Vitamin-D3 (α-1, 25(OH) 2D3) Protects Retinal Pigment Epithelium From Hyperoxic Insults

Ponnalagu Murugeswari,[1] Arman Firoz,[2] Subramani Murali,[1] Anand Vinekar,[3] Lekshmi Krishna,[1] Venkata Ramana Anandula,[4] Nallathambi Jeyabalan,[2] Priyanka Chevour,[2] Chaitra Jayadev,[5] Rohit Shetty,[6] Gilles Carpentier,[7] Govindaswamy Kumaramanickavel,[2] Arkasubhra Ghosh,[2] and Debashish Das[1]

[1]Stem Cell Research Lab, GROW Laboratory, Narayana Nethralaya Foundation, Bangalore, Karnataka, India
[2]GROW Laboratory, Narayana Nethralaya Foundation, Bangalore, Karnataka, India
[3]Department of Pediatric Ophthalmology, Narayana Nethralaya Eye Institute, Bangalore, Karnataka, India
[4]Department of Molecular Diagnostics & Laboratory Services, Narayana Nethralaya Eye Institute, Bangalore, Karnataka, India
[5]Department of Vitreoretinal Services, Narayana Nethralaya Eye Institute, Bangalore, Karnataka, India
[6]Department of Cornea and Refractive Surgery, Narayana Nethralaya Eye Institute, Bangalore, Karnataka, India
[7]Laboratoire CRRET, Faculté des Sciences et Technologie, Université Paris Est Créteil, Créteil Cedex, France

Correspondence: Debashish Das, Stem Cell Research Laboratory, GROW Laboratory, Narayana Nethralaya Foundation, Narayana Nethralaya Eye Institute, Narayana Health City, Bommasandra, Bangalore 560 099, Karnataka, India; drdebashish@narayananethralaya.com; dasdebashish@yahoo.co.uk.

Received: June 27, 2019
Accepted: November 22, 2019
Published: February 7, 2020

Citation: Murugeswari P, Firoz A, Murali S, et al. Vitamin-D3 (α-1, 25(OH) 2D3) protects retinal pigment epithelium from hyperoxic insults. *Invest Ophthalmol Vis Sci.* 2020;61(2):4. https://doi.org/10.1167/iovs.61.2.4

Purpose. Oxidative stress affects the retinal pigment epithelium (RPE) leading to development of vascular eye diseases. Cholecalciferol (VIT-D) is a known modulator of oxidative stress and angiogenesis. This in vitro study was carried out to evaluate the protective role of VIT-D on RPE cells incubated under hyperoxic conditions.

Methods. Cadaver primary RPE (PRPE) cells were cultured in hyperoxia (40% O_2) with or without VIT-D (α-1, 25(OH) 2D3). The functional and physiological effects of PRPE cells with VIT-D treatment were analyzed using molecular and biochemical tools.

Results. Vascular signaling modulators, such as vascular endothelial growth factor (VEGF) and Notch, were reduced in hyperoxic conditions but significantly upregulated in the presence of VIT-D. Additionally, PRPE conditioned medium with VIT-D induced the tubulogenesis in primary human umbilical vein endothelial cells (HUVEC) cells. VIT-D supplementation restored phagocytosis and transmembrane potential in PRPE cells cultured under hyperoxia.

Conclusions. VIT-D protects RPE cells and promotes angiogenesis under hyperoxic insult. These findings may give impetus to the potential of VIT-D as a therapeutic agent in hyperoxia induced retinal vascular diseases.

Keywords: retinal pigment epithelium, vitamin D, hyperoxia, vascular endothelial growth factor (VEGF), tubulogenesis

Oxidative stress is the result of an imbalance between the synthesis of reactive oxygen species (ROS) and the levels of antioxidants in the cells. It plays a major role in the pathophysiology of various ocular diseases, such as age-related cataract, macular degeneration, glaucoma, diabetic retinopathy, and retinitis pigmentosa, by affecting cellular and vascular physiological aspects.[1,2]

Antioxidants, such as enzymatic antioxidants, vitamins, minerals, carotenoids, and flavonoids, are the primary scavengers of ROS reducing levels of oxidative stress.[3] Cholecalciferol (vitamin-D3 (VIT-D)) has diverse functions, including modulation of inflammation, angiogenesis, oxidative stress, and fibrosis.[4,5] Recent studies have demonstrated an association between VIT-D and retinal pathophysiological conditions, such as age-related macular degeneration (AMD), diabetic retinopathy, and retinopathy of prematurity (ROP).[6–8] These studies implicated deficiency of VIT-D to higher risk for early/late AMD, whereas a supplementation leads to delay or prevention in the progression of AMD.[6,9]

Although VIT-D receptors as well as enzymes for VIT-D metabolism are present in the retina, choroid, and retinal pigment epithelium (RPE) cells, their functions are still not well understood.[6] VIT-D has been studied as a potential inhibitor of angiogenesis in a mouse model of oxygen-induced ischemic retinopathy (OIR).[10] A correlation of VIT-D and vascular endothelial growth factor (VEGF) has been observed in retinal pericytes.[8] In ocular pathology, not much is known about the role of VIT-D in hyperoxia-induced oxidative stress. Concurrently, in early stages of AMD and ROP, local hyperoxic stress results in retinal degeneration.[11] Hence, determining the role of VIT-D under hyperoxia-induced oxidative stress conditions might provide insight for a potential therapeutic aspect. We have, therefore,

investigated the effect of VIT-D (1α,25-Dihydroxyvitamin D3) on primary RPE (PRPE) cells cultured under hyperoxic conditions.

Materials and Methods

Human PRPE Cell Culture

PRPE cells were isolated and cultured within 24 hours of enucleation from 10 noninfectious human cadaver eyes obtained from Shankar Anand Eye Bank, Narayana Nethralaya, Bangalore, India.[12] The age of the donor eyes range from 2 to 45 years (mean ± SD, 32 ± 15.8). Cells were cultured with 2% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) in an incubator with 21% O_2 for normoxia (HERAcell 240i; Heraeus, Germany) and 40% O_2 for hyperoxia (SMA-80DS/165; ASTEC, Japan) with 5% CO_2 at 37°C. In this study, early passages (3–5) of PRPE were used for the experiments.

VIT-D Supplementation on PRPE Cells

PRPE cells were seeded on 6-well tissue culture plates (Thermo Fisher Scientific Inc., Franklin, MA, USA) at a density of 3×10^5 cells/well. Cultures at 70% to 80% confluence were washed and supplemented with 20 nM of 1α, 25-Dihydroxyvitamin D3 (VIT-D; Sigma-Aldrich, St. Louis, MO, USA), in 2% FBS DMEM, for 5 days in normoxia (21% O_2) and hyperoxia (40% O_2). The culture supernatants and cells were then collected for further analysis.

Reverse Transcriptase Quantitative Polymerase Chain Reaction

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed on cultured PRPE cells using specific primers, as described previously[12,13] (See Supplementary Table S1).

Secreted VEGF Level Estimation

The VEGF levels in cell supernatants from different experimental conditions (normoxia, hyperoxia, normoxia + VIT-D, and hyperoxia + VIT-D) were estimated using the human VEGF DuoSet ELISA (R&D Systems, Minneapolis, MN, USA), as described previously.[12,13]

Immunofluorescence Staining

PRPE cells were cultured on cover slips (12 mm; Blue Star, India) in a 12-well plate with a cell density of 1×10^4 cells/well in normoxia and hyperoxia with or without VIT-D for 5 days. Immunofluorescence staining was carried out with specific antibodies (See Supplementary Table S2), as previously described.[12,13] For VEGF and DLL4, fluorescent images were captured using the ProgRes Capture Pro version 2.5 software on an Olympus BX41 fluorescent microscope (Olympus, Tokyo, Japan). For other proteins, fluorescent images were captured using the Olympus BX41 Qimaging software. Fluorescence intensity was quantified using the Olympus Qimaging micropublisher version 3.3 software (Olympus).

The ZO-1 staining of cells was used as an estimate for membrane stability in PRPE cells. The number of intact and disintegrated ZO-1 stained cells along with the total number of DAPI-positive nucleus was counted. The percentage of stable cells was calculated as the number of intact/disintegrated ZO-1-positive cells divided by the total number of DAPI positive nucleus. F-actin cytoskeleton staining was quantified using Particle Analyzer Plugin ImageJ software, as described earlier.[14] The epithelial cell shape with large cell volume and roundness of the cell was calculated as described earlier.[15,16]

Tubulogenesis Assay

Primary human umbilical vein endothelial cells (HUVECs) were purchased (American Type Culture Collection, Manassas, VA, USA) and cultured with an endothelial cell basal medium (PromoCell Gmbh, Germany) in T-25 Nunclon culture flasks (Nalgen Nunc International, Wiesbaden, UK). The in vitro tubulogenesis assay was performed using a µ-slide angiogenesis (ibidi GmbH, Germany) as per the manufacturer's instructions. After polymerization of the Matrigel (Biosciences, Inc., Blauvelt, NY, USA), 2×10^4 cells/well were added to 50 µl of experimental conditioned PRPE medium (normoxia, hyperoxia, normoxia + VIT-D, and hyperoxia + VIT-D). The µ-slide wells with the cells were incubated at 37°C in a cell culture incubator. After 5 to 6 hours, tube formation was observed and phase contrast images were captured using an Olympus CKX41miscroscope (Olympus, Shinjuku, Tokyo, Japan). Tube assay was analyzed using the Angiogenesis Analyzer plugin for ImageJ.[17] The assays were performed in triplicate. Length of the tubes and segments were measured in pixels. HUVEC cells of passage 4 to 6 were used for the experiments.

