PEDF Gene Deletion Disrupts Corneal Innervation and Ocular Surface Function

Zhenying Shang,[1] Chenxi Li,[1] Xuemei Liu,[1] Manhong Xu,[1] Xiaomin Zhang,[1] Xiaorong Li,[1] Colin J. Barnstable,[1–3] Shaozhen Zhao,[1] and Joyce Tombran-Tink[1–3]

[1]Tianjin Key Laboratory of Retinal Functions and Diseases, Tianjin Branch of National Clinical Research Center for Ocular Disease, Eye Institute and School of Optometry, Tianjin Medical University Eye Hospital, Tianjin, China
[2]Department of Neural and Behavioral Sciences, Penn State College of Medicine, Hershey, Pennsylvania, United States
[3]Department of Ophthalmology, Penn State College of Medicine, Hershey, Pennsylvania, United States

Correspondence: Joyce Tombran-Tink, Penn State College of Medicine, 500 University Drive, Harrisburg, PA 17033, USA; jttink@aol.com, jxt57@psu.edu. Shaozhen Zhao, Tianjin Key Laboratory of Retinal Functions and Diseases, Tianjin Branch of National, Clinical Research Center for Ocular Disease, Eye Institute and School of Optometry, Tianjin Medical University Eye Hospital, Fukang Road 251, Tianjin 300380, China; zhaosz1997@sina.com.

ZS and CL contributed equally to the work presented here and should therefore be regarded as equivalent authors.

PURPOSE. The cornea is richly innervated by the trigeminal ganglion (TG) and its function supported by secretions from the adjacent lacrimal (LG) and meibomian glands (MG). In this study we examined how pigment epithelium–derived factor (PEDF) gene deletion affects the cornea structure and function.

METHODS. We used PEDF hemizygous and homozygous knockout mice to study effects of PEDF deficiency on corneal innervation assessed by beta tubulin staining, mRNA expression of trophic factors, and PEDF receptors by adjacent supporting glands, corneal sensitivity measured using a Cochet-Bonnet esthesiometer, and tear production using phenol red cotton thread wetting.

RESULTS. Loss of PEDF was accompanied by reduced corneal innervation and sensitivity, increased corneal surface injury and tear production, thinning of the corneal stroma and loss of stromal cells. PEDF mRNA was expressed in the cornea and its supporting tissues, the TG, LG, and MG. Deletion of one or both PEDF alleles resulted in decreased expression of essential trophic support in the TG, LG, and MG including nerve growth factor, brain-derived neurotrophic growth factor, and GDNF with significantly increased levels of NT-3 in the LG and decreased EGF expression in the cornea. Decreased transcription of the putative PEDF receptors, adipose triglyceride lipase, lipoprotein receptor–related protein 6, laminin receptor, PLXDC1, and PLXDC2 was also evident in the TG, LG and MG with the first three showing increased levels in corneas of the *Pedf*^{+/−} and *Pedf*^{−/−} mice compared to wildtype controls. Constitutive inactivation of ERK1/2 and Akt was pronounced in the TG and cornea, although their protein levels were dramatically increased in *Pedf*^{−/−} mice.

CONCLUSIONS. This study highlights an essential role for PEDF in corneal structure and function and confirms the reported rescue of exogenous PEDF treatment in corneal pathologies. The pleiotropic effects of PEDF deletion on multiple trophic factors, receptors and signaling molecules are strong indications that PEDF is a key coordinator of molecular mechanisms that maintain corneal function and could be exploited in therapeutic options for several ocular surface diseases.

Keywords: cornea, corneal nerves, tear, lacrimal gland, meibomian gland, trigeminal ganglion, corneal sensitivity, MAPK pathway, ERK1/2, AKT, corneal epithelium, PEDF knockout, neurotrophic factors: NGF, BDNF, NT-3, GDNF, EGF, PEDF receptors: laminin receptor, ATGL, PLXDC1, PLXDC2, LRP6

The cornea is a dome-shaped transparent structure that allows light to enter the eye and lies directly in front of the iris and pupil. Unlike most tissues in the human body, the cornea contains no blood vessels and receives nutritive, trophic, and homeostatic support from the lacrimal gland tear fluid, meibomian gland (MG), aqueous humor, and nerve fibers from the trigeminal ganglion that innervate it.[1–3]

The cornea is one of the most richly innervated tissues in the body with nerves emanating from the trigeminal ganglion. Major nerve bundles lose their myelin sheath soon after entering the corneal stroma at the limbus.[4,5] The nerves then penetrate upward and terminate at the corneal epithelium to form nerve fiber networks for each layer of the tissue.[6,7] Corneal nerves are exquisitely sensitive and respond to damaging external stimuli by inducing protective blinking and by stimulating tear production and secretion.[8] These nerves also deliver many neurotrophic factors (NTFs) to the cornea that enable wound healing and provide balance to the corneal microenvironment.[4,9,10]

