Int J Clin Exp Pathol 2015;8(11):13928-13944
www.ijcep.com /ISSN:1936-2625/IJCEP0016669

Original Article

Evidence of a novel gene *HERPUD1* in polypoidal choroidal vasculopathy

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Received September 22, 2015; Accepted October 25, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Polypoidal choroidal vasculopathy (PCV) is an exudative maculopathy, with clinical features distinct from neovascular age-related macular degeneration (nAMD) which is the leading cause of irreversible blindness in the elderly. Our studies focused on the genetic background and function of a novel gene *HERPUD1* in PCV. *HERPUD1* has been reported to increase the level of amyloid β (Aβ), which is a component of drusen deposits underlying the retinal pigment epithelium (RPE) layer. To verify the genetic functional associations of *HERPUD1* with PCV, exome sequencing of *HERPUD1* was performed in unrelated Chinese individuals, including nAMD patients, PCV patients and control subjects. Immunohistochemistry assays for HERPUD1 were performed in the subretinal membranes of PCV patients. The relationship between *HERPUD1* and amyloid beta precursor was determined using real-time PCR in *HERPUD1*-overexpressing RPE cells. The gene expression patterns of angiogenesis cytokines and chemokines in both Aβ-treated RPE cells and in Brown Norway rats that received Aβ subretinal injections were determined. We showed that *HERPUD1* rs2217332 is significant associated with Chinese PCV, and HERPUD1 was expressed in PCV subretinal membranes. Besides, Plasma Aβ42 protein was significantly higher in PCV patients compared to nAMD and control subjects. Aβ could upregulate angiogenic factors, chemokines and matrix metalloproteinases both in RPE cells and in a rat model of subretinal Aβ injection. The imbalance of the cytokines may be one of the mechanisms for the formation and development of PCV. Our results strongly suggest that *HERPUD1* is highly associated with PCV patients.

Keywords: HERPUD1, single-nucleotide polymorphism (SNP), amyloid beta, polypoidal choroidal vasculopathy, angiogenesis

Introduction

Age-related macular degeneration (AMD), the leading cause of blindness in the elderly population, is characterized as the chronic and progressive loss of central vision [1, 2]. Based on clinical findings, AMD can be divided into "dry" and "wet" AMD; "wet" AMD is also termed neovascular AMD (nAMD) [2]. Polypoidal choroidal vasculopathy (PCV) is categorized by some experts as a subtype of nAMD, but others consider PCV a different disease entity [3]. Clinically, PCV is characterized by hyalinization and either peripapillary macular or peripheral subretinal pigment epithelium (RPE) polypoidal dilations of a network of abnormal choroidal vessels [4]. Although indocyanine green angiography (ICGA) can differentiate PCV from nAMD, the etiology and pathogenesis of PCV are largely unknown, and it remains controversial whether or not PCV represents a subtype of nAMD [4-6]. Our studies focused on genetic background and function of genes in PCV, to determine whether this phenotype can be attributed to differences in genetic components.

Previous articles have reported that PCV has some similarities to arteriosclerotic changes, including basement membrane disturbances and hyalinized vessels [7, 8]. Other reports have suggested that drusen, which underlies the RPE layer, shares several clinical and pathological features with Alzheimer's disease (AD), including the mechanism of Aβ deposition [9, 10]. However, the association between Aβ deposition and PCV has not been investigated comprehensively. The homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like do-

Table 1. Demographic distribution of the study subjects

	nAMD	PCV	Controls
Total	453	174	425
Males, n (%)	253 (55.8%)	94 (54%)	225 (52.9%)
Females, n (%)	200 (44.1%)	80 (46%)	200 (47.1%)
Age* range (Years)	50-90	50-85	51-95
Mean age ± SD (Years)	67.12±6.81	64.52±6.78	68.41±7.32

nAMD, neovascular AMD; SD, standard deviation; *Age of presentation.

main member 1 (Herp) protein, which is encoded by the *HERPUD1* gene, is reported to play a role in the unfolded protein response (UPR) and in endoplasmic reticulum (ER) stress, but the association of Herp with Aβ is still controversial [11-14]. Some reported that *HERPUD1* increased the level of Aβ and was found to be expressed in neurons and vascular smooth muscle cells.

In this study, the exons of HERPUD1 were assessed using a whole exome sequencing technique, and the expression of *HERPUD1* in the subretinal membranes of PCV patients was tested. Plasma amyloid protein precursor (APP) and Aβ42 were detected. Additionally, the relationship between *HERPUD1* and APP and the functions of Aβ in RPE cells and in rat models of subretinal injection were also evaluated. Our results showed that *HERPUD1* is highly related to PCV, and the imbalance in the secretion of cytokines could be one of the mechanisms behind the formation and development of the abnormal vessels in PCV through the up-regulation of Aβ. The hypothesis for our present study was that *HERPUD1* may play a role in the development of PCV, partially through the production of amyloid beta. While the genetic differences in *HERPUD1* between nAMD and PCV need to be illustrated in further studies.

Materials and methods

Subjects

The study protocol was approved by the Ethical Committee of Peking University People's Hospital and was conducted in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from each study subject. All of the participants provide their written informed consent to participate in this study. In this study, all participants were unrelated Chinese subjects and were recruited at the Department of Ophthalmology of the Peking University People's Hospital. All subjects received a standard ophthalmic examination by a retinal specialist. All cases with nAMD and PCV underwent fluorescein angiography (FFA), optic coherence tomography (OCT), and indocyanine green angiograms (ICGA). The diagnosis of nAMD was made according to the International Classification System for ARM [15]. The diagnosis of PCV was made according to evidence-based guidelines for clinical diagnosis and treatment of PCV [4]. Exclusion criteria included eyes with other macular abnormalities, such as pathologic myopia, idiopathic CNV, presumed ocular histoplasmosis, angioid streaks or other secondary CNV. Normal controls were defined as individuals with no clinical evidence of early or late AMD in either eye or any other eye disease except mild age-related cataracts. A total of 1052 individuals were included for exome sequencing (**Table 1**), including 453 nAMD patients, 174 PCV patients and 425 normal controls.

Genomic DNA extraction and genotyping by sequencing

Genomic DNA was extracted from peripheral venous blood leukocytes with a genomic extraction kit, according to the manufacturer's instructions (Beijing eBios Biotechnology Co., Ltd). The purified PCR products were directly sequenced using an ABI 3730XL DNA sequencer. Variants in the *HERPUD1*gene were identified using an ABI automatic allele calling program. The genotyping was conducted with 99% completeness and 99% accuracy, as determined by random re-sequencing of 10% of the samples.

Immunohistochemistry assays in PCV patient membranes

Human PCV subretinal membranes were surgically excised from patients after proper consent and approval. Immunohistochemistry was performed with 6 mm frozen serially sections across the whole specimen. Briefly, tissue sections were fixed in 4% paraformaldehyde and blocked with 10% normal goat serum, and anti-HERPUD1 monoclonal antibody (ab56742, 1:200 dilution, Abcam Inc., Cambridge, MA, USA) was applied to the tissue sections at 4°C overnight. The slides were then exposed to

horse radish peroxidase (HRP)-conjugated secondary antibody (A0216, 1:1000 dilution, Beyotime, Jiangsu, China) for 30 min, and the sedimentary type of tetramethylbenzidine (TMB, P0211, Beyotime, Jiangsu, China) was applied for 10 min. Sections were washed with phosphate buffer saline (PBS) between all staining procedure steps. For each case, normal mouse IgG (A7028, Beyotime, Jiangsu, China) was used as a negative control. Images of slides were viewed at 400× and captured on a Nikon microscope (Eclipse E800).

