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Subtilisin-like Proprotein Convertase Expression, Localization, and Activity in the Human Retina and Optic Nerve Head

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Abstract

Purpose—Subtilisin-like proprotein convertases (SPCs) are a family of calcium-dependent cleavage enzymes that act on dibasic sites of various peptide/protein substrates. The purpose of this study was to investigate the expression, localization, and activity of SPCs in the human retina and optic nerve head.

Methods—mRNA expression of the SPC family in the human retina and optic nerve head tissues was evaluated by quantitative reverse transcription polymerase chain reaction (QRT-PCR). Double immunofluorescence staining was performed on paraffin-embedded human posterior sections to localize SPC family members. Western blot analysis was used to identify PACE4 isoform expression within the optic nerve head and retina. In addition, a fluorogenic SPC substrate-based assay was used to elucidate SPC enzyme activity within human retina and optic nerve head (ONH) tissues.

Results—QPCR results indicated that PC1 and PC2 were expressed 4.1- and 5.7-fold higher in retina compared to optic nerve head, whereas PACE4 was expressed 4.1-fold higher in the ONH. PC1 and PC2 were localized primarily in neuronal cells, whereas PACE4 and PC5 were limited to the glia of the retina and optic nerve head. SPC activity in ONH lysate was significantly higher than that of retinal lysate; however, when an SPC inhibitor was added, activity in ONH decreased more than that in retina.

Conclusions—These results indicate that the SPCs are expressed in distinct patterns throughout the human retina and ONH. PC1 and PC2 were primarily expressed in neurons, whereas PACE4 appeared to be largely restricted to glia. Thus, elevated PACE4 may modulate the bioactivity of proteins secreted in the ONH and retina.

Optic nerve head (ONH) excavation, cupping, and subsequent extracellular matrix changes are hallmarks of damage observed in primary open-angle glaucoma (POAG).[1–3] Although elevated intraocular pressure (IOP) is a well-characterized risk factor for retinal ganglion

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cell (RGC) death, the mechanisms responsible for ONH morphologic and biochemical changes are poorly understood. Among the hypothesized mechanisms are mechanical stress to the ONH due to high IOP, ischemia and subsequent reperfusion, and biochemical changes due to differential activity of growth factors produced by and acting on the cells of the glaucomatous ONH.[4–8] Although the actual mechanics of ONH changes have yet to be elucidated, it is apparent that the cell and molecular activity of the cells localized within the ONH (e.g., ONH astrocytes and lamina cribrosa cells), have profound effects on the extracellular environment and subsequent RGC survival.

The subtilisin-like proprotein convertases (SPC/PCs) are a family of Ca^{2+}-dependent serine endoproteases responsible for prodomain cleavage and subsequent protein maturation. The SPC family consists of furin, PC1/3 (referred to herein as SPC1), PC2, PC4, PACE4, PC5/6A (referred herein as PC5), and PC7. SPCs enzymatically process peptide substrates at single or paired dibasic residues.[9,10] Structurally, the SPC family members all possess a signal peptide, prodomain, subtilisin-like catalytic domain, and a homo-B domain (P domain).[11,12] PC1 and PC2 are expressed in neuroepithelium, and PC4 expression is limited to reproductive tissues.[13–18] The remaining PCs are expressed to a certain degree in all tissues.[9,19] However, expression patterns are unique to each tissue and have not been fully characterized.[9,15,20–22] Furthermore, spatiotemporal analysis in animal models has demonstrated unique expression patterns in development.[23–27] Although there is a degree of overlap of substrate processing within the family, each SPC processes different substrates with different affinities.[10,28–32] The unique expression patterns of the SPCs within each cell type and tissue may confer unique protein and peptide processing capabilities.

PC1, PC2, and PC7 are known to remain primarily active within the trans-Golgi network (TGN) and within secretory granules in the cytosol.[9,16,33,34] Furin predominantly localizes to the TGN, but can cycle to the cell surface.[35] PC5 and PACE4 are primarily secreted, and bind to extracellular heparan sulfate proteoglycans (HSPG) via their cysteine-rich domain.[36,37] PACE4 expression can be increased in hypoxia, a hallmark of tumorigenesis, and one of the hypothesized causal mechanisms for glial cell activation in CNS disorders including glaucoma.[38,39] Recent studies suggest that increased SPC activity may induce extracellular matrix changes in multiple cell lines.[40–45] Extracellular matrix remodeling may occur by directly altering the maturity and conformation of the extracellular adhesion molecules, and by altering growth factor as well as matrix metalloproteinase bioactivity.

Aside from cancer biology, literature concerning SPC expression and activity within other pathologic conditions is limited. However, SPC-mediated processing may influence a wide variety of acute and chronic diseases. We hypothesize that the SPCs are crucial for the maintenance of protein maturation and turnover in the retina and ONH. Furthermore, we believe that PACE4 is an important intermediate to the processing of proteins secreted into the extracellular milieu by ONH glia and that modulation of its bioactivity may have important consequences with respect to growth factor processing and maturation as well as extracellular matrix deposition in the ONH. In this study, we characterized for the first time the mRNA expression, protein localization, and enzymatic activity of SPC family members within the human retina and ONH.

Materials and Methods

Human Tissue Samples

Tissue samples were acquired through the Florida Lions Eye and Tissue Bank (Tampa, FL), Alcon Research, Ltd. (Fort Worth, TX), or the Willed Body Program at the University of North Texas Health Science Center. All samples analyzed were obtained after a postmortem interval of 8 hours or less. Samples for mRNA analysis were either flash frozen in an isopentane/dry-ice slurry, or transported in an RNA stabilization solution (RNAlater; Applied Biosystems, Inc. [ABI], Foster City, CA). Samples used for immunohistochemistry were fixed in 4% paraformaldehyde and paraffin embedded before sectioning. The donor demographic characteristics for the samples analyzed are presented in Table 1. Human tissues were obtained in compliance with the Declaration of Helsinki.

Total RNA Extraction and cDNA Synthesis

Human retina and ONH tissues were homogenized in extraction reagent (TRIzol; Invitrogen, Carlsbad, CA), and extracted according to the manufacturer's instructions. Isolated total mRNA was treated with DNase (Turbo-Dnase I; ABI). Total RNA (1.5 μg) was used to synthesize cDNA (High-Capacity cDNA Synthesis Kit; ABI), containing RNase inhibitor per the manufacturer's instructions.

Primer Design

Primers for furin, PC1, PC2, PACE4, PC5, and PC7, and TBP were designed by using Primer3 (http://frodo.wi.mit.edu/ provided in the public domain by the Whitehead Institute, MIT, Cambridge, MA) with 60°C annealing temperatures. Primer pairs were tested by using In-Silico PCR (http://genome.ucsc.edu/ provided in the public domain by the University of California at Santa Cruz) to verify negative genomic amplification. Validated amplicons were subsequently submitted to BLAST (www.ncbi.nlm.nih.gov/blast/ provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) to ensure specificity. Designed primer pairs are listed, along with the expected product size in Table 2.

Quantitative RT-PCR

Real-time PCR was performed as described previously.[46] Briefly, 2.5 μL of cDNA was used in a reaction consisting of 1.5 units per reaction of antibody-bound *Taq* enzyme (Hot Start; Sigma-Aldrich, St. Louis, MO), 1× PCR buffer, 1.5 mM $MgCl_2$, 200 nM dNTP mix, 100 to 300 nM respective primers, 2.5 μL green nucleic acid dye (EvaGreen; Biotium, Hayward, CA), as well as 30 nM passive reference dye (Rox; USB, Cleveland, OH) per 50-μL reaction. PCR was performed on a real-time thermal cycler (model Mx3000p; Stratagene, La Jolla, CA), with cycling parameters of initial denaturation at 95°C; 40 cycles of 95°C 30 seconds, 60°C 30 seconds, and 72°C 60 seconds, and a denaturation cycle for creation of a dissociation curve. Reactions for each sample and gene of interest were run in duplicate, and cycle thresholds (C_t) were normalized to TATA binding protein (TBP) expression, a low-abundance housekeeping gene, and comparative quantitation was performed (MxPro ver. 4.0 software; Stratagene).

