Short Pulse of Clinical Concentration of Bevacizumab Modulates Human Retinal Pigment Epithelial Functionality

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Purpose. Cross-talk between Notch signaling and vascular endothelial growth factor (VEGF) is a major driver of angiogenesis. Here we investigated the temporal effect of bevacizumab (BEV) on Notch signaling and the functional features of cultured primary retinal pigment epithelial (PRPE) cells.

Methods. Human (cadaver) PRPE cells were treated with clinical concentrations of BEV (0.25 mg/mL). Notch signaling pathway receptors, ligands, and downstream target genes were analyzed with quantitative real-time polymerase chain reaction (qRT-PCR). Cell proliferation along with phagocytosis and transmembrane potential was analyzed by fluorescent activated cell sorter (FACS) and immunofluorescence.

Results. Bevacizumab-treated PRPE cultures revealed a significant temporal downregulation of *notch4* ($P < 0.05$) and Delta-like-4 ($P < 0.005$) gene (16% reduced) and protein (29.7% reduced) expression only at the 2-hour exposure, though secreted VEGF levels were significantly blocked ($P < 0.005$) at all the time points (2, 4, 6 hours). Further, a significant downregulation ($P < 0.005$) in cell cycle (reduced by 34.1%) and a concurrent ($P < 0.005$) upregulation of F-actin staining (increased by 2.5-fold) could be detected. Bevacizumab-treated PRPE cells revealed an elevated transmembrane potential (by 63%) and significant decrease ($P < 0.01$) in phagocytosis (by 19.25%) in comparison to untreated controls.

Conclusions. There is temporal interaction between BEV and the Notch signaling pathway, specifically with Notch4 and Delta-like-ligand-4 in PRPE cultures. This transient decrease in Notch signaling can impact the functionality of RPE cells. These findings can help to provide a better understanding of the effect of long-term usage of anti-VEGF agents in the treatment of retinal degenerative and vitreoretinopathy diseases.

Keywords: retinal pigment epithelium, bevacizumab, Notch4, DLL4, neovascularization, VEGF

The retinal pigment epithelium (RPE) is a polarized pigmented monolayer, essentially maintaining a physical barrier between the neural retina and choroid layer. Absorption of light, transport of ions, exchange of retinaldehyde, phagocytosis, and secretory functions are some of salient features of the RPE.[1] Apart from cytokines, inflammatory molecules, and other growth factors, the RPE also secretes VEGF, one of the major angiogenic factors regulating the development and maintenance of the retinal and choroidal vasculature.[1–3] While an equilibrium between angiogenic and antiangiogenic factors maintains the homeostasis in vasculogenesis, any deviation results in diseased states.[3–6]

Raised levels of VEGF are seen in most vasoproliferative ocular diseases. Hence, intravitreal injections of anti-VEGF agents are the preferred treatment for diseases such as proliferative diabetic retinopathy (PDR) and age-related macular degeneration (AMD).[7,8] Bevacizumab (BEV) (Avastin; Genentech/Roche, San Francisco, CA, USA) and ranibizumab (RAN) (Lucentis; Novartis Pharma Stein AG, Stein, Switzerland) are the most widely used VEGF-binding proteins in clinical practice. Treatment with BEV surpasses RAN in ophthalmic clinics in moderate- to low-income countries due to its low cost and comparable efficacy.[9] Some of the major adverse effects of long-term BEV treatment are endophthalmitis, vitreous hemorrhage, tractional retinal detachment, RPE tears, and fibrotic membrane formation.[10,11] It would therefore be interesting to determine if there exists any response immediately after BEV injections that precludes pathologic long-term outcomes. There is also a cohort of patients that remain nonresponsive (~40% PDR, ~45% AMD) to anti-VEGF injections.[12,13] This necessitates investigating the cross-talk between anti-VEGFs and various signaling pathways.[14] Such studies could provide the basis for

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Table 1. Primers Used for RT-PCR

Serial Number	Primer Name	Sequences, 5′–3′	Size, bp	Gene Accession No.
1	*gapdh*	FP: GCCAAGGTCATCCATGACAAC RP: GTCCACCACCCTGTTGCTGTA	498	NM_001289746.1
2	*rpe65*	FP: TCCCCAATACAACTGCCACT RP: CCTTGGCATTCAGAATCAGG	369	NM_000329.2

developing novel targeted therapies with minimal adverse effects.

Notch signaling is a highly conserved and developmentally regulated signaling pathway involved in multiple spatiotemporal physiological functions including cell fate, cell-to-cell communication, proliferation, and angiogenesis.[15–17] During ocular angiogenesis, Notch pathway proteins reveal a distinct expression pattern. Specifically, a Delta-like ligand-4 (DLL4) promotes vascular sprouting and maturation in the endothelial tip cells.[18,19] Several studies have demonstrated the interaction between Notch signaling and VEGF.[20,21] Notch signaling pathway is also crucial for modulating migration and proliferation of cultured RPE cells.[22,23] Recently, Zhang et al.[24] showed that incubation of ARPE-19 cells with BEV for longer durations resulted in epithelial–mesenchymal transition (EMT), which was most likely through the regulation of Notch signaling.[24] There have been no studies that have investigated the temporal regulation of Notch signaling and modulation of RPE functions after short pulses of BEV. Hence, we attempted to demonstrate the novel temporal relationship of Notch-VEGF regulation in RPE cells and the functional implication in modulating physiological processes of RPE cells such as EMT, membrane potential, and phagocytosis.

Materials and Methods

Human PRPE Cell Culture

This study was approved by the institutional ethics committee and was conducted in concordance with the tenets of the Declaration of Helsinki. Primary retinal pigment epithelial (PRPE) cultures were established from noninfectious human cadaver eyes that were obtained from a certified and registered eye bank. Human PRPE cells were isolated from 10 donor eyes (aged 21–67 years) within 24 hours of enucleation and cultured with minor modifications to a previously described technique.[25] Briefly, after surface sterilization with povidone iodine solution, the donor eye anterior segment, vitreous, and retina were removed. The leftover eye cup was incubated with 0.25% trypsin (Gibco, Life Technologies, Carlsbad, CA, USA) for 20 to 30 minutes at 37°C. The dissociated PRPE cells were then cultured in tissue culture flasks in the presence of Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) (Gibco, Life Technologies) and 1% antibiotic solution (10,000 U penicillin, 10 mg streptomycin, and 25 µg amphotericin B in 1 mL 0.9% normal saline; Himedia, Mumbai, India) and incubated at 37°C with 5% CO_2. After the initial attachment and proliferation, the PRPE cells were maintained with DMEM containing 10% FBS and 1% antibiotic solution at 37°C with 5% CO_2. In this study, for each experiment, a minimum of three different donor PRPE cells were used within passages 3 to 6 for all experiments.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for PRPE Characterization

Reverse transcriptase-PCR was performed for *rpe65* gene to confirm the cell identity of the cultured PRPE cells. Total RNA was extracted using TRIzol reagent (Ambion, Carlsbad, CA, USA) and converted to cDNA from cultured cells as described previously.[26] Briefly, equal amounts of extracted RNA were converted to cDNA using High-Capacity cDNA Reverse Transcriptase Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and stored at −20°C. Reverse transcriptase-PCR was performed on an Applied Biosystems Veriti (Life Technologies, Foster City, CA, USA) with *rpe65* and *gapdh* gene-specific primers (Table 1). The PCR was set in a total volume of 25 µL as described previously.[26] The products were resolved on 1.5% agarose gel and documented on a UV transilluminator (Vilber Lourmat, Mumbai, India).

BEV Treatment on PRPE Cells

Primary RPE cells were seeded onto 12-well tissue culture plates at a density of 2×10^5 cells per well. Semiconfluent cultures (70%–80% confluency) were serum starved for 24 hours[25] and treated with a clinical concentration (0.25 mg/mL) of BEV (Roche Diagnostics, GmbH, Penzberg, Germany) for 2, 4, and 6 hours. The supernatants as well as the cells were collected for measurement of secreted VEGF levels by sandwich ELISA (R&D Systems, Minneapolis, MN, USA) and other molecular/biochemical analysis. Untreated and anti-human IgG isotype (Abcam, Cambridge, MA, USA)-treated cells served as controls for the experiments.