Transmembrane Potential Assay

Transmembrane potential assay was performed on PRPE cells in different experimental conditions (normoxia, hyperoxia, normoxia + VIT-D, and hyperoxia + VIT-D), as previously described.[12,13]

Phagocytosis Assay

The photoreceptor outer segments (POS) were isolated from fresh goat eyes, with a few modifications in a previously described protocol.[18] In brief, the retinae isolated from goat were homogenized and filtered through gauze. The homogenized solution was then added onto a discontinuous sucrose gradient (70% and 30%), prepared by flash freezing each layer followed by overnight incubation at 4°C. POS (pinkish orange color band) obtained after ultracentrifugation was collected with a sequential wash and labeled with FITC (Sigma-Aldrich). The isolated POS were aliquoted and stored at -80°C. To avoid the bleaching of the rhodopsin in rods, the isolation procedure of POS was performed in dim red light. Aluminum foil was used to cover tubes during centrifugation steps of the protocol. For the phagocytosis assay, human PRPE cells cultured on coverslips were kept in hyperoxia and normoxia with/without VIT-D supplement for 5 days. On day 3, cells were incubated with FITC labeled POS, and the results were read out on day 5, as described earlier.[12,13] To count the number of cells with internalized POS, post-phagocytosis assay cells were quenched with trypan blue (to avoid the POS bound on to the membrane). Cells were then fixed and the nucleus stained with DAPI. The coverslips with cultured

Investigative Ophthalmology & Visual Science

cells were mounted and then observed under a fluorescence microscope.[18]

Reactive Oxygen Species

The intracellular ROS level was assayed during hyperoxia, normoxia with/without VIT-D supplementation. Cells cultured on coverslips were stained with a fluorescent probe 2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen, Molecular Probes, Thermo Fisher Scientific), as per the manufacturer's instructions. The fluorescent intensity of cells was captured using the Olympus BX41 Qimaging version 3.3 software and quantified using the Olympus Qimaging micropublisher version 3.3 software.

Statistical Analysis

For statistical analysis, the 1-way ANOVA with post hoc Tukey's test was carried out to compare the multiple experimental conditions. All the experiments were performed in triplicate and the results of at least three independent experiments were used for the statistical analysis. The P values for all the experiments are represented in Supplementary Table S3 ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$).

Results

VEGF and VEGF-R2 are downregulated in hyperoxia and restored in the presence of VIT-D

The gene expression levels of *Vegf-A* and *Vegf-R2* showed no difference in hyperoxia when compared with normoxia (Fig. 1A). However, the secreted VEGF-A protein in hyperoxia (561 ± 15.4 pg/mL) when compared with normoxia (698.05 ± 15.4) was significantly low (Fig. 1B). The gene expression levels of *Vegf-A* and *Vegfr-R2* increased with VIT-D supplementation (Fig. 1A). Additionally, VIT-D supplementation significantly upregulated the secreted VEGF levels in normoxic (1048 ± 15.3 pg/mL) and hyperoxic conditions (980 ± 44.7 pg/mL) compared with those without supplementation (Fig. 1B). Intensity of immunofluorescence staining for intracellular VEGF and VEGF-R2 levels was low in hyperoxia compared with normoxia (Figs. 1C, 1D (i, ii), 1E, 1F). In the presence of VIT-D in hyperoxia, the intensity of VEGF and VEGF-R2 levels was significantly upregulated compared with cells in hyperoxia without VIT-D supplement (Figs. 1C, 1D (ii, iv), 1E, 1F). Normoxia cell cultures with VIT-D also showed an apparent increase in VEGF and VEGFR2 levels (Figs. 1C, 1D (ii, iii), 1E, 1F) compared with hyperoxia.

Hyperoxic Conditioned Media Impaired Vessels are Restored by VIT-D

The tube formation using primary HUVEC cells in the hyperoxic conditioned PRPE medium showed a significant reduction in tube length, number of segments, segment length, number of junctions, and number of meshes when compared with those in normoxia. Interestingly, VIT-D supplemented hyperoxia-conditioned medium showed recovery of the assayed tubulogenesis parameters compared with those with hyperoxia insult (Figs. 2A (i–iv), 2B, 2D, 2E, 2G, 2H; see Supplementary Table S4). The length and numbers of isolated segments were significantly higher in cells cultured in hyperoxia when compared with those cultured in normoxia conditioned medium (Figs. 2A (i, ii), 2C, 2F; see Supplementary Table S4). With VIT-D supplementation, a reduction in the length and number of isolated segments was detected compared with those grown with hyperoxia alone (Figs. 2C, 2F). Results obtained from cells cultured in normoxia with or without VIT-D supplementation for the analyzed parameters were similar (Figs. 2A (i, iv), 2B–H).

VIT-D Modulates Notch Signaling

In this study, a significant downregulation of *Notch-1* receptor, *Jag-2* ligand, and the downstream target *Hes-1* mRNA in PRPE cells grown in hyperoxia compared with normoxia was observed (Figs. 3A, 3B). Moreover, *Dll-4* and the downstream targets *Hes-5* and *Hey-1* were also downregulated in cells cultured in hyperoxia compared with those cultured in normoxia, although not significant. However, cells cultured in hyperoxia with VIT-D supplementation had an upregulated mRNA level for Notch signaling with a significant difference in receptor (*Notch-1*) ligands (*Dll-4* and *Jag-2*) and downstream target (*Hes-1*) compared with those cultured in hyperoxia (Figs. 3A, 3B). Further, the regulation of gene expression was corroborated with immunofluorescence staining. A decreased mean fluorescence intensity was observed in cells cultured in hyperoxia for NOTCH-1 receptor, the ligands DLL-4 and JAG-2, compared with those cultured in normoxia. However, with VIT-D supplementation in hyperoxia, there was an increase in the fluorescence intensity of the Notch receptor (NOTCH-1; Figs. 3C (i, ii, iv), 3F) and ligands (DLL-4 and JAG-2; Figs. 3D, 3E (i, ii, iv), 3G, 3H) when compared with cells grown in hyperoxia alone. The fluorescent intensity of NOTCH-1, DLL-4, and JAG-2 in cells cultured in normoxia with VIT-D supplementation were similar to those cultured under normoxia alone (Figs. 3C–E (i, iii), 3F–H).

Effects of VIT-D on Cell Proliferation in Hyperoxia

A significant reduction was observed in mRNA expression of *Cyclin-D1*, *Cyclin-B*, and *Cyclin-E* in cells grown in hyperoxia compared with normoxia (Fig. 4A). A significant downregulation of mRNA expression levels was observed in *Cdk-2*, *Cdk-4*, *Cdk-6*, and *Cdc-25* in cells grown in hyperoxia compared with normoxia (Fig. 4B). In the presence of VIT-D, cells grown under hyperoxia showed an upregulation of *Cyclin* and *Cdk* mRNA expressions compared with those grown under hyperoxia without VIT-D (*Cyclin-D1*, *Cyclin-B*, *Cyclin-E*, *Cdk-2*, *Cdk-4*, and *Cdc-25*; Figs. 4A, 4B). Cells cultured under hyperoxia showed a significant decrease in the percentage of Ki67 immunofluorescence-positive cells compared with normoxia (Figs. 4C (i, ii), 4D). In the presence of VIT-D, there was a significant increase in the percentage of cells positive for Ki67 compared with hyperoxia (Figs. 4C (ii, iv), 4D). The percentage of Ki67 positivity in the presence of VIT-D and normoxia was similar to cells cultured in normoxia without VIT-D supplementation (Figs. 4C (i, iii), 4D).