Immunofluorescence

Immunohistochemical localization of SPC family members was performed on paraffin-embedded tissues as published previously.[46,47] Briefly, 7 μm sections were deparaffinized and heat treated for 10 minutes in 10 mM sodium citrate (pH 6) containing 0.05% Tween-20. The sections were then blocked with 5% goat serum and 1% BSA for 30 minutes and subsequently incubated in primary antibody at 4°C over-night. Secondary staining was performed with either goat anti-rabbit Alexa 488- or goat anti-mouse Alexa 594-conjugated secondary antibodies (Invitrogen). Alternatively, secondary staining was performed with donkey anti-mouse Alexa 488 and donkey anti-rabbit Alexa 647 for confocal microscopy. Antibodies used and respective dilutions are provided in Table 3. Images were taken with an epifluorescence microscope (model BX51; Olympus America, Center Valley, PA) with the attached digital imaging system at 20 × magnification or with a confocal imaging system (model 415; Carl Zeiss Meditec, Inc., Thorn-wood, NY) at 40 × magnification.

Primary ONH Astrocyte Culture

Primary ONH astrocyte cultures were generated by using a modified protocol.[48] Briefly, explants isolated with a postmortem interval under 6 hours were isolated and cultured in DMEM-F12 supplemented with 3% FBS, 4 mM L-glutamine, 100 μg/mL ascorbic acid, 50 ng/mL insulin, 10 ng/mL EGF, 10 U/mL penicillin-streptomycin, and 1.25 μg/mL amphotericin B (Fungizone; Bristol-Meyers Squibb, New York, NY) for 7 to 10 days. Explants were then removed from the culture dish and briefly rinsed in Dulbecco's PBS without Ca^{2+}/Mg^{2+} (Hyclone, Logan, UT) and placed in a dissociation enzyme (TrypLE; Invitrogen) two times for 20 minutes each. The tissue was then triturated with a transfer pipette and strained through a 70-μm cell strainer (BD Biosciences, San Diego, CA). The cells were allowed to grow on culture plates (Primaria Cultureware; BD Biosciences) until the third passage. GFAP immunoreactivity was assessed, and the cells were found to be >97% GFAP positive.

Immunoblot Analysis

Frozen human tissues were homogenized with disposable tissue processors in MPER buffer (Pierce Biotechnology, Rockford, IL) containing protease inhibitors (Sigma-Aldrich) and centrifuged at 10,000*g* for 2 minutes. Lysates from human retina and ONH were electrophoresed under reducing conditions and immunoblotted onto a PVDF membrane. Blots were probed with anti-PACE4 (1:5000; Abcam, Cambridge, UK), secondary staining was performed with goat anti-rabbit (1:20,000) and was imaged with ECL reagent (Femto; Pierce). Images were taken with a fluorescence imaging system (FluorChem 8900; α-Innotech, San Leandro, CA).

Fluorogenic SPC Activity Assay

SPC activity in postmortem human tissue was measured with a modified protocol.[49,50] Unfixed human eyes were enucleated and flash frozen in an isopentane/dry ice slurry in OCT compound. Thirty-five-micrometer sections were cut, and optic nerve and retina tissue were dissected. The tissue was placed in 100 mM HEPES [pH 7.5], 1 mM $CaCl_2$, 0.5% Triton X-100, and 1 mM 2-mercaptoethanol and disrupted by sonication. The samples were

centrifuged at 10,000*g* for 5 minutes and supernatant was taken. The lysates were then pretreated with or without PC inhibitor (Hexa-D-Arginine [HDR] at 580 nM; Axxora, San Diego, CA) for 30 minutes at 4°C. The fluorogenic PC substrate Boc-RVRR-AMC was added for a 100-μM final concentration (EMD Bioscience, San Diego, CA) and incubated at 37°C for 2 hours. Activity was determined using a 20/20n luminometer with UV fluorescent add-on module (Turner BioSystems, Sunnyvale, CA).

Statistical Analysis

Analysis of PC mRNA expression and inhibition of PC activity was performed with the Mann-Whitney U test. PACE4 mRNA levels in individual samples were determined with a two-way analysis of variance (ANOVA) with Bonferroni's post hoc test. PC activity was analyzed by ANOVA. Significance was assessed at $P < 0.05$ (GraphPad Prism ver. 5. 00; Graph Pad Software, San Diego, CA).

Results

Expression of PC1, PC2, and PACE4 in the Retina and ONH

We evaluated mRNA and protein expression for the SPC family members in the human retina and ONH. To compare mRNA levels of members of the SPC family between the ONH and retina, we performed qRT-PCR (Fig. 1A). Overall, PC1 and PC2 message levels were significantly higher in the retina, and PACE4 was significantly higher in the ONH. mRNA for PC1 and PC2, the neuroendocrine-specific convertases, demonstrated 5.7- and 5.2-fold higher expression in the neural retina than in the ONH ($P < 0.001$, 0.04 respectively). PACE4 mRNA expression was 3.8-fold higher in the ONH than in the retina ($P < 0.001$). Evaluation of PACE4 mRNA levels in the five individual donor samples revealed significantly higher expression in the ONH in four of the five samples (Fig. 1B, $P < 0.001$). Expression of the closest homologue to PACE4, PC5, was only slightly (0.5-fold) higher in the ONH than in the retina, but not at a significant level. Furin and PC7 mRNA levels were not significantly different between the tissues.

PACE4 and PC5 in Glia in the ONH

Having shown a difference in mRNA expression for PC1, PC2, and PACE4 in human ONH and retina, we then determined SPC protein localization to GFAP-positive glia of the ONH. PC1 and PC2 immunofluorescence was slightly above background levels throughout the ONH (Figs. 2A–D). However, PACE4 demonstrated strong expression in the prelaminar ONH (Figs. 2E, 2M). Furthermore, PACE4 was strongly colocalized with GFAP (Fig. 2F). Of interest, PC5 immunofluorescence was found in what appeared to be nerve fiber bundles traversing the pre-laminar ONH and continuing through the lamina cribrosa (Figs. 2G, 2H, 2N).

Expression of SPCs in Neural and Glial Cells in the Retina

PC1 and PC2 have been reported to be broadly expressed throughout neuroendocrine tissues and have been localized to numerous neurons within the CNS.[17,18] However, there have been no previous reports of SPC expression within the human neural retina. Double immunohistochemistry was used to determine the localization of the SPC family within the

retina. Each SPC was counterstained with GFAP, which stains Müller cells and astrocytes in the retina, and Hoechst 33342 was used to identify nuclei. Similar to mRNA results, PC1 and PC2 expression was localized throughout the neurons of the retina (Figs. 3A–D). In contrast, PACE4 and PC5 immunoreactivity was strongest in the INL of the retina (Figs. 3E–H). Furthermore, PC5 colocalized strongly with GFAP in the ILM and nerve fiber layer (NFL; Figs. 3G, 3H). The NFL is composed of nerve fiber bundles exiting the neural retina, as well as retinal astrocytes and Müller cell endfeet, and so it is likely that these cell types are the source of PACE4 and PC5 in the retina, rather than neuronal cells. Immunoreactivity for furin and PC7 was weak throughout the retina (data not shown).

Colocalizing of PC5 and PC2 with Neurofilament in the GCL and NFL of the Retina and ONH

To determine whether PC1, PC2, and PC5 immunoreactivity colocalizes to the nerve fiber of the retinal ganglion cells, we stained for each respective PC as well as neurofilament (NF), a nerve fiber intermediate filament, and performed confocal microscopy. Strong colocalization of PC1, PC2, and PC5 was found in the ganglion cell layer (GCL) as well as the NFL of the inner retina. (Fig. 4).