Quantitative RT-PCR

Total cellular RNA was extracted and converted to cDNA from BEV-treated as well as control (untreated and anti-IgG treated) PRPE cultures. Quantitative RT-PCR (qRT-PCR) was performed on the Bio-Rad CFX Connect (Bio-Rad, Hercules, CA, USA) sequence detector using the KAPPA SYBER FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), and the results were analyzed according to manufacturer's instructions. A list of gene-specific primers is provided in Table 2.

Measurement of VEGF Levels

The secreted VEGF levels in cell supernatant of untreated and BEV-treated cultures were estimated using the sandwich Human VEGF DuoSet ELISA (R&D Systems). Per the manufacturer's instructions, the samples and standards were run in triplicate. The reaction product was quantified with microplate reader 680 (Bio-Rad) at a wavelength of 450 nm with the reference filter at 570 nm.

Bromodeoxyuridine (BrdU) Assay

The effect of BEV treatment on the PRPE cell proliferation was determined using BrdU labeling kit (BD Biosciences, San Diego CA, USA). Cells (1.5×10^5) were seeded for BrdU experiments in 6-well plates. After BEV treatment, the semiconfluent cells (approximately 70% confluency) were incubated with BrdU each day (days 1–4) for 5 hours and then collected and stained per the manufacturer's instructions. Stained cells were analyzed by flow cytometry using FACSCalibur (BD Bioscienc-

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Table 2. Primers Used for qRT-PCR

Serial Number	Gene Name	Sequences, 5′–3′	Size, bp	Gene Accession No.
1	*gapdh*	FP: ACCCACTCCTCCACCTTTGAC RP: TGTTGCTGTAGCCAAATTCGTT	100	NM_001289746.1
2	*vegfa*	FP: TGCAGATTATGCGGATCAAACC RP: TGCATTCACATTTGTTGTGCTGTAG	81	NM_001287044.1
3	*vegfr1*	FP: CAGGCCCAGTTTCTGCCATT RP: TTCCAGCTCAGCGTGGTCGTA	82	NM_002019.4
4	*vegfr2*	FP: CCAGCAAAAGCAGGGAGTCTGT RP: TGTCTGTGTCATCGGAGTGATATCC	87	NM_002253.2
5	*notch1*	FP: ATCCAGAGGCAAACGGAG RP: CACATGGCAACATCTAACCC	106	NM_017617
6	*notch2*	FP: GGACCCTGTCATACCCTCTT RP: CATGCTTACGCTTTCGTTTT	150	NM_024408.3
7	*notch3*	FP: GTGTGTGTCAATGGCTGGAC RP: GTGACACAGGAGGCCAGTCT	150	NM_000435.2
8	*notch4*	FP: GAGGACAGCATTGGTCTCAAGG RP: CAACTCCATCCTCATCAACTTCTG	61	NM_004557.3
9	*jagged1*	FP: AAGGCTTCACGGGAACATAC RP: AGCCGTCACTACAGATGCAC	120	NM_000214
10	*jagged2*	FP: GGTCGTACTTGCACTCACAATACC RP: GTAGCAAGGCAGAGGGTTGC	51	NM_145159.2
11	*dll1*	FP: GGCTACTCCGGCTTCAACT RP: ATCACCGAGGTCCACACACT	90	NM_005618.3
12	*dll3*	FP: AGCTCGTCCGTAGATTGGAA RP: AGCGTAGATGGAAGGAGCAG	81	NM_016941.3
13	*dll4*	FP: AAGGCTGCGCTACTCTTACC RP: AAGTGGTCATTGCGCTTCTT	90	NM_019074.3
14	*hes1*	FP: GAGAGGCGGCTAAGGTGTTT RP: GTGTAGACGGGGATGACAGG	118	NM_005524
15	*hes3*	FP: GAAAGTCTCCCTGGCTCGTC RP: CCAAATAGGGAGCGCCTTCA	146	NM_001024598
16	*hes5*	FP: AGAGAATGTGTGTGCAGAGTCC RP: GGTCAGACACTTGGCAGAAGA	70	NM_001010926
17	*hey1*	FP: GACCGTGGATCACCTGAAAA RP: TCCCAAACTCCGATAGTCCA	91	NM_012258
18	*bax*	FP: TTGCTTCAGGGTTTCATCCA RP: AGACACTCGCTCAGCTTCTTG	113	NM_138761
19	*bcl2*	FP: TGGCCAGGGTCAGAGTTAAA RP: TGGCCTCTCTTGCGGAGTA	143	NM_000633
20	*caspase 9*	FP: CCAGAGATTCGCAAACCAGAGG RP: GAGCACCGACATCACCAAATCC	88	NM_001229
21	*pcna*	FP: GCCAGAGCTCTTCCCTTACG RP: TAGCTGGTTTCGGCTTCAGG	87	NM_002592
22	*cyclin d1*	FP: AGCTCCTGTGCTGCGAAGTGGAAAC RP: AGTGTTCAATGAAATCGTGCGGGGT	133	NM_053056
23	*ki67*	FP: CTTTGGGTGCGACTTGACG RP: GTCGACCCCGCTCCTTTT	199	NM_002417
24	*cdc20*	FP: GTTCGGGTAGCAGAACACCA RP: CCCCTTGATGCTGGGTGAAT	187	NM_001255
25	*col1a1*	FP: TTGTGCGATGACGTGATCTGT RP: TTGGTCGGTGGGTGACTCTG	111	NM_000088.3
26	*col4a1*	FP: GCAAACGCTTACAGCTTTTGG RP: GGACGGCGTAGGCTTCTTG	69	NM_001845.5
27	*α-sma*	FP: GCTGGCATCCATGAAACCAC RP: TACATAGTGGTGCCCCCTGA	104	NM_001613.2
28	*fibronectin*	FP: TGGCCAGTCCTACAACCAGTA RP: CTCGGGAATCTTCTCTGTCAGC	119	NM_001306129.1
29	*vimentin*	FP: ACACCCTGCAATCTTTCAGACA RP: GATTCCACTTTGCGTTCAAGGT	76	NM_003380.3
30	*ctgf*	FP: GACTGGAAGACACGTTTGGC RP: GCGTTGTCATTGGTAACCCG	130	NM_001901.2
31	*e-cadherin*	FP: AAGGAGGCGGAGAAGAGGAC RP: CGTCGTTACGAGTCACTTCAGG	87	NM_004360.3

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es) in the FL1 channel using the BD CellQuest Pro software (BD Biosciences).

Propidium Iodide Staining for Cell Cycle Analysis

The PRPE cells were left untreated or treated with BEV for 2 hours and collected on various days (days 1–4) for propidium iodide (PI) staining per the manufacturer's instructions (Molecular Probes, Life Technologies, Foster City, CA, USA). DNA content of the cells was analyzed by flow cytometry using FACSCalibur and the BD CellQuest Pro software.

Western Blot

Cells were lysed in RIPA (radioimmunoprecipitation assay) lysis and extraction buffer (25 mM Tris, 150 mM sodium chloride, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [G-Bioscience, St. Louis, MO, USA]). Lysates were clarified by centrifugation at 12.4 × 1000*g* for 30 minutes. The total protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Thermo Scientific, Rockford, IL, USA). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane by semidry transfer (Bio-Rad). Membranes were blocked with 5% bovine serum albumin and incubated with rabbit monoclonal anti-β-actin and anti-cleaved Notch4-Notch intracellular domain (NOTCH4-NICD; 1:1000, Cell Signaling, Danvers, MA, USA) overnight. Anti-rabbit horse radish peroxidase conjugate (1:2000; Cell Signaling) was used as the secondary antibody. Enhanced chemiluminescence detection kit (Pierce ECL Plus; Thermo Scientific) was used for protein detection. Gel documentation analysis was done using the Image Quant LAS 500 (GE Healthcare Life Science, Uppsala, Sweden).

