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Age-related Macular Degeneration (AMD) mitochondria modulate epigenetic mechanisms in retinal pigment epithelial cells

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Abstract

Mitochondrial damage and epigenetic modifications have been implicated in the pathogenesis of Age-related Macular Degeneration (AMD). This study was designed to investigate the effects of AMD/normal mitochondria on epigenetic regulation in human transmitochondrial retinal pigment epithelial cybrid cells (RPE) *in vitro*. Human RPE cybrid cell lines were created by fusing mitochondria-deficient (Rho0) ARPE-19 cells with platelets obtained from either AMD patients (AMD cybrids) or normal subjects (normal cybrids). Therefore, all cybrids had identical nuclei (derived from ARPE-19 cells) but mitochondria derived from either AMD patients or age-matched normal subjects. AMD cybrids demonstrated increased RNA/protein levels for five methylation-related and four acetylation-related genes, along with lower levels of two methylation and three acetylation genes compared to normal cybrids. Demethylation using 5-Aza-2'-deoxycytidine (DAC) led to decreased expression of *VEGF-A* gene in AMD cells. Trichostatin A (TSA), an HDAC inhibitor, also influenced protein levels of VEGF-A, HIF1α, NFκB, and CFH in AMD cells. Our findings suggest that retrograde signaling leads to mitochondria-nucleus interactions that influence the epigenetic status of the RPE cells and perhaps may be future potential therapeutic targets for AMD.

Keywords

Age-related Macular Degeneration; AMD; Epigenetics; AMD mitochondria; Methylation; Acetylation

1. Introduction

Epigenetic regulation is one of the critical risk factors associated with age-related macular degeneration (AMD), [1] a retinal degenerative disease which is a primary cause of irreversible vision loss in the United States and currently has limited treatments.[2]

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Epigenetic modifications include DNA methylation, histone modifications (such as acetylation, methylation, and phosphorylation), and chromatin remodeling.[3]

DNA methylation, a process wherein methyl groups are added to the DNA molecule, involves maintenance and de novo activity. Maintenance methylation activity that uses DNMT1 (DNA methyltransferase 1) is required to preserve DNA methylation after every cellular DNA replication cycle. During DNA replication, DNMT1 copies the DNA methylation patterns that are passed onto the daughter strands. De novo methyltransferase activity, using DNMT3A and DNMT3B enzymes, is required to set up DNA methylation patterns early in development.[4] A recent study has identified genes with altered methylation patterns in certain promoter regions in AMD patients [5] and confirmed the association between a hypomethylated promoter and the onset of AMD. Oliver *et al* compared genome-wide DNA methylation profiling of AMD patients and control subjects, and reported that alterations in DNA methylation profiles in retina and blood samples could have critical functional implications in AMD pathology.[6] Furthermore, Bellizzi *et al* have shown that the mtDNA control region has unique CpG methylation patterns and mtDNA variants can modulate total global methylation levels.[7, 8]

In addition to DNA methylation, an essential part of gene regulation is acetylation and deacetylation of N-terminal lysine residues on histone proteins in DNA. This process involves histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes.[9] HDACs play key roles in regulating gene transcription in retinal degeneration, and have been targeted to improve retinal functions.[10]

We have previously shown that AMD mitochondria influence the expression of nuclear genes associated with apoptosis, complement, inflammation, autophagy, ER stress, and oxidative stress pathways. [11,12] In addition, mtDNA variants can significantly influence methylation profiles and transcription of inflammation, angiogenesis and various cell signaling genes.[13]

Although evidence exists for the involvement of epigenetics in AMD, the role of AMD mitochondria-driven epigenetic regulation in retinal pigment epithelial cells has not yet been established. Therefore, the goal of this study was to: 1) examine the influence of AMD mitochondria on epigenetic patterns in retinal pigment epithelial cells, and 2) examine the effects of inhibitors of methylation i.e., 5-aza-2′-deoxycytidine (DAC) and/or histone deacetylation i.e., Trichostatin A (TSA) upon the AMD mitochondria. Our results demonstrate that epigenetic modifications of the AMD versus normal mtDNA can be contributing factors for regulation of angiogenesis, inflammation, and apoptosis, which are key pathways involved in AMD pathogenesis. Therefore, our data shows epigenetic profiles as potential therapeutic targets for AMD. Delving into the epigenetic mechanisms that control the risk of AMD might aid in the development of treatment options.

2. Materials and Methods

2.1. Human subjects

The Institutional Review Board of the University of California, Irvine (protocol # 2003–3131) approved all research with human subjects. Written informed consent was obtained from all subjects and clinical investigations were conducted according to the tenets of Declaration of Helsinki.

2.2. Cell culture

All experiments were conducted *in vitro* using AMD and age-matched normal cybrid cell lines, which had mitochondria derived from either AMD patients (AMD cybrids) or normal subjects (normal cybrids), and nuclei from ARPE-19 cell lines.[12] Passage 5 normal and AMD cybrid cell lines were used for all experiments. Cells were grown in DMEM/Ham's F12 1:1 (Invitrogen-Gibco, NY, USA) cell culture medium containing 24 mM sodium bicarbonate, 10% dialyzed fetal bovine serum, and 1.0 mM sodium pyruvate. Passage 5 cybrids were used for all experiments. Age-matched normal cybrids served as controls.

2.3. Quantitative real-time PCR (qRT-PCR)

Cells were grown in 6-well plates, and RNA was extracted at 72 hr using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's protocol. The extracted RNA was quantified using NanoDrop 1000 Spectrophotometer (Thermoscientific, Waltham, Massachusetts, USA). RNA at 100 ng/μL was reverse transcribed into cDNA using Superscript VILO Master Mix (Cat. # 11755–050, Invitrogen, Grand Island, NY, USA). To examine the expression of all genes, qRT-PCR was performed using StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA), QuantiTect Primer Assays (Qiagen) (Supplementary Information (SI) Table 1), and Power SYBR® Green PCR Master Mix (Cat. # 4367659, Life Technologies, Grand Island, NY, USA). Each sample was run in triplicate. QRT-PCR data were analyzed using ΔΔCt method. [12] Fold change was calculated using the following formula: Fold change = $2^{\Delta\Delta Ct}$.

2.4. Western blotting

Cells were plated in 6-well plates for 72 hr followed by lysis using RIPA buffer (Cat. # 89900, Life Technologies). Protein extraction, immunoblotting, and imaging were performed as previously described. [12] We captured images of the blots using the Chemidoc imager (Bio-Rad) instrument that has a program which signals when the intensities are over exposed. Therefore, we felt confident that during the analyses of the bands, the values were within the linear range. Our instrumentation can indicate if the blots are oversaturated and none of our analyses were performed in the saturated range. The images were analyzed using the ImageJ software (NIH image). The primary and secondary antibodies used herein are listed in SI Table 2.

2.5. Treatment with 5-Aza-2'-deoxycytidine (DAC)

Lyophilized 5-Aza-2'-deoxycytidine was obtained from Sigma-Aldrich (Cat. # A3656). Stock solution of 0.219 M concentration was prepared by adding 100 uL of DMSO to 5 mg

DAC powder. DAC stock was further diluted in culture media to obtain a final working concentration of 250 uM. All cybrids were treated with 250 uM DAC in this study.

2.6. Treatment with Trichostatin A (TSA)

TSA solution (5mM) was obtained from Sigma-Aldrich (Cat. # T1952). TSA stock solutions of 100uM and 10uM concentrations were prepared in culture media, and subsequently diluted to obtain working concentration of 0.3uM (300 nM) TSA. All cybrids were treated with 0.3uM TSA solution in this study.

2.7. Global DNA Methylation Assay

The MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) was obtained from Epigentek (Catalog # P-1030) and the assay was performed as per manufacturer's instructions. In this assay, DNA is bound to strip-wells that are specifically treated to have a high DNA affinity. The methylated fraction of DNA is detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer at 450 nm. The percentage of methylated DNA is proportional to the OD intensity measured. Since 5-methylcytosine (5-mC) is formed by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNA methyltransferases in the process of DNA methylation, we quantitated the percentage of methylated DNA i.e., 5-mC% between normal and AMD cybrids.

2.8. HDAC Activity Assay

The Epigenase™ HDAC Activity/Inhibition Direct Assay Kit (Colorimetric) was obtained from Epigentek (Catalog # P-4034) and the assay was performed as per manufacturer's instructions. In this assay, an acetylated histone HDAC substrate is stably coated onto the microplate wells. Active HDACs bind to the substrate and removes acetyl groups from the substrate. The HDAC-deacetylated products can be recognized with a specific antibody. The ratio or amount of deacetylated products, which is proportional to the enzyme activity, can then be colorimetrically measured by reading the absorbance in a microplate spectrophotometer at 450 nm. The activity of the HDAC enzyme is proportional to the OD intensity measured.

2.9. Statistical analysis

Non-parametric Mann-Whitney test (for 2 groups) or one-way ANOVA (for 3 or more groups) followed by post-hoc Tukey–Kramer test (GraphPad Prism 5.0; GraphPad Software, CA, USA) were performed to analyze data between groups. P values < 0.05 were considered statistically significant.

3. Results

3.1. AMD mitochondria mediate differential expression of methylation-associated genes and proteins

3.1. a. Gene expression—Compared to normal cybrids, AMD cybrids showed significant changes in the expression of genes associated with methylation (Table 1, Figure

1). Two of the methylation-related genes showed decreased expression i.e., ***DNMT1*** (p=0.01, 18.78 % decrease) and ***MBD2*** (p=0.03, 24.02 % decrease). The other genes showed increased expression levels in the AMD cybrids compared to age-matched normal cybrids i.e., ***DNMT3A*** (p=0.01, 141 % increase), ***DNMT3B*** (p=0.03, 104.2 % increase); ***MAT1A*** (p=0.03, 32.8 % increase), ***MAT2B*** (p=0.03, 47.9 % increase), and ***MBD4*** (p= 0.029, 69.2 % increase). These results suggest that although the NL and AMD cybrids have identical nuclei, the presence of AMD mitochondria can influence the differential expression of methylation genes compared to age-matched normal mitochondria.

