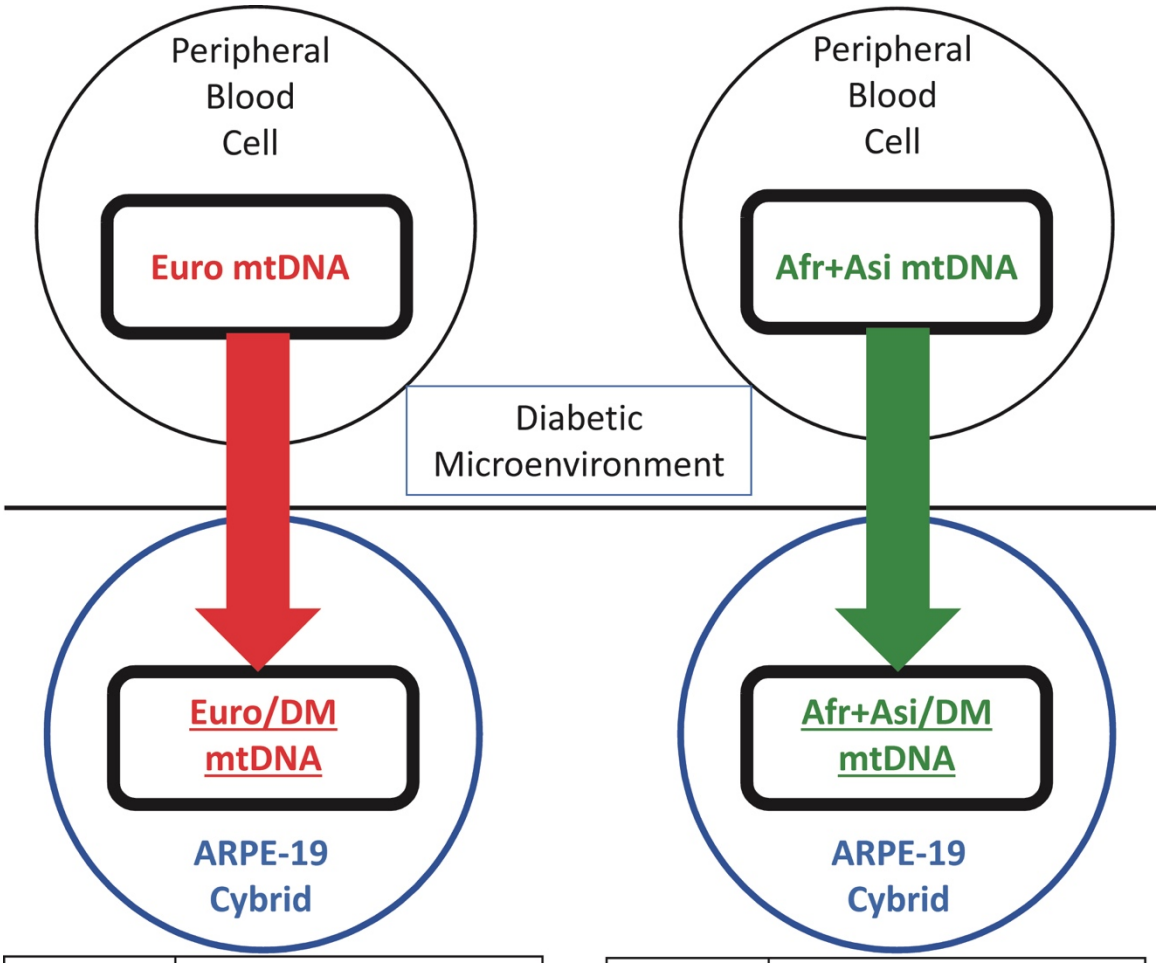


Figure 1.5. Euro and [Afr+Asi] cybrids have different levels of DNA methylation after treatment with 5-Aza-dC. Amount of DNA methylation of Euro and [Afr+Asi] cybrids grown ± 5-Aza-2-deoxycytidine (5-Aza-dC), a DNA methyltransferase inhibitor, as measured by an ELISA against 5-methylcytosine. (Euro/Non-DM 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 1.6. 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) * = $p < 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

Figure 1.6.


A.
DNMT1 Fold-Change
B.
DNMT3A Fold-Change
C.
DNMT3B Fold-Change
D.
MAT2B Fold-Change
E.
TRDMT1 Fold-Change
F.
MBD2 Fold-Change
Fold-Change
Euro Non-DM room air
Euro Non-DM HYPOX
Euro DM room air
Euro DM HYPOX
Afr+Asi Non-DM room air
Afr+Asi Non-DM HYPOX
Afr+Asi DM room air
Afr+Asi DM HYPOX



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

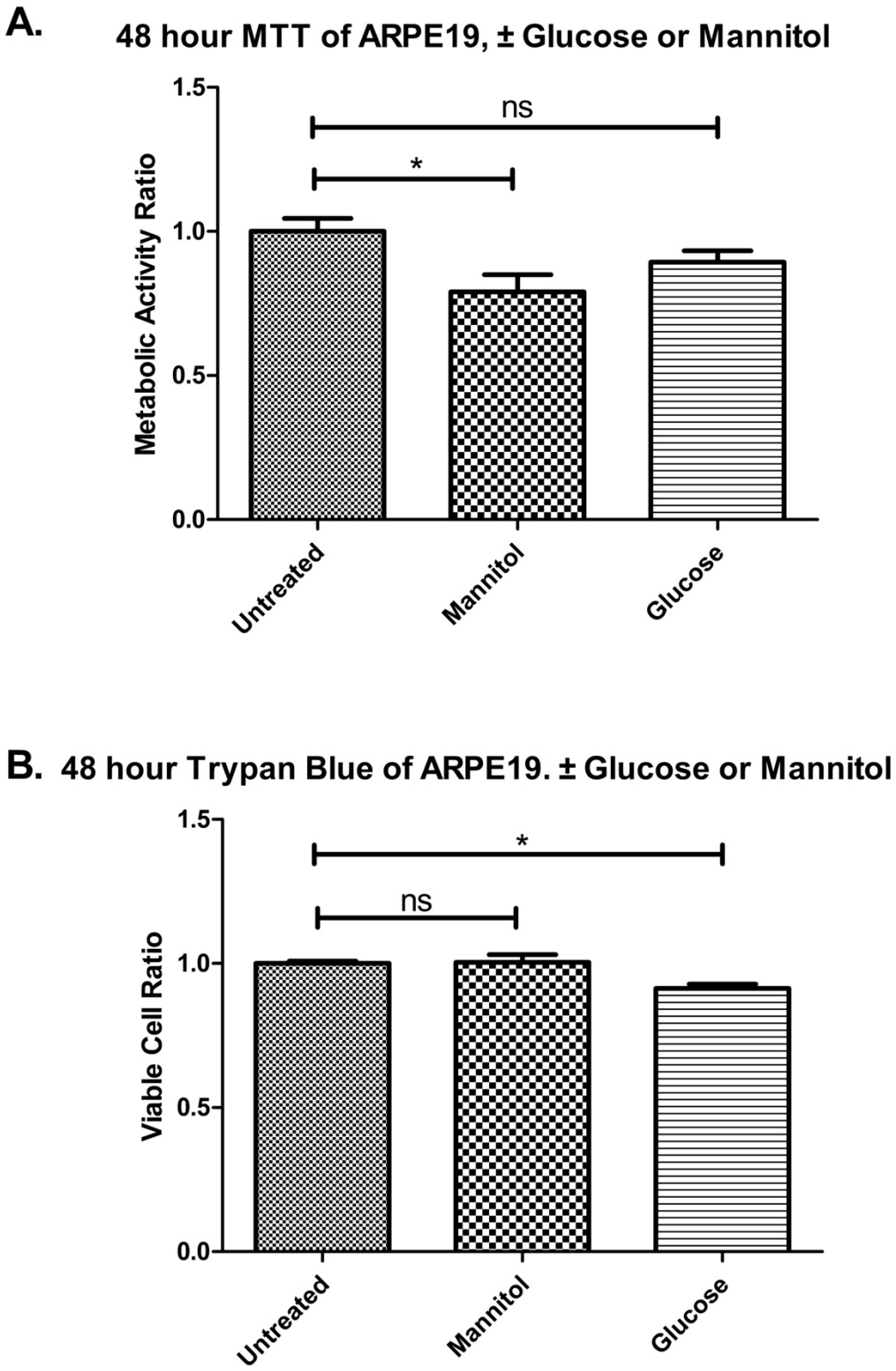
Stressor	Afr+Asi/DM Result
High Glucose	• **Resist** ⬇ Metabolic Activity • **Resist** ⬇ Live cells
Hypoxia	• Metabolic Activity – Resist ⬇ • Live cells – ⬆ • Transcripts – **Unique, Different From** Non-DM
5-Aza-dC	• DNA methylation **persists**