Fluorescent Activated Cell Sorting (FACS) Analysis for NOTCH4 and DLL4

While one group of PRPE cells was treated with BEV for 2 hours and incubated further for 1, 2, 3, and 4 days, the other group was left untreated. The cells were stained for NOTCH4 and DLL4 using mouse anti-human NOTCH4 and rabbit anti-human DLL4 antibodies (Cell Signaling). Donkey anti-rabbit IgG-fluorescein isothiocyanate (FITC) and goat anti-mouse IgG-Dylight 488 were used as secondary antibodies (BioLegend, San Diego, CA, USA). Cells were stained for FACS analysis as mentioned previously.[26] Briefly, cells were trypsinized and fixed with 4% paraformaldehyde before being permeabilized with 0.1% Triton X-100 (Fisher Scientific, Qualigens, Mumbai, India). The permeablized cells were stained with antibodies for NOTCH4 and DLL4. Unstained cells and cells stained with secondary antibodies alone were used as controls. The fluorescence emitted by cells in FL1 channel was recorded and analyzed using BD CellQuest Pro software.

Apoptosis Assay

Cultured PRPE cells were untreated or incubated with BEV for 2, 4, and 6 hours. Cells were collected; total RNA was extracted and an equal amount of RNA was converted to cDNA. Quantitative RT-PCR was performed for *bcl2*, *bax*, and caspase 9 genes, and the results were analyzed. Active caspase staining was performed using a fluorochrome inhibitor of caspases (FLICA) kit (Imgenex, San Diego, CA, USA) per the manufacturer's instructions on cultured PRPE cells with/without BEV for 2 hours. The stained cells were observed and documented using the ProgRes Capture Pro 2.5 software on an Olympus BX41 fluorescent microscope (Olympus, Shinjuku, Tokyo, Japan).

Transmembrane Potential Assay

Membrane potential assay was performed on cultured PRPE cells with and without incubation with BEV for 2 hours. Membrane potential assay was performed according to the manufacturer's instructions. Briefly, after BEV treatment, cells were isolated and incubated with 20 nM bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC4(3)] for 30 minutes at 37°C. Cells depolarized with 30 mM potassium chloride (Fisher Scientific, Fair Lawn, NJ, USA) followed by incubation with 20 nM bis-(1,3-dibutylbarbituric acid) trimethine oxonol were used as positive control and analyzed by FACS. The fluorescence emitted by cells in FL1 channel was recorded and analyzed using BD CellQuest Pro software.

Immunofluorescence Staining

Primary RPE cells were stained with anti-NOTCH4 and anti-DLL4 antibodies with/without BEV treatment for 2 hours. Cultured cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Permeablized cells were stained with mouse anti-human NOTCH4 and rabbit anti-human DLL4 antibodies as mentioned above in the section on FACS analysis. Cells were stained with secondary antibodies (see same section) and mounted with VECTASHIELD containing 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were documented on ProgRes Capture Pro 2.5 software on an Olympus BX41 fluorescent microscope. Fluorescence intensity was quantified using ImageJ 1.48 version software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Phagocytosis Assay

Cultured PRPE cells were treated with or without BEV for 2 hours. The cells were then incubated with FITC-labeled latex beads (1:50; Cayman Chemicals, Ann Arbor, MI, USA) and followed up on different days (days 1–4). Each day the cells were trypsinized and analyzed in FL1 channel by the FACSCalibur. For immunofluorescence, the PRPE cells were grown to confluence in 0.2% gelatin (Himedia)-coated coverslips with DMEM medium. Cells were treated with BEV for 2 hours, followed by incubation with FITC-labeled latex beads for 48 hours at 37°C per the manufacturer's instruction. The coverslips were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories) and observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The number of DAPI-positive cells with latex beads was counted manually and represented graphically.

Epithelial–Mesenchymal Transition Assay

Quantitative RT-PCR was performed on cultured PRPE cells with and without treatment with BEV for 2 hours and followed up on different days (days 1–4). On each day, cells were trypsinized and collected for total RNA extraction, and cDNA conversion was performed as stated in the previous section. Quantitative RT-PCR was performed for collagen I, collagen IV, alpha-smooth muscle actin (*α-SMA*), vimentin, connective tissue growth factor (*CTGF*), and fibronectin genes and the data were analyzed. Quantitative RT-PCR was also performed for e-cadherin for PRPE cells, with or without treatment with BEV. Analysis of the samples was performed for cells of days 1 through 4. F-actin staining was performed

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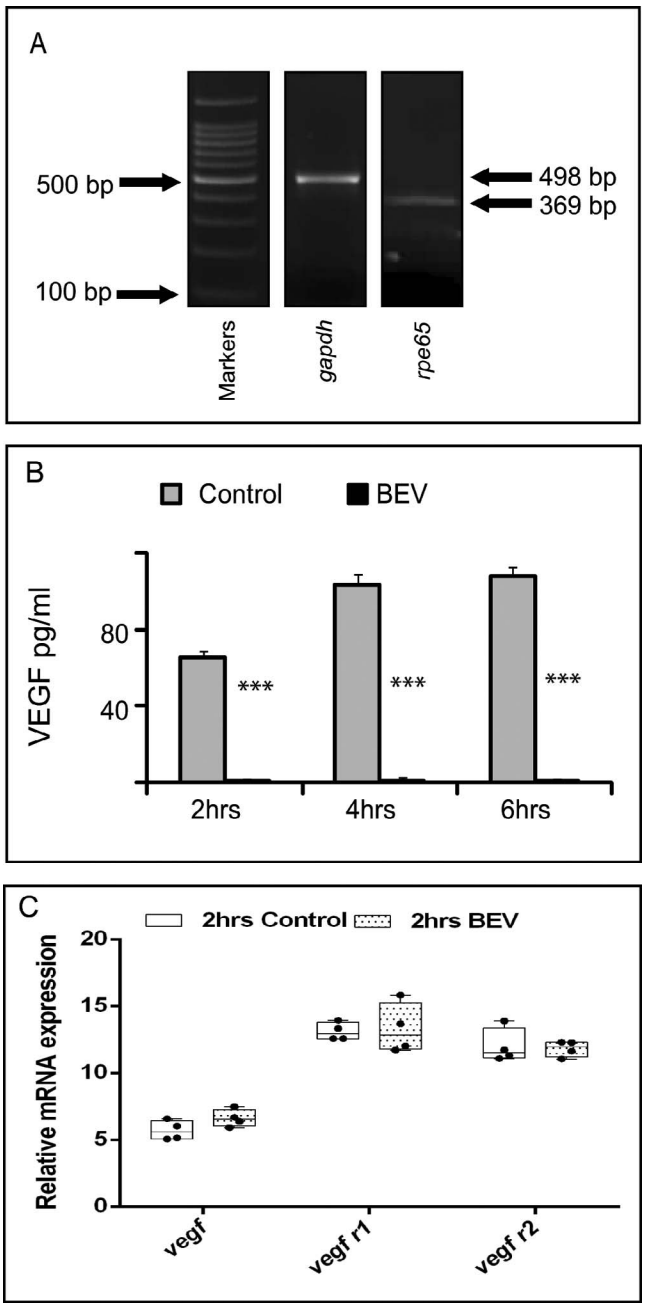

FIGURE 1. Effect of time-dependent incubation with BEV on VEGF levels. RT-PCR of *rpe65* and *gapdh* gene expression for PRPE cells (**A**). Secreted VEGF levels were estimated by ELISA after BEV treatment at different time intervals (2, 4, and 6 hours) in PRPE cells (**B**). Gene expression levels were estimated by qRT-PCR for *vegf*, *vegf-r1*, and *vegf-r2* in PRPE cells (**C**) with 2 hours of BEV treatment. The results of the qRT-PCR were normalized with *gapdh* levels. Statistical analysis was performed using Student's *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$) ($n \geq 3$).

on PRPE cells exposed to a short pulse of BEV for 2 hours. Cells after the BEV treatment were followed for days 4 to 7, and on each day, controls as well as treated cells were collected and stained with Alexa Fluor 488 Phalloidin (Life Technologies, Carlsbad, CA, USA). Positive cells were detected on FACSCalibur and the mean fluorescence intensity was calculated.