Pigment epithelium–derived factor (PEDF or Serpin F1) is a neurotrophic molecule that is important to corneal nerve

function.[11] This glycoprotein is a noninhibitory member of the serpin protease inhibitor family of genes and was first discovered in the eye in 1989.[12] Since then, it is shown to be a multifunctional glycoprotein with established neurotrophic, antiangiogenic, anti-inflammatory, and tumor suppressive properties and is widely distributed in almost all tissues of the body.[13–17] In the adult human eye, PEDF exists in the retina, choroid, ciliary body, and the corneal epithelium and endothelium.[18]

The protein exerts its biological function by binding to one of several putative receptors. Adipose triglyceride lipase (ATGL),[19] low-density lipoprotein receptor–related protein 6 (LRP6) also known as Wnt coreceptor,[20] the plexin domain containing 1 (PLXDC1) and 2 (PLXDC2),[21] and the laminin receptor (LR)[22,23] have all been identified as putative receptors through which PEDF transduces its signals. There is growing evidence for PEDF as a therapeutic agent in various ophthalmic diseases including AMD,[24] diabetic retinopathy,[14] glaucoma,[25] and diabetic keratopathy,[26] mainly through its neurotrophic 44 amino acid domain (44-mer)[27] and antiangiogenic 34-mer.[28]

In corneal nerve injury, topical treatment with PEDF in conjunction with DHA promote corneal nerve regeneration and improves SP-positive nerve density in diabetic mice.[26] It was also found that subconjunctival injections of PEDF or its 44-mer Nterminal bioactive peptide increases corneal nerve density in HSV-1–infected mice[29] and in postoperative nerve injury.[30]

PEDF can exert its neurotrophic and neuroprotective effects by inducing expression of other NTFs including the neurotrophin family, glial cell line–derived factor family and neurokines.[31] NTFs play a crucial role in the growth, survival, differentiation and regeneration of neurons in both the central and peripheral nervous systems.[32] The neurotrophin family including nerve growth factor (NGF), brain-derived neurotrophic growth factor (BDNF), and neurotrophin-3, -4/5 (NT-3, NT-4/5)[33,34] are all expressed in various parts of the cornea and play key roles in corneal physiology and regeneration. NGF is mainly localized to the basal epithelial layer of the human limbus, BDNF in all epithelial layers of the cornea, limbus and the limbal stromal cells while NT-3 and NT-4 are detected in the uppermost layer of the cornea epithelium and limbus.[35] Together these trophic factors work to support the function of the cornea.

In the regenerating corneal epithelium, an up-regulation of NGF and GDNF expression is consistent with the progress of corneal sub basal nerve recovery.[36] After lamellar flap surgery, expression levels of BDNF are increased in the cornea, and this correlates positively with increased nerve regeneration.[37] Treatment with NGF plus DHA after photorefractive keratectomy in rabbits yielded faster corneal nerve recovery compared to vehicle controls,[38] whereas topical administration of NGF eyedrops can promote corneal epithelium wound healing and improve tear function to maintain ocular surface homeostasis.[39] NGF and BDNF also have therapeutic potential for several neurodegenerative diseases, including glaucoma,[40] spinal cord injury,[41] and Alzheimer's disease.[42] GDNF, a vitally important member of the GDNF family of ligands is also important to corneal nerve growth and regeneration.[35,43,44] GDNF and its receptors are distributed in the basal layer of the limbal epithelium and activates neuroprotective signaling cascades for corneal nerve growth function.[35]

Because several studies show that exogenous PEDF treatment promotes nerve regeneration,[28–30] the question

that begs to be answered then is does a deficiency in PEDF have negative effects on corneal nerve growth and tissue function? This article focuses on the impact of a loss of one or both PEDF alleles on nerve innervation and function of the cornea and expression of other corneal-dependent trophic support from the lacrimal glands (LG) and MG using a PEDF knockout mouse model.

MATERIALS AND METHODS

PEDF Deficient Mouse Colonies

The PEDF deficient mice (*Pedf*^{−/−}, *Pedf*^{+/−}) used in these experiments were derived from a C57BL/6 mouse strain background and generated by Regeneron Pharmaceuticals. They were a gift to our laboratory by Professor Cai (Sun Yat-sen University, Guangzhou, China).[45] PEDF homozygous knockout (KO) mice were bred with C57BL/6 females to obtain PEDF heterozygotes (*Pedf*^{+/−}) which were crossed to give homozygous (*Pedf*^{−/−}) knockouts. C57BL/6 male and female wildtype mice were purchased from Vital River (Beijing, China) and used as controls in the experiments below. All animal studies used age-, gender-, and weight-matched wildtype mice as controls and five to eight mice enrolled in each group in the studies. We used only male mice in this study to remove potential variability caused by the estrous cycle. Estrogen is known to alter the function of several glands including those supporting the cornea. The trigeminal ganglion is particularly rich in estrogen receptors, and estrogen has been shown to affect nerve growth from the trigeminal ganglion (TG) and secretion of neurotrophic factors in tears.[46,47]