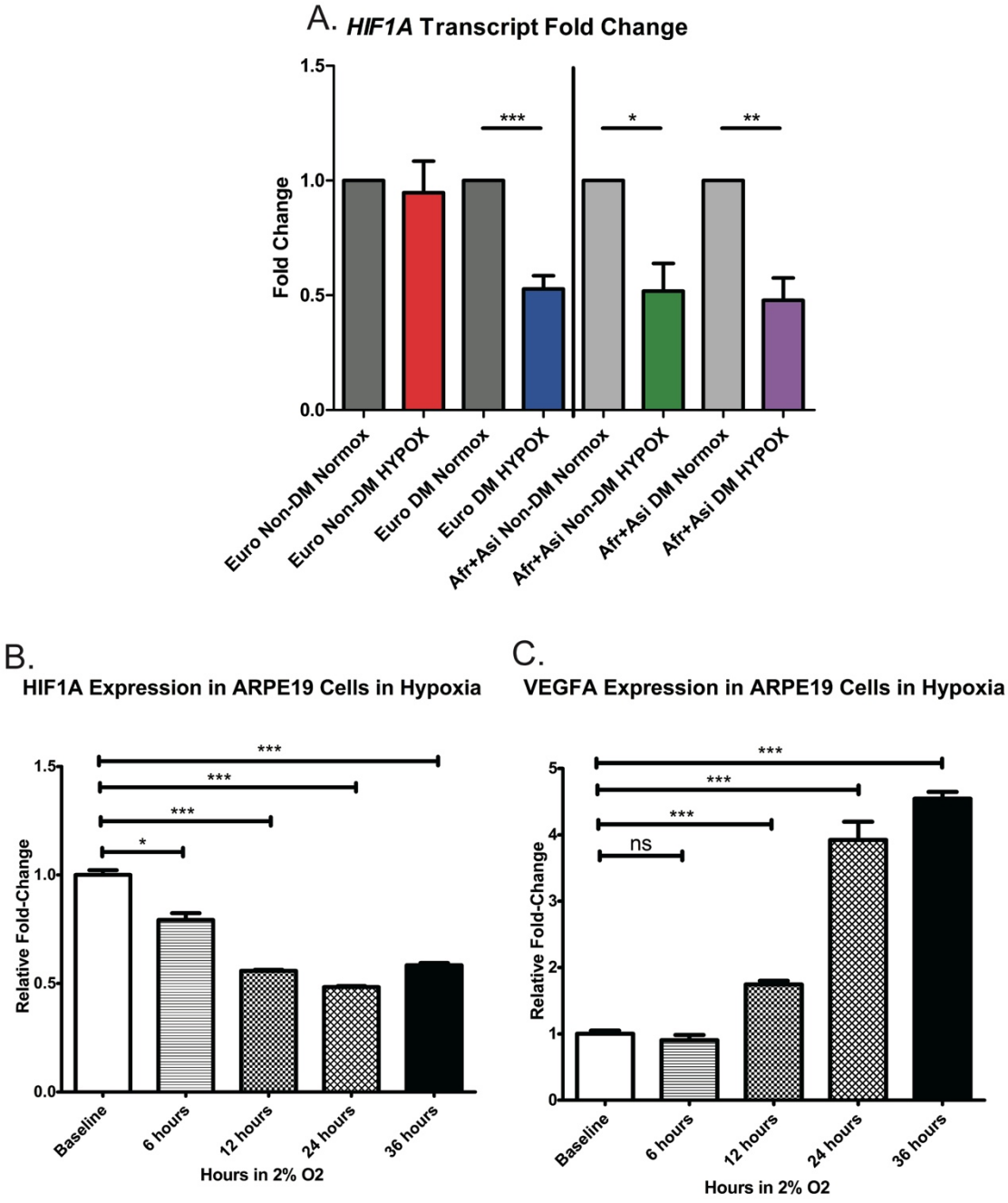
Figure 1.7. [Afr+Asi]/DM mitochondria confer transferable resistance to both high glucose and hypoxia. Summary of data from this study.

SUPPLEMENTAL FIGURES

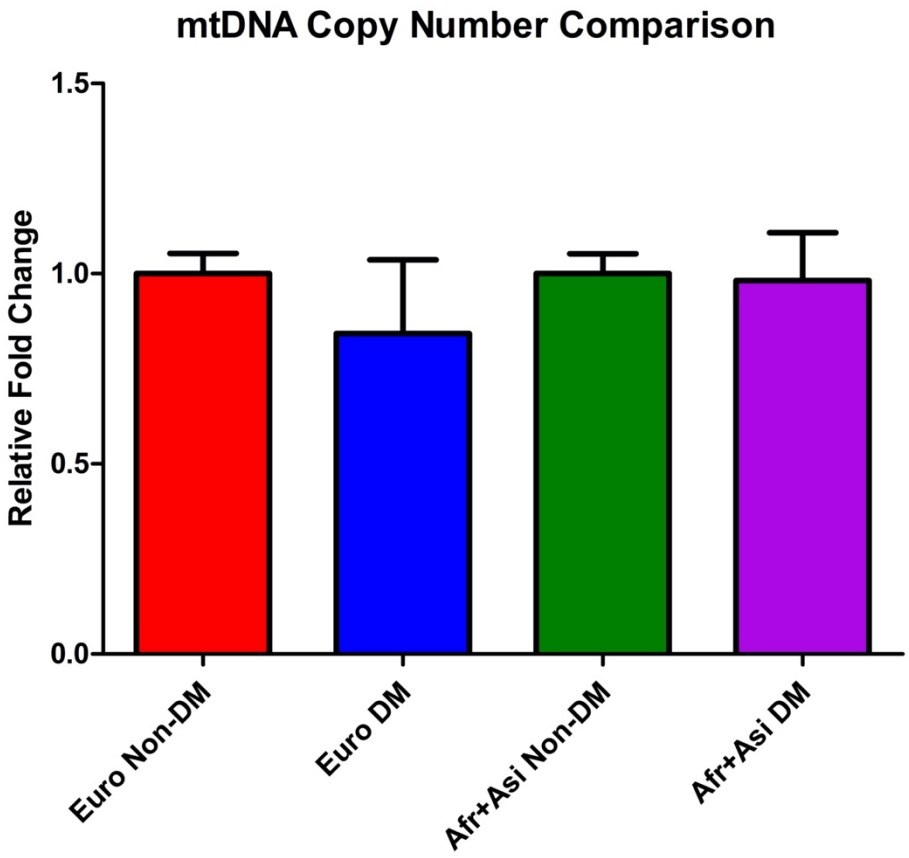
Supplemental Figure S1.1. Effect of High Mannitol vs. Glucose on ARPE19 Cells. (A) Changes in viability after 48 h normalized to untreated (standard glucose) controls, measured by MTT assay. Experiment was done with 6 replicates. (B) Changes in viability after 48 h normalized to untreated (standard glucose) controls, measured by trypan blue dye exclusion assay. Experiments were done in duplicate, repeated twice.

Supplemental Figure S1.1.





Supplemental Figure S1.2. HIF-1α Transcript Decreases In Cultures of Cybrids and ARPE-19 Cells Grown in Extended Hypoxia. RNA from cybrids grown 48 h ±2% O2 was measured by qRT-PCR against (A) *HIF1A*. (n = 4 Euro/Non-DM, n = 4 Euro/DM, n = 4 Afr+Asi/Non-DM, n = 3 Afr+Asi/DM; 8 independent experiments total) RNA from ARPE-19 cells grown in ±2% O2 for certain periods of time was measured by qRT-PCR against (B) *HIF1A* and (C) *VEGFA*. Experiments were done in triplicate. * = $p < 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$



Supplemental Figure S1.3. Cybrids Used in These Experiments Have Similar mtDNA Copy Numbers. DNA from cybrids grown in room air was measured by qRT-PCR against the mitochondrial gene *MT-ND2* and the nuclear gene *18S*, and the ratio *MT-ND2* / *18S* was calculated for each cybrid. Within each mtDNA background, values were normalized to those of the non-diabetic cybrids. (n = 7 Euro/Non-DM, n = 4 Euro/DM, n = 4 Afr+Asi/Non-DM, n = 4 Afr+Asi/DM; 4 independent experiments total) * = $p < 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

Table 1.1. Euro and [Afr+Asi] cybrids used in these experiments have a similar average age.

	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		

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.

Table 1.2. Diabetes and diabetic retinopathy status of patients whose mitochondria were used in our cybrids.

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 1.3. 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

Supplemental Table S1.1. Primers Used In These Experiments.

Primer Name	Function	Manufacturer
H_VEGFA_1	Growth factor that induces blood vessel growth and endothelial cell migration	MilliporeSigma
Hs_BAX_1_SG QuantiTect Primer Assay	Pro-apoptotic indicator, induces cytochrome c release in apoptosis	Qiagen
H_HPRT1_1	Enzyme involved in purine production [used as housekeeping gene]	MilliporeSigma
H_PPARGC1A_2	Master regulator of metabolism, induces mitochondrial replication	MilliporeSigma
H_TFAM_1	Enzyme involved in mitochondrial replication	MilliporeSigma
H_DNMT1_1	DNA methyltransferase, usually for maintenance of 5-methylcysteines (5-mC)	MilliporeSigma
H_DNMT3A_2	DNA methyltransferase, usually for de novo 5-mC formation	MilliporeSigma
H_DNMT3B_1	DNA methyltransferase, usually for de novo 5-mC formation	MilliporeSigma
H_MAT2B_1	Enzyme that catalyzes formation of S-adenosylmethionine (SAM), a precursor metabolite for 5-mC formation	MilliporeSigma
Hs_MBD2_1_SG QuantiTect Primer Assay	Enzyme that binds 5-mC, repressing transcription	Qiagen
H_TRDMT1_1	Methyltransferase of Aspartic Acid tRNA, has some 5-mC function on DNA	MilliporeSigma
H_HIF1A_1	Master regulator of hypoxia response	MilliporeSigma

CHAPTER 2

Hypoxia-Induced Transcriptional Differences in African and Asian Versus European Diabetic Cybrids

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ABSTRACT

Diabetic retinopathy (DR) is the most common diabetic microvascular complication as well as the most common cause of blindness in adults under the age of 65. Our results suggest that, when comparing transcriptomes of cultures grown in hypoxic conditions versus room air, cybrids containing mitochondria from African and Asian diabetic subjects ([Afr+Asi]/DM) have some uniquely different transcriptome profiles compared to European/diabetic (Euro/DM) cybrids (eg. Fatty acid metabolism: EnrichR rank 10 in Afr+Asi, rank 85 in Euro; Endocytosis: rank 25 in Afr+Asi, rank 5 in Euro; Ubiquitin mediated proteolysis: rank 34 in Afr+Asi, rank 7 in Euro). As determined by both RNA-seq and qRT-PCR results, transcription of the gene encoding oleoyl-ACP hydrolase (*OLAH*) was significantly increased in [Afr+Asi]/DM cybrids compared to Euro/DM cybrids in hypoxic conditions. Additionally, our results show that in hypoxic conditions, Euro/DM and [Afr+Asi]/DM cybrids show similar decreases in ROS production. All cybrids showed decreased ZO1-minus protein levels, but their phagocytic functions were not significantly altered in hypoxic conditions. In conclusion, our findings suggest that the "molecular memory" imparted by [Afr+Asi]/DM mtDNA may act through one of the

molecular pathways seen in transcriptome analysis, such as fatty acid metabolism, without significantly changing essential RPE functions.