Assessment the amyloid protein precursor (APP) and amyloid beta 42 (Ab42) level in the serum

The concentration of APP and Aβ42 in the serum of nAMD, PCV and control subjects was measured by enzyme-linked immunosorbent assays (ELISA) using a human APP ELISA kit (Human APP ELISA Kit; KHB0051; Invitrogen, Frederick, Maryland, USA) and a human Aβ42 ELISA kit (Human Aβ42 ELISA Kit; KHB3544; Invitrogen, Frederick, Maryland, USA). Each assay was performed according to the manufacturer's instructions. A total of 270 individuals were included for serum detection, including 90 nAMD patients, 90 PCV patients and 90 normal controls. There is no dyslipidemia disease in all of these subjects,

Procedure of Aβ oligomerization

Aβ(1-42) (H-1368), its inactive reverse control peptide, Aβ(42-1) (H-3976), and Aβ(1-40) (H-1194) and its inactive reverse control peptide, Aβ(40-1) (H-2972), were used in the experiments and supplied by Bachem (Weil am Rhein, Germany). The preparation of the oligomerization has been previously described [16] (technical notes for the solubilization and oligomerization of Aβ peptides by Bachem). Briefly, non-oligomerized Aβ(1-40) and Aβ(1-42) were incubated at 37°C for 5 days and stored at -20°C until use. Each aliquot was used only once.

RPE cell cultures and cell treatment

The human ARPE19 cell line (ATCC) is a non-transformed human RPE cell line that displays many differentiated properties typical of RPE in vivo [17]. ARPE-19 cells were cultured in DMEM:F12 (Invitrogen, Cergy-Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum (FCS) [17, 18]. The culture medium was replaced every 2 days until confluence. At confluence, ARPE-19 cultures were treated with 1 μM concentrations of different Aβ peptides for 30 min, 1 h, 3 h, 6 h, 12 h or 24 h and their respective inactive reverse peptides, which served as controls. Cells were also treated with PBS (calcium- and magnesium-free), which served as a vehicle control.

RNA extraction and real-time PCR of APP after overexpression of HERPUD1

ARPE19 cells transfected with *HERPUD1*-pCMV6-C-GFP plasmid DNA (OriGene, RG-200693) were lysed in Trizol, and RNA was extracted according to the manufacturer's protocol. Reverse transcriptase (RT) reactions were performed using the RevertAid™ First Strand cDNA Synthesis Kit with oligo-dT primer (Fermentas, EU). Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Real-time PCR reactions were performed with the SYBR Green PCR mix (Thermo, Switzerland) using the ABI7300 real-time PCR system. The primers used in RT-PCR were APP (amyloid protein precursor): forward 5'-GCCAACCAACCA-GTGACCAT-3'; reverse 5'-TTTCGCAAACATCCA-TCCTCT-3'; and GAPDH: forward 5'-GAGTCCACT-GGCGTCTTCAC-3'; reverse 5'-GTTCACACCCAT-GACGAACA-3'. Data were normalized to the housekeeping gene GAPDH, and the results were expressed as fold amplifications. Each experiment was repeated five times.

Animals and subretinal injection treatment in the Brown Norway rat animal model

To investigate the in vivo effects of Aβ on the retina, Brown Norway rats were used as a unilateral subretinal injection animal model. All procedures involving the animal model adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were performed in accordance with the guidelines provided by the Animal Care Use Committee of Peking University (Beijing, China). The rats were anesthetized with sodium pentobarbital (5 mg/100 g) and underwent unilateral subretinal injection with Aβ peptides (10 nmol/2 μl) in PBS [16]. A 5 ml Hamilton syringe was used for subretinal injection. The mice injected with Aβ peptides were compared with litter mates that were subretinally injected with only a PBS vehicle control. The retina and choroid layers of the

Table 2. Genotypic and allelic distribution of *HERPUD1* rs2217332 in patients with either nAMD or PCV and in control subjects

	nAMD (n=453)	PCV (n=174)	Control (n=425)	*P* value [†,#]	Odds Ratio[†] (95% CI)	*P* value[‡,#]	Odds Ratio[‡] (95% CI)	*P* value[§,#]	Odds Ratio[§] (95% CI)
Genotype									
GG	373 (82.34)	125 (71.84)	345 (81.18)				Reference		
AG	77 (17.00)	49 (28.16)	76 (17.88)	7.6 e-1	0.947 (0.662, 1.354)	6.4 e-3	1.78 (1.173, 2.703)	2.6 e-3	1.881 (1.2423, 2.847)
AA	3 (0.66)	0 (0.00)	4 (0.94)	6.3 e-1	0.691 (0.154, 3.110)	—	—	—	—
Allele									
G	766 (90.12)	299 (85.92)	823 (90.84)				Reference		
A	84 (9.88)	49(14.08)	83(9.16)	6.1 e-1	0.920(0.669, 1.265)	.1.3 e-2	0.615 (0.423, 0.902)	4.0 e-2	0.669(0.460, 0.981)

[†]: AMD group vs. control group. [‡]: PCV group vs. control group. [§]: PCV group vs. AMD group. [#]: P<0.05 was considered significant.

Table 3. Statistic results of the plasma concentration of Amyloid protein precursor (APP) and Amyloid beta 42 (Aβ42) in PCV patients, nAMD patients and control subjects

	Control	AMD	PCV
Number of values	90	90	90
Minimum	0.3274	0	1.164
25% Percentile	1.251	0.03199	2.74
Median	2.051	1.208	3.676
75% Percentile	2.691	2.002	9.586
Maximum	4.39	5.067	15.19
Mean	2.199	1.265	5.89
Std. Deviation	1.077	1.275	4.486
Std. Error	0.1136	0.1344	0.4729
Lower 95% CI	1.973	0.9983	4.95
Upper 95% CI	2.424	1.532	6.829

rats were collected. The few eyes with subretinal bleeding were excluded from the study.

Gene expression of chemokines and receptors in ARPE-19 cells and BN rats using the QuantiGenePlex 6.0 reagent system

Target-specific RNA molecules of ARPE-19 cells and BN rats (**Tables 3** and **4**) were detected using the QuantiGenePlex6.0 Reagent System according to the manufacturer's protocol (Affymetrix, Fremont, CA). Briefly, RNA from the cells or the retina-choroid layer lysates was captured by fluorescent microspheres. Signals of cascade amplification were detected by the Luminex 100 xMAP technology and Bio-Plex 5.0 software (Bio-Rad Laboratories, Hercules, CA). The geometric means of two housekeeping genes, PPIB (NM_011149) and HPRT1 (NM_013556) for the ARPE19 cells and PPIB (NM_022536) and HPRT1 (NM_012583) for the BN rats, in each sample were used for normalizations. Fold-changes were the relative ratios between the normalized values of the four infected groups and the values of the untreated group. In the in vitro study, ARPE19 cells from 3 different cell samples were combined for one detection, and the experiments were repeated 3 different times. In the in vivo study, the retina-choroid layer samples from 3 BN rats were combined for one detection, and the experiments were repeated 3 different times.

Statistics

All the identified polymorphisms were assessed for Hardy-Weinberg equilibrium using x^2 analyses. Allelic and genotypic distributions among different groups were compared using the x^2 test or Fisher's exact test, and logistic regression analysis was performed to identify the strongest associated SNPs in the *HERPUD1* locus (SPSS, version 16.0; SPSS Science, Chicago, IL). All experiments were repeated 3 times. Statistical analyses for QuantiGene analysis were performed using the GraphPad Software. Differences between groups were compared with the non-parametric Kruskal-Wallis test, and paired comparisons were conducted using the Mann-Whitney test. Other statistical analyses were performed with the Student's t test. Data are expressed as means ± SEM. $P < 0.05$ was considered statistically significant.