Animal Care

Animal care and experimental procedures were carried out according to the ARVO Guidelines for the Use of Animals in Ophthalmic and Vision Research. Mice were purchased from SPF Biotechnology Co., Ltd. (Beijing, China), housed and bred at the Laboratory Animal Center of the Tianjin Medical University Eye Institute, Tianjin Medical University Eye hospital (Tianjin, China). Mice were kept in a 12- hour on/off light cycle and fed standard mouse chow and water ad libitum. Mice were bred and pups genotyped as previously described.[48] The PCR primers used for genotyping *Pedf*^{−/−}, *Pedf*^{+/−} are as follows:

PEDF Forward: 5′-AAGACCTCAAGTCAAGGGTC-3′;
PEDF Reverse: 5′-CTGCCTCCCTGCACTGTCTC-3′;
Vector Forward: 5′-TCATTCTCAGTATTGTTTTGCC-3′;
Vector Reverse: 5′-CATAAGGACCAGTTTCTCTCC-3′.

Corneal Nerve Length Measurement

Mice in all groups (wt, *Pedf*^{−/−}, *Pedf*^{+/−}) were sacrificed at each developmental time point (two, four, six, and 12 weeks of age; n = 8 mice/age group/time point). These time points were chosen to provide regular time intervals within a three-month developmental period to allow us to determine pathologically events occurring in the cornea immediately after eye opening (PN12-13) to adult stage (12 weeks). Corneas were dissected for nerve length measurements and the TG, LG, and meibomian glands (MG) dissected and stored at −80°C for PCR or Western blot analysis.

Eyes were dissected, fixed in 4% paraformaldehyde (PFA) for one hour and corneas dissected along the sclerocorneal rim in ice-cold paraformaldehyde PFA. Four equidistant radial cuts were made in the corneas and samples washed three times (10 minutes each) in PBS containing 0.3% Triton-X 100 (Solarbio, Beijing, China), then incubated for two hours at room temperature in block solution containing 5% goat serum (Solarbio, Beijing, China), 1% BSA (Solarbio) and 0.3% Triton-X 100 diluted in PBS (PBS-TX) to block nonspecific antigen binding. The corneas were then incubated at 4°C overnight in rabbit anti-β tubulin antibody (1:500; Abcam, Cambridge, MA, USA) diluted in block solution with constant shaking. After three extensive washes (one hour each) using PBS-TX, corneas were incubated with Alexa Fluor 488 goat anti-rabbit IgG (H+L; 1:300) (Abcam) diluted in block solution at 4°C overnight with constant shaking, followed by several PBS-TX washes (three times, one hour each). The corneas were then flat mounted onto glass slides using Prolong Gold Antifade Mountant (ThermoFisher, St. Louis, MO, USA) with the endothelium face oriented anteriorly. Serial and consecutive fluorescent images of the corneal nerves were taken using a laser scanning confocal microscopy (Zeiss LSM800; Zeiss, Oberkochen, Germany). Corneas were imaged using an objective ×5 and an intensity of 3%, and images were acquired using the tiles scan function to encompass the entire cornea. Fifteen serial optical Z-plane frames were taken at a constant depth of 5 µm to visualize all nerves in the cornea epithelium and stroma. The Z-stacked series were merged to render images representing a 75 µm depth of the corneal nerve architecture from the surface of each cornea. Confocal acquisition settings and immunolabeling procedures were all held constant among the groups at each developmental age and samples prevented from photobleaching using an anti-fade mountant.

To measure total stromal nerve length in the central and peripheral corneal stroma, a circled area of the same size was drawn to fit the central region of the cornea at each developmental stage. We used the same area of measurement for groups at each age but slightly increased the circle size to fit the central cornea to accommodate increased cornea size with aging. The length of each of the large nerves was then traced and calculated within the circle (central nerves) and outside the circle (peripheral nerves) using the manual trace function of NIH ImageJ software and its NeuronJ Plugin. Total nerve length was calculated in millimeters according to scale as described.[49] Peripheral nerve measurements were taken in all four corneal quadrants outside the circle. Averages were taken for corneas in each group and the data for eight corneas/group were graphically presented as the average total nerve length in the central or peripheral cornea at each developmental stage per group.