PACE4 on Müller Cells and Astrocytes in the Retina

Counterstaining with CRALBP, a Müller cell marker, was performed to determine whether the PACE4 inner retina immunoreactivity was present primarily in Müller cells or retinal astrocytes. Intense colocalization was found within the INL and ILM, which is composed of Müller endfeet (Figs. 5A–C). Of note, the INL, where the Müller soma reside, did not contain significant colocalization with PACE4. PACE4 has been shown to be predominantly localized to the cell surface and attached to the ECM.[36,37] CRALBP is a membrane-bound protein; thus, strong colocalization to CRALBP rather than GFAP may be due to cellular localization of these marker proteins. Therefore, we cannot rule out the possibility that retinal astrocytes residing in the GCL/NFL may also contribute to PACE4 expression in the retina. When the retina and ONH were observed under low magnification, we noted a contiguous expression of PACE4 throughout the inner retina and prelaminar ONH. To further examine PACE4 expression within the GCL/NFL, we used NeuN, a marker of retinal ganglion cells. There was no colocalization of PACE4 and NeuN, suggesting that PACE4 expression is limited to glia of the inner retina (Figs. 4D–F).

PACE4 in the ONH and in Primary Human ONH Astrocytes

To further study the expression of PACE4 in the ONH, we analyzed the colocalization of PACE4 and CRALBP at the ONH. CRALBP expression halted abruptly at the intermediary tissue of Kuhnt (Fig. 6A), whereas PACE4 expression was contiguous across the prelaminar ONH (Fig. 6B). Higher magnification images showed PACE4 and GFAP colocalizing within the prelaminar ONH (Fig. 6C).

Primary ONH astrocytes were more than 97% GFAP positive (Fig. 6D). Furthermore, nearly all the cells stained were positive for PACE4 (Fig. 6E).

Differential Protein Expression and Activity of PACE4 in the Retina and Optic Nerve

PACE4 is expressed as a 122-kDa prepro peptide that is processed to a mature 106-kDa peptide and is further processed to a predicted 66-kDa isoform. We showed by Western immunoblot analysis that the predominant species in both the retina and ONH was the 66-kDa isoform, with several additional bands in the retina (Fig. 7A). Cellular lysate isolated from tissues stored (RNAlater; ABI) and extracted (TriReagent protocol; Invitrogen), as well as freshly isolated porcine retina and ONH, yielded similar expression patterns (data not shown). In addition, we used a monoclonal antibody that recognizes the C terminus of PACE4. This antibody also recognized the prominent 66-kDa band (data not shown) as well as the smaller forms. Therefore, it is possible that these smaller bands are truncated and/or proteolytically processed PACE4 isoforms rather than nonspecific degradation products.

To elucidate PC activity in these two tissues, we used a fluorogenic SPC substrate that was incubated with cellular lysates with or without HDR, an SPC with a K_i of 580 nM.[51] When adjusted for total protein, SPC activity in the ONH lysate was approximately 25% higher than in the retinal lysate. However, when the ONH and retina lysates are preincubated with 580 nM HDR, the amount of convertase activity dropped significantly in the ONH lysate compared with that in the un-treated control (Fig. 7B). This observation is indirect evidence that PACE4 enzymatic activity is present in higher amounts in the ONH.

Discussion

We report for the first time mRNA expression, protein localization, and enzyme activity of the SPC calcium-dependent cleavage enzyme family members in the human retina and optic nerve. We have determined that the neuroendocrine-specific SPCs, PC1, and PC2, had higher mRNA expression in the retina and were strongly expressed throughout cells of the retina, whereas PACE4 and possibly PC5 were more prevalent in the glia of the ONH and retina. Previously, PACE4 mRNA has been reported via in situ hybridization to localize to optic nerve glia in rat eyes.[26] As the ONH is devoid of neuron cell somas, our results suggest that PACE4 rather than PC1 or PC2 may be the predominant SPC for glia. PACE4 and PC5 demonstrated strong expression in the NFL, ILM, and the ONH and colocalized with GFAP.

In addition, staining with CRALBP and NeuN demonstrate that PACE4 is expressed by Müller cells in the NFL/ILM, but does not colocalize to RGCs. We also demonstrate that the expression for PACE4 in the ILM is contiguous throughout the transition into the prelaminar ONH, although CRALBP expression halts at the maculopapillary bundle. Primary human astrocytes were also found to be positive for PACE4 staining. Therefore, ONH astrocytes are presumably the principle cells responsible for PACE4 immunoreactivity in the prelaminar ONH. Of particular interest is the localization of PC5 to nerve bundles in the retinal NFL and ONH axon bundles. The localization of PACE4 is limited to prelaminar ONH and LC columns. Although PC5 and PACE4 are highly homologous and demonstrate many biochemical similarities, the unique expression pattern for PC5 suggests that it may have functions distinct from PACE4. PACE4 and PC5 display differential expression patterns in development.[26,27,52] Although the source of the PC5 is not known, this could

have important consequences for the activity and processing of growth factors and other proteins that interact with the nerve fiber bundle in the ONH.

Immunoblot analysis revealed differential expression patterns for PACE4 in the ONH and retina. Although PACE4 mRNA was higher in the ONH relative to the retina, immunohistochemistry showed a more defined stain in the ILM of the retina. Colocalization in the ONH was demonstrated in astrocytes in the prelaminar and glial columns, albeit with an intensity that appeared lower than Müller cell immunoreactivity. It is unclear why there was this discrepancy in tissue mRNA levels compared with protein localization. It is possible that the protein turnover for this enzyme is higher in the ONH astrocyte. Alternatively, as the ONH contained dense ECM and PACE4 was bound extracellularly, it is possible that there was a hindrance in epitope accessibility. Furthermore, it is unclear whether the expression pattern influenced total PACE4 activity and whether the PACE4 found in the retina and ONH was fully active. Previous reports have suggested that smaller PACE4 splice variants may be proteolytically inactive.[53,54]

In addition to message and protein, we report for the first time SPC enzyme activity in both the human retina and optic nerve. We found that ONH activity was 51% higher than that in the retina. Although specific substrates for each SPC are not available, we used the inhibitor HDR at the K_i for PACE4, and observed a decrease in activity in the retina and an even greater decrease in ONH activity. Although all SPCs process this substrate, this finding may suggest that PACE4 has higher activity within the ONH. Thus, future research will necessitate specifically targeting PACE4 activity. A more selective PACE4 and PC5 inhibitor has been recently discovered that does not affect the other SPC family members.[55]

PACE4 has been primarily linked to tumor cell invasiveness due to enhanced bioactivity of extracellular substrates such as the MMPs, as well as increased bioactivity of growth factors such as VEGF.[23,40,42,43] We hypothesize that PACE4 acts as a central regulator of growth factor bioactivity and extracellular matrix processing and secretion (Fig. 8). Altered expression of PACE4 may be an intermediate in glaucomatous ONH pathophysiology. In this study, PACE4 appeared to be localized to the glia of the retina as well as the astrocytes of the prelaminar ONH. Studies have previously demonstrated glaucomatous morphologic changes in the ONH and retina, including increased GFAP staining and retinal and optic nerve glia activation and proliferation.[1,56–59] We believe that PACE4 may be involved in glaucomatous glial activation. Although tumor metastasis and glial activation are two very distinct pathologic mechanisms, many similarities with respect to protein maturation and activity are plausible. Furin is proposed to be involved in similar events with respect to tumorigenesis, most notably by increasing the availability of molecules such as TGFβ1, VEGF, and MT1-MMP, which simultaneously enables ECM degradation, proliferation, and thus cancer metastasis.[41]