3.1. b. Protein levels—Western blot analyses of the normal and AMD cybrids demonstrated that changes in protein levels (Table 2, Figure 2) were consistent with the RNA expression values. The AMD cybrids showed significantly lower protein levels of **DNMT1** (p=0.01, 36.74 % decrease) and **MBD2** (p=0.014, 42.33 % decrease). The levels of the other methylation related proteins were increased in the AMD cybrids compared to normal cybrids: **DNMT3A** (p=0.01, 267 % increase), **DNMT3B** (p= 0.01, 320.6 % increase), **MAT1A** (p=0.001, 573.3 % increase), **MAT2B** (p=0.03, 117.6 % increase); and **MBD4** (p=0.0007, 79.4 % increase). These results demonstrate that the methylation proteins are differentially produced in AMD cybrid cells compared to cybrids possessing age-matched normal mitochondria.

3.2 AMD mitochondria mediate differential expression of histone deacetylase (HDAC) genes and proteins

3.2.a. Gene expression—Four of the seven HDACs studies showed up-regulation in the AMD cybrids compared to the age-matched normal cybrids (Table 3, Figure 3). The AMD cybrids showed higher gene expression levels of ***HDAC1*** (p=0.01, 40.4 % increase), ***HDAC2*** (p=0.03, 21.2 % increase), ***HDAC3*** (p=0.01, 23 % increase), and ***HDAC11*** (p=0.029, 20.6 % increase) compared to age-matched normal cybrids. In contrast, AMD cybrids showed reduced gene expression levels of ***HDAC6*** (p=0.029, 24.5 % decrease), ***HDAC9*** (p=0.029, 27.2 % decrease), and ***HDAC10*** (p=0.03, 27.52 % decrease). These findings are significant because HDACs play a significant role in gene regulation associated with differentiation, metabolic stress and responses to DNA damage, pathways that may be involved in AMD pathogenesis.

3.2.b. Protein levels—The western blot analyses for the proteins were consistent with the RNA results. The AMD cybrids showed higher protein levels for **HDAC1** (p= 0.03, 180.9 % increase), **HDAC2** (p= 0.0087, 811.2 % increase), **HDAC3** (p= 0.02, 188.2 % increase), and **HDAC11** (p= 0.48, 13.8 % increase) compared to the normal cybrids (Table 4, Figure 4). AMD cybrids showed reduced protein levels for **HDAC6** (p= 0.02, 55.81 % decrease); **HDAC9** (p= 0.73, 41.49 % decrease), and **HDAC10** (p= 0.02, 98 % decrease). Since the AMD and normal cybrid cell lines all have identical nuclear genomes and are cultured under identical conditions, the data support the idea that the AMD mitochondria can modulate the expression of the histone deacetylase enzymes.

3.3 Effects of 5-Aza-2'-deoxycytidine (DAC), a methylation inhibitor

We next examined the gene expression levels of three genes that are known to have methylation-sensitive regulation (*NFκB*, *VEGF*, and *CFH*) before and after treated with DAC (Table 5, Figure 5A). The ***NFκB*** gene expression level was 101% higher in the untreated AMD cybrid compared to untreated NL cybrid (p=0.0008). The demethylation with DAC did not changes the gene expression of *NFκB* in AMD or NL cybrids (p=0.97).

The ***VEGF*** gene was upregulated 113% in the untreated AMD cybrids compared to untreated Normal cybrids (p= 0.04, Table 5, Figure 5B, bar 1 versus bar 3). After treatment with DAC, the AMD cybrids showed 47% decreased level of *VEGF* (p=0.04, Figure 5B, bar 3 versus bar 4), returning to expression levels similar to the untreated Normal cybrids. There was no change in the *VEGF* expression levels between the untreated Normal and DAC-treated Normal cybrids (p=0.39, Figure 5B, bar 1 versus bar 2).

The untreated Normal cybrids (Figure 5C, bar 1) had significantly higher ***CFH*** gene expression levels (p=0.0047) compared to the DAC-treated Normal cybrids (Figure 5C, bar 2,), the untreated AMD cybrids (Figure 5C, bar 3) and the DAC-treated AMD (Figure 5C, bar 4). There was no significant change in *CFH* gene expression was observed in untreated AMD cybrids versus DAC-treated AMD cybrids (p=0.8).

3.4 Effects of Trichostatin A (TSA), an HDAC inhibitor

The AMD and Normal cybrids were treated with TSA, an inhibitor of the class I and II HDACs, and then protein levels of VEGF, NFκB, HIF1α, and CFH were analyzed by western blotting. TSA-treated AMD cybrids showed a significant decline in the protein levels of **NFκB** (p=0.04, 19% decrease) (Figure 6A, bar 3 versus bar 4), **VEGF-A** (p=0.03, 42% decrease) (Figure 6B, bar 3 versus bar 4), and **HIF1α** (p=0.04, 48% decrease) (Figure 6D, bar 3 versus bar 4), compared to the untreated cybrids (Table 4). In contrast, the **CFH** protein levels increased after treatment with TSA (p=0.001, 84% increase) (Figure 6C, bar 3 versus bar 4).

In contrast to the changes in protein levels seen in the AMD cybrids after TSA treatment, the untreated Normal and TSA-treated Normal cybrids showed similar protein levels to each other. Treatment with TSA did not cause any significant change in the NFκB protein levels (p= 0.66) (Figure 6A, bar 1 versus bar 2); VEGF-A (p= 0.62) (Figure 6B, bar 1 versus bar 2); CFH (p= 0.97) (Figure 6C, bar 1 versus bar 2); and HIF1α (p= 0.39) (Figure 6D, bar 1 versus bar 2), compared to the untreated cybrids.

3.5. Total Global Methylation Assay

The total percentage of methylated DNA (i.e., 5-mC%) was measured using DNA isolated from normal and AMD cybrids. No significant difference (p= 0.63) in global DNA methylation levels was observed between normal (NL UN= 1 ± 0.123; n=7) and AMD (AMD UN= 0.9049 ± 0.082; n=6) cybrids.

3.6. HDAC activity Assay

The activity of total HDAC enzyme was measured using protein extracts from normal and AMD cybrid cells. We observed no significant difference in total HDAC activity ($p= 0.37$) between normal (NL UN=1 ± 0.055; n=7) and AMD (AMD UN=1.068 ± 0.042; n=6) cells.

4. Discussion

The present study demonstrates that AMD mitochondria are able to influence the gene expression as well as protein levels of methyltransferases, methyl-adenosyl transferases, and histone deacetylases. In the transmitochondrial AMD cybrids, the nuclear content is the same and the cells differ because they contain mitochondria from either AMD subjects or age-matched normal subjects. Therefore, changes in the nuclear-encoded epigenetic genes and proteins could be attributed to modulatory signaling from mitochondria originally from AMD patients or normal subjects. Furthermore, blocking methylation using DAC and HDAC inhibition via Trichostatin-A in AMD cells caused differential expression of downstream signaling genes/proteins known to be regulated by methylation and associated with AMD disease. These findings support our hypothesis that the epigenetic system is one of the molecular pathways affected by AMD mitochondria retrograde signaling. This AMD mitochondria-mediated epigenetic regulation may be associated with AMD pathogenesis.

The process of DNA methylation requires DNA methyltransferases (DNMTs) that are enzymes critical to genomic integrity in mammalian development because they influence gene regulation by establishing and maintaining DNA methylation patterns at distinct genomic regions.[14] DNMT1 is a key maintenance DNA methyltransferase that targets hemimethylated DNA and catalyzes the transfer of methyl groups from S-adenosyl-L-methionine to cytosine mainly at CpG sites.[15] DNMT1 is highly expressed during development and is detected in inner retina neurons.[16] DNMT1 knockdown results in defects in RPE and photoreceptor differentiation and reduces levels of interphotoreceptor retinoid binding protein.[17] It has also been shown that DNMT1 deficiency leads to rapid death of neurons and photoreceptors in postnatal retina.[18] The present study demonstrated significantly reduced expression of DNMT1 gene in the AMD cybrids compared to their normal counterparts. Evidence is mounting on a link between deficiency/inactivation of DNMT1 and DNA fragility, as it has been shown that DNMT1 is recruited in DNA Damage Response and DNA Mismatch Repair.[19] DNMT1 inactivation triggers PARP signaling cascade and cell cycle arrest.[19] Therefore, these lower gene expression and protein levels of *DNMT1* associated with the AMD cybrids might explain the mtDNA lesions and damage observed previously in the transmitochondrial AMD ARPE-19 cybrid cells.[12] Ferrington *et al* have also shown that the mtDNA of AMD RPE cells is significantly degraded compared to normal.[20–22] DNMT3A and DNMT3B are essential for de novo methylation in mammals predominantly during early development and can methylate unmethylated and hemimethylated DNA at the same rate.[19] Loss of these enzymes results in progressive loss of DNA methylation and early embryonic lethality.[23] DNMT3A and DNMT3B are highly homologous and are highly expressed in the developing optic cup. However, their expression in the retina declines with age.[16] Knockdown of DNMT3B leads to abnormal RPE and disorganized retinal laminations.[24] Herein, we also observed upregulated gene and protein

levels of *DNMT3A* and *DNMT3B* in AMD cybrids compared to normal cybrids, suggested a feedback loop since DNMT1 was down-regulated in AMD cells.

Furthermore, AMD cybrids showed elevated gene and protein levels of *MAT1A* (Methionine AdenosylTransferase 1 Alpha) and *MAT2B* (Methionine AdenosylTransferase 2 Beta). MAT1A and MAT2B catalyze the transfer of an adenosyl moiety from ATP to methionine, aiding biosynthesis of the universal methyl donor, S-adenosyl methionine.[25] AMD cybrids had reduced gene expression and protein levels of *MBD2* (Methyl-CpG Binding Domain protein) and higher *MBD4* (Methyl-CpG Binding Domain protein). *MBD2* specifically binds methylated DNA, co-localizes with methylated sequences. Both *MBD2* and *MBD4* are likely to be mediators of the effects of DNA methylation in mammalian cells. These changes might be attributed to the differential global DNA methylation levels, although not statistically significant, in AMD cybrids compared to normal cybrids.