VIT-D Maintains the Epithelial Phenotype in Hyperoxia

Gene expression of *E-cad* and *N-cad* showed no significant difference in cells grown in hyperoxia compared

Investigative Ophthalmology & Visual Science

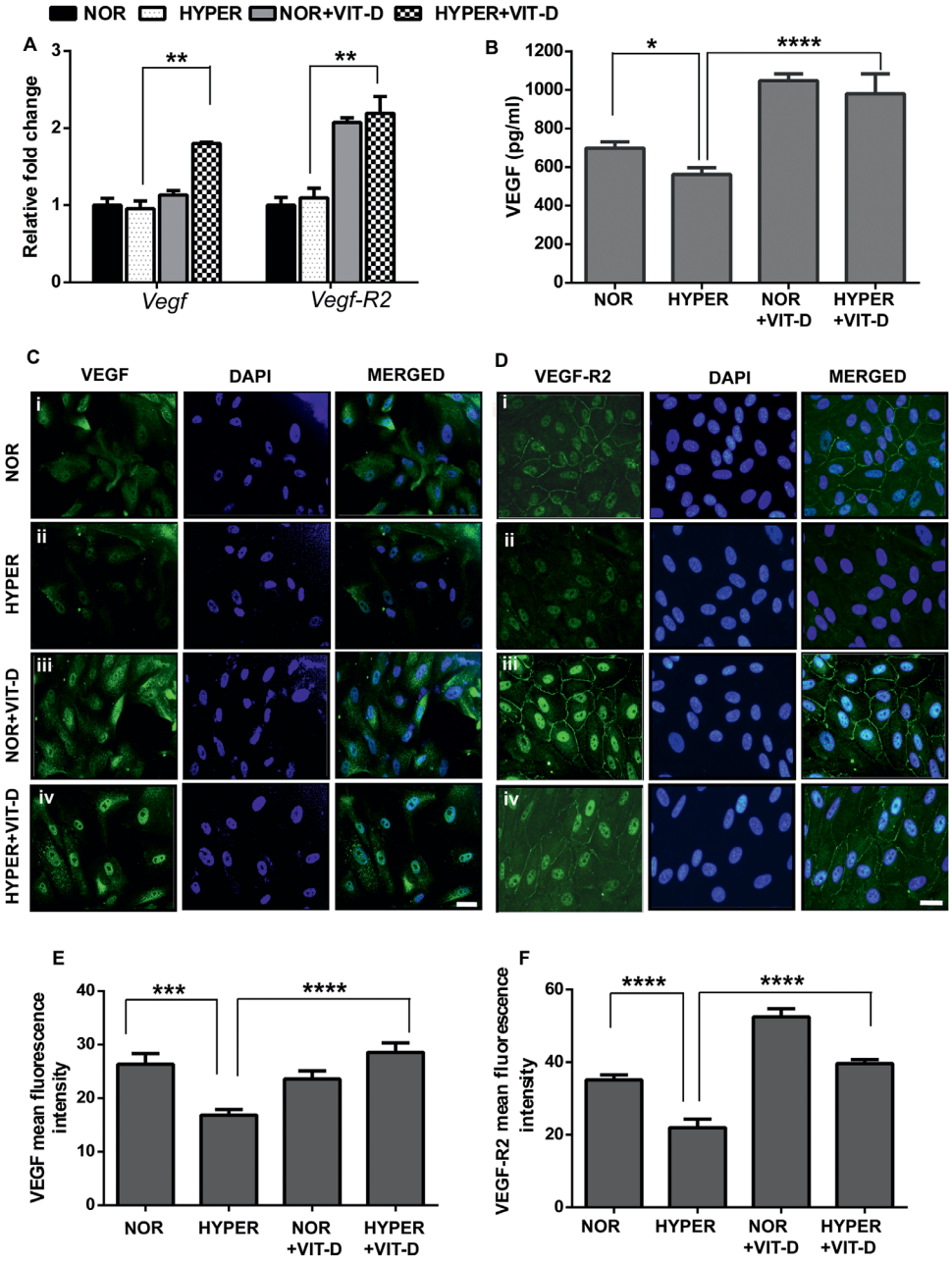

Figure 1. VEGF proteins are upregulated by VIT-D in hyperoxic conditions. PRPE cells are cultured in hyperoxic condition (40% O_2) with and without VIT-D (10 nM) for 5 days. VEGF and VEGF-R2 mRNA expressions analyzed using RT-qPCR with and without VIT-D in comparison to cells incubated under hyperoxia (**A**). Line graph shows the secreted levels of VEGF measured from 5 days conditioned medium using sandwich-enzyme-linked immunosorbent assay (ELISA) (**B**). Representative immunofluorescence images for VEGF (green) (**C** (i–iv)) and VEGF-R2 (green) (**D** (i-iv)). The nucleus is counterstained with DAPI (blue). Bar graphs showing the corresponding mean fluorescence intensity for VEGF (**E**) and VEGFR2 (**F**) in different experimental conditions. *$P \leq 0.05$, ***$P \leq 0.001$, ****$P \leq 0.0001$. Scale bar = 5 µm. NOR = Normoxia, HYPER = Hyperoxia, VIT-D = Vitamin D.

with normoxia. However, with VIT-D supplementation, cells cultured in hyperoxia showed an upregulated *E-cad* gene expression (Fig. 5A) compared with those cultured in hyperoxia alone. No significant modulation was observed in *N-cad* gene expressions across all the experimental conditions. Immunofluorescence staining intensity of ZO-1 was significantly reduced in PRPE cells cultured in hyperoxia in comparison with normoxia (Figs. 5B (i, ii), 5D). However, with VIT-D supplementation, cells had significantly higher intensity of ZO-1 staining compared with cells grown in hyperoxia alone (Figs. 5B (ii-iv), 5D). The increase in the intensity of ZO-1 staining in cells grown under hyperoxia in the presence of VIT-D was similar to those cultured in normoxia environment (Figs. 5B (i, iv), 5D). In hyperoxia, the percentage of stabilized cells, as depicted by ZO-1 intact membrane staining, were significantly lower when compared with normoxia (see Supplementary Fig. S1A). However, the percentage of cells with stabilized membrane when cultured under hyperoxia with VIT-D supplementation increased and was similar to those cultured under normoxia