PACE4 may directly influence ECM turnover by altering MMP activity both within the ONH and at the NFL. PACE4 and PC5 are unique SPCs, in that they are anchored to the ECM in ternary complexes with TIMP-2 and heparan sulfate proteoglycans (HSPGs) in the extracellular matrix.[36,37] In vitro and in situ studies have suggested that TIMP2 and HSPG

molecules are upregulated in experimental models of glaucoma as well as human glaucoma tissues.[60–63]

In the glaucomatous ONH, factors causing increased glial reactivity, due to mechanical, ischemic, or other unknown molecular processes, appear to increase the expression of PACE4. PACE4 may increase the bioavailability of growth factors believed to be involved in POAG pathophysiology such as NGF and TGFβ.[47,64–66] Of particular interest is the secretion and activity of proNGF, which is increased in vitro, in vivo, and in situ in various CNS pathologic and aging models.[67–73] Of interest, NGF is secreted extracellularly in an immature pro form and then is processed and degraded by a cascade involving active plasmin, MMP-2, and TIMP-1.[74] Although there is controversy within this area of research, the presence of un-processed proNGF may induce apoptosis. Our laboratory has previously demonstrated that NGF induces human ONH astrocyte proliferation in vitro.[47] Thus, PACE4 may influence astrocyte proliferation and neuronal survival via growth factor processing.

Increased growth factor availability may have profound effects on extracellular matrix synthesis, turnover, and deposition in the ONH. Members of the TGFβ superfamily are also candidate substrates for PACE4.[75,76] In development, the expression patterns of TGFβ member proteins, notably the BMPs, mirror expression patterns for PACE4.[24,55,76] One family member, TGF-β2, is believed to be involved in POAG pathophysiology by upregulating the synthesis of extracellular matrix proteins produced by cells of the ONH and lamina cribrosa.[64,77–81] It has not been demonstrated that PACE4 processes pro/latent TGF-β2 into the active form. However, evidence has suggested that SPC inhibitors prevent the processing of TGF-β2.[49] Although furin is the predominant physiological cleavage enzyme for TGF-β1, furin processes TGF-β2 poorly.[29,81–83] This is surprising, as the prodomains and dibasic cleavage sites for TGF-β1, -β2, and -β3 are similar. TGF-β2 is primarily secreted in the latent form, where it binds to extracellular HSPGs. It is possible that processing and subsequent modulation of TGF-β2 activity occurs via the HSPG-bound PACE4 and/or PC5. Therefore, PACE4 may influence ONH cupping and related biochemical changes by regulating growth factors involved in ECM remodeling.

In conclusion, we have reported expression patterns of the SPC family in the adult human retina and optic nerve. We have shown that PC1, PC2, and PC5 are preferentially expressed in retinal neuronal cells, whereas PACE4 is astrocyte and Müller cell specific. We believe that PACE4 may be an enzyme that enables altered growth factor bioactivity and increased extracellular matrix biosynthesis by activated glia within the ONH.

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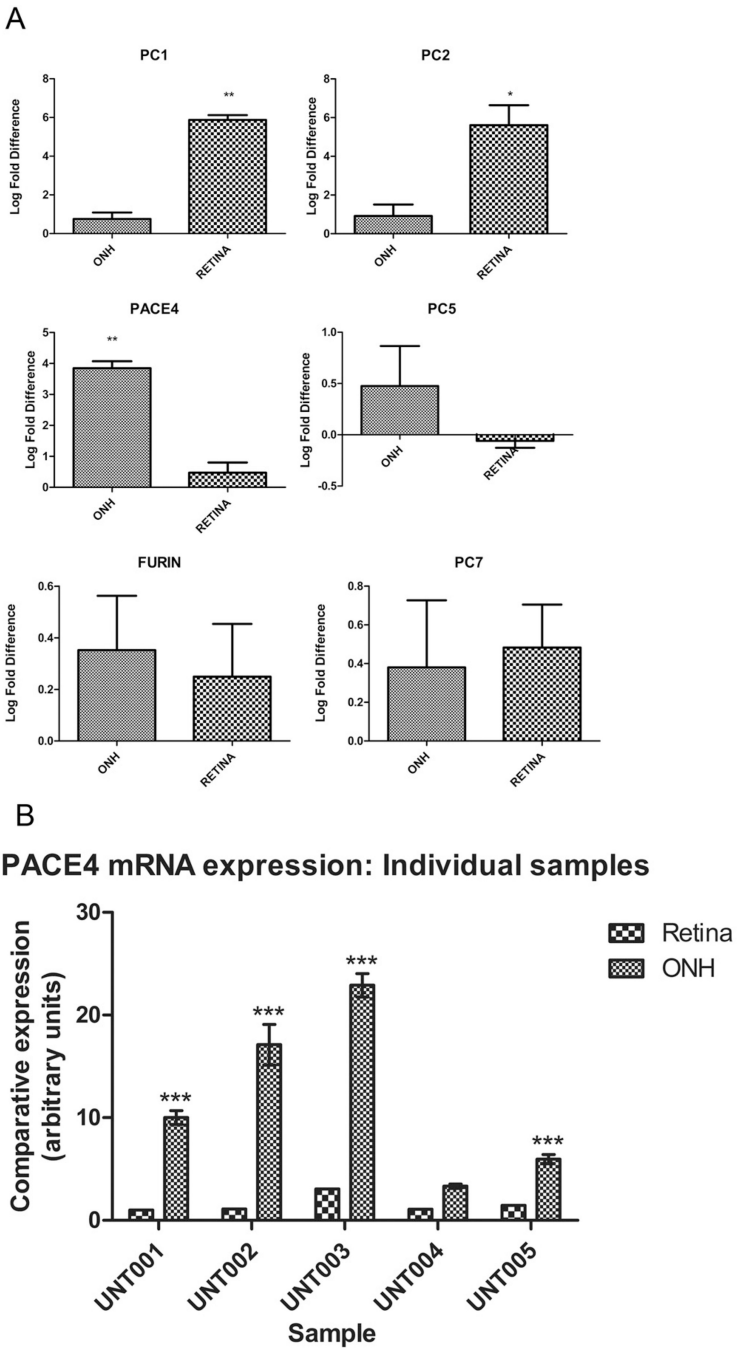

Figure 1.

PC mRNA expression in postmortem ONH and retina. The neuroendocrine-specific PC1 and −2 demonstrated 51- and 4.7-fold higher expression in postmortem retina, respectively. PACE4 demonstrated 3.37-fold higher expression in ONH (n = 4). Furin and PC7 did not demonstrate significant expression differences between tissues (**A**). Individual sample mRNA expression showed normalized PACE4 ranging from 3.3- to 22.9-fold relative expression compared with retina and retinal expression ranging from 1.0- to 307-fold (**B**).

*P< 0.05; **P< 0.005; ***P< 0.001 (Mann-Whitney U test for grouped data, two-way ANOVA with Bonferroni's post hoc for individual PACE4 samples).