Corneal Sensitivity, Tear Production, and Injury

Corneal sensitivity among the experimental and control mouse groups was measured using Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, Chartres Cedex, France) as described.[50] Mice were tested at two, four, six, and 12 weeks of age using nylon monofilaments with a maximal length of 60 mm and diameter of 0.12 mm. The central cornea of each eye was touched once using the full-length filaments and then by reducing the monofilament by 5mm for successive measurements when there was no blink response until one was achieved.[51] Each monofilament length was measured four times and a positive response was considered when more than half of the measurements in each length caused a blink response (n = 8/group).

Tear production at two, four, six, and 12 weeks of age was measured using the phenol red cotton threads (Jingming, Tianjin, China) technique as previously described.[52] Briefly, the threads were held with a pair of jeweler forceps and placed in the lateral cantus of the eye for 30 seconds. Red wetting of the threads by tears was measured in millimeters and tear production calculated as the average of three replicate measurements for each eye (n = 8/group).

To measure injury to the corneal epithelium, 2uL of 1% fluorescein sodium solution (Alcon, Geneva, Switzerland) was instilled into the inferior conjunctival sac of each mice in the three groups (n = 8/group) at four weeks of age. After 30 seconds, corneas were photographed under a slim-lamp with cobalt blue light. Corneal fluorescein staining scores based on fluorescent intensities were used to assess the extent of corneal injury as previously described.[53]

Corneal Thickness Measurements and Quantification of Stromal Cell

To measure the corneal epithelial and stromal thickness, whole eyeballs were collected from wt, *PEDF*^{+/−}, and *Pedf*^{−/−} at four weeks of age, fixed in 4% paraformaldehyde at room temperature overnight, dehydrated with a graded series of ethanol (100%–80%) and embedded in paraffin. Paraffin-embedded tissue sections of 6 µm thickness were stained with hematoxylin and eosin (H&E) for standard histology examination using bright-field microscopy at magnification ×20 and ×40 (Olympus BX51; Olympus, Tokyo, Japan). Total cornea and stromal thickness measurements were taken from three sections/mouse/group (n = 5 mice/group; 15 sections/group) at three separate intervals in the central cornea using ImageJ Software.[54] Stromal cells were counted using NIH ImageJ multipoint function in three H&E corneal sections/mouse (15 sections) and the average presented graphically.

Gene Expression mRNA Levels

The TG, LG, MG, and cornea were harvested and evaluated by PCR for expression of the neurotrophic/growth factors NGF, BDNF, NT-3, GDNF, and EGF and the five putative PEDF receptors (PEDF-R) LRP6, ATGL, PLXDC1, PLXDC2, and LR for all animals enrolled in the study. Samples were harvested when the mice were two weeks of age (n = 3/group) when corneal innervation pathology was evident and each sample from these cornea support tissues evaluated in triplicate by quantitative PCR. All primers used in these analyses were designed using GenBank sequences and Primer Design software. Total RNA from mouse tissues was isolated using universal RNA purification kit (EZBioscience, Roseville, MN, USA) according with the manufacturer's protocol and concentration and quality of RNA examined spectrometrically(Nanodrop 2000; ThermoFisher). RNA 1 µg RNA was used to synthesize cDNA using the Color Reverse Transcription Kit (EZBioscience) according to the manufacturer's guidance. Quantitative RT-PCR (qPCR) was performed in triplicates for each sample using SYBR Green (EZBioscience). PCR parameters used were as follows: cDNA: 300 ng; Primer: 0.5 µM; Cycle number: 35 after a Hot

Start at 95°C for 10 minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference in each reaction, and $2^{\Delta\Delta C_T}$ calculated from the cycle threshold (ct) values of the reference and target genes were used for relative quantitation and graphical representation of gene expression changes.

Protein Analyses

Corneas or trigeminal ganglions tissues were harvested when the mice were two weeks of age and mouse samples (n = 3/group) evaluated in triplicate by Western blots to determine expression and phosphorylation of ERK and AKT signaling molecules. Protein was extracted from the three separate pooled groups in cold RIPA buffer containing PMSF (Solarbio) and phosphatase inhibitor (Cell Signaling Technology, Danvers, MA, USA) for 20 minutes and total protein concentration measured using Bicinchoninic Acid (BCA) protein assay (Cwbiotech, Jiangsu, China). Ten micrograms of protein for each sample were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (EpiZyme, Shanghai, China) for one hour, and gels were transblotted to a polyvinylidene fluoride membrane (Millipore, Burlington, MA, USA). Membranes were subsequently blocked in 5% nonfat dry milk (BD Difco; BD Bioscience, Franklin Lakes, NJ, USA) for two hours at room temperature, then incubated overnight at 4°C in one of the following primary antibodies: p-ERK (1:1000), p-Akt (1:1000), ERK (1:1000), Akt (1:1000) (Cell Signaling Technology) and β-actin (Abcam; 1:5000), which was used as an internal reference. Membranes were then washed and further incubated in an appropriate secondary antibody for two hours at room

temperature. The processed blots were developed using Immobilon ECL reagent (Millipore) and imaged using a transilluminator (Tanon, Shanghai, China). The membranes were then stripped in Stripping Buffer (Cwbiotech, Shanghai, China), and reprobed with another primary antibody used or actin. Proteins band densities were calculated using NIH ImageJ software and the average protein estimation taken for graphical representation of protein levels.