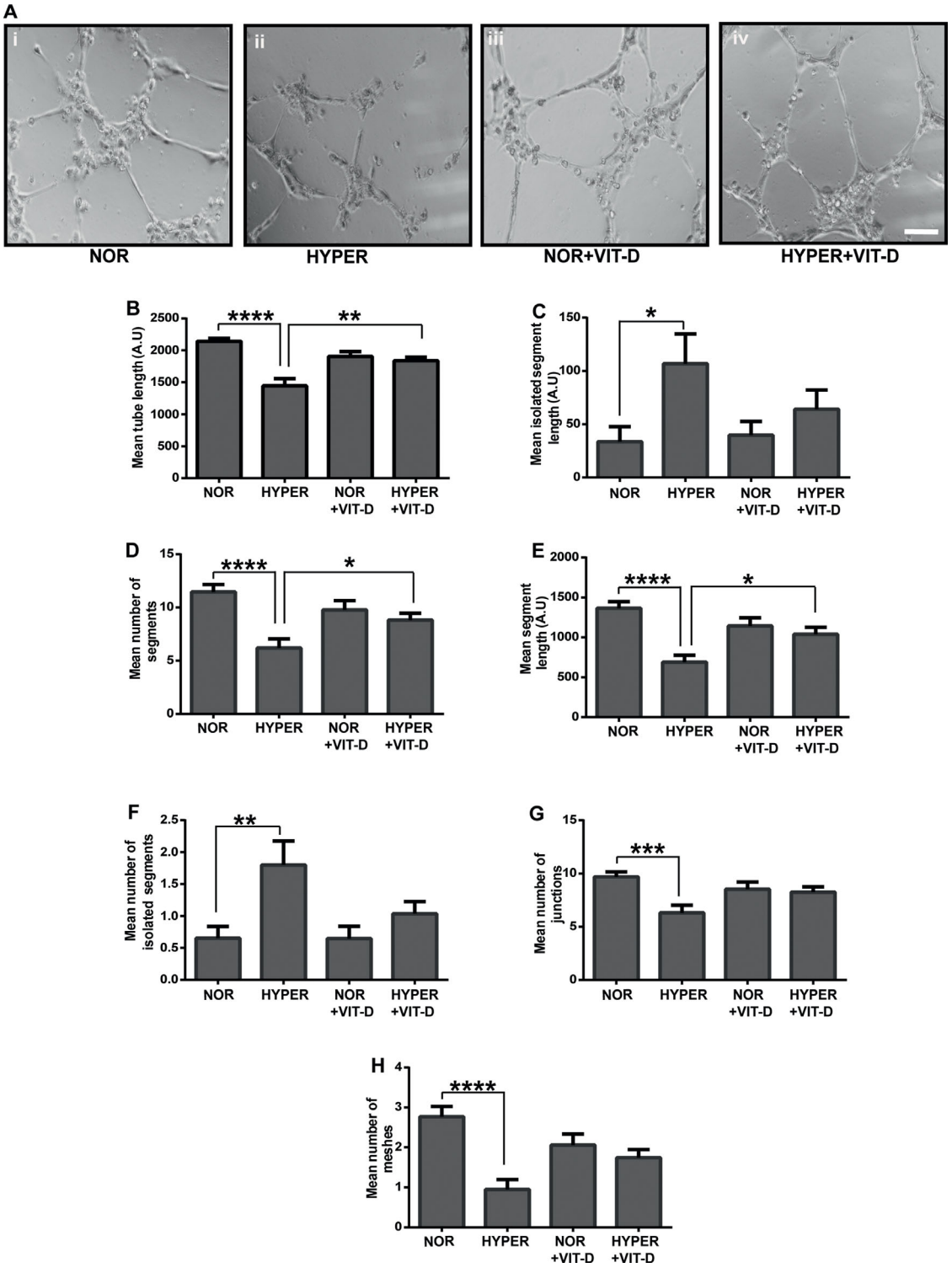

Figure 2. Tube formation assay on hyperoxia and VIT-D supplementation. Cell supernatants of PRPE cells cultured for 5 days in hyperoxia +/− VIT-D_3 supplement were incubated on HUVEC cells for tube formation. Representative images of tube formation assay (**A**) in normoxia (i), hyperoxia (ii), normoxia + VIT-D (iii) and hyperoxia + VIT-D (iv). Bar graphs depicting various parameters for mean total tube length (**B**), mean isolated segment length (**C**), mean number of segments (**D**), mean segment length (**E**), mean number of isolated segments (**F**), mean number of junctions (**G**), mean number of meshes (**H**), measured using Image-J, Angiogenesis Analyzer plugin software. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. Scale bar = 5 µm. NOR = Normoxia, HYPER = Hyperoxia, VIT-D = Vitamin D.

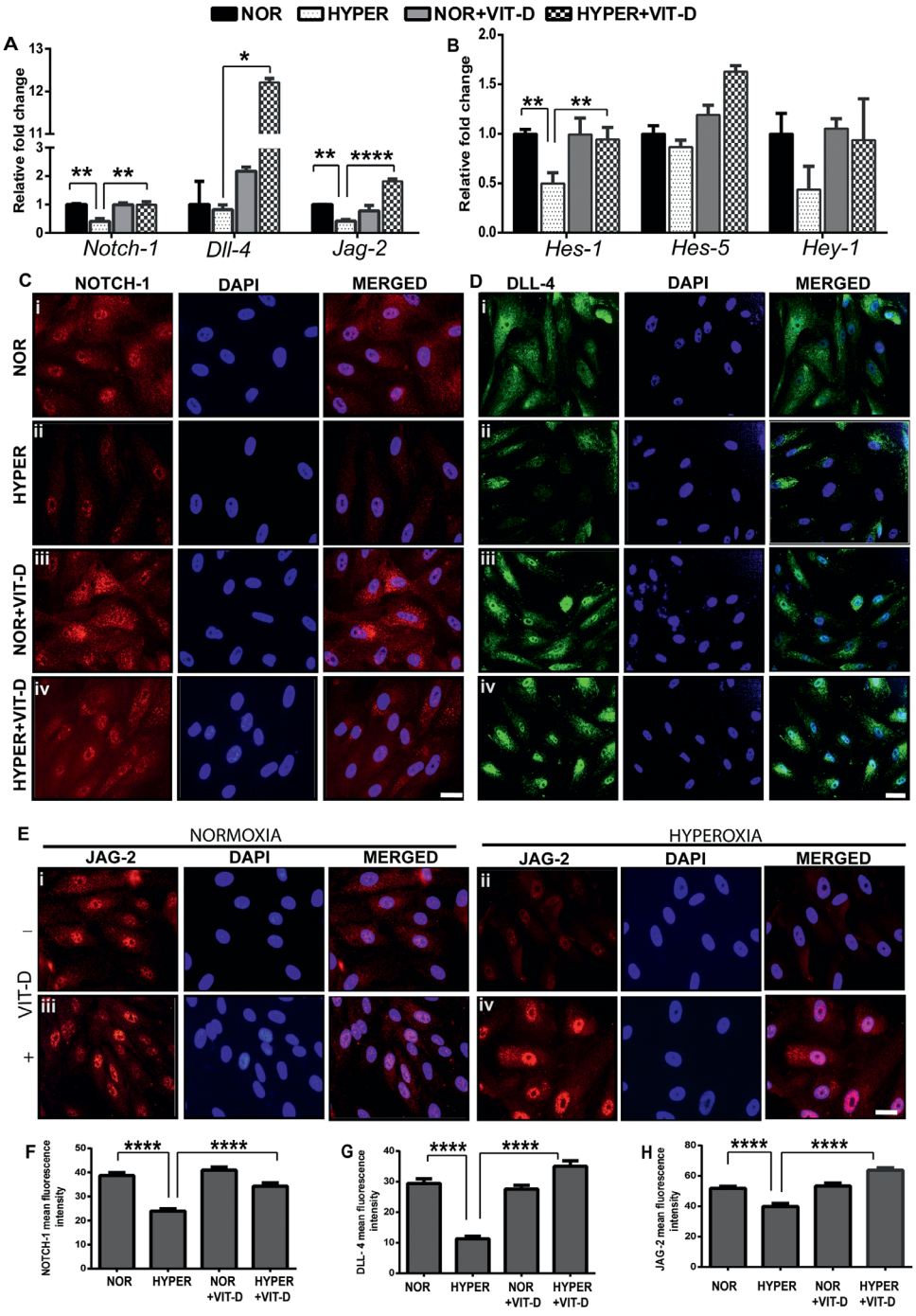

Figure 3. Notch signaling modulated in hyperoxia +/− VIT-D supplementation. Gene expression and proteins were analyzed for Notch receptors, ligands, and downstream targets in 5 day cultured PRPE cells in hyperoxia condition +/− VIT-D supplement. mRNA levels of *Notch-1* receptor, *Dll-4*, and *Jag-2* ligand (**A**) and down-stream targets (*Hes-1*, *Hes-5*, and *Hey-1*) (**B**). Immunofluorescence images depicting NOTCH-1 (red) (**C** (i-iv)), DLL-4 (green) (**D** (i-iv)), and JAG-2 (red) (**E** (i-iv)) staining in normoxic and hyperoxic conditions +/- VIT-D. The nucleus is counterstained with DAPI (blue). Graphical representation showing the mean fluorescence intensity for NOTCH-1 (**F**), DLL-4 (**G**), and JAG-2 (**H**). *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$. Scale bar = 5 µm. NOR = Normoxia, HYPER = Hyperoxia, VIT-D = Vitamin D.

(see Supplementary Fig. S1A). Conversely, the percentage of destabilized cells was high in hyperoxia compared with normoxia and VIT-D supplementation in hyperoxia reduced the percentage of destabilized cells (see Supplementary Fig. S1B). In hyperoxia-conditioned cells, F-actin immunofluorescence had a higher intensity in cell periphery with an enlarged cell volume compared with cells in normoxia (Figs. 5C (i, ii), 5E). Supplementation with VIT-D showed a rearrangement of the F-actin filament with less peripheral fluorescent intensity and a reduced cell volume (Figs. 5C (iv), 5E). Cells incubated in normoxia with VIT-D showed no difference in cell shape when compared with those incubated without VIT-D (Figs. 5C (iii), 5E).The percentage of rounded cells were less in hyperoxia compared with