INTRODUCTION

Over the past 40 years, diabetes mellitus (DM) has become one of the most widespread diseases worldwide. Within the past decade, its prevalence increased from 366 million people in 2011 to 451 million in 2017, and it is predicted to further increase to 693 million people by 2045.[1,2] The characteristic hyperglycemia of this disease induces an inflammatory response that causes damage to blood vessels, resulting in the macrovascular complications, such as myocardial infarction, and microvascular complications, such as nephropathy and peripheral neuropathy.[3] Diabetic retinopathy (DR) is the most common diabetic microvascular complication as well as the most common cause of blindness in adults under the age of 65.[4] In DR, high glucose promotes non-enzymatic glycation of proteins, leading to oxidative stress and inflammation. Together, these elements damage the intraretinal vasculature, which supports the neurons of the inner retinal layers, and the choriocapillaris, which nourishes the photoreceptors in the outer retina.[5]

In healthy individuals, a blood-retina barrier prevents vascular contents from leaking into the retina. The endothelial cells that line the intraretinal vessels tightly bind to each other through tight junctions proteins; however, in DR and related conditions, such as diabetic macular edema (DME), this barrier becomes more permeable.[6,7] The choriocapillaris, which provides nutrients to the outer retina, is fenestrated and consequently more prone to leaking its contents. To prevent vascular leakage from entering the outer retina, retinal pigment epithelial (RPE) cells form a barrier that governs molecular transport between the outer retina and the choroid vessels. In diabetes, the RPE acquires damage that impairs its barrier functions, and these dysfunctions are implicated in the development of DR and DME.[7,8]

The RPE performs multiple functions in order to maintain a homeostatic balance with photoreceptors and preserve the architecture of the outer neural retina. Normally, photoreceptors shed their outer segments full of retinals, and the RPE phagocytoses these segments to convert the *all-trans*-retinal into 11-*cis*-retinal that photoreceptors can use to form new rhodopsins.[9,10] In diabetes, RPE cells are suspected to have decreased phagocytosis as shown in one study that reported decreased phagocytosis of rod outer segments in RPE cells exposed to high glucose *in vitro*.[11] In order to constantly recycle photoreceptor outer segments, RPE cells are highly metabolic, generating a substantial amount of reactive oxygen species (ROS) from mitochondrial oxidative phosphorylation in the process.[12] Diabetes leads to a decline in the ability of retinal cells to reduce ROS as levels of antioxidants, such as glutathione, are decreased.[13] Additionally, the RPE forms the outer portion of the blood-retina barrier through tight junctions proteins, such as zonula occludens-1 (ZO-1) and occludin.[14,15] In diabetes, these proteins are depleted, contributing to a decrease in barrier function.[16-18]

While many studies have examined the effect of diabetes-induced changes in mitochondrial function[19,20] or the effect of mt DNA damage[21,22] on cellular health, none have examined the role of mitochondrial haplogroups on RPE function in diabetes. Individuals can be categorized into haplogroups, which are sets of single nucleotide polymorphisms (SNPs) in mtDNA that arose from an ancestral origin.[23] We have previously shown that mitochondria from diabetic patients of African or Asian ancestry confer resistance against hyperglycemic and hypoxic stressors in RPE cells.[24] In this investigation, we used our cellular hybrid (cybrid) RPE cells with mitochondria from diabetic (DM) or nondiabetic (non-DM) patients of African and Asian ([Afr+Asi]) or European (Euro) haplogroups to explore how mtDNA affects the transcriptomes and functions of RPE cells. Our findings from this study suggest that cybrids containing mitochondria from African and Asian diabetic subjects have significantly different transcriptomes than Euro/DM cybrids cultured in hypoxic conditions. Findings herein also show that RPE cybrids with Euro/DM or [Afr+Asi]/DM exhibit similar changes in phagocytic function

and tight junctions protein expression when cultured in hypoxia. Overall, our findings suggest that the unique "molecular memory" imparted by [Afr+Asi]/DM mtDNA may occur through one of the molecular pathways observed in transcriptome analysis without changing the essential RPE functions.

MATERIALS AND METHODS

Human Subjects

Research involving human subjects was approved by the Institutional Review Board of the University of California, Irvine (#2003-3131). All enrolled patients provided written, informed consent. Clinical investigations were performed based on the ethical principles of the Declaration of Helsinki.[26]

Cybrid Generation

Patient blood was collected in tubes containing 3.2% sodium citrate before being subjected to multiple centrifugations to isolate platelets. Platelet pellets were resuspended in Tris buffered saline (TBS). ARPE-19 cells, an RPE cell line (American Type Culture Collection (ATCC), Manassas, VA), were sequentially passaged in low-dose ethidium bromide to depleted cells of mtDNA (Rho_0). Rho_0 ARPE-19 cells were then cultured in standard culture media (10% FBS, 100 units/mL Penicillin, 100 mcg/mL Streptomycin, 25 µg/mL Fungizone (Amphotericin B: Omega Scientific, Torzana, CA), and 50 µg/mL Gentamycin in DMEM/F12) supplemented with uridine.[25] Then, platelets were fused with Rho_0 cells in polyethylene glycol-containing media in order to generate cybrids, as per a modified protocol by Chomyn.[26] Cybrids were cultured in standard culture media alone to select for RPE cells that successfully integrated mitochondria. mtDNA incorporation was verified using a combination of polymerase chain reaction (PCR) and restriction enzyme digestion of these PCR products. In addition, mtDNA inclusion was verified

through mtDNA sequencing to identify the mtDNA haplogroup for each cybrid.[27] The cohorts of Euro and Afr+Asi cybrids were generated from individuals of similar ages (p = 0.11), genders, and DM versus Non-DM status **(Supplemental Table S2.1)**. In the present study, our cybrids were cultured in media containing 17.5 mM glucose.

RNA-Seq

RNA Extraction – Cybrids (Euro/Non-DM, n = 4; Euro/DM, n = 4; Afr+Asi/Non-DM, n = 4; and Afr+Asi/DM cybrids, n = 4) were cultured in 6-well plates at 500,000 cells/well in duplicate and incubated overnight at 37°C with 5% CO_2, then in 2 mL standard media at 37°C with 5% CO_2 in either room air (~21% O_2) or in a 2% O_2 incubator (MCO-18M O_2/CO_2 Incubator, Sanyo, Osaka, Japan) for 48 hours. 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).

RNA Library Preparation and RNA-Seq – RNA from each sample was analyzed for quality control through a combination of 2100 Bioanalyzer Nano RNA chip (Agilent Technologies, Santa Clara, CA) and NanoDrop ND-1000 (Thermo Scientific) absorbance ratios (260/280nm and 260/230nm). Library construction was performed as per the TruSeq Stranded mRNA protocol (Illumina, San Diego, CA). For each sample, 500 ng of total RNA was enriched for mRNA using oligo-dT magnetic beads and subsequently chemically-fragmented for three minutes. Then, first-strand synthesis was performed, using random primers and reverse transcriptase to make single-stranded cDNA. After second-strand synthesis, the resultant double-stranded cDNA was cleaned using AMPure XP beads (Beckman Coultier, Brea, CA). Cleaned cDNA was end-repaired, and then the 3' ends were adenylated. Illumina barcoded adapters were ligated on the ends of the cDNA and the adapter-ligated fragments were

enriched by nine cycles of PCR. The resulting libraries were validated by qPCR and sized by Agilent Bioanalyzer DNA high sensitivity chip. The concentrations for the libraries were normalized before multiplexing the libraries together and then sequenced using single end 100 cycles chemistry on a HiSeq 4000 sequencer (Illumina).

RNA-Seq Output Analysis – After acquiring the reads, the data were analyzed for quality control using *FastQC*. Reads were then quality and adaptor trimmed using *Trimmomatic*.[28,29] Data were then aligned to the human hg19 reference genome using a short read sequence alignment tool that can account for splice variation, *TopHat2*.[30] Gene expression was quantified by raw read counts using *featureCounts* and Reads-per-Kilobase-per-Million matched reads (RPKM) using *Cufflinks*.[31,32]

Quantified data was then grouped into 8 groups based on whether the cybrid from which the RNA originated had Euro or Afr+Asi mtDNA, was from a DM or Non-DM patient, or was grown in room air or hypoxia. Differential gene expression between selected groups was performed using an *R*-based tool, *DESeq2*.[33]

Differentially-expressed gene lists from comparisons of DM cybrids in room air with their respective hypoxic samples ([Afr+Asi/DM/Room Air versus Afr+Asi/DM/Hypoxia] and [Euro/DM/Room Air versus Euro/DM/Hypoxia]) were analyzed for pathway enrichment terms using EnrichR.[34-36] The top 10 differentially-expressed pathways for each comparison were presented, using the Kyoto Encyclopedia of Genes and Genomes 2016 (KEGG 2016) classification where unique pathways in the top 10 lists were highlighted.

ROS Assay

ROS levels were measured in our cybrids (n = 4 for all groups) using a variation of the methods we have previously described.[37] Experiments were performed in sextuplicate. Briefly, cybrids were plated in 100 µL of standard culture media at a density of 10,000 cells/well in 2

separate 96-well, black well, clear-bottom plates, incubated overnight at 37°C with 5% CO_2, and subsequently incubated 48 hours at 37°C with 5% CO_2 in either room air or 2% O_2. After incubation, media were replaced with 100 µL of 10 uM dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen) in Dulbecco's PBS (DPBS; Gibco, ThermoFisher Scientific, Waltham, MA), and plates were incubated at at 37°C without additional CO_2 for 30 minutes. Subsequently, H_2DCFDA solution was removed and replaced with 100 µL DPBS. Fluorescence was measured using a SpectraMax Gemini XPS fluorescent plate reader (Molecular Devices, San Jose, CA) with excitation at 492 nm and emission at 520 nm. For each cybrid, values were normalized to the average fluorescence of samples cultured in room air.

Phagocytosis Assay

Phagocytosis was measured in our cybrids (n = 4 Euro/Non-DM, n = 5 Euro/DM, n = 3 Afr+Asi/Non-DM, n = 3 Afr+Asi/DM) using a variation of the methods described by Vo et al.[38] All experiments were performed in triplicate.

Culture of Cybrids With Fluorescent Beads – For each cybrid, cells were seeded at a density of 500,000 cells/well in 2 mL of standard culture media in 2 separate 6-well plates and then incubated overnight at 37°C with 5% CO_2. After carefully removing media from wells, media were replaced with 2 mL of a solution of 1 µm, fluorescently-tagged latex microbeads (Fluoresbrite® YG Microspheres 1.00 µm; Polysciences, Inc., Warrington, PA) diluted 1:3,000 in standard culture media. For each cybrid, 1 plate was incubated 48 hours at 37°C with 5% CO_2 in 2% O_2 while the other plate was incubated 48 hours at 37°C with 5% CO_2 in room air.