Statistical Analysis

All the experiments were performed in triplicate, and results of three independent experiments were used for statistical analysis. Data were represented as the mean ± standard deviation (SD) and were analyzed with Student's *t*-test. Significance value is denoted as $P < 0.05$, $P < 0.01$, $P < 0.005$.

RESULTS

Effect of BEV on VEGF and VEGF-Receptor Regulation

The identity of the cultured PRPE cells was confirmed by the expression of *rpe65* gene using RT-PCR (Fig. 1A). Secreted VEGF levels were analyzed in confluent PRPE cultured supernatants in the presence and absence of BEV at different incubation time points (2, 4, and 6 hours) by sandwich ELISA. Bevacizumab at clinical concentration (0.25 mg/mL) neutralized the secreted VEGF levels to lower than the detectable limit (<31.3 pg/mL) of the assay at all time points (2, 4, and 6 hours). In untreated PRPE cells the secreted VEGF concentrations were 65.3, 102.9, and 108.1 pg/mL for 2, 4, and 6 hours, respectively (Fig. 1B). Phase contrast images of the BEV-treated PRPE (data not shown) cells showed no aberrant morphologic changes with respect to the untreated/control cells. No significant difference was detected in the relative gene expression levels of *vegf*, *vegfr1*, and *vegfr2* in PRPE cells in the presence and absence of BEV for 2 (Fig. 1C), 4, and 6 hours (data not shown). Gene expression analysis of *vegf*, *vegfr1*, and *vegfr2* on anti-human IgG (isotype control)-treated PRPE cells (2 hours) showed no difference when compared to the untreated cells (Supplementary Fig. S1A).

Modulation of Notch Signaling After BEV Treatment

Interestingly, of the analyzed Notch receptors and ligands, *notch4* ($P = 0.03$) and *dll4* ($P = 0.003$) gene expression levels were significantly downregulated at 2 hours of BEV-treated cultures (Figs. 2A, 2B). There was a 16% decrease in gene expression of both *notch4* and *dll4* gene expression with short exposures of BEV. This was further corroborated with a downregulation, though not significant, of the Notch downstream targets (*hes3*, *hes5*) except for *hey1* ($P \leq 0.05$) in 2-hour BEV-treated cultures compared to controls (Fig. 2C). There was no significant difference in the gene expression of Notch pathway receptors, ligands, and downstream targets at longer incubation with BEV (data not shown). Further, the temporal downregulation of gene expression modulations of NOTCH4 and DLL4 proteins in cells was analyzed with immunofluorescence and FACS in PRPE cells after BEV treatment (2 hours); immunofluorescence analysis revealed a temporally significant decrease by 35% in the fluorescence intensity on day 2 for both NOTCH 4 ($P = 0.007$, Figs. 2D–I) and DLL4 ($P = 0.03$, Figs. 2J–P). Flow cytometry analysis revealed significant downregulation in the percentage of NOTCH4 ($P = 0.005$)- and DLL4 ($P = 0.001$)-positive cells by 29.7% on day 2 post BEV treatment in PRPE cells (Figs. 2Q, 2R). However, the transient decrease observed on day 2 following incubation with BEV for 2 hours reverted to normal levels on longer follow-ups. Analysis of Western blot revealed a decrease in the levels of NOTCH4-NICD specifically on day 2 in PRPE cells treated with 2 hours of BEV (Fig. 2S). This further confirmed a short-term exposure of BEV that resulted in a decrease in the levels of NOTCH4 and DLL4 along with a decrease in Notch activity. Gene expression analysis of Notch

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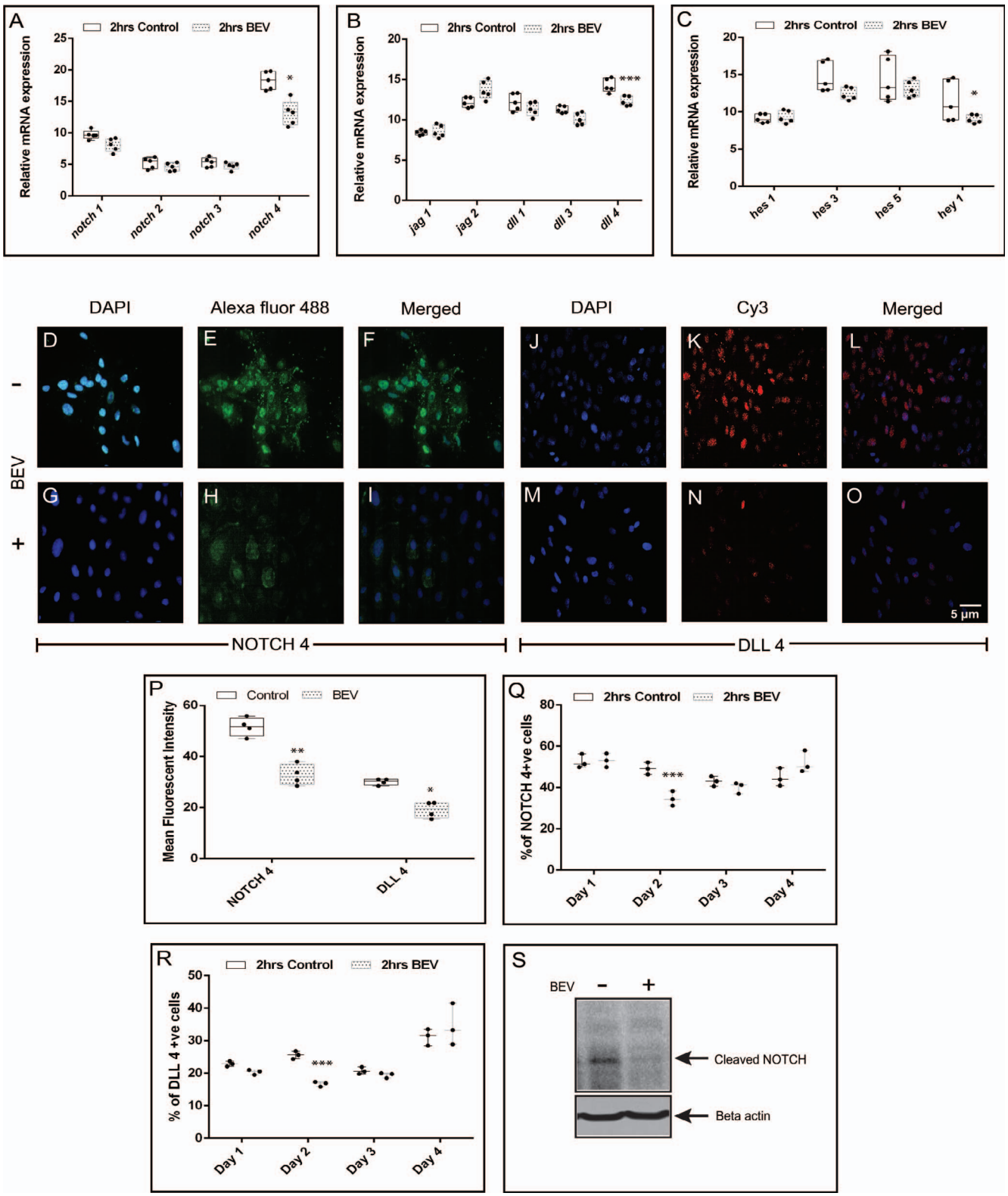

Figure 2. Effect of 2-hour incubation with BEV on Notch pathway. Box-whisker graph depicting the regulation of gene expression profile of Notch receptors *notch1*, *notch2*, *notch3*, *notch4*; ligands *jagged1*, *jagged2*, *dll1*, *dll3*, *dll4*; and downstream targets *hes1*, *hes3*, *hes5*, *hey1* in cultured PRPE (**A–C**) cells in the presence/absence of BEV for 2 hours. Immunofluorescence-stained images of cultured PRPE cells portray the decreased protein expression on day 2 for NOTCH4 (*green*) (**D–I**) and DLL4 *(red)* (**J–O**) with/without 2 hours of BEV incubation. Nuclear staining was performed with DAPI (*blue*). Graphical representation of quantification of intensity estimated by ImageJ in untreated and BEV-treated PRPE cells (**P**). Fluorescent activated cell sorter analyses of the cumulative results are represented graphically with and without BEV on cultured PRPE cells. The box-whisker graph reveals the temporal decrease in protein intensity of NOTCH4 (**Q**) and DLL4 (**R**) in PRPE cells on day 2. Western blot with anti-NOTCH4-NICD on BEV-treated and untreated PRPE cultures on day 2 (**S**). Anti-β-actin antibodies were used as internal controls and followed on days 1 to 4. The results of the qRT-PCR were normalized with *gapdh* levels. Statistical analysis was performed using Student's *t*-test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$) ($n \geq 3$).