Histone deacetylases (HDACs) regulate the acetylation process and are involved in retinal degeneration, and therefore have been identified as therapeutic targets.[10, 26] HDACs are classified in four groups based on function and DNA sequence homology – Class I, IIA, IIB, III, and IV. Class I comprises of HDACs 1, 2, 3, and 8; Class II includes HDACs 4, 5, 6, 7, 9, and 10; Class III consists of Sirtuins (SIRTs) 1, 2, 3, 4, 5, 6, and 7; Class IV includes HDAC11. Classical HDACs, which possess a zinc dependent active site, belong to classes I, II, and III. Their activity can be blocked by Trichostatin A (TSA). Class IV HDACs, which are called Sirtuins, are NADPH-dependent proteins and unaffected by TSA.[27] Our study revealed that AMD cybrids had significantly higher gene expression of *HDAC1, HDAC2, HDAC3*, and *HDAC11* compared to normal, suggesting that AMD mitochondria influence the histone deacetylase activities. Previous studies have demonstrated elevated HDAC1 activity in retinal degeneration models both *in vitro* and *in vivo*.[28, 29] It is also known that *HDAC1* binds and represses the transcriptional activity of *PAX6* during retinal development.[30] The repressive effect of HDAC1 on *PAX6*, a transcription factor critical to development of eye, could be blocked by TSA or by knockdown of HDAC1. Elevated HDAC2 activity has been associated with degeneration and dysfunction of neurons, an event that can be blocked by selective HDAC2 inhibitors.[31] Fan *et al* demonstrated that HDAC2, which was shown to be involved in ischemic retinal degeneration in mice, could be a target for therapy.[32] Furthermore, activation of HDAC3 via phosphorylation causes neurodegeneration, and both HDAC3 and HDAC2 are known to negatively regulate memory.[33–36] HDAC11 is predominantly localized to the nucleus and performs tissue-specific functions. HDAC11 influences immune function by regulating the expression of interleukin 10.[37] Therefore, AMD-mitochondrial DNA mediated up-regulation of *HDAC1, HDAC2, HDAC3*, and *HDAC11* in AMD cybrid cells might be a mechanism that contributes to enhanced retinal damage observed in AMD disease pathology.

Our study also demonstrated significantly reduced expression of *HDAC6, HDAC9*, and *HDAC10* in AMD cells compared to the normal group. HDAC6 is known as an alpha-tubulin deacetylase. Global deacetylation of alpha-tubulin and higher alpha-tubulin acetylation correlates with HDAC6 overexpression and reduced HDAC6, respectively, in animal models.[38] Moreover, in human cells, HDAC6 is a part of the aggresome (an aggregation of misfolded proteins in the cell) that binds ubiquitin-tagged misfolded proteins

thereby recruiting them to the aggresome.[39] Cells deficient in HDAC6 are unable to clear misfolded protein aggregates from the cytoplasm, leading to hypersensitivity to the accumulated misfolded proteins.[40] HDAC9 localizes to human foveal outer segments of photoreceptors, where it may be involved in maintaining structural integrity of photoreceptor cells.[41] HDAC 9 also mediates some pro-angiogenic effects, such as retinal vessel growth, blood flow recovery, and endothelial cell sprouting.[42] HDAC10 harbors an amino-terminal catalytic domain and a carboxyl pseudo-repeat that shares significant homology with its catalytic domain.[43] HDAC10 mediates a survival response to cytotoxic drug-mediated cellular and metabolic stress.[44]

5-aza-2'-deoxycytidine (DAC/Decitabine), a nucleoside analog, covantly binds to DNA where it inhibits DNA methyltransferase (DNMTs) causing DNA demethylation or hemi-demethylation. Specifically, 5-aza-dC causes reduction in cytosine methylation in newly replicated DNA leading to transcriptional activation of genes silenced by addition of 5-methylcytosine. DAC regulates gene expression by relaxing chromatin structure. This remodeling of chromatin structure allows transcription factors to bind to promoter regions, transcription complex assembly, and subsequent gene expression. Decitabine is in clinical use as a drug for cancer treatment because of its efficacious demethylation property, which reactivates tumor suppressor genes silenced by DNA methylation.[45–47] In addition to being a DNA methyltransferase inhibitor (DNMTi), Decitabine induces a DNA-repair response.[48] In this study, we examined the expression of genes that are regulated by methylation i.e., *NF-κB, VEGF*, and *CFH*.

NF-κB (Nuclear Factor κB) is a transcription factor that responds to stimuli such as stress, cytokines, free radicals, and immune responses to infection.[49, 50] NF-κB is involved in aging and longevity along with being a master regulator of innate immune responses that are triggered during aging. Constitutive activation of NF-κB has been reported in aging tissues.[51, 52] Activated NFκB is suggestive of glycoxidation product formation in AMD pathogenesis.[53] NF-κB activation has been reported in retinal degenerations,[54–56] and neurodegenerative diseases. NF-κB influences axonal growth in the central nervous system,[57, 58] and it has been demonstrated that Sirtuins and FoxOs regulate NFκB signaling associated with inflammatory responses.[59] Methylation of lysine and arginine residues in the p65 subunit of NFκB regulates its expression and activation.[60–62] In the current study, AMD cybrids had significant upregulation of *NFκB* gene expression compared to their untreated counterparts, implying that AMD mitochondria influence *NFκB* signaling and this might in turn correlate with aging and AMD pathogenesis. Treatment with DAC did not result in any difference in *NFκB* gene in either AMD or normal cells.

The ***VEGF*** (Vascular Endothelial Growth Factor) gene encodes a signal protein that can induce angiogenesis (i.e., growth of pre-existing blood vessels) and vasculogenesis (i.e., de novo blood vessel formation) and therefore is critical for vessel repair and development.[63] VEGF was originally known as Vascular Permeability Factor (VPF) because it is also a vasodilator that increases microvascular permeability.[64] In mammals, five members of the VEGF family are known: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF (Placental Growth Factor). To date, VEGF-A, the most potent inducer of angiogenesis, is involved in endothelial cell mitogenesis and cell migration. Hypoxic cellular environment stimulates the

production of VEGF-A. In macular degeneration, due to loss of choriocapillaries, the adjacent RPE cells become hypoxic and release angiogenic factors such as VEGF triggering new blood vessel formation and choroidal neovascularization,[65] which is characteristic of the wet form of macular degeneration.[66] To date, intravitreal injections of anti-VEGF drugs such as Ranibizumab, Bevacizumab, and Aflibercept are the most widely used therapy for neovascular AMD.[67–69] **HIF1α** (Hypoxia-inducible factor 1-alpha), a transcription factor, is a master regulator of cellular response to hypoxic stress.[70] Activation of HIF-1α is critical and leads to up-regulation of VEGF.[71] Pisani *et al* suggested a strong correlation between VEGF upregulation and the methylation status of HIF1α-binding site in the *VEGF* gene promoter.[72] Previous studies have demonstrated that the expression of VEGF receptor genes is strongly influenced by epigenetic modifications, including DNA methylation.[73–76] In our study, we observed a 113% upregulation of *VEGF-A* gene in the AMD group compared to normal. However, addition of DAC to AMD cybrids reduced *VEGF-A* gene expression by 47%, thereby bringing the *VEGF-A* levels back to normal. These findings are supported by recent studies wherein treatment with DAC mitigated *VEGF-A* expression in human RPE cells *in vitro*. [77] Moreover, DAC-mediated demethylation is known to cause *VEGF-A* downregulation in human lung cancer cell lines. [78]

CFH (Complement Factor H) is a major inhibitor of the alternative complement pathway and a high-risk gene for AMD.[79] The Tyr402His mutation in CFH gene poses a 5-fold higher risk of AMD, and other less common CFH variants are associated with AMD.[80] The current study reported a significant downregulation of CFH gene and protein in AMD cybrids compared to their normal counterparts, suggesting activation of complement and proinflammatory signaling responses. These findings agree with our previous studies wherein AMD cybrids showed reduced CFH protein levels.[11] Cybrids possessing the J haplogroup which is high risk for AMD, [81] had higher total global methylation, and also lower *CFH* gene expression.[13] Our results are also in concordance with another study reporting an inverse correlation between the CFH transcript levels and the amount of CpG methylation in CFH.[82] Moreover, our study reported that DAC-treated AMD cybrids do not show any difference in gene expression of *CFH* in either AMD or normal cybrids.