After incubation, wells were briefly washed 3 times with 2 mL PBS-EDTA per wash and trypsinized. For each well, 2 mL of standard media was added before pipetting each the contents into a separate 15 mL conical tube. Then, 1.5 mL of standard media was added to each well and then added to its respective 15 mL conical tube. Cell suspensions were then

triturated with a 5 mL pipette to separate into single cells, strained through separate 35 µm nylon filters into separate 5 mL test tubes (Corning™ Falcon™ Test Tube with Cell Strainer Snap Cap;Corning Inc., Corning, NY), and centrifuged for 5 minutes at 1000 RPM. For each tube, media were carefully removed, and the pellet was resuspended in 50 µL of PBS-EDTA.

Flow Cytometry Analysis of Phagocytosis – Each sample was loaded into an ImageSteamX Mark II Imaging Flow Cytometer (Luminex Corp., Austin, TX), excited using a 488 nm laser, and imaged at 40x magnification. 5,000 images were collected for each sample.

Imageset data were then analyzed using IDEAS software (Luminex Corp.) Briefly, images were first gated by object diameter to isolate images with single cells. Then, this subset was gated by fluorescence to determine the images containing microspheres. Finally, this smaller subset was gated to identify images where microspheres had been internalized. To do this, the software generates a “mask” of the cell area, “erodes” this area by a few pixels from the outer edge, and then determines if a fluorescent signal is located within the eroded mask. An internalization ratio was then calculated as follows: [Single Cells that Internalized Beads] / [Total Single Cells]. For each cybrid and condition, this ratio was then normalized to the ratio of its age-matched and haplogroup-matched, Non-DM cybrid cultured in room air.

Western Blot of Tight-Junctions Proteins

Protein Extraction from Cybrid Cultures – For each cybrid, cells were seeded at a density of 500,000 cells/well in 2 mL of standard culture media in all wells of 2 separate 6-well plates and then incubated at 37°C with 5% CO_2 for 19 days. Then, for each cybrid, 1 plate was incubated 48 hours at 37°C with 5% CO_2 in 2% O_2 while the other plate was incubated under same conditions in room air. Each well was lysed in 250 µL of a radioimmuniproecipitation assay (RIPA) buffer-based solution (RIPA Lysis Buffer (MilliporeSigma, Burlington, MA), PhosphataseArrest™ I (Gbiosciences, St. Louis, MO), and Protease Inhibitor Cocktail

(MilliporeSigma). For each plate, lysates from wells in the same column were combined and placed in a 1.5 mL microcentrifuge tube. Lysates were centrifuged for 15 minutes at 14,000 x g at 4°C and supernatants were collected. Clarified lysate protein concentrations were measured using the Pierce™ BCA Protein Assay kit (Thermo Scientific). Experiments were done in triplicate.

Immunoblotting for Tight Junctions Proteins – For each sample, 75 µg of protein was denatured in protein loading buffer (lauryl dodecyl sulfate (Bolt™ LDS Sample Buffer; Life Technologies, ThermoFisher Scientific); dithiothreitol (Bolt™ Sample Reducing Agent; Life Technologies) at 95°C for 5 minutes. Twenty µg of protein in loading buffer of each sample was loaded into the wells of 4-12% Bolt™ Mini Gels (Life Technologies) with protein ladder (Precision Plus Protein™ Dual Color Standards; BioRad, Hercules, CA) and subjected to electrophoresis for 1-2 hours at 100 V. Proteins were then transferred from the gels onto PVDF membranes (ImmunBlot®; BioRad) using wet transfer for 1 hour at 20 V. Membranes were blocked in 5% milk in 1x TBST (Tris Base (Trizma®; MilliporeSigma), 0.1% Tween-20 (Fisher Scientific, ThermoFisher Scientific)) at room temperature for 1 hour and then incubated overnight at 4°C in primary antibodies **(Supplemental Table S2.2)** in 5% milk in 1x TBST.

Membranes were washed in 1x TBST and incubated with secondary antibody **(Supplemental Table S2.2)** in 5% milk in 1x TBST for 1 hour at room temperature. Membranes were washed again in 1x TBST. Protein bands were detected using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) as per manufacturer's instructions. β-actin was used as a housekeeping protein control. Chemiluminescent images were captured using a ChemiDoc MP imager (BioRad) and quantified using ImageJ (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Unless otherwise indicated, 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). Results were adjusted using a Bonferonni correction where appropriate. Results with $p \leq 0.05$ were considered statistically significant.

RESULTS

African+Asian and European Cybrids Show Unique Differences in Transcriptomes Between Hypoxic and Room Air Cultures

We further characterized the molecular mechanisms underlying our previous observations by performing high-throughput RNA-seq on our cybrid population in room air and hypoxic conditions. We first examined the transcriptomes of the DM cybrids in room air and compared them to the transcriptomes of these same cybrids in hypoxia: Afr+Asi/DM/Room Air versus Afr+Asi/DM/Hypoxia **(Table 2.1)** and Euro/DM/Room Air versus Euro/DM/Hypoxia **(Table 2.2)**. Genes that were significantly expressed between the two conditions were then analyzed using the EnrichR online software to determine the top-ranked, differentially-expressed pathways, as categorized by the KEGG 2016 enrichment terms. For both the Euro and Afr+Asi comparisons, differential gene expression of certain pathways was highly-ranked, such as those involving protein processing in the endoplasmic reticulum (3^{rd} for Afr+Asi, 2^{nd} for Euro), metabolic pathways (2^{nd} for Afr+Asi, 3^{rd} for Euro), and carbon metabolism (4^{th} in both lists) **(Tables 2.1 and 2.2)**. However, other pathways were more highly-ranked for the Euro cybrids than the Afr+Asi cybrids. In particular, more genes in pathways associated with endocytosis (25^{th} for Afr+Asi, 5^{th} for Euro) and degradation of ubiquitylated proteins (34^{th} for Afr+Asi, 7^{th} for Euro) were differentially-expressed in the Euro cybrids comparison than that of the Afr+Asi cybrids. In addition, more genes in pathways associated with fatty acid metabolism

were differentially-expressed in the comparison of Afr+Asi/DM cybrid transcriptomes than in the comparison of Euro/DM transcriptomes (10^{th} for Afr+Asi, 85^{th} for Euro).

In addition, we performed a pairwise comparison of transcriptome data between Euro/DM and Afr+Asi/DM cybrids cultured in hypoxia. The analysis revealed that the transcript for oleoyl-ACP hydrolase (OLAH), an enzyme involved in medium fatty acid chain synthesis, was significantly more enriched in Afr+Asi/DM cybrids than Euro/DM cybrids **(Table 2.3)**. qRT-PCR for *OLAH* in our DM/Hypoxia samples revealed that *OLAH* was approximately 4-fold higher in Afr+Asi samples than Euro samples ($p = 0.045$) **(Figure 2.1)**. Overall, these results suggest that genes in the pathways of endocytosis, ubiquitylated protein degradation, and fatty acid metabolism (such as *OLAH*) are differentially-expressed when different mtDNA haplogroup profiles are present.

ROS Levels Decrease in Hypoxia for European and African+Asian Cybrids.

To determine if mtDNA haplogroup and diabetes status affect the cellular functions of RPE cells, we first performed an assay to detect ROS levels in our cybrids **(Figure 2.2)**. When we cultured cybrids in hypoxic conditions, ROS levels significantly decreased for all groups as compared to cultures grown in room air (Euro/Non-DM: 0.73 ± 0.05 -fold of ROS levels in room air cultures, $p = 0.0020$; Euro/DM: 0.77 ± 0.08 -fold, $p = 0.029$; Afr+Asi/Non-DM: 0.62 ± 0.05 -fold, $p = 0.0003$; Afr+Asi/DM: 0.77 ± 0.09, -fold $p = 0.039$) **(Figure 2.2)**. For both Euro and Afr+Asi haplogroup cohorts, the relative ROS levels were not significantly different between DM and Non-DM cybrids cultured in hypoxia (Euro/Non-DM/Hypoxia vs. Euro/DM/Hypoxia, $p > 0.05$; Afr+Asi/Non-DM/Hypoxia vs. Afr+Asi/DM/Hypoxia, $p > 0.05$). Altogether, these findings suggest that ROS levels similarly decrease in RPE cells in hypoxia, regardless of mtDNA haplogroup and diabetes history.

European and African+Asian Cybrids Retain Phagocytic Function in Hypoxia

To determine if mitochondrial background differentially influences phagocytic activity, cybrids were cultured in the presence of 1 μm fluorescent beads for 48 hours in either room air or 2% O_2, and bead internalization was measured using flow cytometry. Phagocytic activity of African and Asian cybrids, both diabetic and non-diabetic, was mostly preserved in hypoxic conditions (Afr+Asi/Non-DM/Hypoxia: 0.92 ± 0.06 –fold of internalized beads in Afr+Asi/Non-DM/Room Air cultures, p = 0.22; Afr+Asi/DM/Hypoxia: 0.95 ± 0.05 –fold, p = 0.33), and phagocytic activity in Afr+Asi/DM cybrids cultured in room air was comparable to that in similarly-treated Afr+Asi/Non-DM cybrids (0.98 ± 0.07 –fold, p = 0.84) **(Figure 2.3B)**. Euro/DM cybrids cultured in room air did not show significant changes in phagocytic activity compared to similarly-treated Euro/Non-DM cybrids (0.88 ± 0.13 –fold of internalized beads in Euro/Non-DM/Room Air cultures, p = 0.42). In addition, phagocytic activity showed a decreased, though not statistically significant, trend in phagocytosis when Euro/Non-DM or Euro/DM cybrids were cultured in hypoxia (Euro/Non-DM/Hypoxia: 0.98 ± 0.04 –fold, p = 0.81; Euro/DM/Hypoxia: 0.72 ± 0.17 –fold, p = 0.20) **(Figure 2.3A)**. In our RNA-Seq data, transcripts of genes involved in phagocytosis, such as clathrin (*CTLA*), *MERTK*, and *RAC1*, showed similar changes between room-air and hypoxic cultures for all cybrids **(Supplemental Table S2.3)**. Overall, these data suggest that phagocytosis is not significantly altered in hypoxia, regardless of mitochondrial diabetic status and mtDNA haplogroup.