Statistical Analysis

GraphPad Software (GraphPad Prism7.0; La Jolla, CA, USA) was used for statistical analysis and mapping charts. The Shapiro-Wilk normality test was used to verify the normal distribution of the data. A one-way analysis of variance was used for multigroup comparisons. An unpaired sample t-test was used to compare the results of various ages in a group. Statistical significance was determined when $P \leq 0.05$.

RESULTS

PEDF Deletion Results in Disruption of Corneal Nerve Growth

Because PEDF or a PEDF bioactive peptide can reduce corneal nerve degeneration,[26] we analyzed whether corneal stromal nerve distribution and length were affected in PEDF knockout mice. We compared total stromal nerve length in the central and peripheral cornea among wild-type, *Pedf*^{+/−} and *Pedf*^{−/−} mice at various developmental stages between two to 12 weeks of age. Our analy-
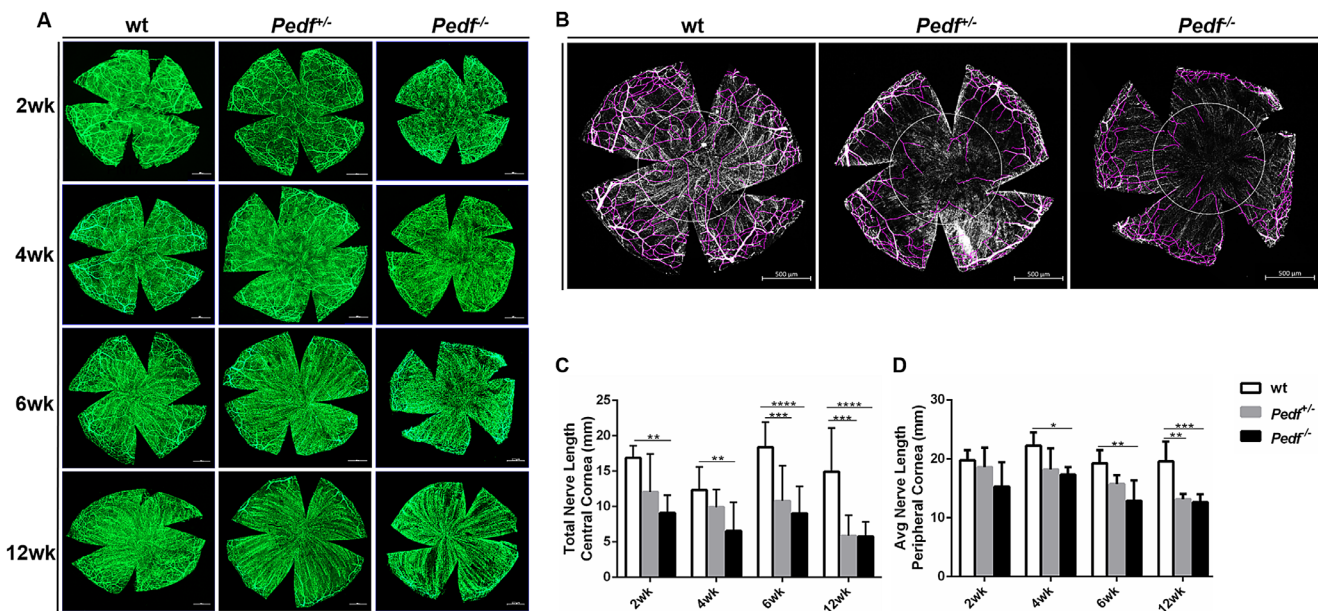