Trichostatin-A (TSA), a prototypical hydroxamic acid-containing compound isolated from the actinomycete *Streptomyces hygroscopicus* was initially used as an anti-fungal drug and was found to inhibit HDACs at nanomolar concentrations. [83–85] TSA is a reversible, pan-HDAC inhibitor of mammalian class I, II and IV HDACs. Mechanistically, TSA mimics the lysine substrate and directly interacts with and chelates the essential Zn^{2+} ion in the enzymatic active site leading to HDAC inhibition. TSA induces terminal differentiation and eukaryotic cell cycle arrest. Furthermore, it induces cell cycle arrest and apoptosis in tumor cell lines and other transformed cells.[83, 86–88] Hoshikawa *et al* have shown in T24 and HeLa carcinoma cell lines, TSA reorganizes actin stress fibers (critical markers of normal cell) and reverts the transformed morphology to normal.[86] In thyroid carcinomas, TSA induces apoptosis and cell-cycle arrest by triggering the caspase cascade and by reducing cdk2- and cdk1- associated kinase activities, respectively. TSA acts synergistically with and amplifies retinoic acid responses.[89,90] Furthermore, in liver fibrosis, TSA retains the

cytoskeleton of hepatic stellate cells, thereby causing an anti-fibrogenic effect.[91] Therefore, TSA is considered a potential anti-cancer agent.[90] TSA promotes revascularization in endothelial cells after ischemia.[92] In spite of its potential role in cancer and vascular diseases, TSA has not yet entered clinical trials, possibly because reports of high cellular toxicity associated with TSA. One such study demonstrated that TSA causes toxicity in neuroblastoma cells (UKF-NB-3 and UKF-NB-4 neuroblastoma cell lines) by suppressing the expression of cytochrome P450 (CYP) biotransformation enzymes. [93] Also, Olaharski AJ *et al* highlighted the genotoxic properties of TSA by demonstrating its potential as an aneugen and clastogen in human lymphoblastoid cells. [93]

Herein, we observed that treatment with TSA led to significant reduction of **NF-κB** protein in AMD cells compared to normal. Normal cells did not respond to TSA treatment and showed no difference in protein levels of NF-κB. Our data is supported by Place *et al* wherein TSA-mediated HDAC inhibition suppresses and reprograms NF-κB signaling in colon cancer cells. [94] Furthermore, AMD cybrids treated with TSA showed a significant decline in protein levels of **VEGF-A** and **HIF1α** compared to their untreated counterparts. These results are in agreement with previous studies showing that TSA suppresses angiogenesis via inhibition of the p300-HAT dependent pathway.[95] TSA-mediated VEGF downregulation has also been demonstrated in human RPE cells lines.[77] TSA induces anti-angiogenic effects both *in vitro* and *in vivo* by down-regulation of HIF1α and VEGF under hypoxic conditions.[96] For instance, a study by Kang *et al* demonstrated that TSA inhibited HIF-1α and VEGF protein expression in the human tongue squamous cell carcinoma SCC-6 cell line *in vitro*.[97] Sawa *et al* showed that TSA reduced VEGF secretion by human glioblastoma cells in conditioned media *in vitro*.[98] Also, TSA suppresses HIF-1α activity and subsequent inhibition of hypoxia-induced angiogenesis in human osteosarcoma *in vitro*.[99] Lastly, similar to *CFH* gene expression levels mentioned earlier, the CFH protein levels were found to be lower in AMD cybrids compared to normal. TSA-mediated HDAC inhibition resulted in 85% increase in CFH protein levels in AMD cybrids compared to their untreated counterparts. Therefore, since TSA can reduce the levels of proteins associated with AMD pathology, it might be used to mitigate the damaging effects observed in AMD.

Having observed substantial gene and protein changes in epigenetic markers in AMD cells, we next examined the total global methylation levels and total HDAC activity in normal and AMD cells. The total HDAC enzyme activity levels were similar between the AMD and Normal cybrid cells, in spite of upregulation of four HDACs (HDAC 1, −2, −3 and −11) and downregulation of three HDACs (HDAC 6, −9 and −10) in the AMD samples. Moreover, we did not find a significant difference in the global DNA methylation levels between AMD and Normal cybrids. This might have been because the AMD and Normal cybrids all had H haplogroup mtDNA. Previous cybrid studies showed that cybrids with different haplogroups demonstrate difference in Total Global Methylation levels. For example, cybrids with J haplogroup mtDNA had a 3.1-fold higher Total Global Methylation compared to those with H haplogroups [13]. Furthermore, the J cybrids and H cybrids had different transcription levels for *CFH*, *VEGFA* and *NF-κB* which became equivalent after treatment with DAC. When comparing the H cybrids with the K haplogroup cybrids, the latter group had a 6.2-

fold higher Total Global Methylation and different expression for acetylation and methylation genes. K cybrids had significantly higher total global methylation levels and differences in expression levels for two acetylation genes and four methylation genes. Demethylation with DAC altered expression levels for *NFκB2*, while *APOE* transcription patterns were unchanged. [100].

While the overall methylation is similar in AMD and Normal cybrids, our RNA/protein data show that AMD cybrids have five upregulated and two downregulated methylation-related genes/proteins, which likely regulates critical pathways differently. This is supported by DAC inhibition studies showing demethylation decreases expression of VEGFA in AMD cybrids but increase levels in the Normal cybrids. Conversely, the demethylation decreases CFH expression in Normal cybrids but has no effect on expression for the AMD cybrids. Further investigation is required to understand if the influence of the mitochondria on epigenetic status is through the mtDNA variants and fragmentation, bioenergetic profiles, oxidative stress, redox status, functional differences between normal and AMD mitochondria [11, 12, 101, 102] or some novel retrograde signaling.

Our recent work with AMD cybrids has demonstrated other factors that may play a role in the differences in epigenetic profiles found in the cell lines. For example, (1) the bioenergetic analyses using the Seahorse flux analyzer showed that cybrids possessing mitochondria from Young-Normal individuals had significantly higher OCR values (indicating higher levels of OXPHOS, an efficient mechanism of ATP production) and ATP turnover, compared to both the Older-Normal and Older-AMD cybrids. In contrast, higher levels of ECAR were found in the Older-Normal and Older-AMD cybrids compared to Young-Normal cybrids, indicating that the old cybrids compensate for loss of mitochondrial function with increase in glycolysis. Our findings are consistent with studies showing that mitochondrial efficiency declines with age and that glycolysis, as a mode of energy production, increases. Since the bioenergetic profiles of the Older-AMD cybrids and Older-Normal cybrids were similar to each other, it suggests that ATP levels or modes of energy production were not influencing the expression of nuclear genes [11]. Other studies have shown different epigenetic profiles for cells using OXPHOS versus glycolysis [103].

(2) Another factor may be the redox status of the cybrids. Our studies have demonstrated that AMD cybrids have: (a) elevated levels of mitochondrial superoxides, the predominant ROS found in the mitochondria, and (b) reduced expression of antioxidant genes, PRDX3 and SOD2, (c) Decreased in mitochondrial GFP fluorescence, (d) lower mtDNA copy numbers and (e) increased mtDNA fragmentation compared to normal cybrids. Furthermore, AMD cybrids had downregulation of *TFAM, POLG, POLRMT*, and *TFB2M* genes, suggesting impaired mitochondrial transcription/replication [12]. Previous studies have shown that redox status can influence the methylation/acetylation profiles of cells [104–106]. Therefore, the differences in levels of OXPHOS, bioenergetics, oxidative stress, and redox profiles between normal and AMD mitochondria may play a role in the varied epigenetic patterns observed in the current manuscript. Moreover, since inhibition of methylation via DAC and Trichostatin A-mediated HDAC inhibition alters the expression of genes associated with AMD pathogenesis i.e., *VEGF*, *CFH, HIF1α*, and *NFκB*, our study

suggest that AMD mitochondria-mediated epigenetic regulation may play a role in AMD pathology.

In conclusion, 1) mitochondria from AMD patients can influence epigenetic regulation i.e., methylation and acetylation status in AMD ARPE-19 cells; and 2) DAC and TSA may be potential targets for therapeutic intervention in AMD. Further studies are required to fully understand mechanisms by which AMD mitochondria regulate epigenetics in transmitochondrial AMD ARPE-19 cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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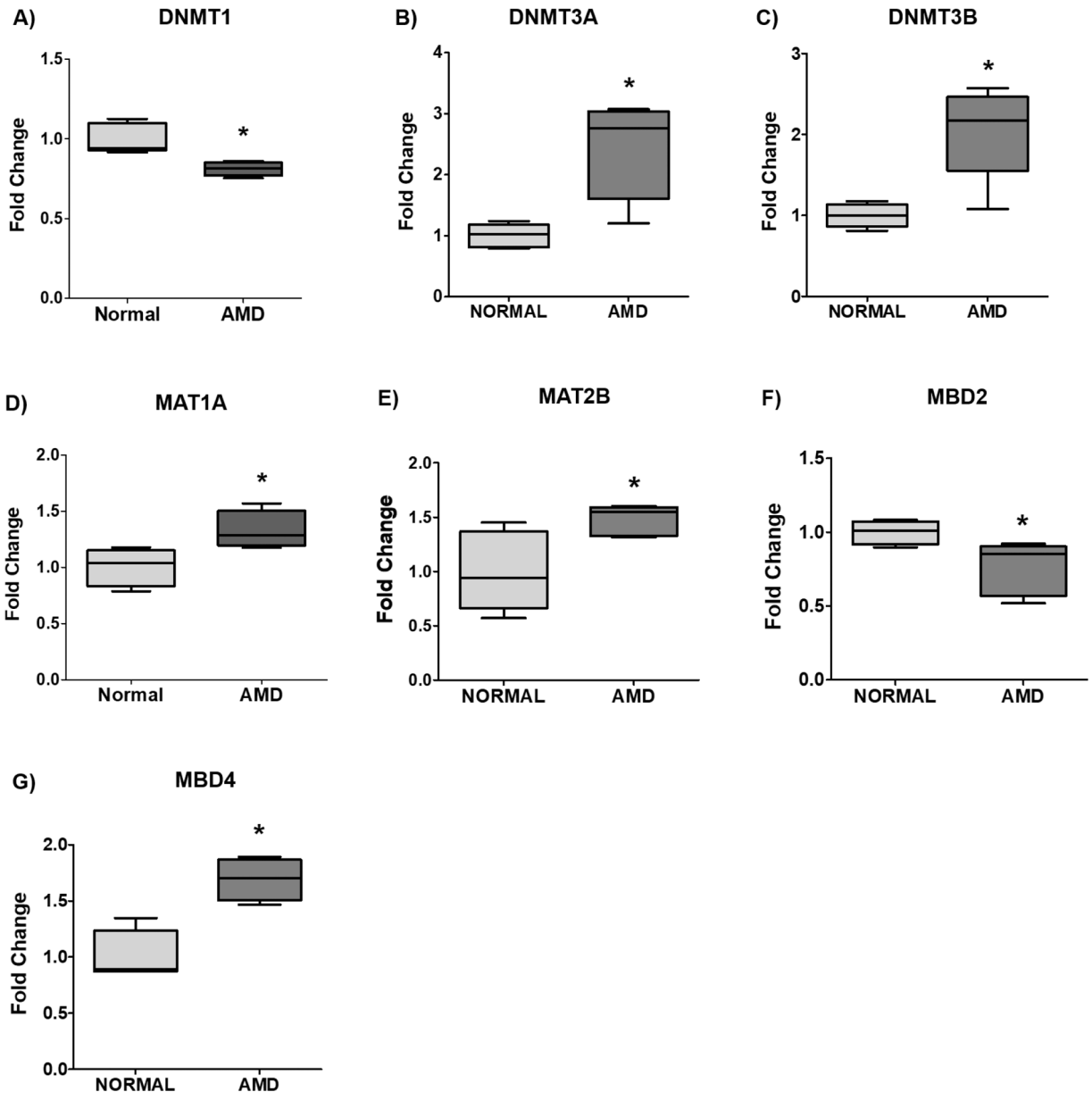

Fig. 1. AMD mitochondria mediate differential expression of methylation-associated genes.