Results

Demographic distribution of the subjects and HERPUD1 SNP analysis

The demographic distribution of the subjects is shown in **Table 1**. Whole exome sequencing of *HERPUD1* was performed in 1052 individuals in this study, including 453 nAMD patients, 174 PCV patients and 425 controls. The success rates of the genotyping for *HERPUD1* rs2217332 were 100%, and the distributions of the genotypes were in Hardy-Weinberg equilibrium ($P>0.05$) for all study groups. We found significant associations with *HERPUD1* rs2217332 in both the additive model and the dominant model between the controls and PCV samples. However, we could not find a significant difference between the control and nAMD samples in any genetic model (**Table 2**).

Furthermore, we calculated the allele and genotype frequencies for rs2217332 among the nAMD, PCV and control samples (**Table 2**). A *HERPUD1* single nucleotide polymorphism (SNP) generated a significant allelic association with PCV (rs2217332, P=0.0064, odds ratio [OR] =1.78 95% confidence interval [CI] =1.173-2.703). There was a significant difference in the allelic frequencies between the nAMD and PCV cases (rs2217332, P=0.0026). The heterozygous AG genotype of rs2217332 conferred a 1.494-fold (95% CI: 1.025-2.178) increased risk for PCV when compared with the homozygous genotypes.

HERPUD1 was highly expressed in the membranes of PCV patients as detected by immunohistochemistry

To evaluate the expression of HERPUD1 in the in vivo tissue, the human PCV subretinal mem-

Table 4. Expression of chemokines and receptors in ARPE19 cells treated with amyloid beta agents

Gene Name*	Accession#	Treatment time	Ab (1-40)		Ab (1-42)		OAb (1-40)		OAb (1-42)	
			MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM
VEGFA	NM_003376	30 min	0.98	0.19	1.13	0.17	0.95	0.13	1.06	0.29
		1 h	0.99	0.23	1.12	0.53	0.98	0.11	0.93	0.54
		3 h	1.49	0.29	1.65	0.13	1.66	0.02	1.46	0.13
		6 h	1.26	0.08	1.33	0.17	1.42	0.27	1.37	0.18
		12 h	1.13	0.14	1.49	0.21	1.41	0.16	3.60	0.18
		24 h	1.16	0.71	1.05	0.06	1.18	0.34	19.29	1.14
FLT	NM_002019	30 min	1.16	0.01	1.00	0.01	0.98	0.01	1.09	0.04
		1 h	0.98	0.11	0.80	0.31	1.02	0.01	0.97	0.01
		3 h	0.75	0.03	1.19	0.22	1.19	0.02	0.92	0.04
		6 h	1.19	0.01	0.98	0.08	1.02	0.01	1.17	0.01
		12 h	0.83	0.41	1.24	0.23	1.02	0.02	0.92	0.01
		24 h	0.99	0.11	0.88	0.02	0.81	0.01	1.12	0.01
KDR	NM_002253	30 min	1.09	0.04	1.07	0.01	1.09	0.01	1.17	0.02
		1 h	1.11	0.03	1.03	0.27	1.18	0.01	1.06	0.07
		3 h	1.04	0.08	0.96	0.02	0.83	0.12	1.04	0.01
		6 h	1.31	0.01	0.85	0.03	1.31	0.04	1.16	0.02
		12 h	1.00	0.15	1.00	0.03	0.77	0.01	1.06	0.01
		24 h	0.54	0.11	0.67	0.01	0.67	0.06	3.42	0.11
TGF b	NM_000660	30 min	1.13	0.28	1.05	0.35	1.16	0.25	1.15	0.23
		1 h	1.15	0.36	1.12	0.25	1.07	0.09	1.03	0.18
		3 h	1.37	0.30	1.31	0.24	1.21	0.13	1.17	0.29
		6 h	1.30	0.37	1.46	0.11	1.54	0.27	1.45	0.27
		12 h	1.32	0.45	1.33	0.36	1.29	0.28	1.90	0.40
		24 h	1.34	0.39	1.49	0.21	1.25	0.18	5.76	0.25
FGF	NM_002006	30 min	1.22	0.14	1.06	0.26	1.14	0.15	1.04	0.19
		1 h	1.08	0.39	1.14	0.24	1.07	0.16	1.07	0.18
		3 h	1.57	0.28	1.40	0.16	1.32	0.12	1.28	0.15
		6 h	1.63	0.43	1.83	0.22	1.79	0.13	1.53	0.25
		12 h	1.67	0.42	1.69	0.12	1.61	0.29	1.63	0.17
		24 h	1.17	0.38	1.29	0.20	1.26	0.19	1.75	0.25
PlGF	NM_002632	30 min	1.25	0.11	0.95	0.02	0.97	0.01	1.34	0.07
		1 h	1.26	0.08	1.50	0.08	1.18	0.01	1.13	0.07
		3 h	1.37	0.01	1.72	0.02	1.45	0.01	1.30	0.02
		6 h	1.04	0.07	1.58	0.01	1.20	0.11	1.16	0.02
		12 h	0.93	0.22	1.44	0.04	1.29	0.01	1.74	0.05
		24 h	1.63	0.10	1.17	0.05	1.52	0.04	27.33	0.03
CXCL12	NM_000609	30 min	0.92	0.20	1.22	0.11	1.22	0.01	1.37	0.04
		1 h	0.72	0.10	0.95	0.16	1.26	0.07	1.13	0.09
		3 h	0.96	0.02	1.22	0.47	1.51	0.06	1.91	0.15
		6 h	0.93	0.41	1.37	0.21	0.87	0.16	2.46	0.24
		12 h	0.89	0.07	1.77	0.31	1.12	0.01	2.34	0.10
		24 h	0.75	0.13	0.90	0.03	0.66	0.11	12.52	0.36
MMP1	NM_002421	30 min	0.58	0.04	0.79	0.01	0.41	0.01	0.72	0.01
		1 h	1.11	0.01	0.60	0.82	0.63	0.01	0.73	0.08
		3 h	1.19	0.10	0.71	0.03	0.64	0.01	0.64	0.03
		6 h	1.65	0.03	1.14	0.03	3.46	0.03	2.78	0.22

		12 h	1.39	0.52	1.69	0.03	2.78	0.02	10.89	0.42
		24 h	5.47	0.64	3.57	0.35	5.92	0.84	21.81	1.13
MMP2	NM_004530	30 min	1.14	0.15	1.10	0.05	1.12	0.18	1.06	0.09
		1 h	1.03	0.10	1.04	0.10	1.01	0.17	0.93	0.15
		3 h	1.38	0.10	1.20	0.14	1.15	0.30	1.09	0.17
		6 h	1.36	0.17	1.50	0.14	1.43	0.28	1.29	0.24
		12 h	1.57	0.21	1.55	0.21	1.50	0.13	1.55	0.20
		24 h	1.24	0.11	1.41	0.11	1.22	0.11	1.57	0.18
MMP3	NM_002422	30 min	1.01	0.12	1.06	0.23	1.14	0.11	1.34	0.21
		1 h	1.17	0.21	1.35	0.17	1.52	0.05	1.41	0.11
		3 h	1.43	0.07	1.53	0.17	1.26	0.03	1.71	0.11
		6 h	1.51	0.01	2.05	0.12	1.53	0.11	1.75	0.01
		12 h	1.52	0.14	2.31	0.21	1.76	0.12	4.18	0.48
		24 h	1.37	0.11	2.74	0.13	1.96	0.11	6.25	0.63
MMP9	NM_004994	30 min	0.95	0.04	1.05	0.04	1.35	0.01	1.01	0.03
		1 h	1.40	0.09	1.59	0.27	1.32	0.21	0.80	0.04
		3 h	0.90	0.08	1.40	0.01	1.55	0.11	1.51	0.11
		6 h	1.70	0.02	1.61	0.06	1.77	0.06	1.61	0.07
		12 h	1.24	0.07	1.20	0.22	1.61	0.03	2.79	0.11
		24 h	2.90	0.23	1.84	0.11	1.94	0.01	10.91	0.36