FIGURE 1. (A) Representative β Tubulin-3 (green) labeled whole-mount images depicting nerve growth in the corneal in wt, *Pedf*^{+/−} and *Pedf*^{−/−} mice ages two to 12 weeks (*Scale bar*: 500 µm). (B) Examples of stromal nerve length measured in the central (circled area) and the four quadrants of the peripheral cornea in the flat mounts. The larger nerves were traced (magenta) and measured in both areas using the NIH ImageJ tracer function (*Scale bar*: 500 µM). (C,D) Comparisons of the average total nerve length in the central and peripheral cornea among PEDF deficient mice and the control groups. In the *Pedf*^{−/−} mice, significant decreases in stromal nerve length was detected between two to 12 weeks in the central and 412 weeks in the peripheral corneas. In the *Pedf*^{+/−}, nerve loss was seen by six to 12 weeks in the central and 12 weeks in the peripheral corneas (n = 8 mice/group). Data are expressed as the mean ± SD. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ***$P \leq 0.0001$
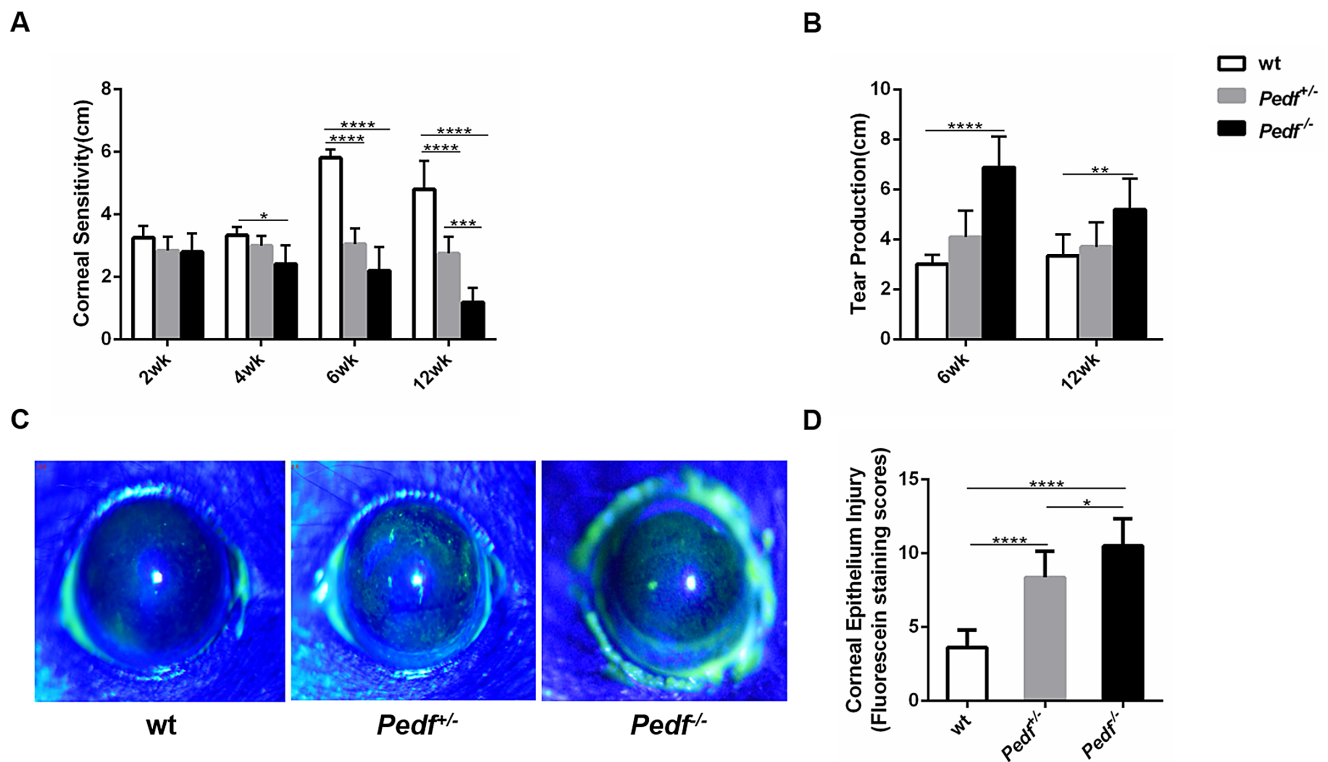
A

B

C

D

FIGURE 2. **(A)** Corneal Sensitivity measured using a Cochet-Bonnet esthesiometer. Significant changes in corneal sensitivity appeared between four to 12 weeks of age for *Pedf*$^{-/-}$ and six to 12 weeks for *Pedf*$^{+/-}$ mice compared to age-matched C57BL/6 controls. The greatest difference in corneal sensitivity for both PEDF genotypes was evident at 12 weeks of age with the decrease more pronounced in the *Pedf*$^{-/-}$ mouse corneas relative to controls (n = 8 mice/group). Data are expressed as the mean ± SD. *$P \le 0.05$; ***$P \le 0.001$; ***$P \le 0.0001$. **(B)** Tear levels, measured using phenol red cotton thread wetting, are increased in *Pedf*$^{-/-}$ mice at six and 12 weeks of age compared to age-matched wildtype C57BL/6 controls. Hemizygous PEDF KO mice did not show significant differences in tear levels relative to wildtype (n = 8 mice/group). Data are expressed as the mean ± SD. **$P \le 0.01$; ****$P \le 0.001$. **(C)** Representative images of corneal fluorescein staining in age-matched PEDF deficient and wildtype mice. The staining shows increased corneal surface retention of the fluorescein dye indicative of injury with loss of PEDF (age: four weeks old). **(D)** Increased fluorescein staining scores indicate damage to the corneal surface with deletion of either one or both PEDF alleles compared to wt. Severity of injury was greater when both PEDF alleles were absent (n = 8 mice/group; age: four weeks old). Data are expressed as the mean ± SD. *$P \le 0.05$; ****$P \le 0.0001$.