Gene expression profiles of methylation-associated genes were measured using qRT-PCR. Cybrids containing AMD mitochondria showed elevated gene levels of *DNMT3A* (Fig.1B), *DNMT3B* (Fig.1C), *MAT1A* (Fig.1D), *MAT2B* (Fig.1E), and *MBD4* (Fig.1G) ($p<0.05$, n=5–6). However, *DNMT1* (Fig.1A) and *MBD2* (Fig.1F) showed reduced gene expression in AMD cybrids compared to normal cybrids ($p<0.05$, n=4–5) (Table 1). Data are presented as Mean ± SEM. All values are normalized to the untreated normal group.

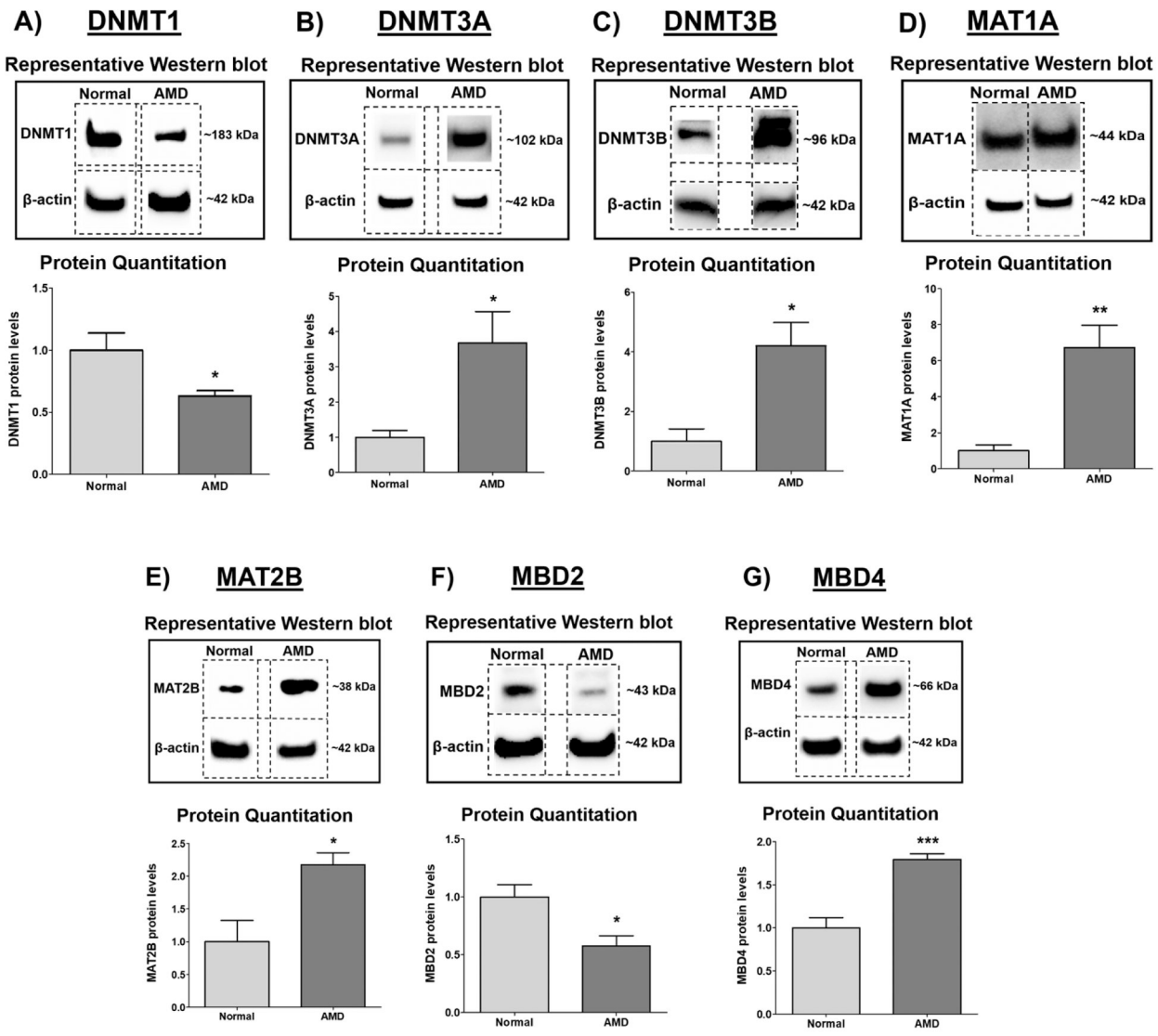

Fig. 2. AMD mitochondria mediate differential protein levels of methylation-associated markers.

Protein levels were measured using Western blotting. Cybrids containing AMD mitochondria showed higher protein levels of DNMT3A (Fig.1B), DNMT3B (Fig.1C), MAT1A (Fig.1D), MAT2B (Fig.1E), and MBD4 (Fig.1G) ($p<0.05$, n=5–6). However, DNMT1 (Fig.1A) and MBD2 (Fig.1F) showed reduced protein levels in AMD cybrids compared to normal cybrids ($p<0.05$, n=4–5) (Table 2). Data are presented as Mean ± SEM. All values are normalized to the untreated normal group.

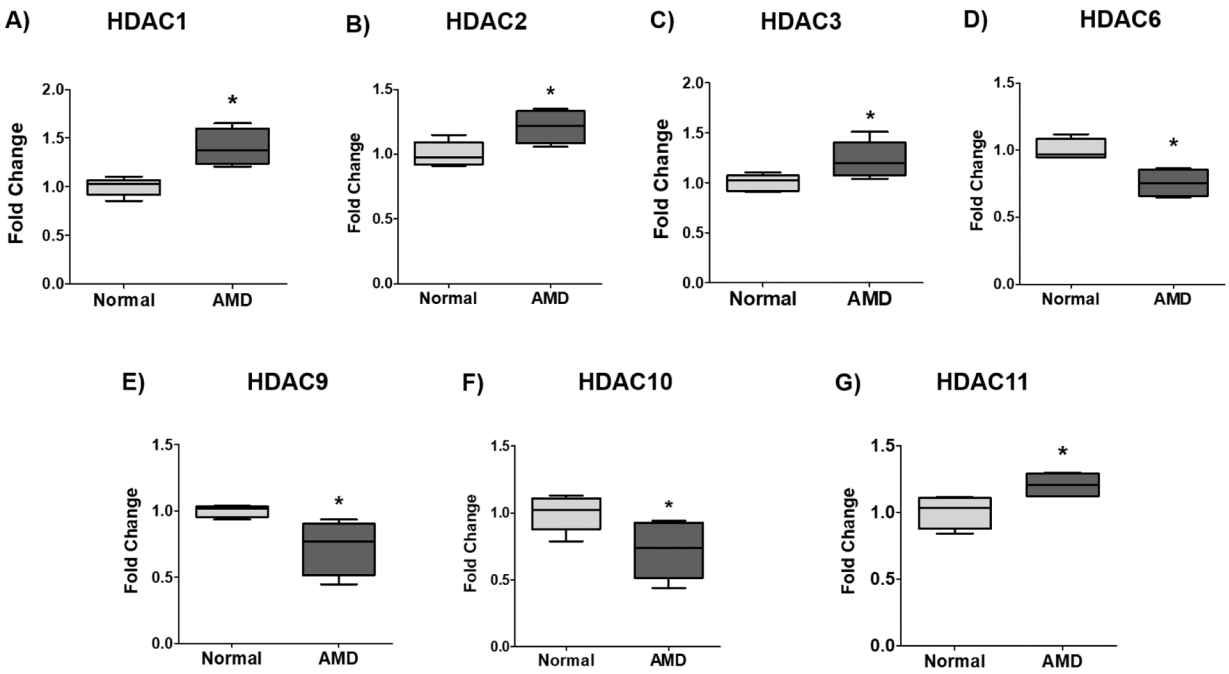

Fig. 3. AMD mitochondria mediate differential expression of histone deacetylase (HDAC) genes.

Analysis of HDAC genes' expression using qRT-PCR revealed significantly elevated gene levels of *HDAC1* (Fig.2A), *HDAC2* (Fig.2B), *HDAC3* (Fig.2C), and *HDAC11* (Fig. 2G) in AMD cybrids. Downregulation of *HDAC6* (Fig.2D), *HDAC9* (Fig.2E), and *HDAC10* (Fig.2F) was observed in AMD cybrids compared to normal ($p<0.05$, n=4–5) (Table 3). Data are presented as Mean ± SEM. All values are normalized to the untreated normal group.