Investigative Ophthalmology & Visual Science

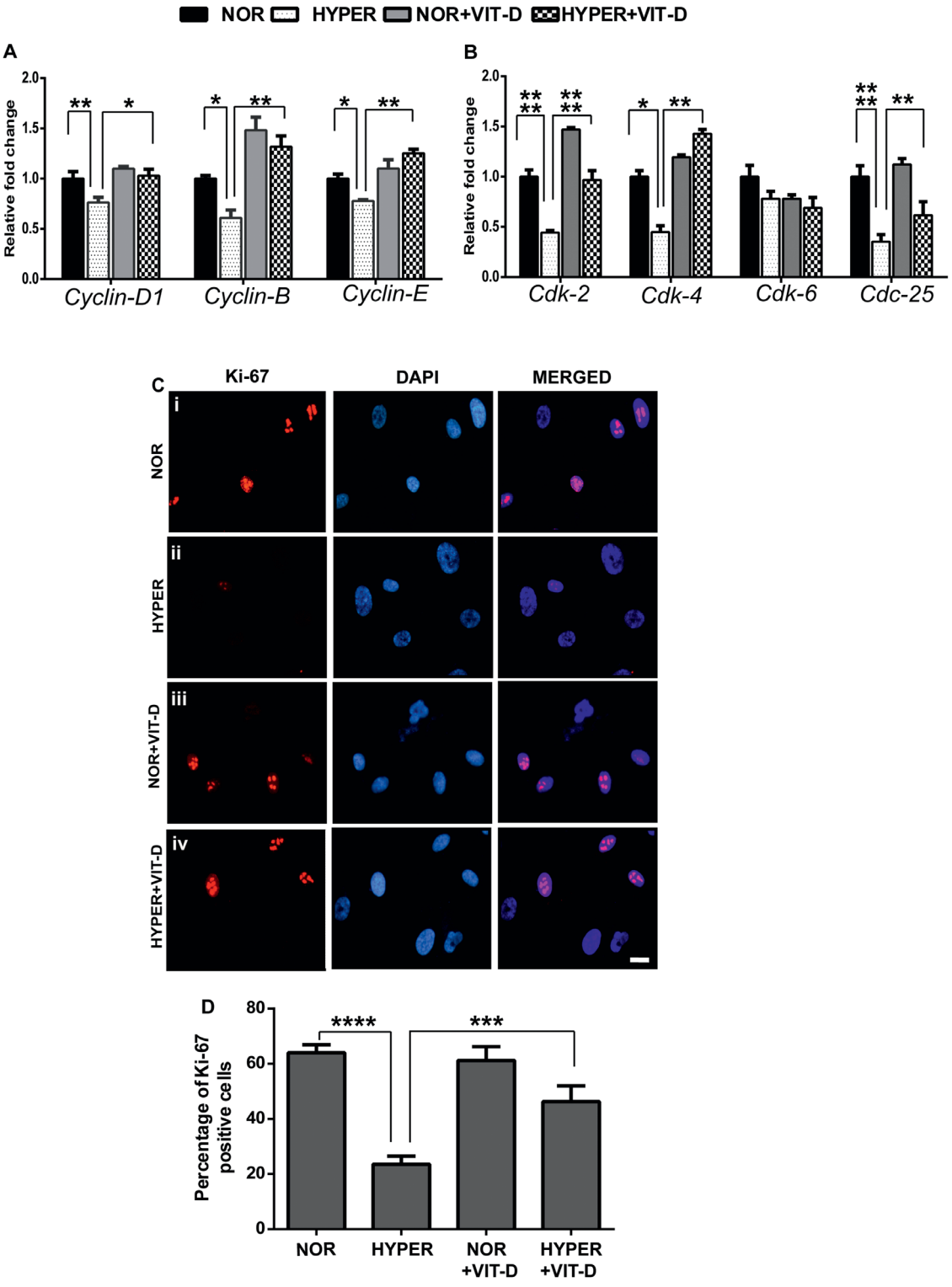

Figure 4. Effects of hyperoxia and VIT-D on proliferation of PRPE cells. PRPE cells were cultured in hyperoxia +/− VIT-D supplementation for 5 days. Graphical representation of the relative gene expressions of *Cyclin-D1*, *Cyclin-B*, and *Cyclin-E* (**A**) and Cdks (*Cdk-2*, *Cdk-4*, and *Cdk-6*) and *Cdc-25* (**B**). Representative immunofluorescence images for cells cultured under various conditions for Ki-67 (red) and counterstained nucleus with DAPI (blue) (**C** (i-iv)). Bar graphs representing the percentage of Ki-67 positive population in cells cultured under different experimental conditions (**D**). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$. Scale bar = 5 µm. NOR = Normoxia, HYPER = Hyperoxia, VIT-D = Vitamin D.

Investigative Ophthalmology & Visual Science

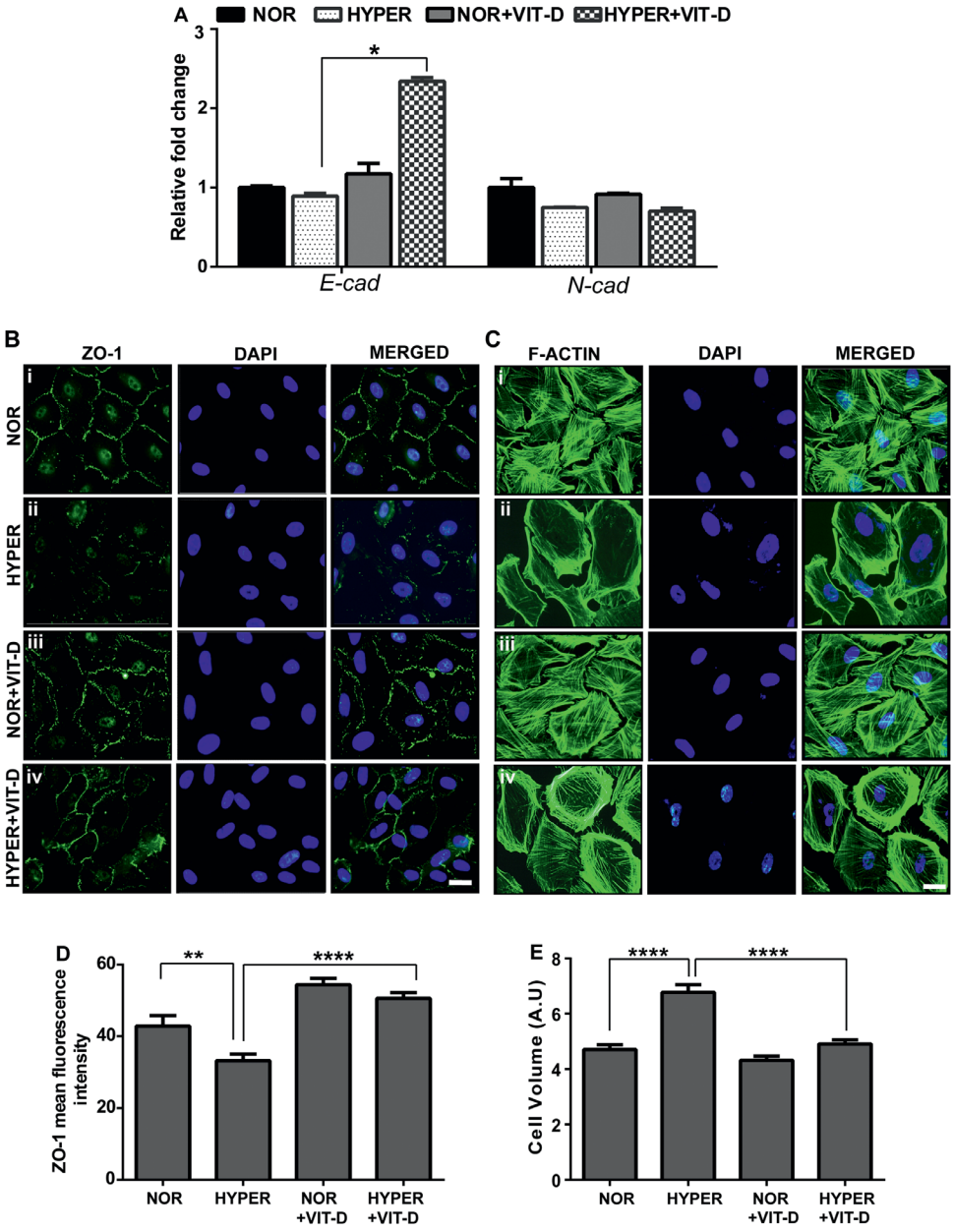

Figure 5. Effects of hyperoxia and VIT-D on junctional and cytoskeletal proteins. Gene expression analysis for E-cadherin and N-cadherin relative gene expression in cells cultured under hyperoxia cultured (**A**). Representative immunofluorescence images for ZO-1 (**B** (i-iv)) and F-ACTIN (**C** (i-iv)) in cells cultured under different experimental conditions. The nucleus is counterstained with DAPI (blue). Graphical representation of the mean fluorescence intensity for ZO-1 (**D**) and cell volume using F-ACTIN stained cells (**E**). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$. Scale bar = 5 µm. NOR = Normoxia, HYPER = Hyperoxia, VIT-D = Vitamin D.

normoxia (see Supplementary Fig. S1C). VIT-D supplementation in hyperoxia increased the number of rounded cells (see Supplementary Fig. S1C).

VIT-D Restores Hyperoxia-Induced Trans-Plasma Membrane Depolarization

The PRPE cells exposed for 5 days in hyperoxia condition revealed a depolarized cell state with an increase in fluorescence intensity of DIBAC4 (3) intake in cells, compared with cells grown in normoxia (Figs. 6A, 6D). In hyperoxia, VIT-D supplementation resulted in a hyperpolarization state of cells in comparison to cells without VIT-D (Figs. 6C, 6D). Similar fluorescence intensity was observed in cells cultured in normoxia or hyperoxia with the presence of VIT-D (Figs. 6B–D).