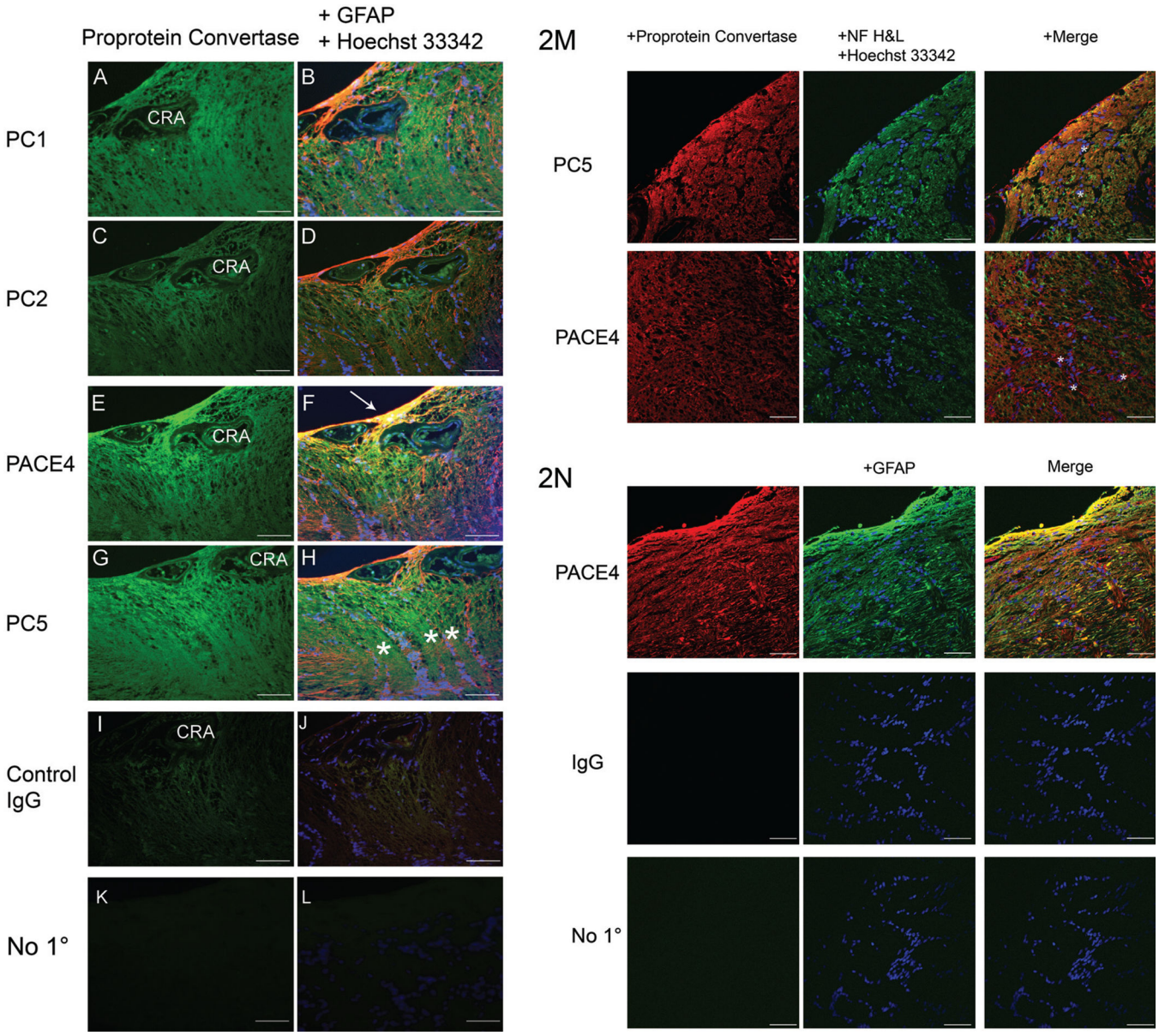

Figure 2.

PC expression in the ONH. Each PC was identified by its respective primary antibody followed by goat anti-rabbit Alexa 488 (*green*; **A, C, E, G, I**), and counterstained with anti-GFAP followed by goat anti-mouse Alexa 594 (*red*). For confocal immunofluorescence, anti-PC5 and PACE4 were stained donkey anti-rabbit Alexa 647 (*red*; **B, D, F, H, J**) and counterstained with either GFAP or NF followed by donkey anti-mouse Alexa 488 (*green*; **M, N**). PC1 and −2 demonstrated nondescript staining through the prelaminar ONH (**A-D**). PACE4 demonstrated strong colocalization with GFAP in the prelaminar ONH (**E, F***arrow*) and in glial columns. (**M**, *) PC5 were absent in glial columns, and staining was evident in nerve fiber bundles. (**M**, *) Control rabbit and mouse IgG antibodies and no primary antibody (**K, L**). CRA central retinal artery. Bar, 50 μm.

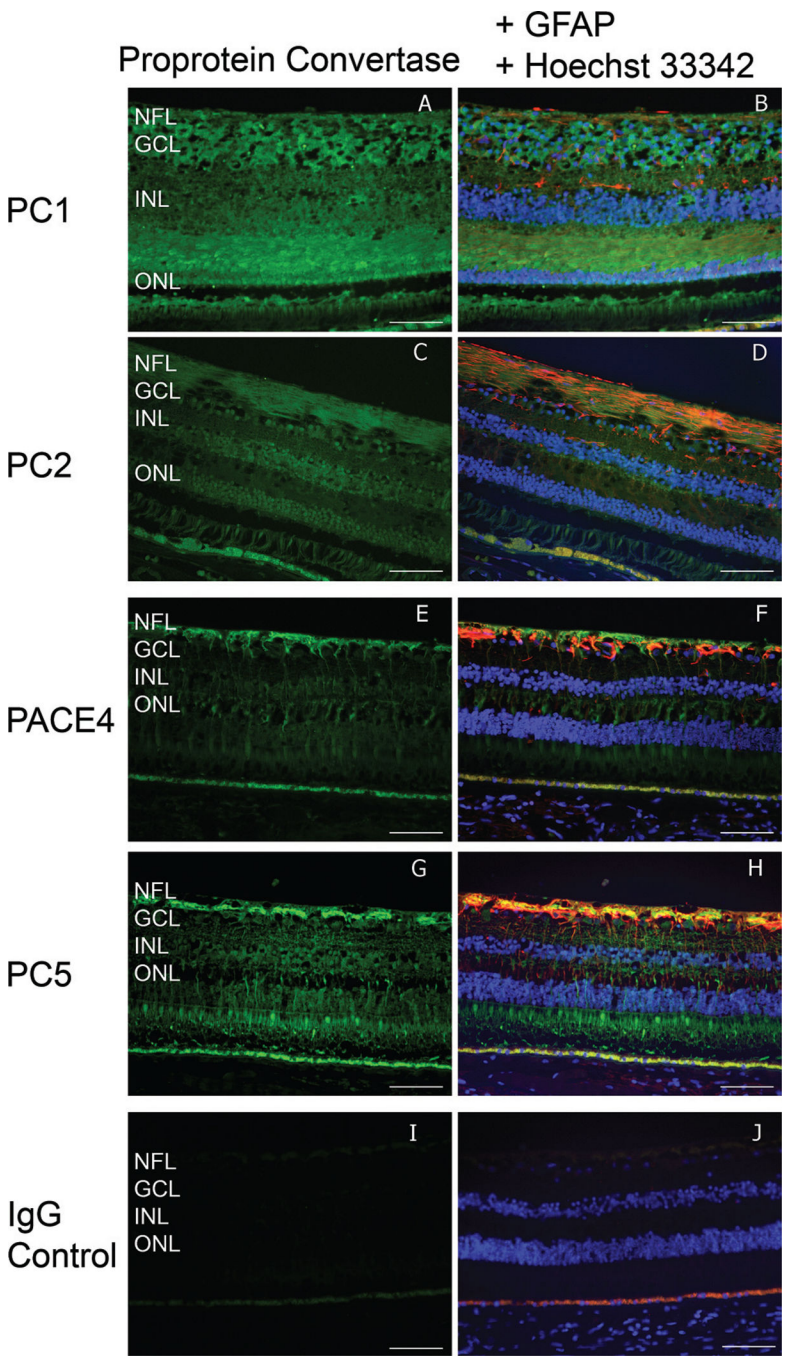

Figure 3.