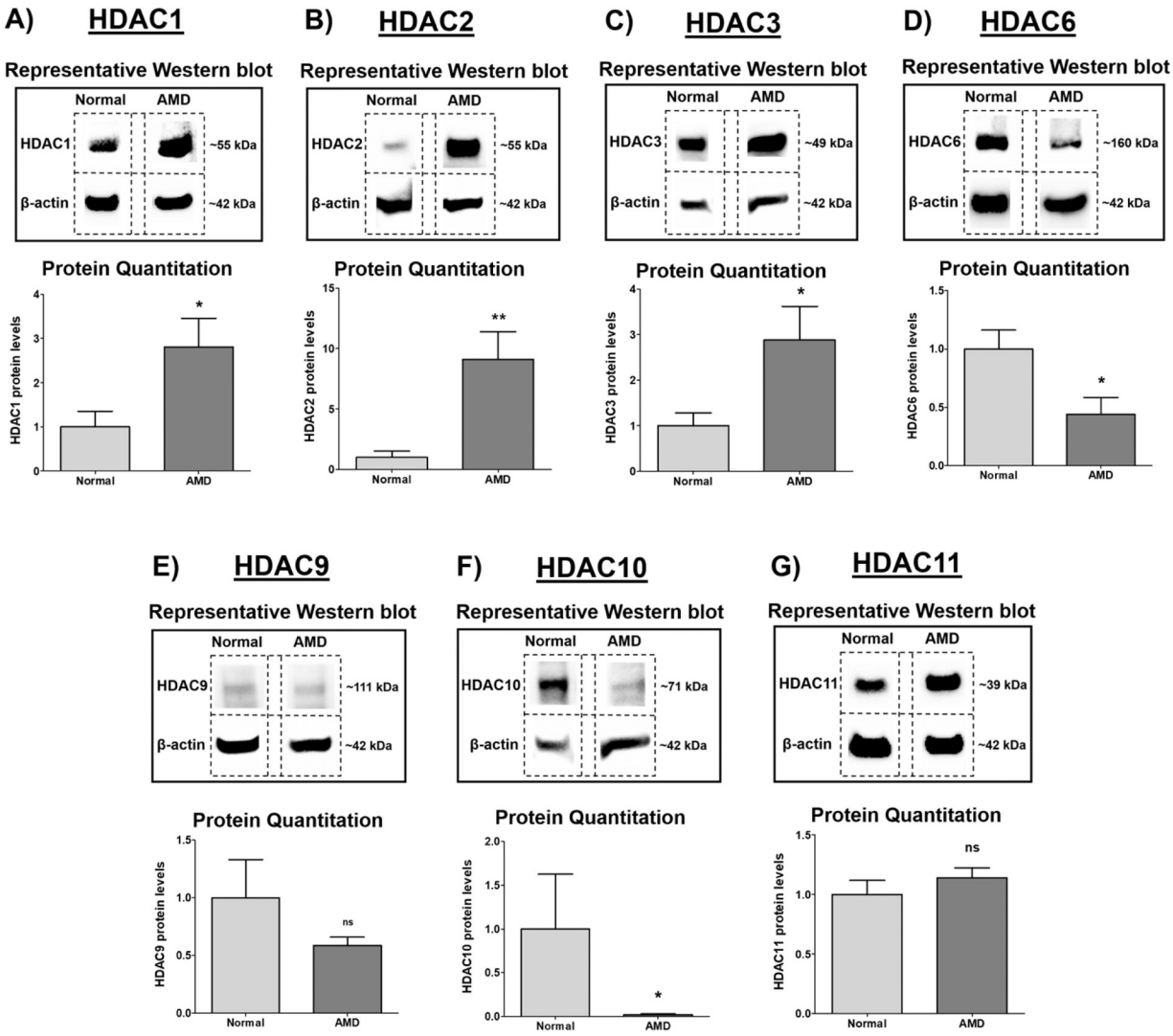

Fig. 4. AMD mitochondria mediate differential HDAC protein levels.

Analysis of HDAC protein levels using Western blotting revealed higher levels of HDAC1 (Fig.2A), HDAC2 (Fig.2B), HDAC3 (Fig.2C) ($p<0.05$, n=4–6), and HDAC11 (Fig. 2G) ($p>0.05$, n=4–6) in AMD cybrids. Downregulation of HDAC6 (Fig.2D) ($p<0.05$, n=4–6), HDAC9 (Fig.2E) ($p>0.05$, n=4–6), and HDAC10 (Fig.2F) ($p<0.05$, n=4–6) was observed in AMD cybrids compared to normal (Table 4). Data are presented as Mean ± SEM. All values are normalized to the untreated normal group.

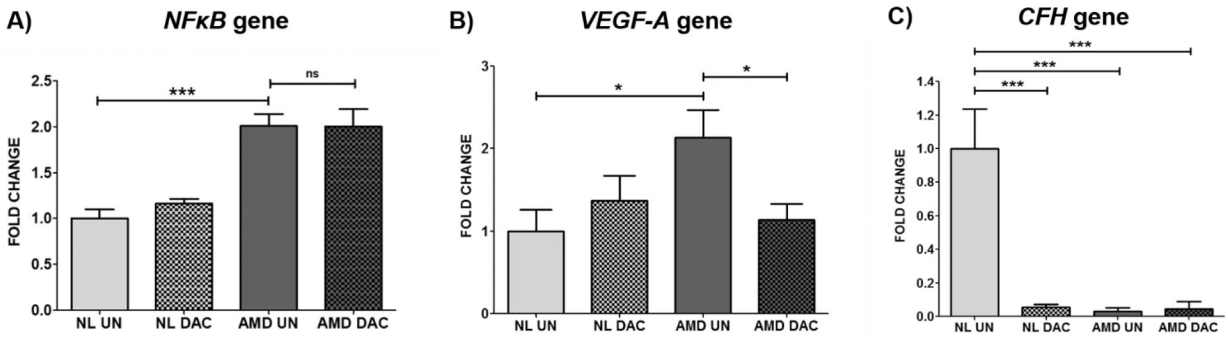

Fig. 5. Effects of 5-Aza-2'-deoxycytidine (DAC).

This figure shows qRT-PCR data for the effects of DAC treatment on AMD and NL cybrid cells. Treatment with DAC caused no change in transcript levels of *NFKB* (Fig. 3A) in AMD cybrids. *VEGF-A* gene expression declined significantly (Fig. 3B), and no difference in *CFH* gene level (Fig. 3C) was observed in DAC-treated AMD cells. Treatment with DAC had no effect on the gene expression of *NFκB* and *VEGF-A* in normal cybrids. However, DAC-treated NL cybrids showed a significant decline in *CFH* ($p<0.05$, n=3–4) (Table 5). Data are presented as Mean ± SEM. All values are normalized to the untreated normal group.

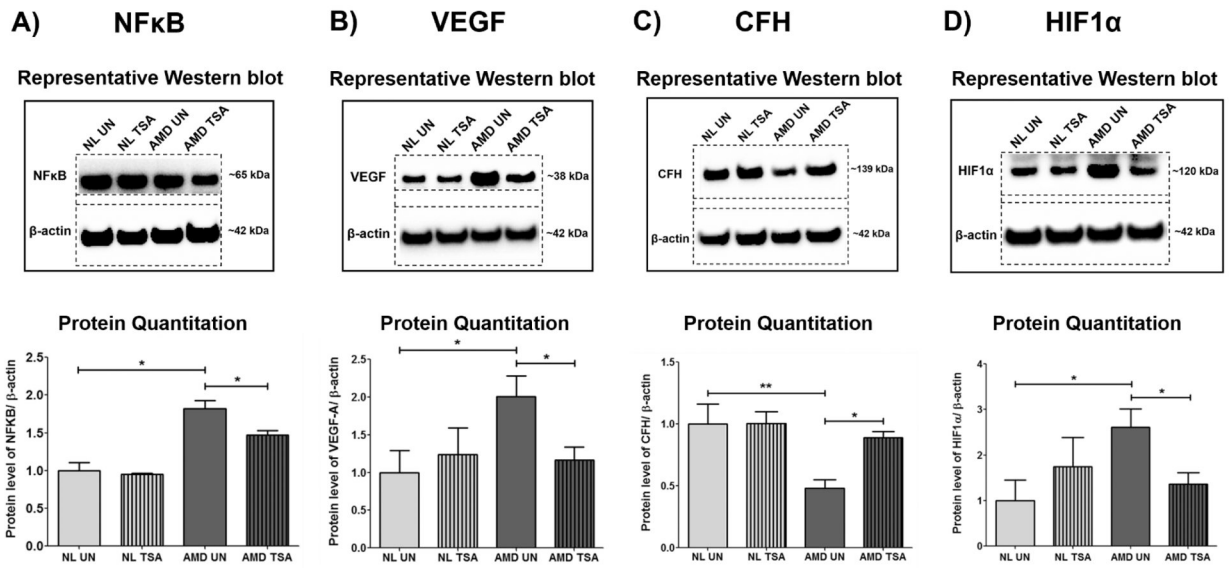

Fig. 6. Effects of Trichostatin A (TSA).

This figure shows Western blot data i.e., representative Western blot images (top panel) and the quantitation graphs (bottom panel), demonstrating the effects of TSA treatment on AMD and NL cybrid cells. Treatment with TSA reduced protein levels of NFKB (Fig. 4A), VEGF-A (Fig. 4B), and HIF1α (Fig. 4D) in AMD cybrids. Protein levels of CFH were significantly elevated in TSA-treated AMD cybrids (Fig. 4C) ($p<0.05$, $n=4$) (Table 6). Data are presented as Mean ± SEM. All values are normalized to the untreated normal group.