*VEGFA: vascular endothelial growth factor alpha; FLT: fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor 1); KDR: kinase insert domain receptor (vascular endothelial growth factor receptor 2); TGFb: transforming growth factor beta; FGF: fibroblast growth factor; PlGF: placental growth factor; CXCL12: Chemokine (C-X-C motif) ligand 12 (Stromal cell-derived factor-1, SDF-1); MMP1: matrix metalloproteinase-1; MMP2: matrix metalloproteinase-2; MMP3: matrix metalloproteinase-3; MMP9: matrix metalloproteinase-9. Expression values are generated from microarray data and are displayed as ratios relative to untreated cells. #: number; "accession#" means "accession number".

branes were surgically excised from patients after proper consent and approval. The membrane we excised was a fibrotic membrane tissue, whereas was not a vascular membrane. Because only in the condition of vascular tissue rupture which leading to retinal hemorrhage and fibrous membrane proliferation, the patients will underwent the surgical treatment. As shown in **Figure 1**, HERPUD1 is highly expressed and localized throughout the subretinal membranes from patients with PCV. No visible staining of HERPUD1 was observed in the negative control membranes. These results indicate that HERPUD1 is a component of PCV subretinal membranes.

Amyloid beta 42 (Ab42) was significantly higher in the PCV patients than nAMD and control subjects

As shown in **Figure 2** and **Table 3**, Box plots show that the plasma concentration of Aβ42 was much higher in the PCV patients, and the level was statistically significant higher compared to the nAMD patients and control subjects (**Figure 2A**). However, the plasma APP level in the three groups had no significant differences (**Figure 2B**). These results indicated that Aβ42 may involve in the occurrence and development of PCV in Chinese patients.

Amyloid protein precursor (APP) was upregulated in ARPE19 cells carrying a HERPUD1 overexpression plasmid

The relationship between HERPUD1 and Aβhas been controversial in previously published articles [13]. In this study, real-time PCR was used to evaluate the relationship between HERPUD1 and Aβ. **Figure 3** showed that APP gene expression significantly increased in ARPE19 cells that were treated with a *HERPUD1* overexpression plasmid when compared with untreated cells, leading us to further investigate the function of *HERPUD1* and the resulting upregulation of Aβ in ARPE19 cells.

Upregulation of angiogenic factors and matrix metalloproteinases in ARPE19 cells treated with different Ab agents

In our in vitro and in vivo studies, we used different kinds of Aβ to stimulate ARPE19 cells and conducted subretinal injections in a rat

Table 5. Expression of chemokines and receptors in BN rats under subretinal treatment with amyloid beta agents

Gene Name*	Accession#	Treatment time	Ab (1-40)		Ab (1-42)		OAb (1-40)		OAb (1-42)	
			MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM
VEGFA	NM_031836	Day 1	1.18	0.07	1.13	0.10	0.97	0.02	1.05	0.12
		Day 2	1.10	0.09	0.93	0.09	1.11	0.15	1.24	0.11
		Day 3	1.23	0.14	1.22	0.20	1.21	0.10	1.52	0.13
FLT	NM_019306	Day 1	1.22	0.13	1.34	0.14	1.11	0.02	1.04	0.26
		Day 2	1.33	0.22	1.35	0.38	1.16	0.17	1.31	0.19
		Day 3	1.13	0.16	1.20	0.19	1.08	0.18	0.96	0.20
KDR	NM_013062	Day 1	0.74	0.03	0.71	0.30	0.78	0.01	1.10	0.19
		Day 2	0.89	0.03	1.08	0.07	1.03	0.16	1.01	0.00
		Day 3	0.88	0.14	0.81	0.17	0.91	0.12	0.86	0.13
TGF b	NM_021578	Day 1	1.76	0.06	1.34	0.07	1.11	0.00	1.39	0.19
		Day 2	0.91	0.16	0.77	0.11	1.15	0.05	1.36	0.16
		Day 3	1.02	0.08	0.83	0.00	1.03	0.17	1.54	0.15
FGF	NM_019305	Day 1	1.43	0.06	1.35	0.07	1.09	0.09	1.01	0.23
		Day 2	1.28	0.16	1.24	0.18	1.17	0.29	1.26	0.22
		Day 3	1.36	0.11	0.91	0.18	1.08	0.18	1.55	0.14
PlGF	NM_053595	Day 1	1.26	0.02	1.19	0.04	0.86	0.01	0.68	0.25
		Day 2	0.95	0.18	1.21	0.18	1.05	0.12	1.26	0.23
		Day 3	1.02	0.17	1.09	0.25	0.75	0.19	1.81	0.12
CXCL12	NM_022177	Day 1	1.04	0.06	0.99	0.00	0.80	0.03	0.87	0.17
		Day 2	1.20	0.27	1.02	0.07	1.19	0.21	1.40	0.15
		Day 3	1.22	0.10	1.25	0.12	1.22	0.20	1.70	0.13
MMP1	NM_001134530	Day 1	0.78	0.04	1.04	0.09	0.85	0.02	0.61	0.05
		Day 2	0.94	0.17	1.17	0.10	0.69	0.08	1.26	0.21
		Day 3	0.57	0.13	1.47	0.15	1.52	0.24	1.57	0.12
MMP2	NM_031054	Day 1	1.86	0.04	2.02	0.09	1.23	0.00	0.80	0.12
		Day 2	1.39	0.23	1.58	0.13	1.50	0.18	1.28	0.05
		Day 3	1.25	0.21	0.99	0.28	1.21	0.11	1.43	0.13
MMP3	NM_133523	Day 1	5.73	0.18	13.48	0.77	10.67	0.53	3.99	0.29
		Day 2	6.24	0.29	11.37	0.79	0.75	0.01	5.92	0.13
		Day 3	1.12	0.09	0.69	0.04	0.95	0.10	1.38	0.16
MMP9	NM_031055	Day 1	1.46	0.13	1.59	0.18	1.16	0.25	3.18	0.20
		Day 2	0.60	0.02	0.75	0.35	0.64	0.27	0.91	0.06
		Day 3	0.80	0.30	1.10	0.00	1.15	0.24	1.07	0.41

*The gene name symbols are the same as in **Table 4**. Expression values are generated from microarray data and are displayed as ratios relative to PBS-treated rats. #: number; "accession#" means "accession number".

model. The types of Aβ used included Aβ(1-40), Aβ(1-42), oligo-Aβ(1-40), oligo-Aβ(1-40) and the negative reverse controls Aβ(40-1), Aβ(42-1), oligo-Aβ(40-1) and oligo-Aβ(42-1), as previously described [16].