VIT-D Restores Phagocytosis in Hyperoxia

The percentage of FITC labeled POS, an indicator of the RPE phagocytosis function, were significantly (Figs. 7A (i, ii), 7B) higher in cells cultured in hyperoxia (56.0 ± 3.5%) when compared with normoxia (42.2 ± 3.3%). The VIT-D

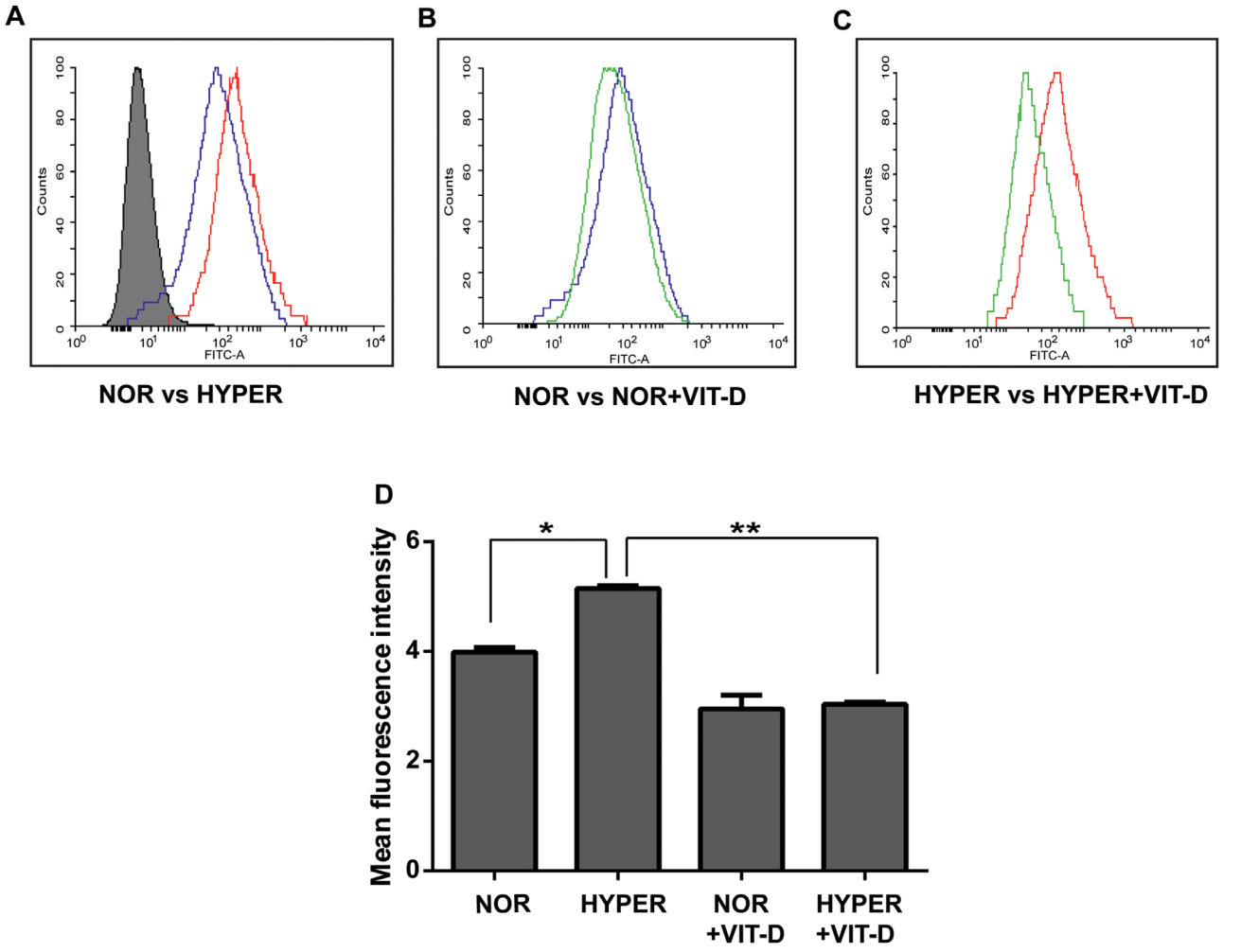

Figure 6. Transmembrane potential is modulated in the presence of VIT-D. PRPE cells cultured in hyperoxic conditions and supplemented with VIT-D were analyzed for membrane potential using DiBAC4(3) by flow cytometry. Representative histogram showing the fluorescent intensity of depolarized cells in hyperoxia (red peak) compared to normoxia (blue peak) (**A**). Representative histogram showing the fluorescent intensity for normoxia (blue) and normoxia + VIT-D supplement (green) cells (**B**). Representative histogram showing the fluorescent intensity for VIT-D supplemented cells under hyperoxia conditions (green) in comparison to hyperoxia alone (red) (**C**). Graphical representation of the mean fluorescence intensity of the internalized DiBAC4 (3) dye (**D**). *$P \leq 0.05$, **$P \leq 0.01$. NOR = Normoxia, HYPER = Hyperoxia, VIT-D = Vitamin D.

supplemented hyperoxia cells showed a significant reduction (34.2 ± 2.1%) in the number of POS-positive cells compared with hyperoxia alone (Figs. 7A (ii, iv), 7B). Supplementation of VIT-D in normoxia conditions (42.5 ± 2.5%) did not have any effect on the percentage of cells with opsonized POS, although there was a mild reduction in hyperoxic conditions compared with untreated normoxia cells (Figs. 7A (i, iii, iv), 7B).

Discussion

Increased production and accumulation of ROS drives cellular oxidative stress. It has been shown that most of the pathological conditions are driven by excess of oxidative stress that leads to damage of intracellular components.[19] Diseases such as AMD and ROP are characterized by vascular defects that are regulated by oxidative stress. Although vitamins are known to be crucial in maintaining cellular homeostasis, the role of VIT-D in modulating hyperoxia-induced oxidative stress in RPE cells is not yet explored. Interestingly, a lower level of VIT-D is associated with progression of advanced AMD. Modulation of VIT-D level can be a potential therapy in retinal disease driven by hyperoxia.[6]

The RPE-340 cells cultured with 40% oxygen (hyperoxia condition) generated modest oxidative stress without cell death and cytotoxicity but altered the phenotype of cells.[20] In this study, the oxidative stress was reduced in PRPE cells cultured in hyperoxia (40% oxygen) with VIT-D (see Supplementary Fig. S2). This further reiterates the potential of VIT-D to reduce oxidative stress.[21]

The active form of VIT-D (α-1,25(OH) 2D3) through VIT-D receptor (VDR), induces upregulation of VEGF receptors (R1 and R2) and antioxidant CuZn-superoxide dismutase levels in HUVEC cells.[22] In the presence of calcitriol (α1,25(OH) 2D3), pericytes express increased levels of VEGF-A.[8] VEGF is known to exert its primary vascular functions by binding to its receptor, VEGF-R2.[23] In rat models, the addition of exogenous VEGF acted as a survival factor for new blood vessel formation in ROP.[24] To assess the role of VIT-D in modulating angiogenesis, we evaluated the

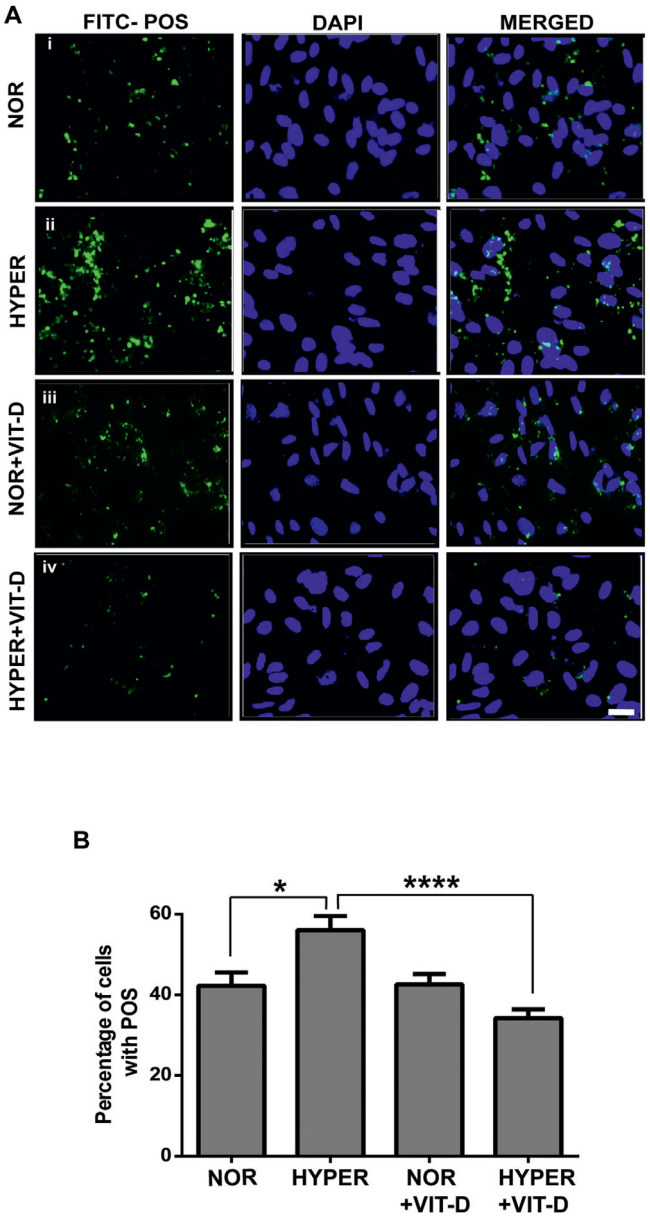

Figure 7. Modulation of phagocytosis in hyperoxia with and without VIT-D. PRPE cells cultured for 5 days in hyperoxia +/− VIT-D. Representative images showing percentage of internalized FITC labeled POS after exposure (hyperoxia +/− VIT-D) (**A** (i–iv)). Graphical representation of the number of cells with opsonized POS (**B**). *$P \leq 0.05$, ****$P \leq 0.0001$. Scale bar = 5 µm. NOR = Normoxia, HYPER = Hyperoxia, VIT-D = Vitamin D.