PC localization in postmortem human retina. Each respective PC was identified by its primary antibody, followed by goat anti-rabbit Alexa 488 (**A, C, E, G, I**), and counterstained with anti-GFAP followed by goat anti-mouse Alexa 594 and Hoechst 33342 nuclear stain (**B, D, F, H, J**). PC1 and −2 demonstrated strong fluorescence throughout the neural retina (**A, B, C, D**). PACE4 demonstrated strong fluorescence on the inner limiting membrane and throughout the NFL (**E, F**). PC5 colocalized with GFAP-positive

immunoreactivity in the NFL (**G, H**). Control rabbit and mouse antibodies (**I, J**). Bars, 50 μm.

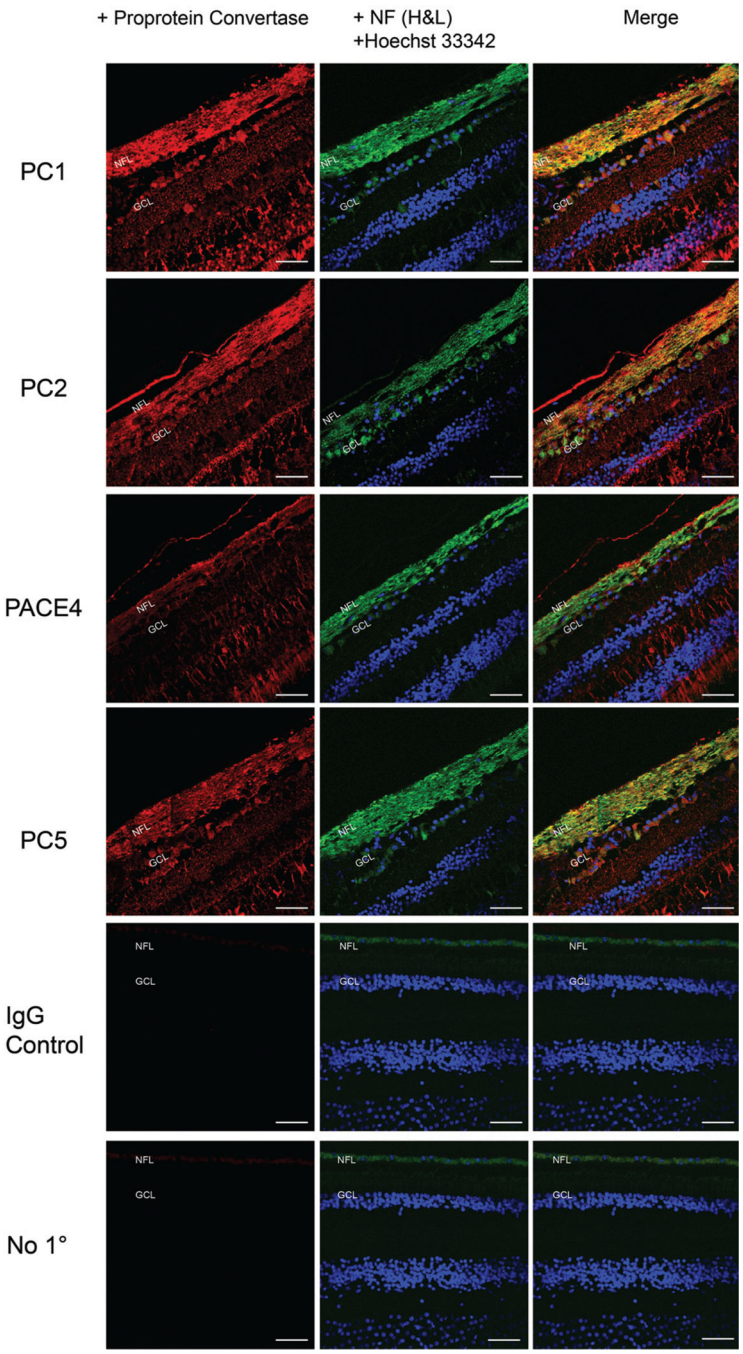

Figure 4.

Confocal PC localization in the GCL and NFL. Each PC was identified by its respective primary antibody followed by donkey anti-rabbit Alexa 647 and counterstained with anti-NF (H&L) followed by donkey anti-mouse Alexa 488 and Hoechst 33342 nuclear stain. PC2 showed strong fluorescence in the RGCs. PACE4 demonstrated strong fluorescence on the inner limiting membrane and NFL. PC5 colocalized with NF-positive immunoreactivity in the NFL. Control rabbit and mouse antibodies. Bars, 50 μm.

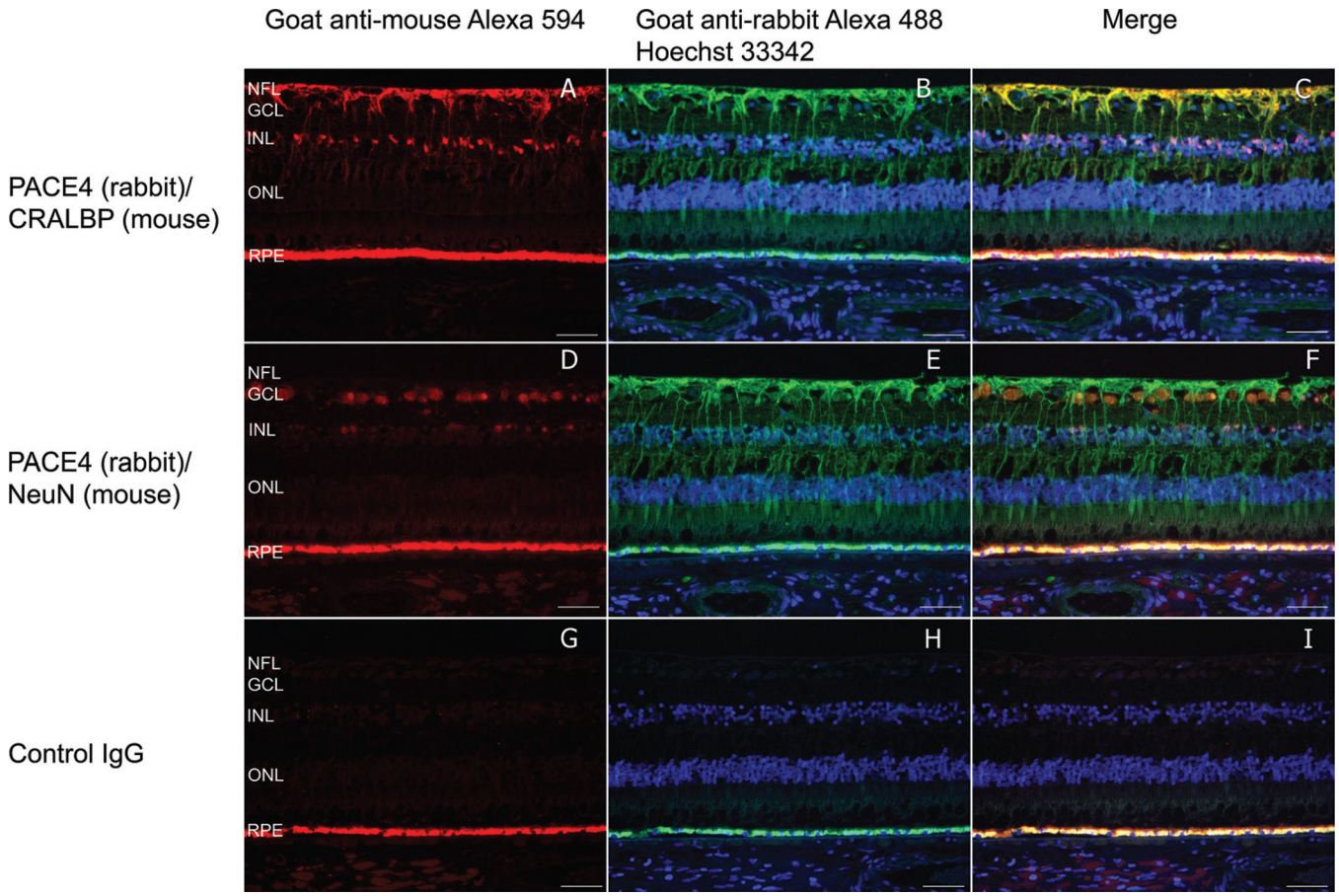

Figure 5.