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receptors, ligands, and downstream targets of anti-human IgG-treated PRPE cells (2 hours) and untreated PRPE cells revealed no difference (Supplementary Figs. S1B–D).

Effect of BEV on Cell Proliferation

Notch signaling pathway is a known regulator of proliferation and apoptosis. Hence, we investigated if the transient decrease in Notch activity dampened cell proliferation status in cultured PRPE cells. Gene expression results of cultured PRPE cells treated with BEV for 2 hours revealed a downregulation of *cyclin D1*, *pcna*, and *ki67* ($P \leq 0.005$) when compared to untreated controls (Fig. 3A). Primary RPE cultures incubated for 4 and 6 hours with BEV or with anti-human IgG (2 hours) did not show any effect (Supplementary Fig. S1E). Gene expression regulation was confirmed by determining the number of proliferative cells using the BrdU assay. A significant reduction of 34.1% in the percentage of BrdU positivity was detected on PRPE cells ($P = 0.005$) specifically on day 3 (Figs. 3B–D) in those treated with BEV when compared to untreated cultures. There was no difference in the BrdU-positive cell population on other days (data not shown). To determine the cell cycle phase distribution on BEV treatment, we performed PI staining, and the results were analyzed by FACS. Analysis of PI-positive cell distribution revealed a significant decrease in the percentage of cells in the S-phase in PRPE cells ($P = 0.04$) with respect to untreated controls (Fig. 3E). The percentage of different phases of the cell cycle was analyzed by FACS and is represented in Figure 3F.

Effect of BEV on Cell Apoptosis

Notch signaling is a known modulator of cell apoptosis. Hence, the effect of short pulse of BEV on cell apoptosis of cultured PRPE cells was assessed. Quantitative RT-PCR revealed a decrease in the expression of *bcl2* alone ($P \leq 0.005$) at 2 hours of exposure to BEV in PRPE cells (Fig. 4A). There was no difference in the expression of apoptotic markers in cells left untreated or treated with anti-human IgG (Supplementary Fig. S1F). Moreover, at longer exposures (6 hours), *bax* and *caspase 9* gene expression were elevated in BEV-treated PRPE cells (Supplementary Figs. S2A, S2B). Additionally, active caspase staining was used to evaluate the number of cells with active caspase enzymatic activity. The results revealed no difference in the active caspase levels in PRPE cultures incubated with BEV for 2 hours compared to untreated controls (Figs. 4B–G).

Effect of BEV on EMT Regulation

To assess the temporal effect of BEV we initially looked at the EMT properties of cultured PRPE cells. The results showed that a short pulse to BEV for 2 hours was sufficient to significantly enhance the expression of collagen 1 ($P = 0.03$), collagen 4 ($P = 0.05$), *α-SMA* ($P = 0.001$), vimentin ($P = 0.04$), and fibronectin (0.05) transiently on day 4 (Fig. 5A). Connective tissue growth factor is a known modulator of EMT. Moreover, there is an inverse correlation between Notch signaling and CTGF.[27] Quantitative RT-PCR results showed that concurrent to increased mRNA levels of EMT markers, *ctgf* gene expression was also increased significantly on day 4 in PRPE cells with respect to untreated controls ($P = 0.01$, Fig. 5B). Results of qRT-PCR showed a decrease of e-cadherin mRNA levels ($P = 0.01$) in PRPE cells treated with BEV for 2 hours (Fig. 5C). The downregulation was specifically detected on day 4 with respect to untreated cultures. Enhanced polymerization of actin filaments is one of the salient features of EMT induction. Polymerized filamentous actin can be detected using F-actin (phalloidin-actin) staining. There was a 2.5-fold increase ($P = 0.005$) in the mean fluorescent intensity of the F-actin staining in the PRPE cells treated with BEV when compared to untreated cells (Fig. 5D).

Effects of BEV on Phagocytosis

One of the salient physiological functions of the RPE is phagocytosis of degenerated photoreceptors. An apparent decrease in the mean fluorescence intensity of phagocytosis could be observed in PRPE cells on treatment with BEV (2 hours) from day 2. A significant decrease ($P = 0.006$) of 19.25% of phagocytosis activity was seen on days 3 and 4 when compared to controls (Fig. 6A). Immunofluorescence staining in BEV-treated PRPE cells revealed a reduction in the number of opsonized latex beads (Figs. 6B–E). Image counting revealed a significant decrease in the number of cells with beads ($P = 0.020$, Fig. 6F) along with the number of beads per cell ($P = 0.022$, Fig. 6G).

Effects of BEV on Plasma Membrane Potential

At day 1 post BEV incubation, increased fluorescence intensity (depolarization) was observed using DiBAC(4) dye intake in cells (Fig. 7A). From days 2 to 4 no difference was observed in the mean fluorescence intensity (data not shown). Along with an increase in fluorescence by 63%, statistically significant increased mean florescence intensity was ($P = 0.04$) observed in PRPE cells (Fig. 7B).

Discussion

The RPE is the single layer of polarized epithelial cells between the retina and the choroid, with a definitive role in maintaining both retinal and choroidal vasculature. Vascular EGF, a major modulator of ocular angiogenesis, is predominantly secreted by the RPE. A deregulated VEGF level is the primary cause of pathologic neovascularization.[28] Intravitreal injections of anti-VEGFs like BEV and RAN are used for the treatment of abnormal neovascularization. A low cost with an efficacy similar to that of RAN has made BEV a popular drug in clinical practice. Though BEV can reduce or cause regression of neovascularization, it is known to cause adverse effects such as increased fibrosis, worsening of tractional retinal detachment, and RPE tear.[10,29–31] It was therefore interesting to study the early changes that could lead to these complications.

Among other angiogenic pathways and their interaction with VEGF, Notch signaling leads the trail with a direct role in angiogenesis.[16,19,20] Hence, we investigated the cross-talk between BEV and Notch signaling in cultured PRPE cells and its cell physiological relevance. Notch1, Notch4, and DLL4 receptors and ligands of the Notch pathway are reported to modulate vasculogenesis as well as arterial-venous specification in health and disease.[32,33] It has been showed that the Notch-VEGF interaction is a bidirectional context-dependent flow of information.[20,24,34–36] There is not much literature on the role of anti-VEGF on Notch signaling beyond its primary function of blocking secretory VEGF in ocular cells.[37] We therefore investigated the temporal effect of BEV on Notch signaling using the PRPE cell culture model. A short-term exposure (2 hours) with BEV resulted in a transient decrease in the expression of Notch4 and DLL4 and significantly reduced the secreted VEGF levels in PRPE cell culture supernatants.