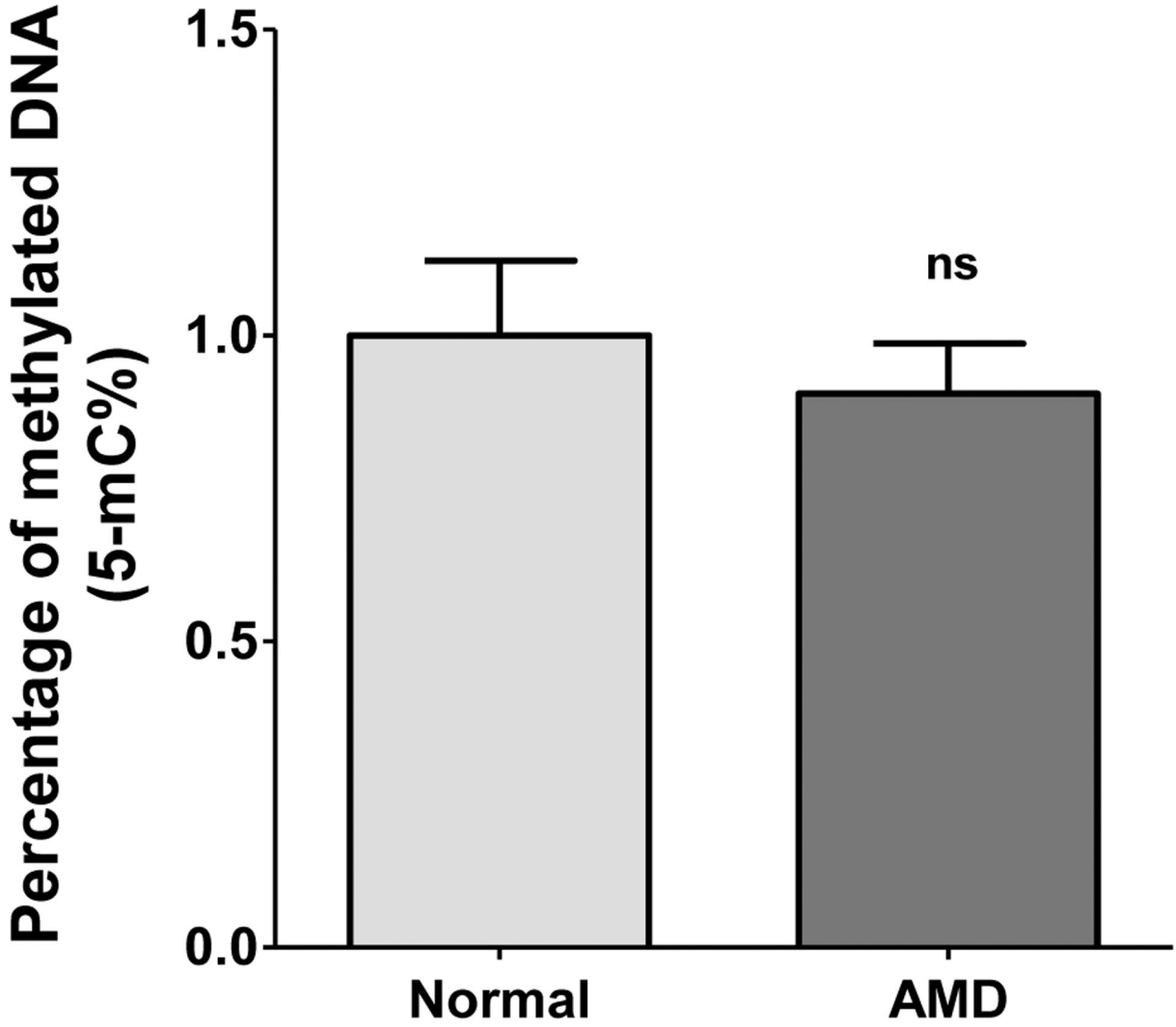

Fig. 7. Total Global DNA Methylation Assay.

This figure shows the percentage of methylated DNA i.e., 5-mC% in normal and AMD cybrids. No statistically significant difference was observed in the total methylation levels between normal and AMD cybrids ($p>0.05$; $n=6–7$). Data are presented as Mean ± SEM. All values are normalized to the untreated normal group.

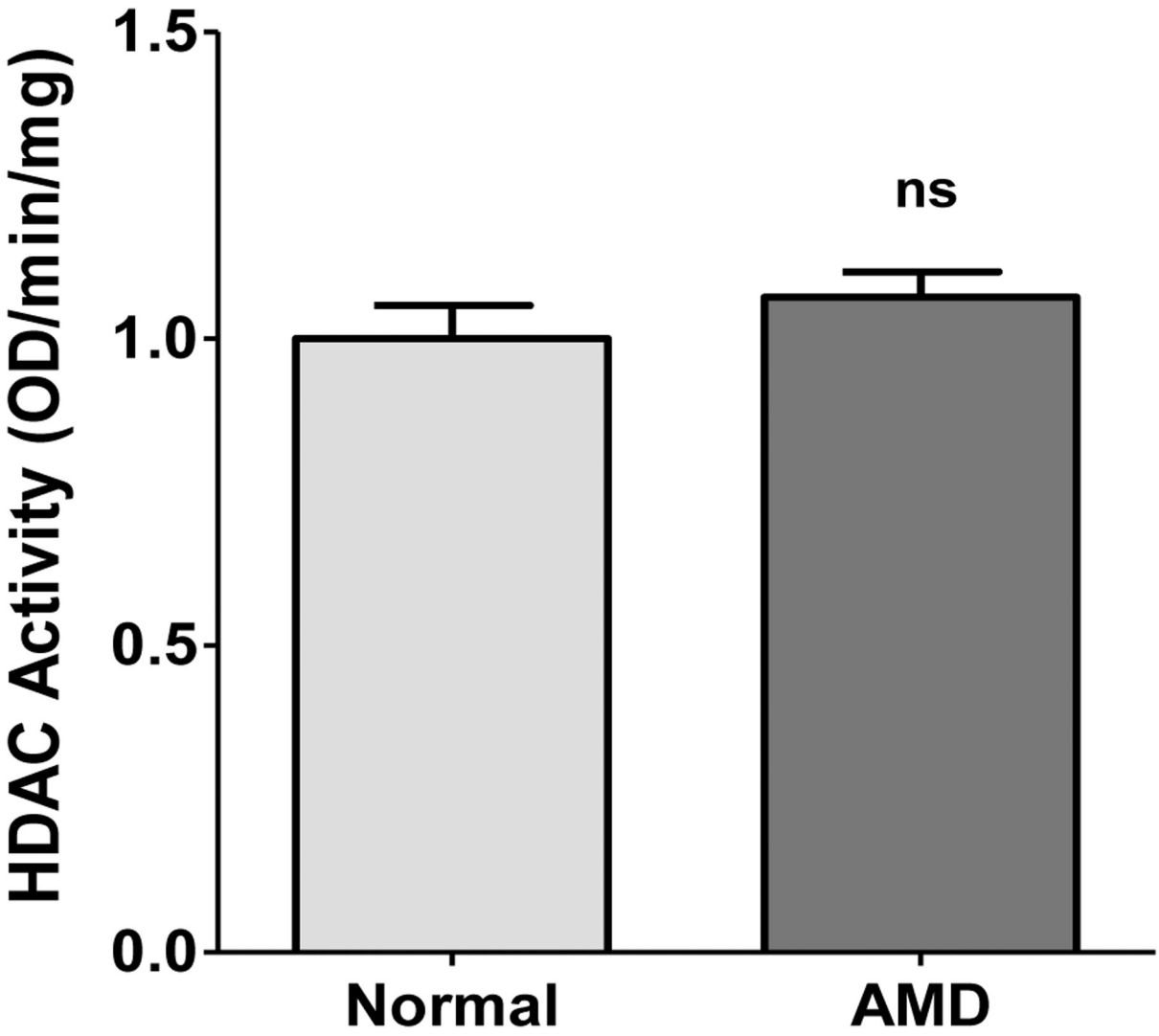

Fig. 8. HDAC Activity Assay.

This figure shows the total activity of the HDAC enzyme (OD/min/mg) in normal and AMD cybrids. No statistically significant difference was observed in the total HDAC activity between normal and AMD cybrids ($p>0.05$; $n=6–7$). Data are presented as Mean ± SEM. All values are normalized to the untreated normal group.

Table. 1.

Methylation genes' expression data.

This table briefly lists the functions of methylation-related genes used in this study, and provides P-values, percent change values, and Mean ± SEM values for the Normal untreated (NL UN) and AMD untreated (AMD UN) groups.

GENE	FUNCTION	NL UN vs. AMD UN P-value	Percent Change	NL UN Mean ± SEM	AMD UN Mean ± SEM
DNMT1 (DNA MethylTransferase 1)	DNMT1 is the most abundant and key maintenance DNA methyltransferase in mammalian cells.	0.01	18.78 %	1 ± 0.0414 n=5	0.8122 ± 0.0223 n=4
DNMT3A (DNA MethylTransferase 3 Alpha)	DNMT3A functions in de novo methylation.	0.01	141 %	1 ± 0.0845 n=5	2.410 ± 0.3567 n=5
DNMT3B (DNA MethylTransferase 3 Beta)	DNMT3B functions in de novo methylation.	0.03	104.2 %	1 ± 0.0637 n=5	2.042 ± 0.2572 n=5
MAT1A (Methionine AdenosylTransferase 1 Alpha)	MAT1A catalyzes the formation of S-adenosylmethionine from methionine and ATP.	0.03	32.8 %	1 ± 0.0742 n=5	1.328 ± 0.085 n=4
MAT2B (Methionine AdenosylTransferase 2 Beta)	MAT2B catalyzes the formation of S-adenosylmethionine from methionine and ATP.	0.03	47.9 %	1 ± 0.1636 n=5	1.479 ± 0.0616 n=5
MBD2 (Methyl-CpG Binding Domain protein 2)	MBD2 acts as transcriptional repressor and plays a role in gene silencing.	0.03	24.02 %	1 ± 0.0401 n=4	0.7598 ± 0.0798 n=5
MBD4 (Methyl-CpG Binding Domain protein 4)	MBD4 functions both in binding to methylated DNA, in protein interactions and in DNA repair.	0.029	69.2 %	1 ± 0.1159 n=4	1.692 ± 0.0942 n=4

Table. 2.

Methylation protein data.

This table briefly lists the functions of methylation-related proteins used in this study, and provides P-values, percent change values, and Mean ± SEM values for the Normal untreated (NL UN) and AMD untreated (AMD UN) groups.