levels of VEGF-A, an important factor for vascular formation. Our study demonstrates that VEGF and VEGF-R2 protein were reduced in hyperoxia that could be rescued with the supplementation of VIT-D. Pierce et al., showed a decrease in VEGF mRNA (55%) and protein (85%) levels in oxygen-induced retinae from animals on postnatal day 7.[25] Interestingly, in cancer cells it has been identified that VIT-D regulates VEGF production through a VIT-D response element (VDRE) on the VEGF promoter.[26] In RPE cells, VIT-D acts most likely through the VDR and VDRE on the VEGF promotor, which may induce the secretion of VEGF. We have previously demonstrated that post anti-VEGF treatment resveratrol (RES) partially rescued secreted VEGF levels in ARPE-19 cells.[13] In the present study, secreted VEGF levels were reduced in cells cultured in hyperoxia conditions compared with those in normoxia. Hence, we investigated if RES similar to VIT-D would be able to rescue secreted VEGF levels in RPE cells cultured in hyperoxia conditions. However, the results revealed distinct outcome of RES and VIT-D in their effort toward rescuing the secreted VEGF levels in PRPE cells cultured in hyperoxia-induced oxidative stress conditions (see Supplementary Fig. S3). RES partially inhibited soluble VEGF levels in comparison with the normoxia levels. Further, in the hyperoxia stress condition, with the supplementation of RES alone and/or in combination with VIT-D, did not show any rescue effects on VEGF secretion levels. Moreover VIT-D did not rescue the RES effects on VEGF secretion. Interestingly, in human cancer cells and animal tissues (kidneys and colon) it has been demonstrated that a combinatorial effects of RES and VIT-D on cell proliferation and ROS production are regulated through VDR.[27,28] Further studies are required to understand the RES effects in combination with VIT-D on the regulation of VEGF in oxidative stress conditions.

Functional implication of VIT-D induced VEGF and VEGF-R2 was evaluated in endothelial by tubulogenesis assay. Hyperoxia-conditioned medium impaired tube formation that could be restored with hyperoxia-conditioned medium containing VIT-D supplementation. VIT-D is known to alter the proliferation and sprouting activity of endothelial cells.[29] Tube formation results imply that most likely VIT-D among other factors through VEGF might be regulating tube formation (Fig. 2). VEGF induced capillary formation in hyperoxia exposed human pulmonary microvascular endothelial cell models.[30]

Apart from the role of VEGF, Notch signaling is also crucial in angiogenesis. RPE acts as a Notch signaling niche for the neural retina and Notch-1 plays a role in migration and proliferation of the RPE cells.[31] Although VEGF acts upstream of Notch signaling, however, their bidirectional signaling mechanism maintains sprouting and vasculature.[12,13] The VEGF-NOTCH axis is pivotal in maintaining vasculature and sprouting of the blood vessels.[32] In an OIR mouse model, DLL-4 remodels the maturing blood vessels as shown by loss-of-function experiments.[32] In the presence of VIT-D, hyperoxia induced alteration of the entire Notch signaling pathway receptors, ligands, and downstream target levels were restored (Fig. 3). Besides functional implications through binding VDR, VIT-D can also induce membrane-based signaling pathways[33] and restore Notch signaling proteins to normoxia (control) levels. All these findings indicate a possible trilateral interaction between VEGF-Notch signaling and VIT-D.

In this study, hyperoxia inhibited the RPE cell proliferation whereas VIT-D restored the proliferative. Studies have shown that higher oxygen delivery to cells inhibit cell proliferation in G1, S, and G2 phase.[34,35] Additionally, in RPE cells it has been reported that oxidative stress-induced telomere shortening is correlated to its proliferation potential.[36,37] Clinical trials with VIT-D supplementation have been shown to not only maintain telomere length but also increase telomerase activity in a study of a population-based cohort of twin women, hemodialysis patients with kidney failure, and overweight African American population, respectively.[38–40] Further, it has been shown that telomerase activity is enhanced in human dental follicle cells upon induction of Notch signaling.[41] Increased proliferation in the presence

of VIT-D could be attributed to multiple factors, including protection of telomere length along with the upregulation of Notch signaling. Although this study revealed the findings in an in vitro oxidative stress condition, it can be envisaged that the beneficial effects of VIT-D supplementation on RPE cell physiology and function remains as a salient feature.

Epithelial mesenchymal transition (EMT) is a cell physiological aspect of RPE cells. VIT-D supplementation in cells cultured in hyperoxia conditions can improve the membrane stability and restore the epithelial property, as demonstrated by enhanced intensity/intactness of ZO-1 membrane staining and actin filament reorganization. EMT is regulated by VEGF, Notch signaling, and oxygen concentration status.[42,43] In the present study, supplementation of VIT-D did not alter EMT status in normoxia. It can be envisaged that under oxidative stress conditions in the presence of VIT-D, Notch and VEGF may induce EMT. Further studies are warranted to delineate the detailed functional implication of Notch and VEGF function on EMT status in the presence of VIT-D under hyperoxia.

RPE cells play a major role in maintaining outer blood retinal barrier function. We found that hyperoxia conditions depolarized PRPE cells whereas VIT-D supplementation hyperpolarized the cells. In murine models, VIT-D can induce the uptake of Ca^{2+} through L-type calcium channels leading to hyperpolarization and regulation of VEGF.[44] Depolarization of the plasma membrane leads to the redistribution of cytoskeletal components and disruption of cell adhesion components.[45]

Phagocytic property of RPE paves the way for the renewal of photoreceptor outer segment.[46] In this study, hyperoxia-induced phagocytosis levels were reduced by VIT-D supplementation (from 56 to 34.2%), similar to that in normoxia conditions (42.2%; Fig. 7). Moreover, the follow-up on POS degradation revealed that VIT-D supplementation improved the degradation of POS in hyperoxic conditions (from 56 to 22.6%; see Supplementary Fig. S4). It has been shown that during hyperoxic stress uptake of retinal outer segments by human RPE increases.[46] Likewise, murine macrophage cells show enhanced phagocytic activity during hyperoxidative stress that was marginalized with VIT-D.[47] It could be concluded that VIT-D might act as a phagocytosis modulator.

In this study, VIT-D treatment induced VEGF secretion, hyperpolarized the cells, and modulated the actin filaments. This effect might have functionally contributed to an increase in tube formation in primary HUVEC cells, increase in the intensity of tight junction protein, and modulated phagocytosis. Hence, VIT-D treatment improved the vascular and cellular properties of RPE cells cultured under oxidative stress.

To conclude, this in vitro study demonstrated that VIT-D could rescue RPE cells exposed to hyperoxia stress and thereby protect their cell physiological properties. These results can provide insight in application of VIT-D as a potential therapeutic molecule for ocular vascular pathologies primarily driven by oxidative stress, such as early stages of AMD and ROP. Further studies with animal models can substantiate our findings for a clinical translational aspect.

Acknowledgments

The authors thank P. Narendra, MBBS and Arkasubhra Ghosh, Ph.D for their administrative support. The authors express their gratitude to K. Bhujang Shetty, MBBS for providing all the logistics needed for this work.

Supported by Department of Biotechnology-BioCARe, Govt. of India [No:(N0.BT/Bio-CARe/07/10017], Department of Science and Technology, Govt. of India [No. SR/SO/HS-228/2012], and Narayana Nethralaya Foundation.