The downregulation in Notch signaling decreases cell proliferation as evaluated by BrdU and PI staining. Though transient, the decrease in Notch signaling enhanced the expression of EMT genes and CTGF. This was further

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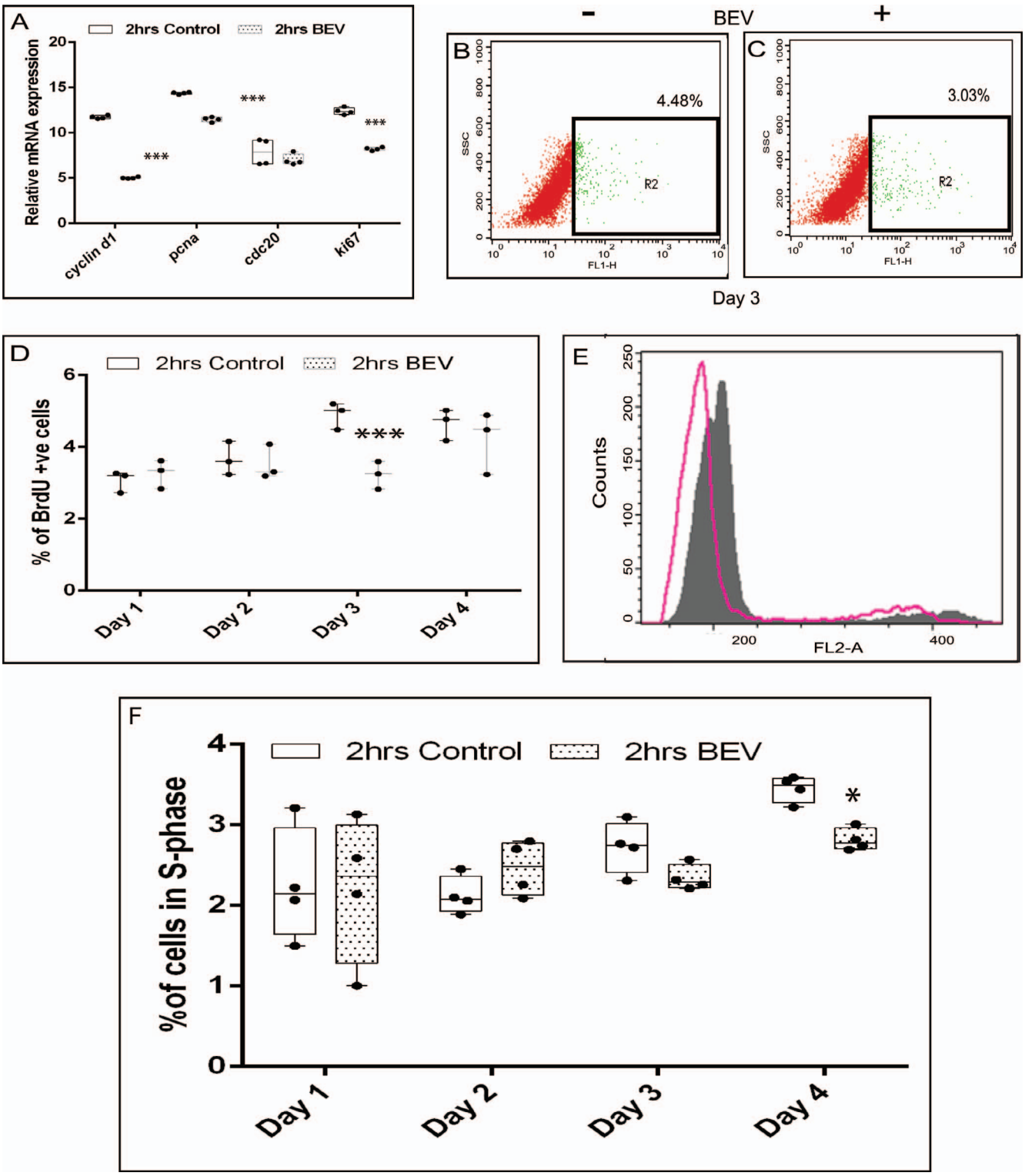

FIGURE 3. Effect of short pulse with BEV on cell proliferation. Box-whisker graph shows the gene-specific mRNA expression of cell cycle markers (*cyclinD1*, *ki67*, *pcna*, and *cdc20*) in cultured PRPE cells (**A**), after 2 hours of BEV treatment. BrdU analysis was performed for days 1 to 4. Fluorescent activated cell sorter analysis of BrdU positivity in cultured PRPE (day 3) cells (**B–D**) in the presence/absence of BEV incubation for 2 hours with representative plots. Propidium iodide-stained BEV-treated (2 hours) PRPE (*pink*) (**E**) cells were analyzed by FACS with representative histogram plots (untreated control, *gray*). The percentage of cells in S-phase of cell cycle is represented graphically (**F**). The results of the qRT-PCR were normalized with *gapdh* levels. Statistical analysis was performed using Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$) ($n \geq 3$).

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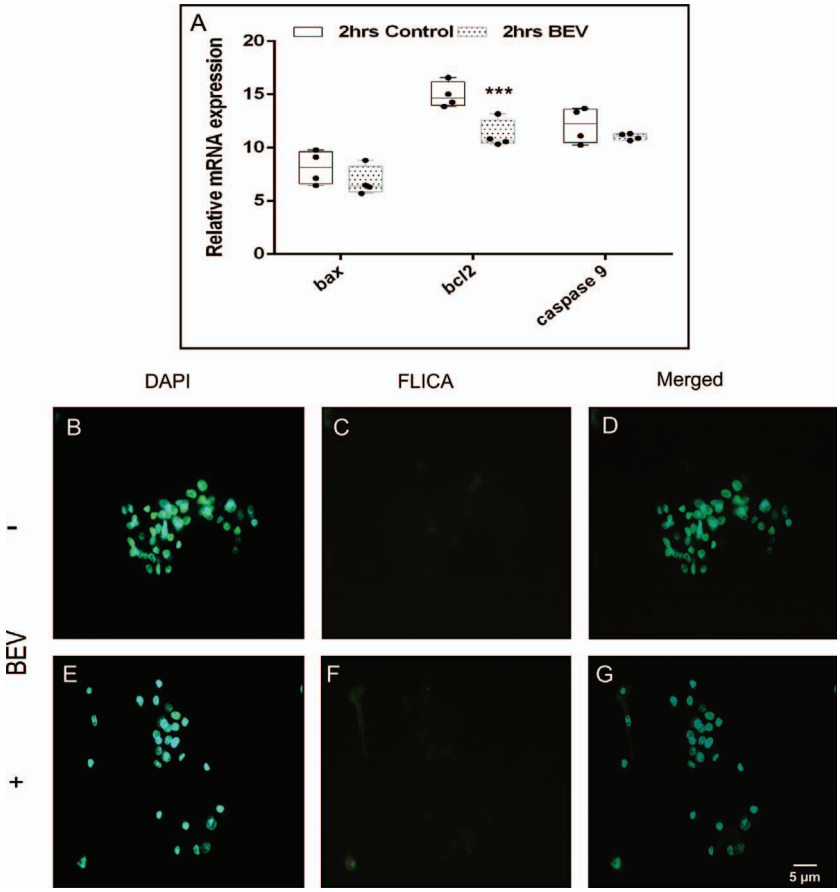

Figure 4. Effect of short incubation with BEV on cell apoptosis. Gene-specific expression pattern of apoptotic genes (*bax*, *bcl2*, caspase 9) was evaluated with qRT-PCR from PRPE cells (**A**) that were incubated with BEV (2 hours) or left untreated. Active caspase staining was performed by FLICA active caspase staining kit. Active caspase staining (*green*) was analyzed with an immunofluorescence microscope on PRPE cells in the presence and absence of BEV (representative images) (**B**–**G**). The results of the qRT-PCR were normalized with *gapdh* levels. Statistical analysis was performed using Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$) ($n \geq 3$).

confirmed by enhanced mean fluorescent intensity of F-actin staining in BEV-treated cultures. However, the apoptosis level did not show any change with short exposures of BEV. Short pulses of BEV enhance the transmembrane potential and dampen the phagocytosis activity in PRPE cultures, thereby having implications in retinal degenerative diseases as well as vascular retinopathies. Cultured human PRPE cells serve as a tool for better understanding of the cellular and molecular events of RPE in disease and treatment regimens.[38] Hence, our study findings can be further validated and translated in disease models.