In the present study, the expression of several angiogenic cytokines, matrix metalloproteinases and VEGF receptors were detected using the QuantiGenePlex6.0 Reagent System, and the results are shown in **Table 4** and **Figure 4.** In this QuantiGene assay, angiogenic factors were upregulated at 3 h time points, including VEGFA (vascular endothelial growth factor alpha), TGF-β (transforming growth factor beta), FGF (fibroblast growth factor), PlGF (placental growth factor), CXCL12 (Chemokine (C-X-C motif) ligand 12) and matrix metalloproteinases 1, 2, 3, 9 (MMP1, 2, 3, 9), especially in the oligo-Aβ(142)-treated group. The VEGF receptors FLT (fms-related tyrosine kinase 1, VEGFR1) and KDR (kinase insert domain receptor, VEGFR2) did not show any significant changes, except for the expression of KDR in the oligo-Aβ(142)-

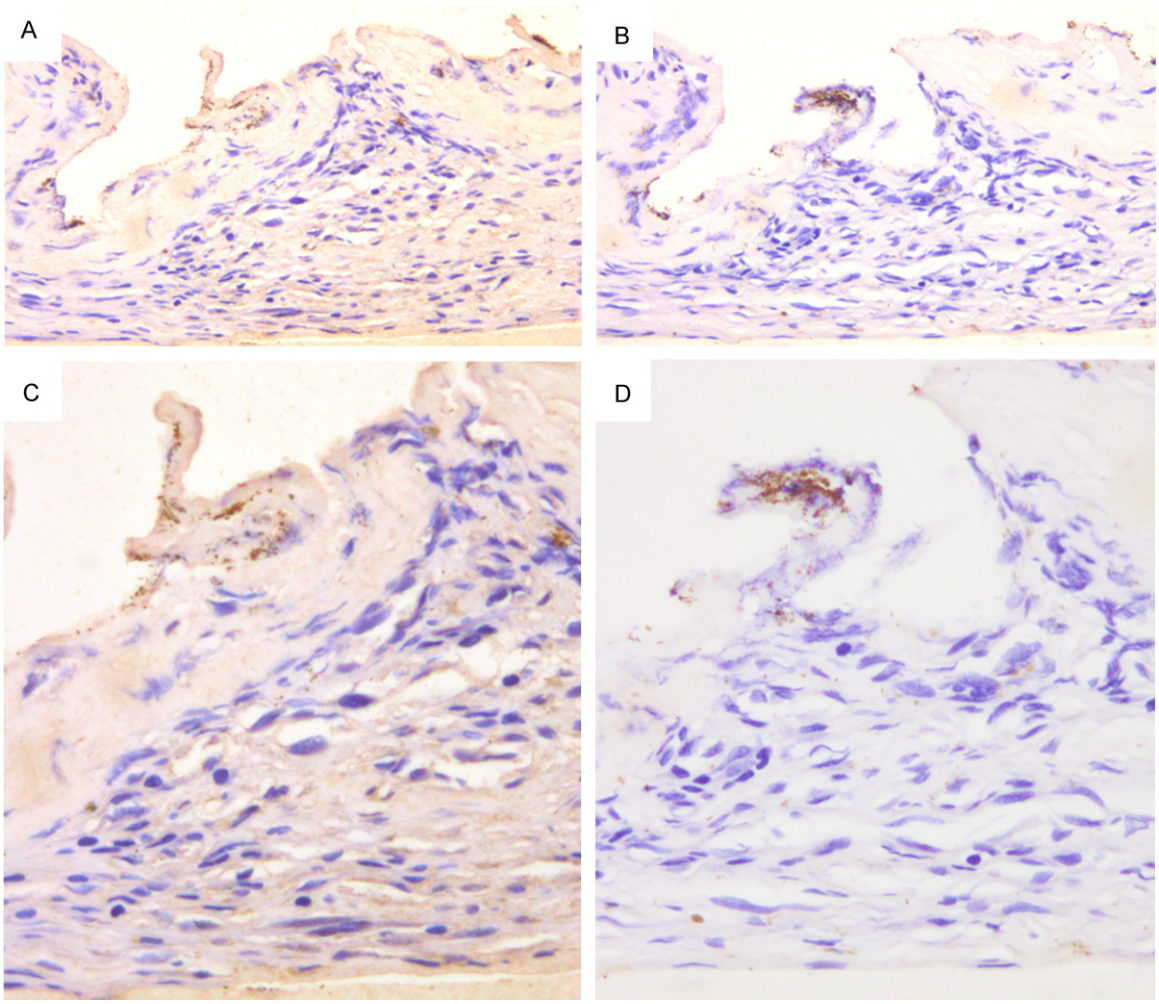

Figure 1. Immunohistochemistry assay for HERPUD1 in the subretinal membranes of PCV patients. HERPUD1 is highly expressed and localized throughout the PCV patient membranes (A & C) compared to the negative control (B & D). (A and B: 200X; C and D: 400×).

treated group at the 24 h time point. There were no statistically significant changes between the negative reverse Aβ controls and the treated groups (Aβ(40-1), Aβ(42-1), oligo-Aβ(40-1) and oligo-Aβ(42-1)) in the expression of either the cytokines or receptors when compared with untreated cells (data not shown).

*Upregulation of angiogenic factors and matrix metalloproteinases in BN rats that were subretinally injected with different A*b *agents*

Similar to our tests on ARPE19 cells that were treated with Aβ agents, the expression levels of angiogenic cytokines, matrix metalloproteinases and VEGF receptors in BN rats that were subretinally injected with Aβ were detected using the QuantiGenePlex6.0 Reagent System. The results are shown in **Table 5** and **Figure 5**. In this in vivo QuantiGene assay, the angiogenic cytokines were upregulated with increased treatment time in the oligo-Aβ(1-42)-treated group, while there were no significant changes in the other Aβ-treated groups. For the matrix metalloproteinase genes, MMP1 became more upregulated with time, while MMP2, 3 and 9 were all upregulated on day 1 of the treatment, though they returned to normal levels by day 3. Similar to the results for the ARPE19 cells, there were no significant changes in either the FLT or the KDR levels. There were no statistically significant changes in the negative reverse Aβ-treated groups (Aβ(40-1), Aβ(42-1), oligo-Aβ(40-1) and oligo-Aβ(42-1)) for these cytokines (data not shown).

Discussion

PCV was first defined by Yannuzzi et al. as idiopathic polypoidal choroidal vasculopathy in

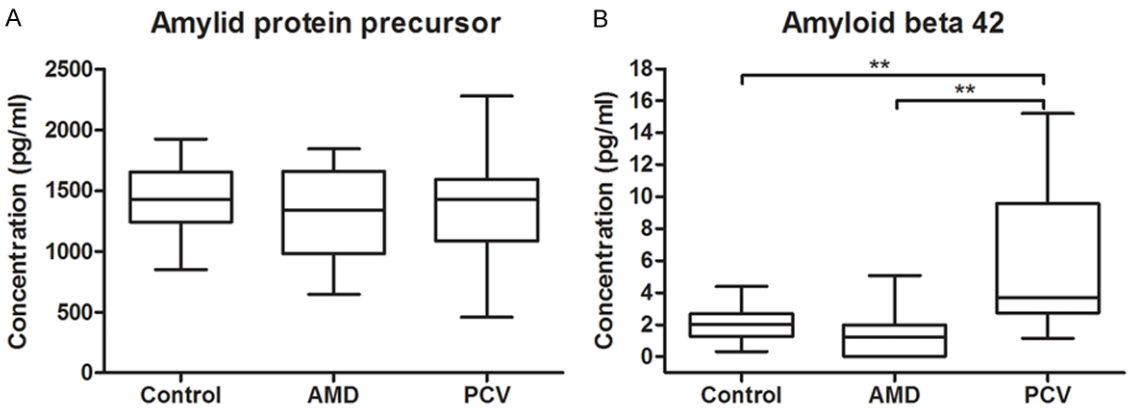

Figure 2. Amyloid beta 42 (Aβ42) was significantly higher in the PCV patients than nAMD and control subjects. Box plots show that the plasma concentration of Aβ42 in the PCV patients had the highest level, and the level was statistically significant compared with that of nAMD patients and control subjects (A). However, the plasma APP level in the three groups had no significant differences (B). **P<0.01, ***P<0.001.