PROTEIN	FUNCTION	NL UN vs. AMD UN P-value	Percent Change	NL UN Mean ± SEM	AMD UN Mean ± SEM
DNMT1 (DNA MethylTransferase 1)	DNMT1 is the most abundant and key maintenance DNA methyltransferase in mammalian cells.	0.01	36.74 %	1 ± 0.1403 n=5	0.6326 ± 0.0429 n=5
DNMT3A (DNA MethylTransferase 3 Alpha)	DNMT3A functions in de novo methylation.	0.01	267 %	1 ± 0.1982 n=7	3.670 ± 0.8951 n=6
DNMT3B (DNA MethylTransferase 3 Beta)	DNMT3B functions in de novo methylation.	0.01	320.6 %	1 ± 0.4201 n=7	4.206 ± 0.7771 n=6
MAT1A (Methionine AdenosylTransferase 1 Alpha)	MAT1A catalyzes the formation of S-adenosylmethionine from methionine and ATP.	0.001	573.3 %	1 ±0.3299 n=7	6.733 ± 1.228 n=6
MAT2B (Methionine AdenosylTransferase 2 Beta)	MAT2B catalyzes the formation of S-adenosylmethionine from methionine and ATP.	0.03	117.6 %	1 ± 0.3258 n=5	2.176 ± 0.1805 n=4
MBD2 (Methyl-CpG Binding Domain protein 2)	MBD2 acts as transcriptional repressor and plays a role in gene silencing.	0.014	42.33 %	1 ± 0.1059 n=7	0.5767 ± 0.0876 n=6
MBD4 (Methyl-CpG Binding Domain protein 4)	MBD4 functions both in binding to methylated DNA, in protein interactions and in DNA repair.	0.0007	79.4 %	1 ± 0.1186 n=8	1.794 ± 0.0652 n=6

Table. 3.

HDAC genes' expression data.

This table briefly mentions the functions of HDAC genes used in this study, and provides P-values, percent change values, and Mean ± SEM values for the Normal untreated (NL UN) and AMD untreated (AMD UN) groups.

GENE	FUNCTION	NL UN vs. AMD UN P-value	Percent Change	NL UN Mean ± SEM	AMD UN Mean ± SEM
HDAC1 (Histone deacetylase 1)	HDAC1 regulates specific gene functions by repressing or activating certain promoters. HDAC1 is involved in transcriptional repression regulated by the retinoblastoma protein Rb. It also functions in the DNA-damage response.	0.01	40.4 %	1 ± 0.0415 n=5	1.404 ± 0.0949 n=4
HDAC2 (Histone deacetylase 2)	HDAC2 is involved in the regulation of neuronal differentiation through a direct interaction with the N-terminal domain of DNMT3b. HDAC3 is involved in transcriptional repression regulated by the retinoblastoma protein Rb. It also functions in the DNA-damage response.	0.03	21.2%	1 ± 0.0433 n=5	1.212 ± 0.0644 n=4
HDAC3 (Histone deacetylase 3)	HDAC3 modulates the transcriptional activities of nuclear receptors.	0.01	23 %	1 ± 0368 n=5	1.230 ± 0 0822 n=5
HDAC6 (Histone deacetylase 6)	HDAC6 possesses a C-terminal ubiquitin-binding domain BUZ that facilitates interaction with Lys63-polyubiquitinated proteins and mediates their transport to the aggresome. HDAC6 functions in various physiological and disease processes.	0.029	24.5 %	1 ± 0.0404 n=4	0.7550 ± 0.0533 n=4
HDAC9 (Histone deacetylase 9)	HDAC9 regulates a variety of physiological functions, including muscle differentiation and development, cardiac growth, T-regulatory cell function, and neuronal disorders.	0.029	27.2 %	1 ± 0.0227 n=4	0.7280 ± 0.1041 n=4
HDAC10 (Histone deacetylase 10)	HDAC10 mediates a survival response to cytotoxic drug-mediated cellular and metabolic stress.	0.03	27.52 %	1 ± 0.0595 n=5	0.7248 ± 0.0950 n=5
HDAC11 (Histone deacetylase 11)	HDAC11 influences immune function by regulating the expression of interleukin 10.	0.029	20.6 %	1 ± 0 0615 n=4	1.206 ± 0.0480 n=4

Table. 4.

HDAC protein data.

This table briefly lists the functions of HDAC proteins used in this study, and provides P-values, percent change values, and Mean ± SEM values for the Normal untreated (NL UN) and AMD untreated (AMD UN) groups.

PROTEIN	FUNCTION	NL UN vs. AMD UN P-value	Percent Change	NL UN Mean ± SEM	AMD UN Mean ± SEM
HDAC1 (Histone deacetylase 1)	HDAC1 regulates specific gene functions by repressing or activating certain promoters. HDAC1 is involved in transcriptional repression regulated by the retinoblastoma protein Rb. It also functions in the DNA-damage response.	0.03	180.9 %	1 ± 0.3513 n=6	2.809 ± 0.6482 n=5
HDAC2 (Histone deacetylase 2)	HDAC2 is involved in the regulation of neuronal differentiation through a direct interaction with the N-terminal domain of DNMT3b. HDAC3 is involved in transcriptional repression regulated by the retinoblastoma protein Rb, It also functions in the DNA-damage response.	0.0087	811.2 %	1 ± 0.5164 n=6	9.112 ± 2.276 n=6
HDAC3 (Histone deacetylase 3)	HDAC3 modulates the transcriptional activities of nuclear receptors.	0.02	188.2 %	1 ± 0.2833 n=7	2.882 ± 0.7385 n=6
HDAC6 (Histone deacetylase 6)	HDAC6 possesses a C-terminal ubiquitin-binding domain BUZ that facilitates interaction with Lys63~polyubiquitinated proteins and mediates their transport to the aggresome. HDAC6 functions in various physiological and disease processes.	0.02	55.81 %	1 ± 0.1631 n=7	0.4419 ± 0.1421 n=6
HDAC9 (Histone deacetylase 9)	HDAC9 regulates a variety of physiological functions, including muscle differentiation and development, cardiac growth, T-regulatory cell function, and neuronal disorders.	0.73	41.49 %	1 ± 0.3295 n=5	0.5851 ± 0.0729 n=4
HDAC10 (Histone deacetylase 10)	HDAC10 mediates a survival response to cytotoxic drug-mediated cellular and metabolic stress.	0.02	98 %	1 ± 0.6282 n=3	0.01937 ± 0.0085 n=6
HDAC11 (Histone deacetylase 11)	HDAC11 influences immune function by regulating the expression of interleukin 10.	0.48	13.8 %	1 ± 0.1169 n=4	1.138 ± 0.08358 n=4

Table. 5.

Effects of 5-Aza-2´-deoxycytidine (DAC), a methylation inhibitor.

This table briefly lists the functions of *NFκB*, *VEGF-A*, and *CFH* genes, and provides P-values and Mean ± SEM values for the Normal untreated (NL UN), Normal DAC-treated (NL DAC), AMD untreated (AMD UN), and AMD DAC-treated (AMD DAC) groups.

GENE	FUNCTION	NL UN Mean ± SEM	NLDAC Mean ± SEM	AMD UN Mean ± SEM	AMD DAC Mean ± SEM	NL UN vs. AMD UN P-value	AMD UN vs. AMD DAC P-value
NFκB (Nuclear Factor Kappa B)	NFκB is a transcription regulator. Activated NFκB translocates into the nucleus and stimulates the expression of genes involved in a wide variety of biological functions.	1 ± 0.097 n=4	0.9269 ± 0.11 n=4	2.01 ± 0.13 n=4	2.004 ± 0.19 n=4	0.0008	0.97
VEGF-A (Vascular Endothelial Growth Factor A)	VEGF-A induces proliferation and migration of vascular endothelial cells, and is essential for both physiological and pathological angiogenesis.	1 ± 0.26 n=4	1.372 ± 0.29 n=4	2.13 ± 0.33 n=3	1.138 ± 0.19 n=4	0.04	0.04
CFH (Complement Factor H)	CFH regulates complement activation, restricting this innate defense mechanism to microbial infections.	1 ± 0.24 n=3	0.055 ± 0.02 n=4	0.032 ± 0.02 n=4	0.045 ± 0.04 n=4	0.0047	0.80

Table. 6.

Effects of Trichostatin A, an HDAC inhibitor.

This table briefly lists the functions of *NFκB*, *VEGF-A*, *CFH*, and *HIF1α* genes, and provides P-values and Mean ± SEM values for the Normal untreated (NL UN), Normal TSA-treated (NL TSA), AMD untreated (AMD UN), and AMD TSA-treated (AMD TSA) groups.

PROTEIN	FUNCTION	NL UN Mean ± SEM	NLTSA Mean ± SEM	AMD UN Mean ± SEM	AMD TSA Mean ± SEM	NL UN vs. AMD UN P-value	AMD UN vs. AMD TSA P-value
NFκB (Nuclear Factor Kappa B)	NFκB is a transcription regulator. Activated NFκB translocates into the nucleus and stimulates the expression of genes involved in a wide variety of biological functions.	1 ± 0.10 n=3	0.9492 ± 0.02 n=3	1.822 ± 0.10 n=3	1.468 ± 0.06 n=3	0.005	0.04
VEGF-A (Vascular Endothelial Growth Factor A)	VEGF-A induces proliferation and migration of vascular endothelial cells and is essential for both physiological and pathological angiogenesis.	1 ± 0.29 n=4	1.239 ± 0.35 n=3	2 ± 0.27 n=5	1.163 ± 0.17 n=5	0.04	0.03
CFH (Complement Factor H)	CFH is secreted into the bloodstream and has an essential role in the regulation of complement activation, restricting this innate defense mechanism to microbial infections.	1 ± 0.16 n=4	1.006 ± 0.09 n=4	0.4829 ± 0.07 n=5	0.8906 ± 0.05 n=5	0.01	0.001
HIF1α (Hypoxia Inducible Factor 1 Alpha)	HIF-1 functions as a master regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes including those involved in energy metabolism, angiogenesis, apoptosis; and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia.	1 ± 0.45 n=3	1.75 ± 0.64 n=3	2.615 ± 0.40 n=4	1.366 ± 0.24 n=4	0.04	0.04