Notch signaling is a developmental conserved signaling pathway with a decisive role in several cell types and at multiple decision time points.[17] It is well known from studies on neural crest differentiation that a short activation of Notch signaling was sufficient to block neural crest neurogenesis.[39] Hayes et al.[40] showed that blockage of Notch signaling at different time points of Müller glia dedifferentiation can have different outcomes. This implies that Notch signaling executes its functions in a temporally regulated pattern, that is, context dependent and time dependent. Hence, Notch signaling exerts differential outcomes at a specific "window." We observed that the effect of Notch signaling was restricted to 2 hours of BEV treatment. Since the effect is subtle, it is possible that only certain RPE cell types are more prone to BEV treatment rather than all showing a global response. The underlying mechanisms of the specific RPE cells that respond to only short-term BEV treatment need further investigation.

Similar to our findings, another study demonstrated that overexpression of VEGF in endothelial cells induces Notch4 and DLL4.[35] Additionally, our results demonstrated a tight temporal regulation of VEGF-Notch interplay. A short pulse of BEV resulted in a transient reduction of the gene and protein expression of Notch4 and DLL4 in cultured PRPE cells. Notch signaling is also a modulator of cell type specification along with proliferation, apoptosis, and differentiation. Delta-like-4 functions as a ligand for Notch1 and Notch4, as shown by coherent expression of DLL4 along with Notch1 and Notch4 during vasculogenesis.[41] Our findings of a transient decrease in Notch4 and DLL4 expression suggest a plausible decrease in cues for vascularization. Notch4 and DLL4 are also known to modulate arterial endothelial cells during angiogenesis.[42] The transient decrease in Notch4 and DLL4 expression that we saw in our study suggests a possible decrease in stimuli for vascularization with a compromise on arterial vascular development and a trigger toward venous vasculature modulation. These results are specific for a short pulse of BEV, as a similar outcome of decreased Notch4 and DLL4 gene/protein expression could not be detected in cells incubated with BEV for longer durations (4–6 hours) or cells incubated with anti-human IgG. Incubation with a clinical concentration of BEV for longer duration in ARPE-19 cells enhances the expression of

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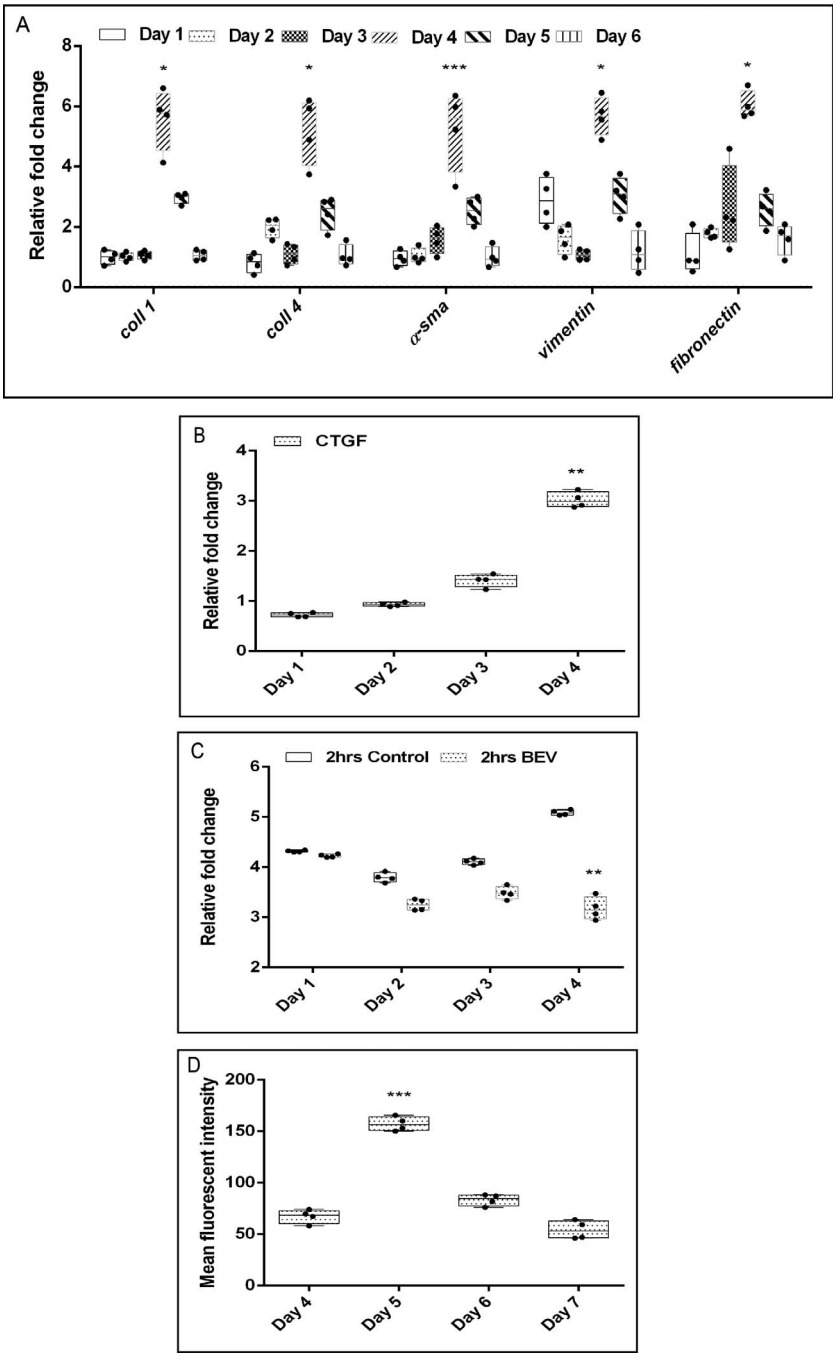

Figure 5. Effect of 2-hour incubation with BEV on EMT regulation. Quantitative RT-PCR analysis for EMT-specific markers (*collagen I*, *collagen 4*, *α-SMA*, *vimentin*, and *fibronectin*) was performed in PRPE cells (**A**) with/without incubation with 2 hours of BEV and followed up from days 1 to 6. Quantitative RT-PCR analysis of *ctgf* (**B**) and *e-cadherin* (**C**) gene expression on days 1 to 4 after 2 hours in BEV-treated and untreated PRPE cultures. Cells were stained for F-actin from days 4 to 7 and analyzed by FACS. The cumulative differences in the intensity of F-actin staining were graphically represented for BEV treated/untreated PRPE cells (**D**). The results of the qRT-PCR were normalized with *gapdh* levels. Statistical analysis was performed using Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$) ($n \geq 3$).

Notch1 and Jagged1 gene expression with a role in EMT.[24] It is well known that cells need to interpret a temporally changing output of a signaling pathway to generate functional outcomes, particularly for the temporal stage.[43]

Notch signaling in health as well as in disease undergoes temporal and spatial regulation.[44,45] The temporal regulation has a role in the proliferation and differentiation of neural progenitors of embryonic stem cells, supporting our findings in PRPE cells.[17] In addition, Liu et al.[22] recently showed that blocking Notch signaling inhibited the proliferation and migration of RPE cells. Though other studies have illustrated the role of Notch signaling in cellular apoptosis, we did not detect any regulation at the cellular apoptosis levels with a transient decrease in Notch4 and DLL4.[15,46] However, at longer incubation periods of BEV (6 hours) there is an increase of proapoptotic mRNA for *bax* and caspase-9 in