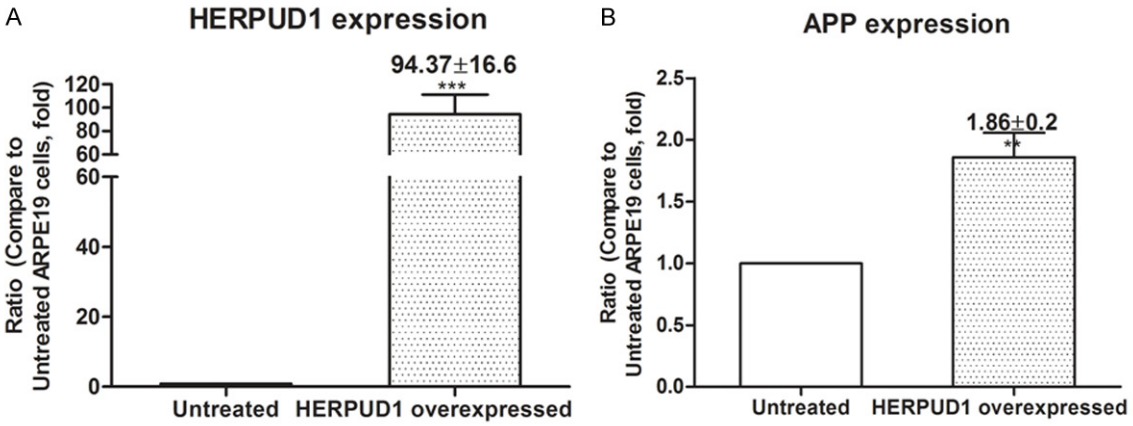

Figure 3. Q-PCR upregulation of amyloid beta in HERPUD1-overexpressing RPE cells. Increases in APP gene expression (B) in HERPUD1-overexpressing ARPE19 cells (A) by real-time PCR. GAPDH was used as the internal control for sample normalization. Each diagram represents 5 independent experiments. The data are represented as means ± SEM. Data are expressed as a percentage of the untreated control. **P<0.01, ***P<0.001.

1990 [19]. Over the last two decades, our understanding of this disease has progressed rapidly. Its prevalence is quite distinct, as reported by different groups, with an approximate prevalence of 4.0% to 9.8% in Caucasian patients [20-23] and substantially higher prevalence rates between 23.9% and 54.7% in Asian patients [24-28]. Because the pathological and mechanisms of PCV are still unclear, PCV is categorized by some experts as a subtype of nAMD [28, 29], but others consider PCV to be a different disease entity [5]. Clinically, PCV is divided into three types, quiescent, exudative and hemorrhagic categories, and it is characterized by hyalinization and either peripapillary macular or peripheral sub-RPE polypoidal dilatations of a network of abnormal choroidal vessels [4, 19, 30]. Given the differences in the clinical presentation between PCV and nAMD, several studies have focused on identifying other genetic components of these two entities to determine whether these two different phenotypes can be attributed to differences in genetic components, which may reveal different underlying pathogenic mechanisms [3, 4].

HERPUD1, which plays a role in both the unfolded protein response (UPR) and endoplasmic reticulum (ER)-associated protein degradation (ERAD), is one of the novel genes we found that is related to PCV but not to nAMD among a number of Chinese individuals (**Tables 1** and **2**). Additionally, we found that HERPUD1 was

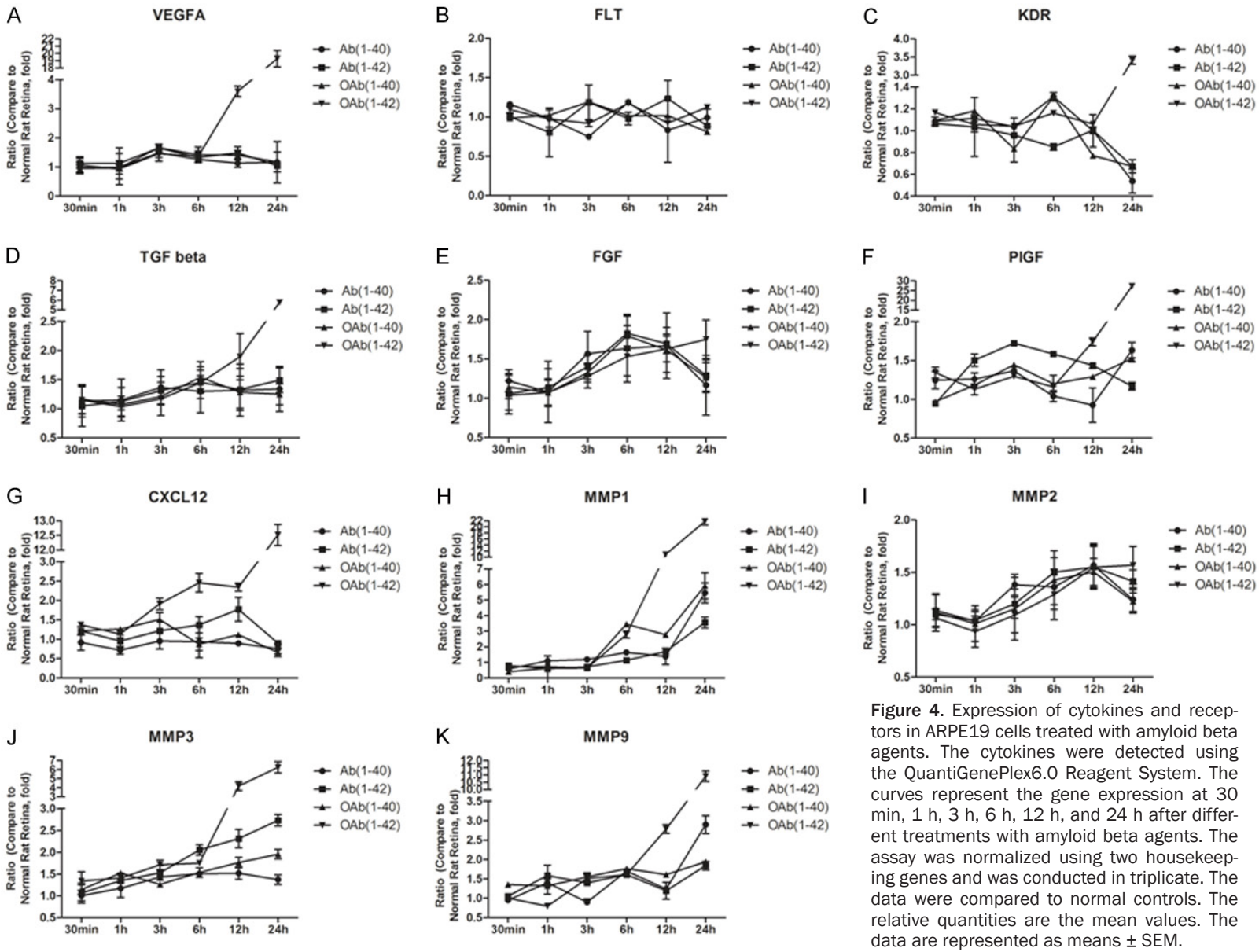

Figure 4. Expression of cytokines and receptors in ARPE19 cells treated with amyloid beta agents. The cytokines were detected using the QuantiGenePlex6.0 Reagent System. The curves represent the gene expression at 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h after different treatments with amyloid beta agents. The assay was normalized using two housekeeping genes and was conducted in triplicate. The data were compared to normal controls. The relative quantities are the mean values. The data are represented as means ± SEM.