PACE4 colocalization in human retina with CRALBP and NeuN. Mouse marker antibodies were detected with goat anti-mouse Alexa 594 (**A, D, G**). Anti-PACE4 was detected with goat anti-rabbit Alexa 488, and the nuclei were stained with Hoechst 33342 (**B, E, F**). PACE4 demonstrated strong colocalization with CRALBP in the ILM (**A–C**). PACE4 immunoreactivity surrounded but did not colocalize with the RGC marker NeuN (**D–F**). Control rabbit and mouse IgG sections (**G–I**). Bar, 50 μm.

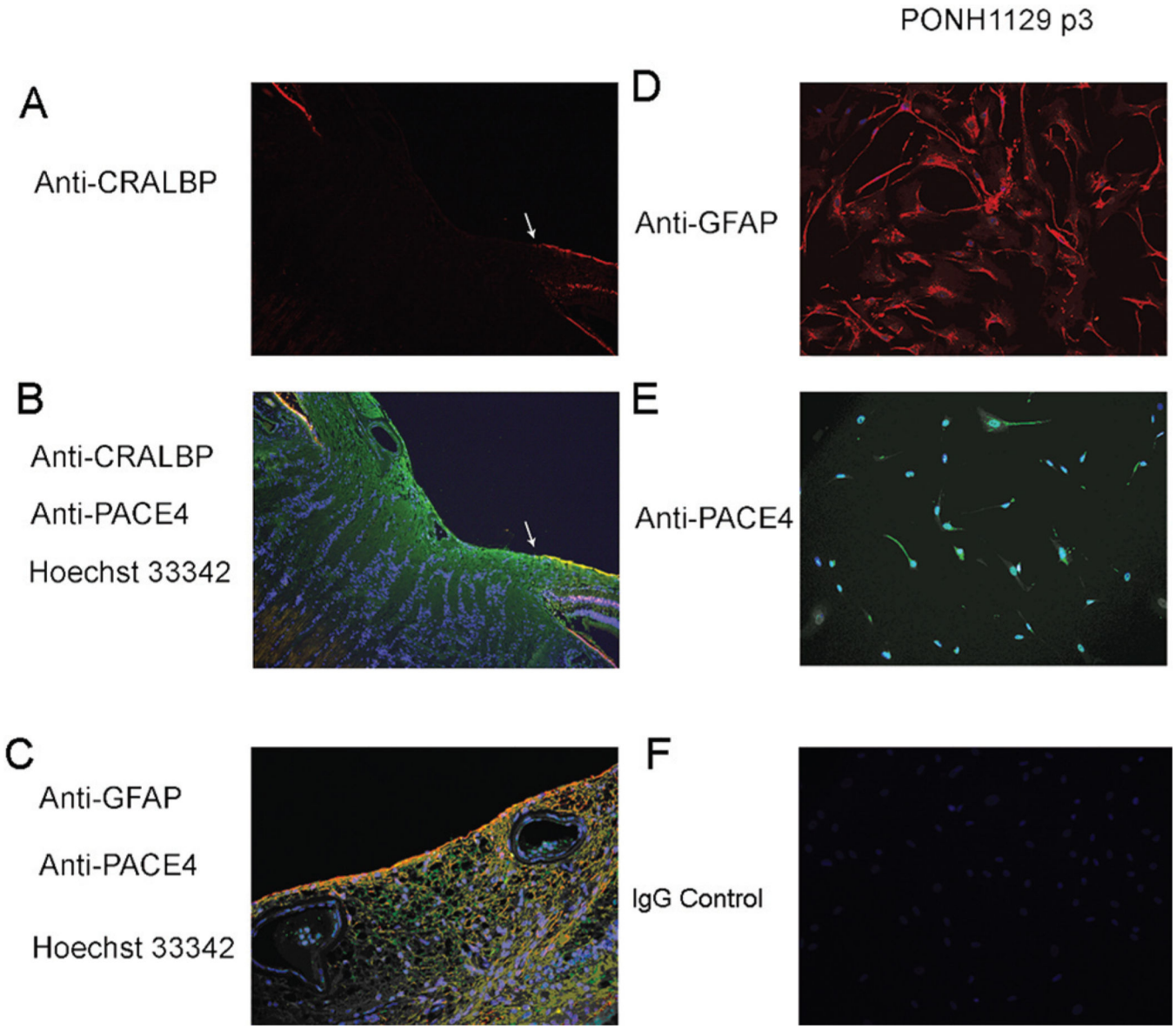

Figure 6.

PACE4 was expressed in primary ONH astrocytes. CRALBP immunoreactivity halted near the maculopapillary bundle (**A**, *arrows*). In contrast, PACE4 was expressed contiguously across the prelaminar ONH (**B**). GFAP and PACE4 colocalized within the ONH (**C**). Primary human ONH astrocytes stained >97% positive for GFAP (**D**), negative for CRALBP (**E**), and also positive for PACE4 immunoreactivity (**F**). CRALBP and GFAP were counterstained with goat anti-mouse Alexa 594 (*red*), and PACE4 was counterstained with goat anti-rabbit Alexa 488 (*green*). Magnification: (**A, B**) ×10; (**C, D, E**) ×40.

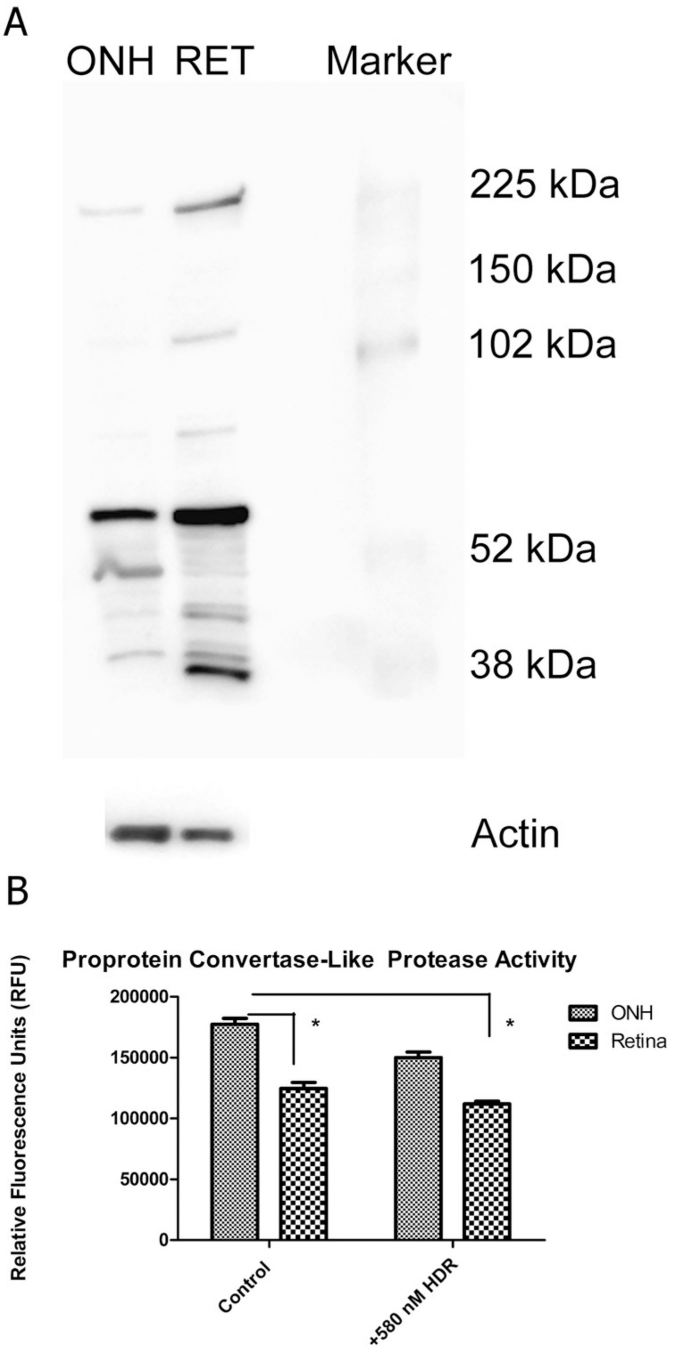

Figure 7.