European and African+Asian Cybrids Show Decreases in ZO-1α-minus protein in hypoxia

Since the RPE forms part of the blood-retina barrier through tight junctions, we investigated whether mitochondrial background and diabetes history affect levels of tight junction proteins, such as ZO-1 and occludin. For all groups, cybrids cultured in 2% O_2 showed levels of occludin that were not significantly different to those in cells cultured in room air (Euro/Non-DM: 0.84 ± 0.25 -fold of protein levels in room air cultures, p ≈ 1; Euro/DM: 0.77 ± 0.16 -fold, p = 0.58; Afr+Asi/Non-DM: 1.65 ± 0.42 -fold, p = 0.57; Afr+Asi/DM: 0.92 ± 0.25, -fold

p ≈ 1) **(Figure 2.4A)**. However, *OCLN* transcripts were lower in all hypoxia-treated cultures than in room air cultures in the RNA-Seq data, suggesting that future production of occluding protein would be similarly decreased among all cybrids cultured in hypoxia. **(Supplemental Table 2.4)**. When we examined ZO-1 levels, we found that our cultures expressed 2 different isoforms, ZO-1α-plus and ZO-1α-minus, which differ by a span of 80 amino acids. While ZO-1α-plus is expressed on the surface of many epithelial cell types, the ZO-1α-minus isoform is only expressed on endothelial cells and specialized epithelial cells, including Sertoli, renal glomerular, and RPE cells.[39,40] For all groups, cybrids cultured in 2% O_2 exhibited levels of ZO-1α-plus that were not significantly different to those in cells cultured in room air (Euro/Non-DM: 1.04 ± 0.29 -fold, p ≈ 1; Euro/DM: 0.98 ± 0.25 -fold, p ≈ 1; Afr+Asi/Non-DM: 2.64 ± 1.84 -fold, p ≈ 1; Afr+Asi/DM: 1.08 ± 0.52, -fold p ≈ 1) **(Figure 2.4C)**. On the other hand, ZO-1α-minus levels were significantly lower in hypoxia-treated cultures than those of cells cultured in room air for Euro/Non-DM (0.27 ± 0.03 -fold, p < 0.0003), Euro/DM (Euro/DM: 0.38 ± 0.07 -fold, p = 0.0003), and Afr+Asi/Non-DM cybrids (0.40 ± 0.08 -fold, p = 0.0048). ZO-1α-minus levels showed a decreased trend in Afr+Asi/DM cybrids, though this difference was not statistically significant (0.47 ± 0.17 -fold, p = 0.11) **(Figure 2.4B)**. This finding is supported by the fact that *TJP1* (ZO-1 gene) transcripts were lower in all hypoxia-treated cultures than in room air cultures, based on the RNA-Seq results **(Supplemental Table 2.4)**. Overall, these data suggest that mtDNA haplogroup and diabetes status do not significantly alter levels of tight junction proteins in hypoxia.

DISCUSSION

In diseases such as DR and AMD, hypoxia induces changes within the RPE. Previous studies have shown that RPE cells exposed to hypoxia have decreased ATP, lower cytochrome oxidase activity, and increased levels of cytokines compared to those exposed to room air.[41-

43] In this study, our RNA-seq data showed that when we compared transcriptomes of DM cybrids cultured in hypoxia to those in room air, certain gene pathways were more highly enriched for African+Asian cybrids than Euro cybrids, and vice-versa. In particular, we found that European cybrids in hypoxia versus room air had more differentially-expressed genes associated with endocytosis and ubiquitin-mediated proteolysis than African+Asian cybrids. This suggests that the initial response of European cybrids to hypoxia uniquely involves modification of these pathways, and that further experiments are needed to clarify how the mtDNA modifies these pathways and which factors are specifically targeted. Similarly, we found that African+Asian cybrids in hypoxia versus room air had more differentially-expressed genes associated with fatty acid metabolism than European cybrids. Since we found that African+Asian/DM cybrids showed increased resistance to hypoxic and hyperglycemic stresses, one may speculate this pathway contributes to the protection imparted by African+Asian/DM mtDNA.

Furthermore, we found that *OLAH* expression was significantly elevated in diabetic Afr+Asi/DM cybrids than in Euro/DM diabetic cybrids. *OLAH* encodes for Oleoyl-ACP Hydrolase, an enzyme involved in medium fatty-acid chain synthesis. Originally called thioesterase II, OLAH was observed to be specifically expressed in normal and tumor-derived breast epithelial tissue, with elevated serum levels in rat models of breast cancer.[44,45] However, expression of *OLAH* can be induced in other cell types in disease conditions. For example, monocytes from patients affected by rheumatoid arthritis expressed significantly higher levels of *OLAH* than monocytes from patients with osteoarthritis.[46] In addition, in a study of children infected by an influenza virus, *OLAH* was significantly enriched in patients who exhibited neurologic symptoms, such as seizures or loss of consciousness, as well as in patients with pneumonia compared to patients without any of these symptoms.[47]

This is particularly interesting as the pathology of retinal diseases, such as accumulation of lipid-rich drusen deposits in the Bruch's membrane in AMD, is associated with altered fat

metabolism of RPE cells.[48] In particular, the Friedlander group generated mouse models of hypoxia that lack Von-Hippel Lindau protein, which inhibits hypoxia-inducible factors (Hifs), and found these mice had thickened choroid with dilated blood vessels. However, double knockouts of *Vhl* and *Hif2a* did not show changes in choroid thickness, suggesting that the process is driven by *Hif2a*. Similarly, microarray analysis revealed that the hypoxia model mouse significantly downregulated levels of thioesterases, such as *Acot7* and *Acot8*, compared to uninduced littermates, while *Vhl* / *Hif2a* double knockouts did not.[49] Their study suggested that enzyme activity similar to that of OLAH is decreased in hypoxic RPE. Since we found that Afr+Asi/DM cybrids thrive in hypoxic conditions while Euro/DM cybrids do not, one may speculate that the increased *OLAH* expression in Afr+Asi/DM cybrids allows them to use fatty acids for metabolism and avoid the accumulation of lipid deposits in RPE correlated with disease.

Since the RPE expend large amounts of energy to maintain the outer retinal microenvironment,[50] it generates high levels of ROS that must be managed. Previous work has shown that elevated ROS develop in RPE models of age-related macular generation and DR, particularly in response to hyperglycemia.[51] In our study, we found that hypoxia induced significant decreases in ROS for all cybrids tested. In addition, the decrease in ROS in hypoxic conditions was similar for both DM and non-DM cybrids, for both Euro and Afr+Asi cybrids. These data are consistent with our previous work where we showed that Euro and Afr+Asi cybrids have similar mitochondrial bioenergetics profiles.[24] One reason why hypoxic conditions induce decreased ROS in our cybrids may be a shared adaptive mechanism. Work by the Semenza group showed that mouse epithelial fibroblast cells decrease ROS when cultured in 1% oxygen; this decrease was revealed to be due to a Hif-1α-induced mitochondrial autophagy pathway.[52] Overall, our data suggest that mtDNA haplogroup and diabetic status do not significantly affect ROS levels in response to hypoxia.

Phagocytosis of sloughed outer segments of photoreceptors is an important function of RPE cells. Previous work has shown that macrophages from diabetic mouse models have impaired phagocytosis that was correlated with increased glycated protein levels.[53] Additionally, mouse RPE cells exposed to high glucose levels *in vitro* exhibit impaired phagocytic function.[18] However, our cybrids did not show significant decreases in phagocytic function in cells exposed to hypoxia compared to untreated cultures. Moreover, when we examined the transcript levels of key phagocytosis pathway factors, we found they changed similarly between hypoxic and room air conditions for all cybrids tested. This finding is similar to results we obtained when examining phagocytic activity in cybrids with mtDNA from patients with AMD or unaffected patients. When cybrids from either group were treated with the anti-VEGF compounds bevacizumab (Avastin©), ranibizumab (Lucentis©), or aflibercept (Eylea©), phagocytic function was decreased, with similar fold-changes in function between AMD and normal cybrids.[38] However, in this investigation, we also found that the differential expression of endocytosis genes in hypoxic cybrids was more highly ranked for Euro/DM than for Afr+Asi/DM. Since Euro/DM cultures demonstrated a decreased trend in phagocytosis (though not significant), we may speculate that the Euro/DM cybrids increase transcription of phagocytic factors in order to recover from an initial decrease in phagocytic function. Overall, these data suggest that the metabolic memory imparted by mtDNA does not significantly impact phagocytic function in the presence or absence of hypoxic stress. However, given the decreased trend in phagocytosis in Euro/DM cybrids, these experiments warrant further investigation.

Another of the main functions of the RPE is to form the outer portion of the blood-retina barrier to prevent leakage of fluid from the choroid vasculature into the neural retina. Experiments in diabetic animal models and human RPE cells exposed to stresses seen in diabetes show that tight junctions proteins, such as occludin and ZO-1, decrease in amount and become disorganized, resulting in increased retinal permeability.[16,54] Consistent with these findings, we observed significant decreases in the ZO-1α-minus protein isoform on western blot

for Euro/Non-DM, Euro/DM, and Afr+Asi/Non-DM cybrids cultured in hypoxia compared to protein levels of cells in room air; Afr+Asi/DM cybrids showed a decreased trend in ZO-1α-minus protein levels. Previous work described that while tight junctions containing ZO-1α-plus exhibit a more constant resistance, tight junctions containing ZO-1α-minus may fluctuate in their resistance and permit greater flow between cells.[39] Given this, one may speculate that the decrease in ZO-1α-minus we observed may indicate an attempt by the RPE to reduce permeability in response to hypoxic stress. In addition, we found *TJP1* transcript levels were decreased in our RNA-Seq analysis for all cybrids cultured in hypoxia compared to room air cultures. Interestingly, we observed no significant change in either the ZO-1α-plus or occludin for all groups of cybrids cultured in hypoxia compared to cultures in room air. Other work in RPE cells has shown that 24 hours of hypoxia can reduce occludin in RPE cells.[55,56] One explanation for our findings is that occludin protein levels may decrease more gradually than transcript levels. While our protein data suggests that 48 hours of hypoxia does not significantly change occludin protein levels in our cybrids, our RNA-seq data shows that *OCLN* transcript levels are decreased for all cybrids cultured in hypoxia, consistent with these previous studies. In both cases, our cybrid populations reacted similarly in response to hypoxic stress. Overall, our study suggests that mtDNA haplogroup and diabetic status do not significantly affect tight junction protein levels in response to hypoxia.