ses show a visible decrease in nerve innervation in the central cornea stroma with loss of PEDF relative to age-matched wild-type controls at all developmental ages studied (Fig. 1A). The nerve length in the central cornea showed a significant decrease in total central corneal nerve length with deletion of both PEDF alleles (*Pedf*$^{-/-}$), at all developmental stages analyzed (Fig. 1B). In the *Pedf*$^{+/-}$ mice, a similar effect was detected at six weeks of age and by the twelfth week both heterozygous and homozygous knockouts showed reduction in nerve length by 52.9% (*P* $\le$ 0.001) in *Pedf*$^{+/-}$ and 61.6% (*P* $\le$ 0.0001) in *Pedf*$^{-/-}$, mice compared to wildtype controls (Fig. 1C). There was a more slowly developing decrease in peripheral nerve length in corneas of the *Pedf*$^{-/-}$ mice with the greatest difference detected at six weeks by 22.1% (0.01) and 12 weeks by 35.4% (*P* $\le$ 0.001). Even the *Pedf*$^{+/-}$ mice showed a significant decrease in nerve length by the twelfth week (Fig. 1D).

Loss of PEDF Promotes a Decrease in Corneal Sensitivity

Corneal sensitivity in PEDF deficient mice was also assessed at two to 12 weeks of age using the classic Cochet-Bonnet

esthesiometer technique. As the normal cornea develops there is an increase in corneal innervation that should correlate with increased sensitivity. We noted that the corneas of PEDF deficient mice were significantly less responsive than wildtype controls between four to 12 weeks of age and never showed the large developmental increase in sensitivity seen with the increased corneal innervation in the wildtype mice (Fig. 2A). Corneal sensitivity was 27.4% (*P* $\le$ 0.05), 62.2% (*P* $\le$ 0.0001), and 75.3% (*P* $\le$ 0.0001) less in *Pedf*$^{-/-}$ mice than controls at four, six, and 12 weeks, respectively and in the *Pedf*$^{+/-}$ mice by 47.5% (*P* $\le$ 0.0001) at six weeks and 42.7% (*P* $\le$ 0.0001) at 12 weeks less than their age-matched wildtype. Consistent with the difference in nerve innervation pattern (Fig. 1), corneal sensitivity was delayed and less acute with loss of a single PEDF allele compared to deletion of both.