Chemiluminescence detection for PACE4 in human post-mortem ONH and retina lysate. Representative blots of three human donors are shown. Anti-actin was used as the loading control (**A**). Total PC activity in the presence of 580 nM HDR in the ONH and retina (**B**). **P*< 0.05 (one-way ANOVA).

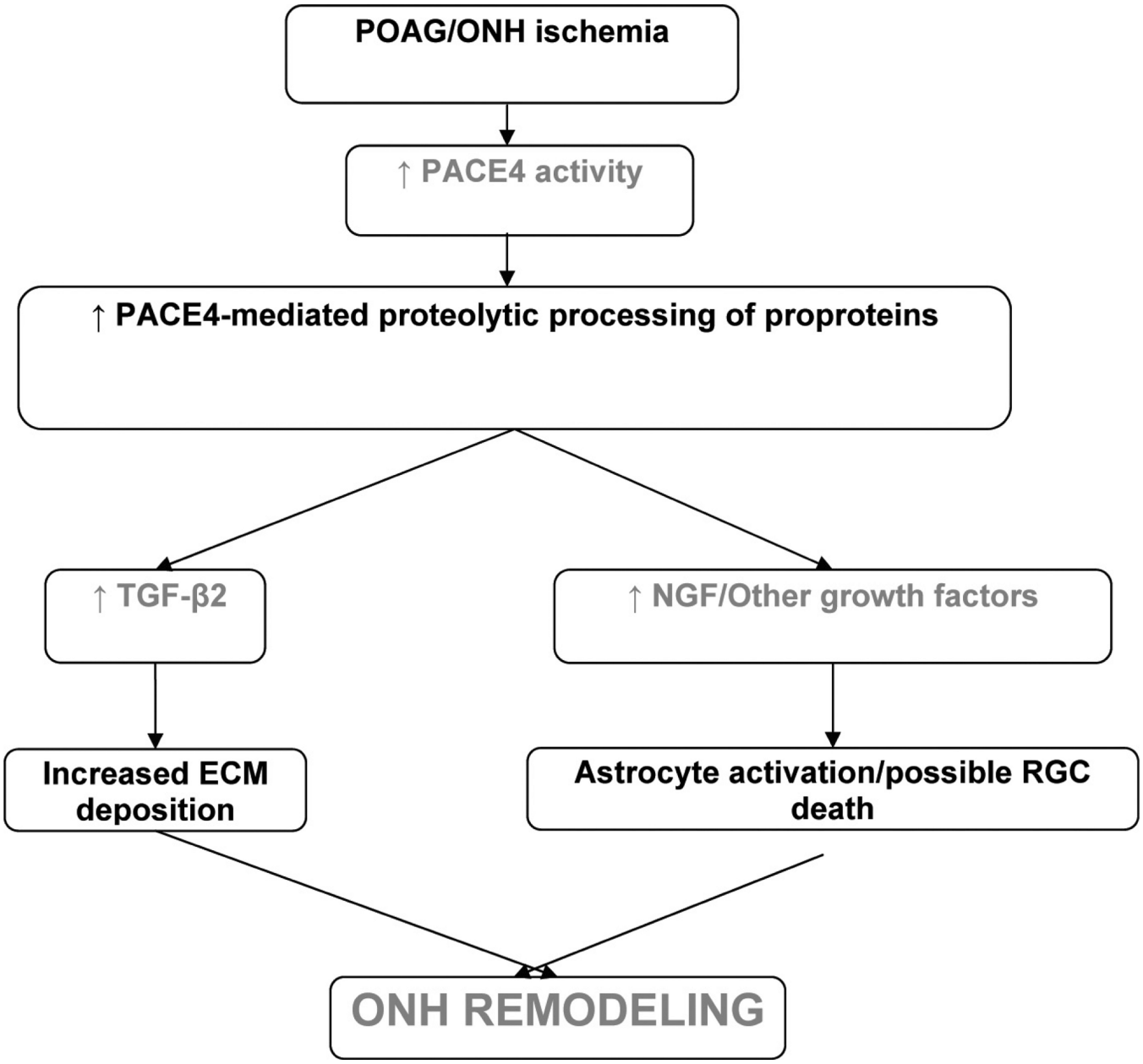

Figure 8.
Proposed role for PACE4 in POAG pathophysiology.

Table 1

Donor Demographic Characteristics

Sample	Age	Sex	Approximate PMI (h)	Technique
UNT001	64	F	2.5	qRT-PCR
UNT002	67	F	4	qRT-PCR
UNT003	64	M	8	qRT-PCR
UNT004	32	F	6	qRT-PCR, FLP
UNT005	55	M	8	qRT-PCR, primary ONA culture
04–057	40	M	<8	IHC
04–138	88	F	<8	IHC
04–220	86	F	<8	IHC
04–122	97	F	<8	IHC
05–180	94	M	<8	IHC
05–368	79	M	<8	IHC
04–102	79	M	<8	IHC
03–100	37	F	<8	IHC

Table 2

Proprotein Convertase Primers Used for qRT-PCR Analysis

Gene	Expected Amplicon Size (bp)	Sense Primer (5′-3′)	Antisense Primer (5′–3′)
PCSK1 (PC1)	131	TGGTTTGGAGTGGAATCACA	ATCTGGTCCCGTGTTTGTTC
PCSK2 (PC2)	225	CCTCCAACTATAATGCCGAAG	AGGCCTCGATGATGTCTGTC
PCSK6 (PACE4)	200	AGTGTGTCGAAGGTGTGACG	GGCAGCAGAACTGAATGAAG
PCSK5 (PC5)	109	CCTGGAAGAGAGGCTACACG	CAACTTGCCAGAGCATCGTA
FURIN	112	GTACAGTGGCTGGAACAGCA	GCTGAGTGACACCAGACAGG
PCSK7 (PC7)	147	ACGCCAACTCCATCTACACC	CCCAGTCAGTGGTCACAATG
TBP	181	GAAACGCCGAATATAATCCCA	GCTGGAAAACCCAACTTCTG

Table 3

Antibodies

Antibody	Cells Identified	Host	Source*	Dilution
GFAP	Astrocytes and Müller glia	Mouse	LabVision	1:250
GFAP	Astrocytes and Müller glia	Rabbit	Promega	1:250
Neurofilament (H&L)	Neurons	Mouse	Chemicon	1:500
NeuN	Retinal ganglion cells	Mouse	Chemicon	1:500
CRALBP	Müller glia	Mouse	Affinity Bioreagents	1:50
PC1		Rabbit	Affinity Bioreagents	1:100
PC2		Rabbit	Affinity Bioreagents	1:100
PACE4		Rabbit	Abcam	1:500 (IHC), 1:5000 (WB)
PC5/6A		Rabbit	Abcam	1:500
Furin		Mouse	Axxora	1:100
PC7		Rabbit	Gift of Robert Day	1:100

* LabVision, Fremont, CA; Promega, Madison, WI; Chemicon, Temecula, CA; Affinity Bioreagents, Golden, CO; Abcam, Cambridge, UK; Axxora, San Diego, CA.