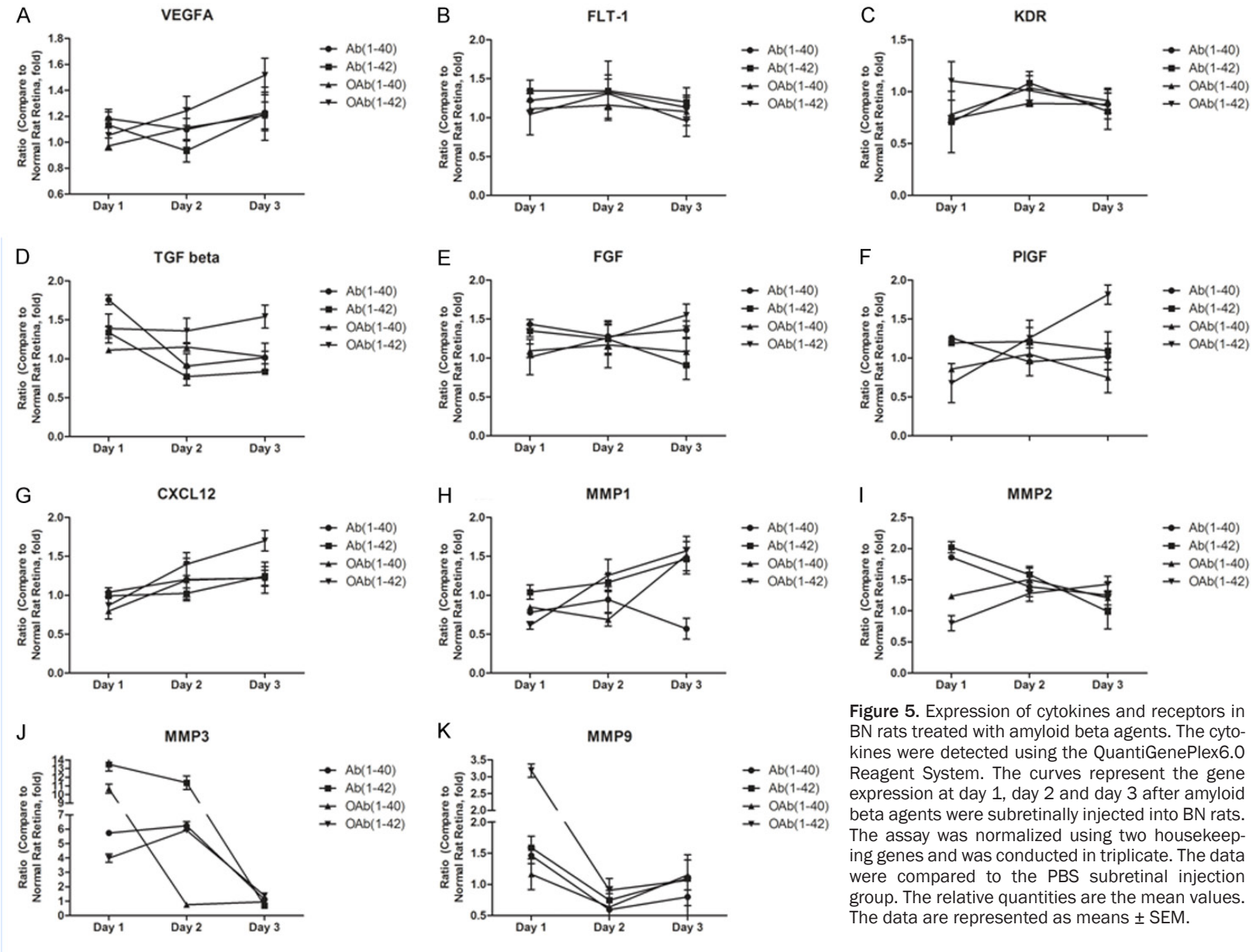

Figure 5. Expression of cytokines and receptors in BN rats treated with amyloid beta agents. The cytokines were detected using the QuantiGenePlex6.0 Reagent System. The curves represent the gene expression at day 1, day 2 and day 3 after amyloid beta agents were subretinally injected into BN rats. The assay was normalized using two housekeeping genes and was conducted in triplicate. The data were compared to the PBS subretinal injection group. The relative quantities are the mean values. The data are represented as means ± SEM.

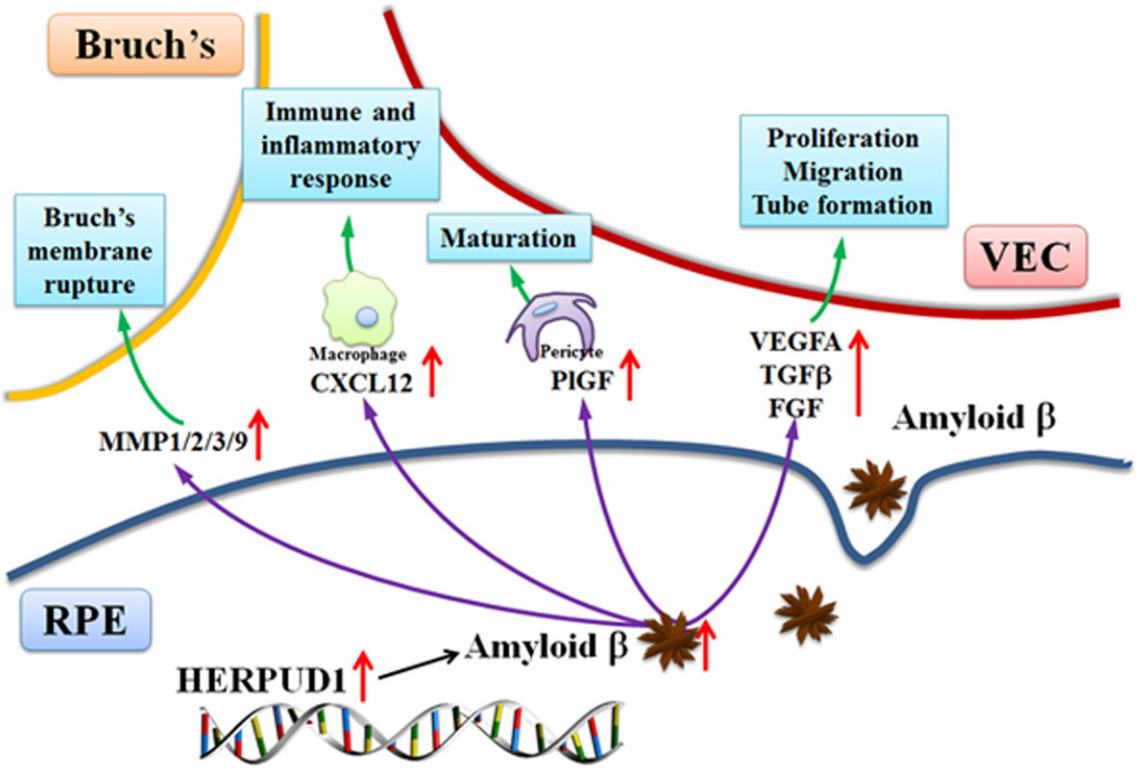

Figure 6. Schematic summary of the role of HERPUD1 and Aβ in RPE, VEC and Bruch's membranes. HERPUD1 overexpression induces the upregulation of amyloid beta (A). The overexpressed A increased the expression of VEGFA, TGF beta, and FGF, induced the proliferation, migration and tube formation effects of vascular endothelium cells (VEC), increased the expression of PlGF, caused the maturation of the pericyte, increased the expression of CXCL12, induced macrophage assembly, which induced the immune and inflammatory response, increase the expression of MMP1, MMP2, MMP3 and MMP9, and led to the rupture of Bruch's membrane.

highly expressed in the surgically excised membranes of PCV patients (**Figure 2**), which further supports the idea that HERPUD1 may be related to PCV formation or development. As investigated in other articles, the Herp protein, which is encoded by the *HERPUD1* gene, was reported to play a role in maintaining ER homeostasis by facilitating the proteasome-mediated degradation of ER-resident Ca^{2+} release channels [31, 32]. Additionally, HERPUD1 was reported to increase the level of Aβ and was found to be expressed in both neurons and vascular smooth muscle cells by immunohistochemical analysis [24]. In our study, we found that the plasma concentration of Aβ42 protein in PCV patients was significantly higher compared to nAMD patients and normal controls. And overexpression of the *HERPUD1* gene upregulated the expression of the APP gene in RPE cells, which lay the theoretical foundation for future studies of the function of HERPUD1 and Aβ in PCV.