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FIGURES

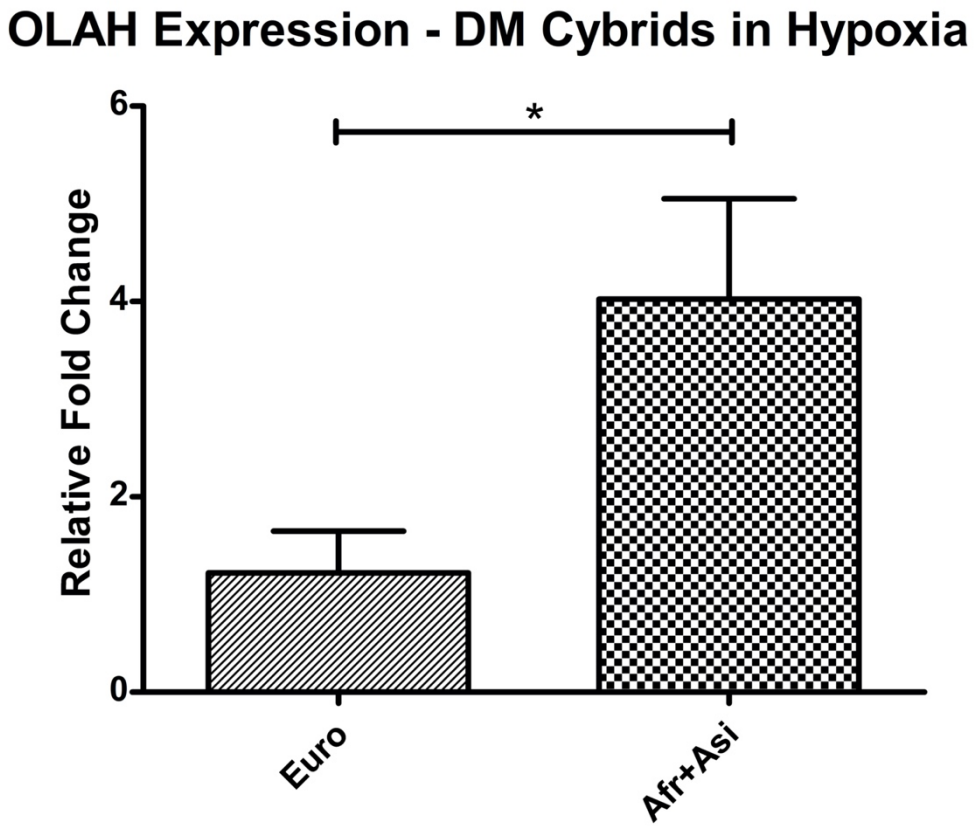


Figure 2.1. Non-Euro/DM cybrids express more OLAH transcript in hypoxia than similarly-treated Euro/DM cybrids. RNA from DM cybrids cultured 48 hours in 2% O_2 was analyzed by qRT-PCR to measure transcript levels of the Oleoyl-ACP Hydrolase (*OLAH*) gene and compare expression between Euro/DM and Non-Euro/DM cybrids. The data was normalized to average OLAH expression in Euro/DM cybrids (Fold-Change = 1).

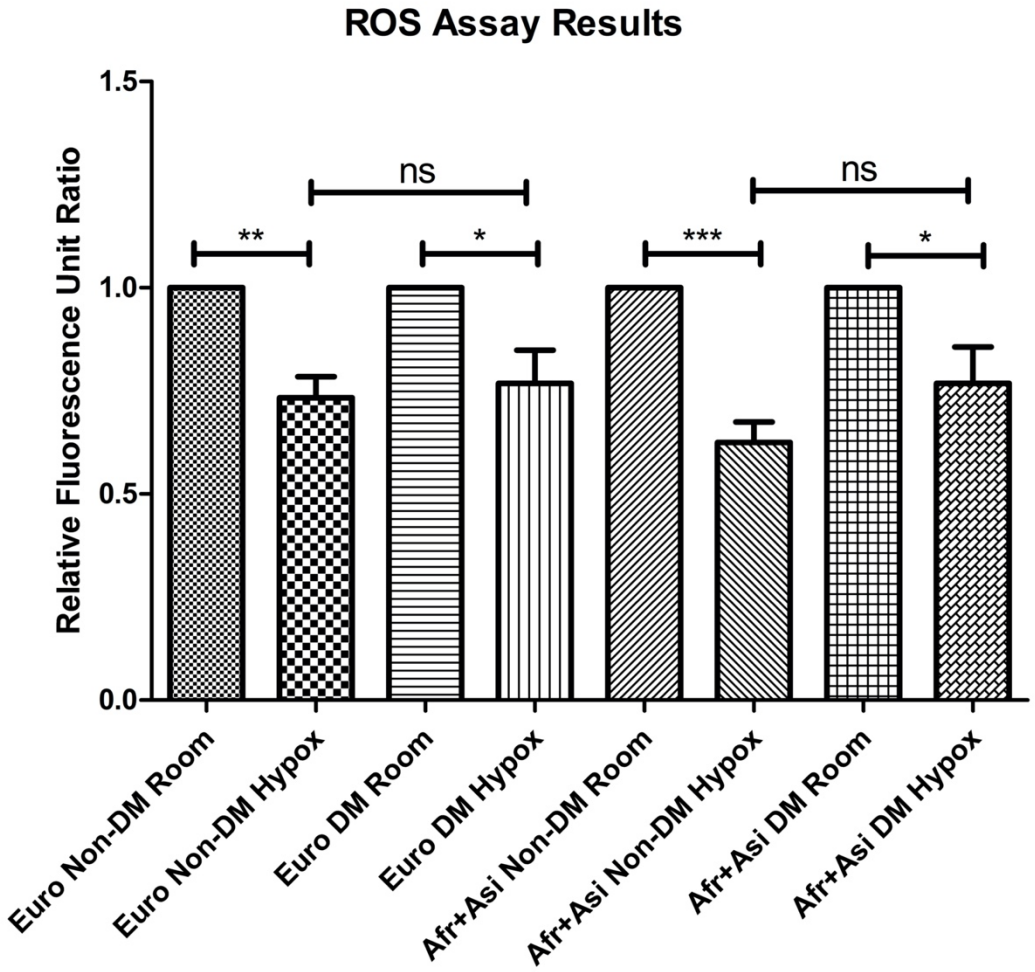


Figure 2.2. ROS levels decrease in hypoxia for European and Afr+Asi, DM and Non-DM cybrids. ROS levels in cybrids cultured 48 hours ± 2% O_2 was measured using an H_2DFCDA-based assay. For each group of cybrids, data was normalized to relative fluorescence of cells cultured in room air.

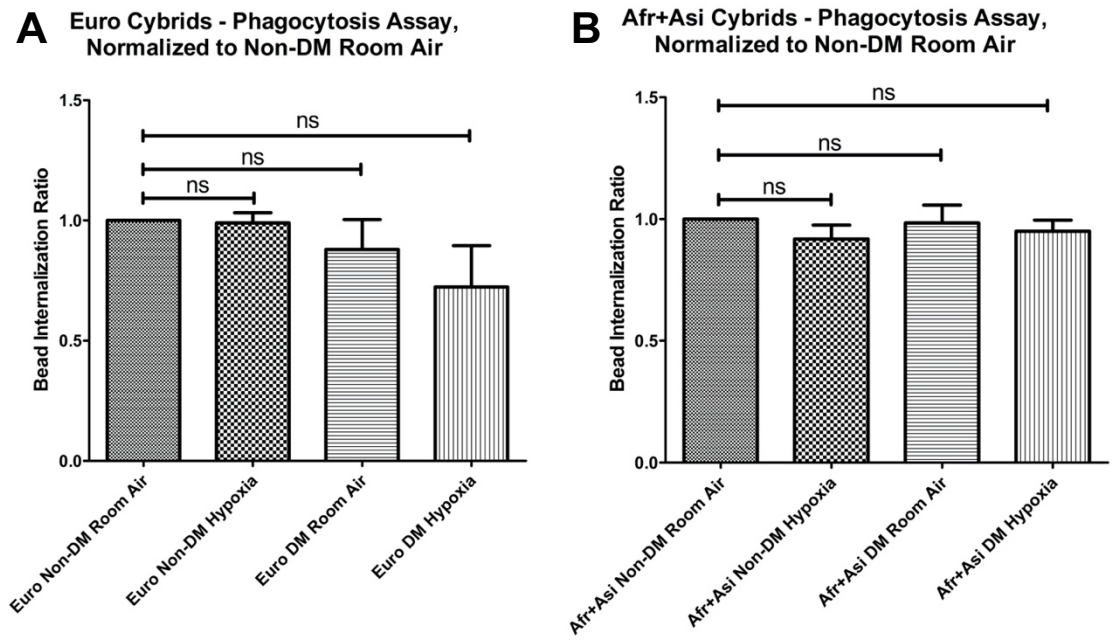


Figure 2.3. Phagocytic function is retained in hypoxia for European and African+Asian, DM and Non-DM cybrids. Phagocytosis in our cybrids cultured 48 hours ± 2% O_2 was measured using a fluorescent bead internalization-based assay for (A) European cybrids and (B) Afr+Asi cybrids. For both groups of cybrids, data was normalized to bead internalization for Non-DM cells cultured in room air.

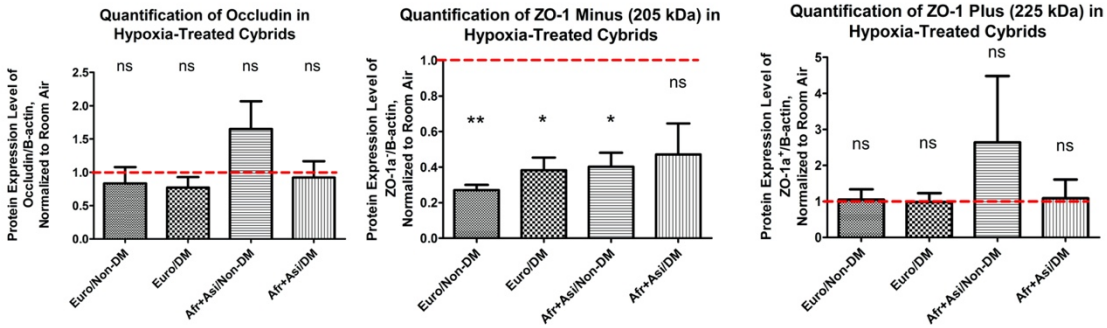


Figure 2.4. Tight junction protein levels are similarly changed in hypoxia for European and Afr+Asi, DM and Non-DM cybrids. Tight junction proteins in our cybrids cultured 48 hours ± 2% O_2 was measured using a western blot assay for (A) occludin, (B) the 205 kDa ZO-1α-minus isoform, and (C) the 225 kDa ZO-1α-plus isoform. For both groups of cybrids, data was normalized to protein levels in cybrids cultured in room air.