Disclosure: **P. Murugeswari,** None; **A. Firoz,** None; **S. Murali,** None; **A. Vinekar,** None; **L. Krishna,** None; **V.R. Anandula,** None; **N. Jeyabalan,** None; **P. Chevour,** None; **C. Jayadev,** None; **R. Shetty,** None; **G. Carpentier,** None; **G. Kumaramanickavel,** None; **A. Ghosh,** None; **D. Das**, None

References

1. Reins RY,McDermott AM. Vitamin D: implications for ocular disease and therapeutic potential. *Exp Eye Res*. 2015;134:101–110.
2. Roberts PA, Gaffney EA, Whiteley JP, et al. Predictive mathematical models for the spread and treatment of hyperoxia-induced photoreceptor degeneration in retinitis pigmentosa. *Invest Ophthalmol Vis Sci*. 2018;59:1238–1249.
3. Poljsak B. Strategies for reducing or preventing the generation of oxidative stress. *Oxid Med Cell Longev*. 2011;2011:194586.
4. Shen CL, Samathanam C, Graham S, et al. Green tea polyphenols and 1-alpha-OH-vitamin D(3) attenuate chronic inflammation-induced myocardial fibrosis in female rats. *J Med Food*. 2012;15:269–277.
5. Iseki K, Tatsuta M, Uehara H, et al. Inhibition of angiogenesis as a mechanism for inhibition by 1alpha-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 of colon carcinogenesis induced by azoxymethane in Wistar rats. *Int J Cancer*. 1999;81:730–733.
6. Layana AG, Minnella AM, Garhofer G, et al. Vitamin D and age-related macular degeneration. *Nutrients*. 2017;9.
7. Luo BA, Gao F,Qin LL. The association between vitamin D deficiency and diabetic retinopathy in type 2 diabetes: a meta-analysis of observational studies. *Nutrients*. 2017;9.
8. Jamali N, Wang S, Darjatmoko SR, Sorenson CM,Sheibani N. Vitamin D receptor expression is essential during retinal vascular development and attenuation of neovascularization by 1, 25(OH)2D3. *PLoS One*. 2017;12:e0190131.
9. Merle BMJ, Silver RE, Rosner B,Seddon JM. Associations between vitamin d intake and progression to incident advanced age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2017;58:4569–4578.
10. Albert DM, Scheef EA, Wang S, et al. Calcitriol is a potent inhibitor of retinal neovascularization. *Invest Ophthalmol Vis Sci*. 2007;48:2327–2334.
11. Fu Y, Dong Y,Gao Q. Age-related cataract and macular degeneration: oxygen receptor dysfunction diseases. *Med Hypotheses*. 2015;85:272–275.
12. Subramani M, Murugeswari P, Dhamodaran K, et al. Short pulse of clinical concentration of bevacizumab modulates human retinal pigment epithelial functionality. *Invest Ophthalmol Vis Sci*. 2016;57:1140–1152.
13. Subramani M, Ponnalagu M, Krishna L, et al. Resveratrol reverses the adverse effects of bevacizumab on cultured ARPE-19 cells. *Sci Rep*. 2017;7:12242.
14. Truong D, Puleo J, Llave A, et al. Breast cancer cell invasion into a three dimensional tumor-stroma microenvironment. *Sci Rep*. 2016;6:34094.
15. Middleton CA, Brown AF, Brown RM,Roberts DJ. The shape of cultured epithelial cells does not depend on the integrity of their microtubules. *J Cell Sci*. 1988;91 (Pt 3):337–345.

16. Flood MT, Gouras P,Kjeldbye H. Growth characteristics and ultrastructure of human retinal pigment epithelium in vitro. *Invest Ophthalmol Vis Sci*. 1980;19:1309–1320.
17. Carpentier G. *ImageJ contribution: Angiogenesis Analyzer*. Available at: http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ (2012).
18. Mao Y,Finnemann SC. Analysis of photoreceptor outer segment phagocytosis by RPE cells in culture. *Methods Mol Biol*. 2013;935:285–295.
19. Lu L, Hackett SF, Mincey A, Lai H,Campochiaro PA. Effects of different types of oxidative stress in RPE cells. *J Cell Physiol*. 2006;206:119–125.
20. Honda S, Hjelmeland LM,Handa JT. The use of hyperoxia to induce chronic mild oxidative stress in RPE cells in vitro. *Mol Vis*. 2001;7:63–70.
21. Basit S. Vitamin D in health and disease: a literature review. *Br J Biomed Sci*. 2013;70:161–172.
22. Zhong W, Gu B, Gu Y, et al. Activation of vitamin D receptor promotes VEGF and CuZn-SOD expression in endothelial cells. *J Steroid Biochem Mol Biol*. 2014;140:56–62.
23. Shibuya M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J Biochem*. 2013;153:13–19.
24. Alon T, Hemo I, Itin A, et al. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med*. 1995;1:1024–1028.
25. Pierce EA, Foley ED,Smith LE. Regulation of vascular endothelial growth factor by oxygen in a model of retinopathy of prematurity. *Arch Ophthalmol*. 1996;114:1219–1228.
26. Cardus A, Panizo S, Encinas M, et al. 1,25-dihydroxyvitamin D3 regulates VEGF production through a vitamin D response element in the VEGF promoter. *Atherosclerosis*. 2009;204:85–89.
27. Dampf Stone A, Batie SF, Sabir MS, et al. Resveratrol potentiates vitamin D and nuclear receptor signaling. *J Cell Biochem*. 2015;116:1130–1143.
28. Uberti F, Morsanuto V, Aprile S, et al. Biological effects of combined resveratrol and vitamin D3 on ovarian tissue. *J Ovarian Res*. 2017;10:61.
29. Mantell DJ, Owens PE, Bundred NJ, Mawer EB,Canfield AE. 1 alpha,25-dihydroxyvitamin D(3) inhibits angiogenesis in vitro and in vivo. *Circ Res*. 2000;87:214–220.
30. Miranda LF, Rodrigues CO, Ramachandran S, et al. Stem cell factor improves lung recovery in rats following neonatal hyperoxia-induced lung injury. *Pediatr Res*. 2013;74:682–688.
31. Ha T, Moon KH, Dai L, et al. The retinal pigment epithelium is a Notch signaling niche in the mouse retina. *Cell Rep*. 2017;19:351–363.
32. Lobov IB, Cheung E, Wudali R, et al. The Dll4/Notch pathway controls postangiogenic blood vessel remodeling and regression by modulating vasoconstriction and blood flow. *Blood*. 2011;117:6728–6737.
33. Larriba MJ, Gonzalez-Sancho JM, Bonilla F,Munoz A. Interaction of vitamin D with membrane-based signaling pathways. *Front Physiol*. 2014;5:60.
34. Helt CE, Rancourt RC, Staversky RJ,O'Reilly MA. p53-dependent induction of p21(Cip1/WAF1/Sdi1) protects against oxygen-induced toxicity. *Toxicol Sci*. 2001;63:214–222.
35. Ko JC, Wang YT,Yang JL. Dual and opposing roles of ERK in regulating G(1) and S-G(2)/M delays in A549 cells caused by hyperoxia. *Exp Cell Res*. 2004;297:472–483.
36. Honda S, Hjelmeland LM,Handa JT. Oxidative stress–induced single-strand breaks in chromosomal telomeres of human retinal pigment epithelial cells in vitro. *Invest Ophthalmol Vis Sci*. 2001;42:2139–2144.
37. Park JK, Kim BH, Han YS,Park IK. The effect of telomerase expression on the escape from M2 crisis in virus-transformed human retinal pigment epithelial cells. *Exp Mol Med*. 2002;34:107–113.
38. Richards JB, Valdes AM, Gardner JP, et al. Higher serum vitamin D concentrations are associated with longer leukocyte telomere length in women. *Am J Clin Nutr*. 2007;86:1420–1425.
39. Borras M, Panizo S, Sarro F, Valdivielso JM,Fernandez E. Assessment of the potential role of active vitamin D treatment in telomere length: a case-control study in hemodialysis patients. *Clin Ther*. 2012;34:849–856.
40. Zhu H, Guo D, Li K, et al. Increased telomerase activity and vitamin D supplementation in overweight African Americans. *Int J Obes (Lond)*. 2012;36:805–809.
41. Chen X, Zhang T, Shi J, et al. Notch1 signaling regulates the proliferation and self-renewal of human dental follicle cells by modulating the G1/S phase transition and telomerase activity. *PLoS One*. 2013;8:e69967.
42. Haase VH. Oxygen regulates epithelial-to-mesenchymal transition: insights into molecular mechanisms and relevance to disease. *Kidney Int*. 2009;76:492–499.
43. Kofler NM, Shawber CJ, Kangsamaksin T, et al. Notch signaling in developmental and tumor angiogenesis. *Genes Cancer*. 2011;2:1106–1116.
44. Zanatta L, Goulart PB, Goncalves R, et al. 1alpha,25-dihydroxyvitamin D(3) mechanism of action: modulation of L-type calcium channels leading to calcium uptake and intermediate filament phosphorylation in cerebral cortex of young rats. *Biochim Biophys Acta*. 2012;1823:1708–1719.
45. Chifflet S, Correa V, Nin V, Justet C,Hernandez JA. Effect of membrane potential depolarization on the organization of the actin cytoskeleton of eye epithelia. The role of adherens junctions. *Exp Eye Res*. 2004;79:769–777.
46. Miceli MV, Liles MR,Newsome DA. Evaluation of oxidative processes in human pigment epithelial cells associated with retinal outer segment phagocytosis. *Exp Cell Res*. 1994;214:242–249.
47. Phan BD, Entezari M, Lockshin RA, Bartelt DC,Mantell LL. Hydrogen peroxide enhances phagocytosis of Pseudomonas aeruginosa in hyperoxia. *J Immunotoxicol*. 2011;8:3–9.

Investigative Ophthalmology & Visual Science