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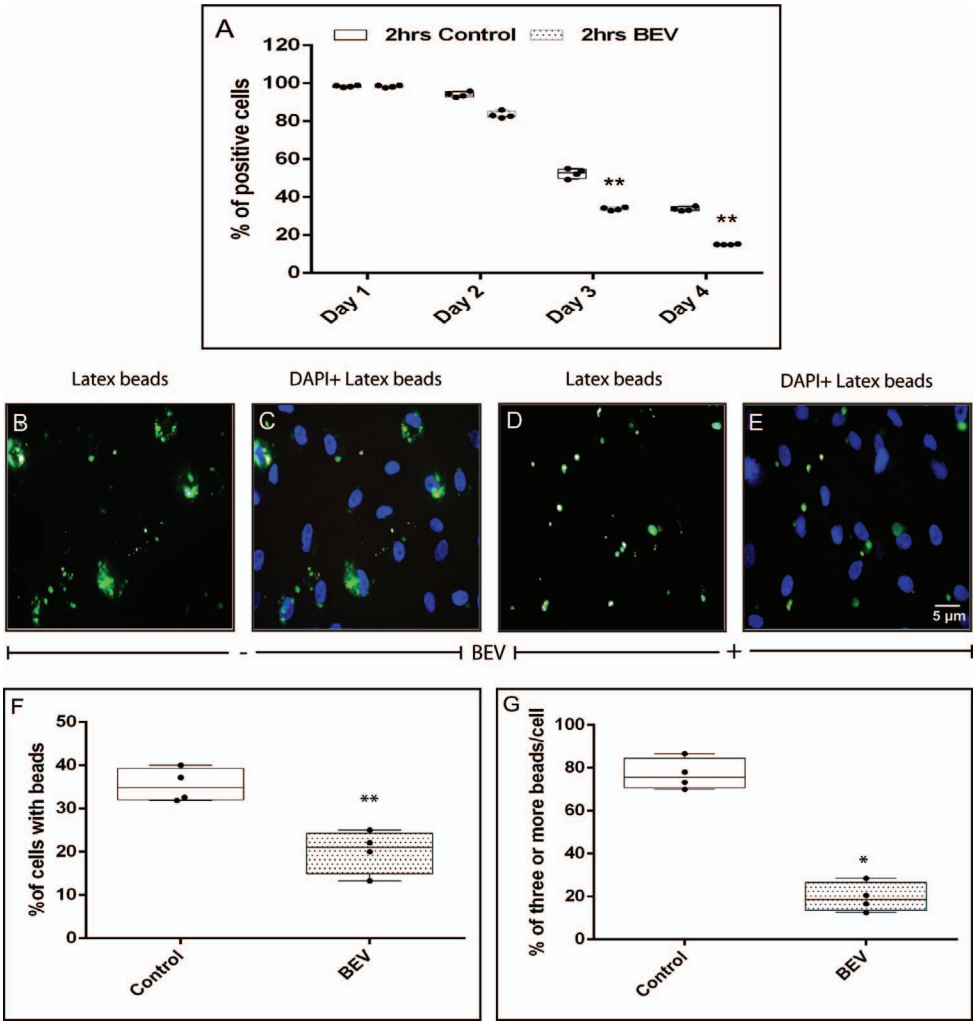

Figure 6. Effects of short incubation of BEV on RPE phagocytosis. Box-whisker graph shows the graphical representation of results from FACS analysis of opsonized beads in cultured PRPE cells (**A**) in the presence/absence of 2 hours of BEV incubation. Analysis was performed from days 1 to 4 after incubation with BEV. Fluorescence microscopic analysis of phagocytosis was performed after 2 hours of BEV treatment followed by incubation of PRPE cells with latex beads (**B–E**). Nuclear counterstain was performed with DAPI (*blue*) in the BEV-treated and control cells; the opsonized FITC-labeled latex beads are in *green*. Cumulative graphical representation of the number of cells with beads (**F**) and with three or more beads per cell (**G**). Statistical analysis was performed using Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$) ($n \geq 3$).

comparison to values in untreated controls.[47] Subsequent to a reduction in proliferation, there was EMT induction in PRPE cells. Another recent study has also shown that BEV regulates EMT via the Notch signaling pathway.[24] The profibrotic effects of BEV are regulated by CTGF, which in turn regulates the Notch signaling.[27,48,49] Notch signaling is crucial not only for migration and proliferation of RPE cells but also for EMT by TGFβ-2 induction.[22] Our results demonstrate that a short pulse of BEV induces an increase in the expression profile of EMT markers and CTGF. Accordingly, the mean fluorescent intensity was elevated with F-actin staining. A longer exposure of BEV induces EMT by upregulating Notch1 and Jagged1, while a short-term exposure with BEV induces EMT most probably by the upregulation of CTGF expression.[24] Activated Notch signaling can induce phagocytosis in microglial cells,[50] and a short exposure of BEV decreases phagocytic activity in cultured RPE cells.[29] Clinically lower phagocytic capacity of RPE results in the accumulation of cellular debris, which could act as a primer for age-related retinal degenerative diseases. The transmembrane potential of the RPE helps in maintaining the blood–retinal barrier function between the retina and choriocapillaris.[51] Loss in the retinal barrier function is secondary to cytoskeleton actin remodeling along with modulation of cadherins leading to the formation of intracellular gaps.[52] Pathophysiologically this could be a trigger for the fibrosis seen in proliferative vitreoretinopathy and edema seen in diabetic retinopathy with infiltration of serum constituents and inflammatory molecules.[53] While hyperpolarization stabilizes cadherins, depolarization leads to destabilization of cadherins and cytoskeleton reorganization.[54] In this study we found increased depolarization of PRPE with a short-duration incubation with BEV, which can cause cytoskeleton remodeling and thereby increase the possibility of generating intercellular gap and formation of fibrotic tissue.

Thus far studies have investigated only long-term outcomes of BEV treatment. To the best of our knowledge, this is the first study that highlights the importance of temporal regulation as well as the physiological effects of the transient regulation of

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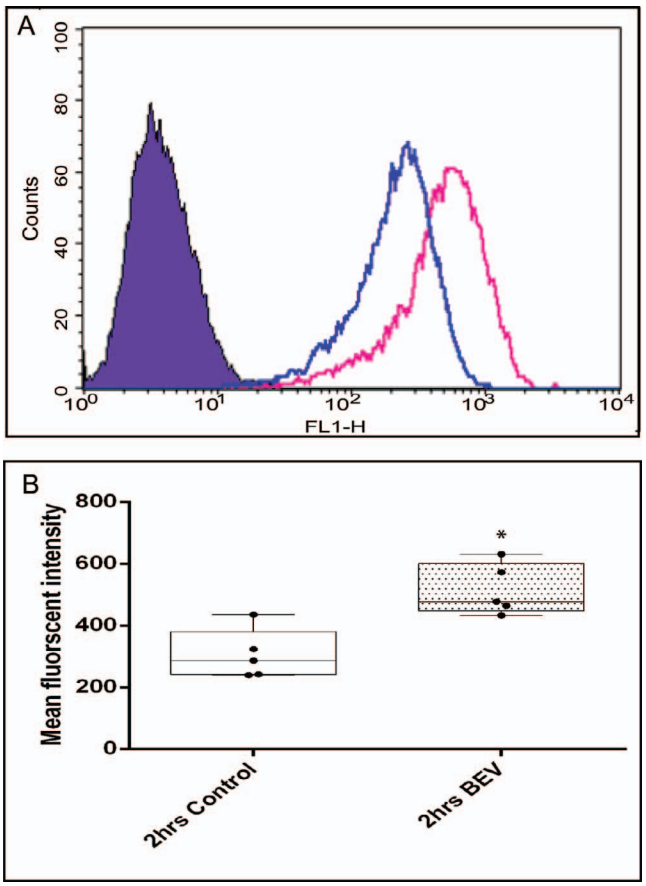

FIGURE 7. Effects of short incubation of BEV on PRPE membrane potential. Flow cytometry analysis of membrane potential was performed by incubating cells with DiBAC4(3). The histogram reveals the increased intensity of internalized dye in cultured PRPE cells (**A**) on day 1 after 2 hours of BEV treatment (*pink*) compared to the control (*blue*) (representative plots). Box-whisker graph represents the statistical significance of the analyzed histogram fluorescence intensity in BEV-treated PRPE cells in comparison to the control (**B**). Statistical analysis was performed using Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$) ($n \geq 3$).

Notch signaling by a short pulse of BEV in PRPE cultures. These findings may provide better insight into the long-term outcomes of BEV treatment with VEGF-binding partners. It is also plausible that the changes in RPE cells exposed to short-term BEV may be completely different from those in RPE cells exposed to long-term BEV treatment as seen in clinical scenarios. Further studies are needed to corroborate our findings to provide a deeper insight into BEV actions in short- and long-term treatments. Our findings can pave the way for comparing the effect of combination therapy with BEV monotherapy for both retinal and choroidal neovascularization diseases.

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