Table 2.1. EnrichR Pathway analysis of differentially-expressed genes between Afr+Asi/DM cybrids cultured in room air versus 2% oxygen. The top 10 pathways, along with others that are much more differently ranked between the Euro/DM and Afr+Asi/DM comparisons are listed.

African and Asian DM, Hypoxia vs. Room Air

Rank	Term	Overlap	Adjusted P-value	Z-score	Combined Score
1	Ribosome_Homo sapiens_hsa03010	83/137	3.36816E-21	-1.74614	92.23118
2	Metabolic pathways_Homo sapiens_hsa01100	378/1239	1.30460E-13	-1.98676	68.85048
3	Protein processing in endoplasmic reticulum_Homo sapiens_hsa04141	70/169	1.77845E-07	-1.76655	35.54995
4	Carbon metabolism_Homo sapiens_hsa01200	49/113	6.29999E-06	-1.63462	26.59338
5	Lysosome_Homo sapiens_hsa04142	50/123	4.15835E-05	-1.67413	23.70320
6	Valine, leucine and isoleucine degradation_Homo sapiens_hsa00280	25/48	1.09485E-04	-1.77390	23.07510
7	TNF signaling pathway_Homo sapiens_hsa04668	43/110	5.61712E-04	-1.75310	19.66767
8	Neurotrophin signaling pathway_Homo sapiens_hsa04722	45/120	9.57908E-04	-1.67261	17.45152
9	Inflammatory mediator regulation of TRP channels_Homo sapiens_hsa04750	38/98	1.15460E-03	-1.66358	16.43489
10	Fatty acid metabolism_Homo sapiens_hsa01212	23/48	9.70756E-04	-1.45318	14.98963
11	HIF-1 signaling pathway_Homo sapiens_hsa04066	39/103	1.39184E-03	-1.43983	13.65642

12	Insulin resistance_Homo sapiens_hsa04931	40/109	2.37148E-03	-1.43738	12.78004
18	AGE-RAGE signaling pathway in diabetic complications_Homo sapiens_hsa04933	36/101	7.02269E-03	-1.43281	10.75077
25	Endocytosis_Homo sapiens_hsa04144	75/259	1.54455E-02	-1.17626	7.47406
34	Ubiquitin mediated proteolysis_Homo sapiens_hsa04120	43/137	2.17697E-02	-0.66996	3.84699

Table 2.2. EnrichR pathway analysis of differentially-expressed genes between Euro/DM cybrids cultured in room air versus 2% oxygen. The top 10 pathways, along with others that are much more differently ranked between the Euro/DM and Afr+Asi/DM comparisons are listed.

European DM, Hypoxia vs. Room Air

Rank	Term	Overlap	Adjusted P-value	Z-score	Combined Score
1	Ribosome_Homo sapiens_hsa03010	85/137	1.47075E-21	-1.74614	93.67801
2	Protein processing in endoplasmic reticulum_Homo sapiens_hsa04141	71/169	5.56805E-07	-1.79043	34.71290
3	Metabolic pathways_Homo sapiens_hsa01100	352/1239	2.40768E-06	-1.96192	34.36975
4	Carbon metabolism_Homo sapiens_hsa01200	46/113	4.07692E-04	-1.63462	19.77707
5	Endocytosis_Homo sapiens_hsa04144	84/259	2.87911E-03	-1.83881	17.86864
6	AGE-RAGE signaling pathway in diabetic complications_Homo sapiens_hsa04933	39/101	3.61021E-03	-1.83334	16.69610
7	Ubiquitin mediated proteolysis_Homo sapiens_hsa04120	51/137	2.00388E-03	-1.54990	15.93823
8	HIF-1 signaling pathway_Homo sapiens_hsa04066	40/103	2.87911E-03	-1.65506	15.64200
9	Lysosome_Homo sapiens_hsa04142	46/123	2.87911E-03	-1.62388	15.51125
10	Insulin resistance_Homo sapiens_hsa04931	41/109	3.93832E-03	-1.59642	14.07932
85	Fatty acid metabolism_Homo sapiens_hsa01212	18/48	6.91730E-02	0.12171	-0.55465

Table 2.3. The *OLAH* transcript is more enriched in Afr+Asi/DM cybrids cultured in hypoxia than similarly-treated Euro/DM cybrids.

DM/Hypoxia, Euro vs. Afr+Asi		
Gene	**Log(2) of Fold-Change**	**Adjusted p Value**
OLAH	-2.459220449	0.009283141

Pairwise analysis of transcripts from Euro/DM/Hypoxia and Afr+Asi/DM/Hypoxia cultures. Results were normalized to the Afr+Asi/DM/Hypoxia transcripts (Log(2) of Fold Change for Non-Euro = 0).

Supplemental Table S2.1. Euro and [Afr+Asi] cybrids used in these experiments have a similar average age.

	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	L1b1a	54	F	DM
13.103	K1a3	25	F	DM	11.31	L2b2	42	M	Non-DM
13.45	H1e1a	47	F	Non-DM	11.18	D4a6	39	M	Non-DM
16.186	H1	48	M	DM	13.55	D4a2b	48	F	DM
10.06	U5	53	M	Non-DM	11.38	L0a1a1	38	F	Non-DM
16.184	U	60	M	DM	13.124	L1b2a	33	M	DM
14.135	H1c3	75	F	Non-DM	13.126	L1b1a7	40	F	Non-DM
16.190	H	80	F	DM	15.176	L0a1a	32	M	DM
16.185	U5a1a1	68	M	DM					
				p (avg age) =	0.11				
	Avg Age	53.33				Avg Age	40.75		
	# Male	5				# Male	4		
	# Female	4				# Female	4		
Non-DM	Avg Age	49.75			Non-DM	Avg Age	39.75		
	# Male	2				# Male	2		
	# Female	2				# Female	2		
DM	Avg Age	56.20			DM	Avg Age	41.75		
	# Male	3				# Male	2		
	# Female	2				# Female	2		

Cybrids are listed in pairs of Non-DM and DM cybrids of similar mtDNA haplogroup and age. The average ages of Euro and [Afr+Asi] cybrids are not significantly different from each other.

Supplementary Table S2.2. Antibodies Used In These Experiments.

Antigen	Antibody	Primary/Secondary	Dilution	Protein Band Size(s)
ZO-1 (TJP1)	ZO-1 Polyclonal Antibody (Cat #40-2200; ThermoFisher Scientific)	Primary	1:1,000	225 kDa (ZO-1 plus) 200 kDa (ZO-1 minus)
Occludin (OCLN)	Occludin Polyclonal Antibody (Cat #71-1500; ThermoFisher Scientific)	Primary	1:1,000	29 kDa (isoform)
Beta-Actin	β-actin (13E5) Rabbit mAb (Cat# 4970; Cell Signaling Technology, Danvers, MA)	Primary (Housekeeping Protein)	1:10,000	45 kDa
Rabbit IgG	Anti-Rabbit IgG, HRP-linked Antibody (Cat# 7074; Cell Signaling Technology)	Secondary	1:10,000	N/A

Supplemental Table S2.3. Transcripts of endocytosis-associated genes show similar changes between room air and hypoxia for all cybrids.

tracking_id	11-31 normoxia	11-31 HYPOXIA	11-30 normoxia	11-30 HYPOXIA	11.38 normox	11.38 HYPOX	13.124 normox	13.124 HYPOX
CLTA	208.019	249	156.142	294.979	186.529	226.353	149.928	220.616
MERTK	0.837731	0.414042	1.22734	0.253667	1.08911	0.762945	2.39124	0.93357
RAC1	152.373	144.824	146.348	145.885	167.052	153.58	143.093	151.286

13.45 H normox	13.45 H HYPOX	16.186 normox	16.186 HYPOX	11.08 normox	11.08 HYPOX	13.103 normox	13.103 HYPOX
248.192	259.53	155.08	283.733	159.779	202.7	153.008	247.348
0.931135	0.38165	0.498627	0.147659	1.64637	0.832872	3.02166	0.756854
149.003	145.005	129.143	165.542	153.968	186.161	130.755	145.597

14-135 normoxia	14-135 HYPOXIA	16-190 normoxia	16-190 HYPOXIA	11-18 normoxia	11-18 HYPOXIA	13-55 normoxia	13-55 HYPOXIA
287.558	270.225	152.73	226.074	158.675	255.759	221.554	251.753
0.326303	0.102863	0.292812	0.114299	1.43579	0.737189	0.953007	0.560644
137.126	173.049	164.228	170.756	141.414	151.667	180.602	169.248

13-126 normoxia	13-126 HYPOXIA	15-176 normoxia	15-176 HYPOXIA	10-06 normoxia	10-06 HYPOXIA	16-184 normoxia	16-184 HYPOXIA
128.969	159.457	138.795	214.157	108.102	143.012	98.1176	157.432
3.2417	0.902009	0.649644	0.291492	2.21566	1.5334	0.528258	0.295764
145.21	179.387	112.474	132.262	154.862	183.681	112.683	166.004

Values are expressed as Reads-per-Kilobase-per-Million matched reads (RPKM).

Supplemental Table S2.4. Transcripts of tight junction protein genes are lower for all cybrids cultured in hypoxia than in room air.

tracking_id	11-31 normoxia	11-31 HYPOXIA	11-30 normoxia	11-30 HYPOXIA	11.38 normox	11.38 HYPOX	13.124 normox	13.124 HYPOX
OCLN	1.0998	1.47645	2.21595	0.650693	2.24395	1.39374	2.49914	2.01838
TJP1	3.39907	2.76345	4.96728	1.96341	4.90931	2.85099	5.74854	3.53912

13.45 H normox	13.45 H HYPOX	16.186 normox	16.186 HYPOX	11.08 normox	11.08 HYPOX	13.103 normox	13.103 HYPOX
0.976347	1.15972	2.29511	1.00747	3.02433	2.72341	2.06283	0.856506
2.4331	2.61054	3.07859	0.999963	6.54895	3.08761	4.47968	2.17855

14-135 normoxia	14-135 HYPOXIA	16-190 normoxia	16-190 HYPOXIA	11-18 normoxia	11-18 HYPOXIA	13-55 normoxia	13-55 HYPOXIA
0.992384	1.20245	2.94434	2.59705	1.94416	1.55445	1.14019	1.40275
1.22128	1.10268	9.6376	2.62006	3.84719	2.46155	2.97211	2.76858

13-126 normoxia	13-126 HYPOXIA	15-176 normoxia	15-176 HYPOXIA	10-06 normoxia	10-06 HYPOXIA	16-184 normoxia	16-184 HYPOXIA
4.27803	2.67753	1.93878	1.46684	5.22227	2.72177	3.28947	2.96846
6.78457	3.75345	3.22741	1.41463	6.56698	5.03422	3.77525	3.87615

Values are expressed as Reads-per-Kilobase-per-Million matched reads (RPKM).