Dentchev T et al. found that Aβ was localized to a subset of drusen in 4 out of 9 AMD eyes [33]. Drusen are extracellular deposits that build up between Bruch's membrane and the retinal pigment epithelium (RPE) layer of the posterior part of the eye [34]. The presence of larger and numerous drusen in the macula is a common early sign of AMD, and present in PCV patients [1, 3, 35]. Based on the location of drusen between the RPE and choriocapillaris, it is possible that drusen deprive the RPE and photoreceptor cells of oxygen and nutrients, causing atrophy of the RPE, disturbance of the transepithelial barrier and photoreceptor death [36, 37]. Previous studies have shown that Aβ(1-42), especially oligo-Aβ(1-42), alters the structure and function of RPE cells, including reducing the mitochondrial redox potential, increasing the production of reactive oxygen species (ROS) and inducing the disorganization of the cytoskeleton. However, Aβ(1-42) did not induce apoptosis in RPE cell cultures in vitro [16].

Additionally, the study showed that Aβ subretinal injections can decrease RPE expression in tight junctions and decrease the number of photoreceptors in vivo [16]. To study the dysfunction of RPE and neovascularization under Aβ treatment, we also conducted in vitro and in vivo studies that focused on the angiogenic factors, chemokine and matrix metalloproteinases that induce the rupture of Bruch's membrane and facilitate the formation of neovascularization.

From our in vitro and in vivo studies (**Tables 4**, **5**; **Figures 4** and **5**), we verified that oligo Aβ(1-42) is the pathological form of Aβ and is responsible for the change in cytokines rather than the other forms of Aβ. We have shown that VEGFA, FGF, TGFβ, PlGF and CXCL12 were upregulated, especially in the oligo-Aβ(1-42)-treated groups. To date, VEGFA (or $VEGF_{165}$) is considered to be the most potent angiogenic factor [38]. FGF and TGFβ play important roles in vascular generation and fibrosis of endothelial cells (ECs), RPE cells and membrane formation [39, 40]. PlGF is important for the maturation of vascular pericytes, which help the maturation of blood vessels [41, 42]. CXCL12 (also known as SDF-1) is a crucial chemokine that is responsible for recruiting macrophages and also plays important roles in neovascularization formation [43, 44]. The above results agree with other studies and suggest that Aβ accumulation affects the balance between VEGF and PEDF in RPE cells, which may be a key contributor to the development of CNV in AMD [45]. Besides the angiogenic factors, MMP1, 2, 3, and 9 were also upregulated in the Aβ-treated group. Matrix metalloproteinases (MMPs) belong to a larger family of proteases that are capable of degrading many extracellular matrix proteins and can also process a number of bioactive molecules [46]. MMPs are also thought to play a major role in cell behaviors such as cell proliferation, migration, adhesion, differentiation, angiogenesis, apoptosis and host defense [47, 48]. During the formation of abnormal retina vessels, the degeneration of Bruch's membrane is a necessary step for vessels ingrowth [49-51]. In our study, the upregulation of MMPs provided the important steps for abnormal retina vessels formation. Here, the expression of the VEGF receptors FLT and KDR did not change significantly, with one exception: KDR was upregulated in the oligo-Aβ(142)-treated group at the 24 h time point. This result could be because the main function of RPE cells is to mediate internal environment changes and induce functional and structural changes to vascular endothelial cells but not to the RPE cell themselves.

Bruban et al. reported that RPE cytoskeleton damage could be observed as early as day 3 post-injection and that there was no difference in damage between day 3 and day 7 or day 14. Additionally, Liu R.T. et al. reported that the cytokine mRNA levels diminished with time and significantly decreased after day 4 in a mouse model of intravitreal injection. Therefore, we chose a 3-day time-course for observation[9,14]. In our in vivo study, the data were similar to the data from our in vitro studies, that is the angiogenic factors and chemokine were upregulated with time, especially in the oligo Aβ(1-42)-treated group. The MMPs were highly expressed on day 1 and decreased over time; the changes in the expression pattern of the MMPs require further investigation.

Molecular angiogenesis is a continuous process that involves several steps [52-54]: (1) triggering of an angiogenic switch by a focal molecular stimulus, e.g., hypoxia or ischemia; (2) stimulation of angiogenic growth factor receptors in ECs, e.g., VEGFR; (3) proteolysis of the supporting basal membrane of the ECs, e.g., MMPs; (4) EC proliferation and migration toward the angiogenic stimulus; (5) invasion and proteolysis of the extracellular matrix (ECM) to facilitate EC invasion toward the target, e.g., MMPs; (6) vessel survival/stabilization by several molecules, e.g., TGFβ and VEGF; (7) pericytes/smooth muscle cell recruitment to surround the immature vessel for further maturation and stabilization, e.g., PlGF; and (8) closing of the arteriovenous loops, e.g., VEGF and angiostatin [51]. It is well known that all those cytokines are involved in the abnormal angiogenesis process. However, it remains unknown whether those cytokines play a role in the development of PCV. One possibility is all those cytokines are upreguleted in both PCV and CNV conditions but with different level. When they are highly upregulated, CNV formed; while when they are moderately or slightly elevated, PCV formed. But this hypothesis needs to be verified in vitreous body humor in further studies.

Taken together, our results strongly suggest that *HERPUD1* is highly associated with PCV but not nAMD patients. This association could be a result of the upregulation of Aβ, which further stimulates RPE cells to secret angiogenic factors, chemokine, and MMPs and facilitates the formation and development of abnormal vessels in PCV (**Figure 6**). Analysis of *HERPUD1* activity in PCV animal models would therefore be of interest for future studies. Though the number of our validation samples was limited, we did find that *HERPUD1* was associated with PCV but not with nAMD; thus, nAMD and PCV patients may have different genetic backgrounds. There may be several explanations for these results. One possibility is that the function of *HERPUD1* may be more important in the development of PCV than in nAMD. Alternatively, the sample numbers for the nAMD and PCV groups may not have been large enough to detect the differences between cases and controls.

Further investigation is warranted to verify the relationship between HERPUD1 and PCV. We are collecting more genetic and plasma samples to verify our results in the future studies. More PCV vascular membrane samples are in great need for the etiology studies of PCV. And more gene function studies need to be performed which may better explain the pathogenesis mechanisms of HERPUD1 and PCV.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 Program, 2011CB510200), Beijing Nova Program (Z131102000413004), the National Natural Science Foundation of China Grant (81470651, 81200690, 81100666) and the Peking University People's Hospital Research and Development Fund (RDB2012-24). The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Disclosure of conflict of interest

None.

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