Conclusion of the Dissertation

African and Asian Mitochondria Promote Retinal Pigment Epithelial Cell Viability Through Distinctive Transcriptional Differences

This dissertation examined how mitochondrial DNA (mtDNA) of either African/Asian ancestry or European ancestry, from patients with or without diabetes (DM), affects RPE cell viability and function. In **Chapter 1**, our lab found that African+Asian/DM mitochondria confer resistance against decreases in RPE cell viability in response to hyperglycemia or hypoxia.[1] In **Chapter 2**, we showed that African+Asian/DM cybrids cultured in hypoxia differentially express factors of certain gene ontology families compared to European/DM cybrids grown in similar conditions. In particular, African+Asian/DM cybrids more significantly altered expression of genes related to fat processing, such as *OLAH*.

Diabetic retinopathy (DR) is the leading cause of blindness in individuals fewer than 65 years-old, and minorities are more vulnerable to developing diabetes and diabetic retinopathy (DR).[2] Currently, DR is managed through strict, systemic control of blood glucose (e.g. insulin and oral medications such as metformin), pan-retinal photocoagulation for severe proliferative or non-proliferative diabetic retinopathy, and intravitreal injections of anti-vascular endothelial growth factor (VEGF) agents.[3] However, clinical studies of anti-VEGF agents have shown that at least 40% of individuals with diabetic macular edema treated with these medications do not effectively treat the condition and decrease macular thickness.[4,5] Our work encourages future studies into the mechanisms behind the molecular memory imparted by African+Asian/DM mtDNA to better understand how we can treat DR in this vulnerable

population by using therapeutics that enhance protective pathways and inhibit detrimental pathways induced by this memory.

The use of our cybrid model was indispensable to allow us to understand the mitochondrial influence on cell health. While our model is not a perfect representation of the RPE and its microenvironment *in vivo*, our model is excellent in that it elegantly isolates the effect of mtDNA on cellular health and function by holding the nuclear genome constant. Furthermore, our model shows consistency with previous data. Cells that have been cultured in a diabetic environment retain altered gene expression and cellular function long after returning to a euglycemic environment;[6-8] similarly, our cybrids showed that mtDNA from African+Asian/DM individuals confers unique resistance to diabetic stressors, even after many months and several passages after collection from our patient donors.[1] Using our model, we have learned about the importance of mtDNA on cell metabolism and how retrograde signaling can induce changes in complement factors, inflammatory markers, and other pathways such as those we found in this work.[9,10] By doing so, we have gained insight into possible pathways and factors that can serve as additional therapeutic targets aside from those affected by current treatments, such as VEGF.

Future studies of DNA methylation in African+Asian/DM cybrids

In **Chapter 1**, we found that African+Asian/DM cybrids are resistant to DNA de-methylation when exposed to the DNMT1 inhibitor, 5-Aza-2'-deoxycytidine (5-Aza-dC). In

addition, African+Asian/DM cybrids cultured in hypoxia showed significant increases in transcription of DNA-methylation factors, such as *DNMT3A, DNMT3B, MBD2*, and *TRDMT1*.[1] Together, these data suggest that African+Asian/DM mtDNA induces resilient DNA methylation that is further increased in hypoxia. We hope to further understand which how African+Asian/DM mtDNA modify the epigenome. Previous studies have shown that mitochondria can alter the nuclear epigenome in disease states. For example, a study by Dr. Wallace's laboratory showed that increasing amounts of mtDNA containing the A3243G mutation within a cell is associated with a dose-dependent induction of nuclear genes associated with neurologic disorders.[11] Increased heteroplasmy for this mutation was also found to be correlated with decreased acetylation of histone 4 and increased methylation of histone 3.[12] To better understand how mtDNA affects the nuclear epigenome, we would like to perform bisulfite sequencing of nuclear DNA and histone modification mass spectrometry in our cybrids. Combined with the transcriptome data from **Chapter 2**, such data would allow us to further elucidate the mechanisms behind the molecular memory imparted by African+Asian/DM mtDNA.

In addition, we are particularly interested in the differences of the mitochondrial epigenome in African+Asian/DM cybrids compared to other cybrids. The extent of mtDNA methylation has been an active subject of debate. Since 5-methylcytosine was discovered in mtDNA, researchers have sought to determine how diseases and other cellular stressors affect mtDNA methylation.[13] Notably, the Cheng laboratory found that insulin resistance in their subjects correlated with increases in methylation of the *ND6* gene and the D-loop as shown by bisulfite sequencing.[14,15] However, other studies suggest that mtDNA methylation had been

overstated in previous studies because the circular structure of mtDNA interferes with bisulfite conversion and artificially inflates 5-mC values.[16-18] To investigate how mtDNA methylation is affected by hypoxia in our cybrids, and to decrease the confounding effects of non-linear DNA on chemical conversion, we hope to perform bisulfite sequencing on linearized mtDNA from cybrids cultured in the presence or absence of hypoxia.

Future studies on the function of OLAH

In **Chapter 1**, we showed that African+Asian/DM cybrids thrived in hypoxic conditions, resisting decreases in cell viability. Of all the differentially-expressed genes we found in our RNA-seq study (**Chapter 2**), the transcript levels of the oleyol-ACP hydrolase (*OLAH*) gene was significantly increased in African+Asian/DM cybrids cultured in hypoxia compared to European/DM cybrids cultured in similar conditions. However, little is known about the function of OLAH in stress conditions. OLAH, a thioesterase, was originally found in lactating rat mammary cells and was shown to shift production of fatty acids from long-chain (14- to 18-carbon) to medium-chain (8- to 12-carbon) products.[19] This enzyme has not yet been characterized in retinal cells such as RPE. However, other members of the thioesterase family have been implicated in neurodegeneration and neuroprotection. One study showed that patients with mesial temporal lobe epilepsy have decreased levels of the ACP-hydrolase Acot7.[20] A mouse knockout model of *Acot7* exhibited ataxia and behavioral dysfunction. [21] Additionally, a mouse model of impaired response to hypoxia in the retina showed increased

choroid thickness and showed decreased expression of the thioesterases *Acot7* and *Acot8*.[22] Conversely, mice that overexpress the slow Wallerian degeneration (Wld^S) transgene exhibit neuroprotection; *Acot7* was found to be 8-fold higher in Wld^S mice than in wild-type littermates, suggesting that thioesterase function is associated with neuroprotection.[23] Given these results, we would like to investigate how OLAH affects the resistance to hypoxia in our cybrids. To do so, we would use siRNA to knock-down *OLAH* transcription in our cybrids, culture them in hypoxia, and examine the effect on cell viability using the trypan blue dye-exclusion assay. In addition, the levels of RNA and protein related to neuroprotection, apoptosis and mitochondrial biogenesis would be examined. Since OLAH is from the same protein family as ACOT7, we would expect to see a decrease in cell viability with decreased *OLAH* expression.

On the other hand, it is possible that upregulation of *OLAH* benefits RPE cells but is detrimental to the rest of the retina. This idea is supported by research that associates *OLAH* expression with pathology. *OLAH* has also been called *AURA1* because it was found to be augmented in rheumatoid arthritis subjects' synovial cells compared to the level found in synovial cells of age-matched osteoarthritis subjects.[24] In addition, a study of children affected by H1N1 influenza found that subjects with more severe CNS symptoms (such as loss of consciousness and convulsions) showed upregulation of *OLAH* in peripheral blood cells.[25] One way to investigate the effect of RPE *OLAH* expression in hypoxia would be to use a mouse model. The Knockout Mouse Project (KOMP, University of California Davis; Davis, CA) has an embryonic stem cell line for a conditional knockout for *Olah* inducible in the RPE using a targeted Cre-recombinase. By using this knockout mouse and subjecting it to transient hypoxia, we can examine the effects of *Olah* loss on retinal cell health. If *Olah* expression in the RPE is

detrimental to the surrounding microenvironment, loss of *Olah* should prevent or decrease retinal pathology. However, if *Olah* expression is meant to ameliorate stress conditions, its loss should result in increased retinal damage in hypoxia.

Future studies on the role of mitochondrial-derived peptides (MDPs) in African+Asian/DM cybrids

Our results suggest an association between the resistance against decreases in cell viability from hypoxia in African+Asian/DM cybrids (**Chapter 1**) and genes involved in fat processing, such as *OLAH* (**Chapter 2**). However, it is unclear how the mitochondria induce changes to nuclear transcription. One way the mitochondria could signal to the rest of the cell is through small, mitochondrial-derived peptides (MDPs) encoded from the mitochondrial genome. The first known MDP, humanin, was found to prevent apoptosis from Aβ peptide toxicity in an *in vitro* model of familial Alzheimer's disease.[26] Importantly, humanin has been shown to increase insulin sensitivity in wild-type mice and decrease blood glucose in a mouse model of diabetes.[27] A related MDP, MOTS-c (mitochondrial open reading frame of the 12S rRNA-c), was found to improve glucose tolerance test response in mice, increase muscular uptake of glucose *in vivo*, and increase the capacity of mouse muscle cells to perform glycolysis *in vitro*. Additionally, MOTS-c was found to alter fatty acid metabolism by decreasing the amount of metabolites with long-chain fatty acids and increasing the amount of metabolites with shorter-chain fatty acids, such as oleoylcarnitine and myristoyl-CoA.[28] Since we found

that African+Asian/DM cybrids significantly modify expression of factors involved in fatty acid metabolism, such as *OLAH*, we are eager to investigate whether African+Asian/DM cybrids have increased levels of MDPs. We would first use qRT-PCR to determine expression of MDPs in our cybrid populations. However, since levels of transcript do not necessarily reflect increases in MDP protein products, as was seen in a study in men with impaired glucose metabolism,[29] we would also measure MDP protein levels using Western blot analyses. To determine if MDPs improve cell viability in hypoxic or hyperglycemic conditions, we would perform assays as done in **Chapter** 1 on cybrids treated with or without MDPs. In addition, we would perform qRT-PCR on cybrids treated with or without MDPs to determine if they affect expression of genes involved in fatty acid metabolism, such as *OLAH*.

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