Tear Production is Increased With PEDF Deficiency

We next evaluated tear secretion among the groups using the phenol red cotton thread measurements and found that mice that completely lacked PEDF expression had
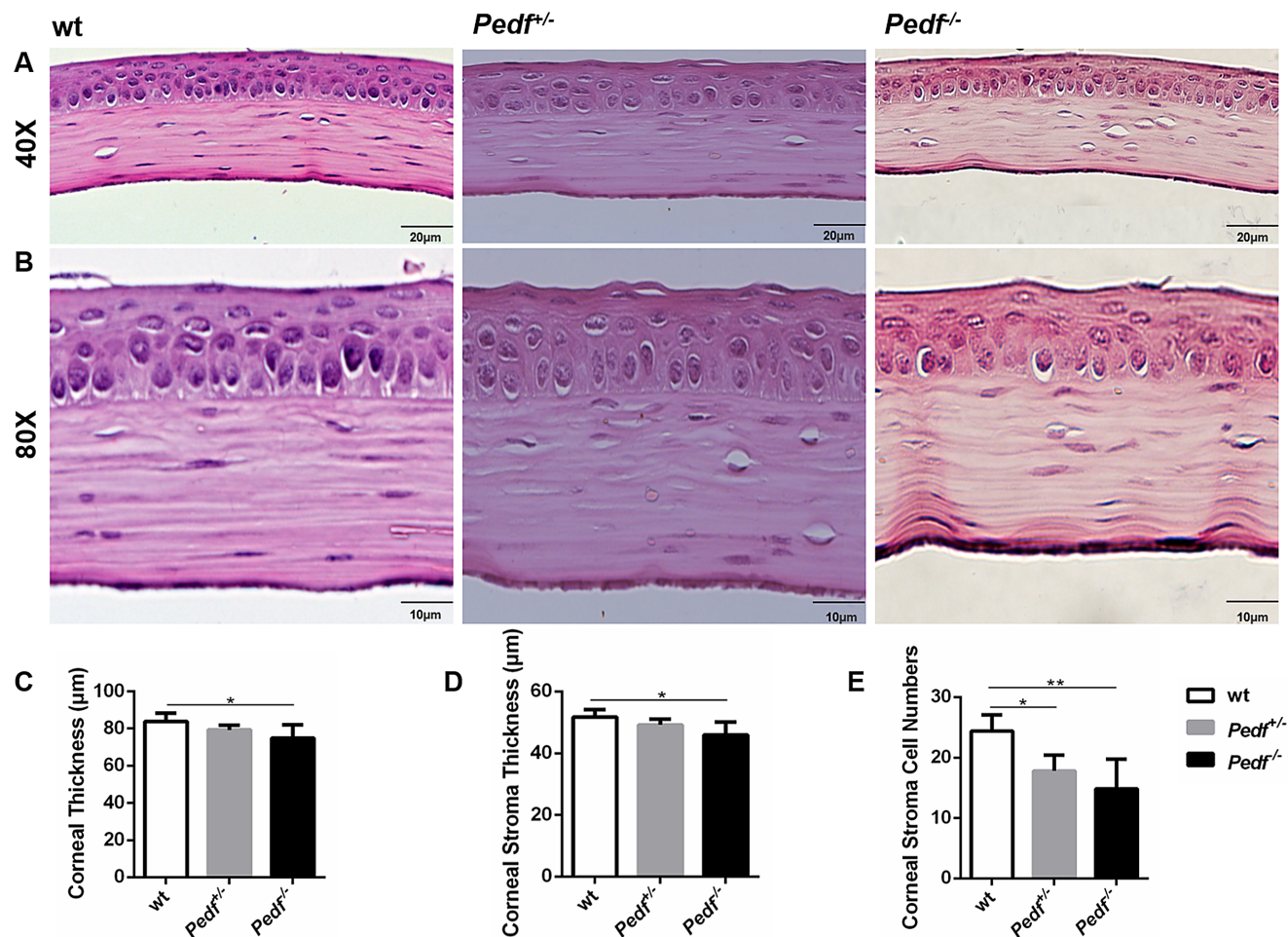
FIGURE 3. (A) Representative light microscopic images of H&E stained corneas of C57BL6 wt, *Pedf*$^{+/-}$ and *Pedf*$^{-/-}$ mice at four weeks of age. (B) Higher magnification (×80) images of corneas of the three groups. (C) Total corneal thickness measurements by NIH ImageJ indicate that corneas were thinner in the PEDF homozygous knockout mice compared to hemizygous and wildtype. (D) Loss of corneal thickness was due to a decrease in corneal stroma depth with deletion of both PEDF alleles. (E) Reduction in corneal stroma cell numbers is evident in both PEDF deficient genotypes with severity greater in the *PEDF*$^{-/-}$ mice (n = 5 mice/group). Data are expressed as the mean ± SD. *$P \leq 0.05$; **$P \leq 0.01$.

significantly increased tearing over their wildtype counterparts by 130% ($P \leq 0.0001$) and 55.2% ($P \leq 0.01$) at six and 12 weeks of age, respectively (Fig. 2B). However, animals that lost a single PEDF allele did not show a significant increase in tear volume relative to controls.

Injury to the Corneal Epithelium Accompanies PEDF Gene Deletion

With a loss of innervation and decreased sensitivity of the cornea in PEDF deficient mice, we evaluated the severity of injury to this tissue with the constitutive endogenous absence of PEDF expression. Our measurements show increased retention of fluorescein, a marker indicative of epithelial damage, on the corneal surface compared to controls (Fig. 2C, D). In the representative images, wildtype corneas have relatively weak fluorescent staining of the epithelial layer but greater fluorescein retention was evident on the epithelial surface of *Pedf*$^{+/-}$ and *Pedf*$^{-/-}$ mice by 131% ($P \leq 0.0001$) and 189.6% ($P \leq 0.0001$), respectively,

compared to wildtype mice indicating a marked increase in corneal surface damage in the absence of PEDF. Thus the loss of expression of neuroprotective PEDF results in corneal surface damage with severity greater when both alleles are absent.

PEDF Deficiency Results in Structural Modification of the Cornea

When we compared the corneal thickness in hemi- and homozygous PEDF knockout mice, we noted a visible difference in the stromal layer compared to controls (Fig. 3A). There was also a visible loss of stromal cells in the *Pedf*$^{-/-}$ corneas compared to the wildtype corneas (Fig. 3B). Our measurements indicate that the total corneal thickness in *Pedf*$^{-/-}$ mice at 4 weeks of age was reduced by 10.7% (p ≤ 0.05), with the stromal thickness alone (~11.4%; p ≤ 0.05) accounting for this reduction relative to controls (Fig. 3C, D). No significant difference in stromal thickness was seen with a loss of a single PEDF allele. The number of stromal cells
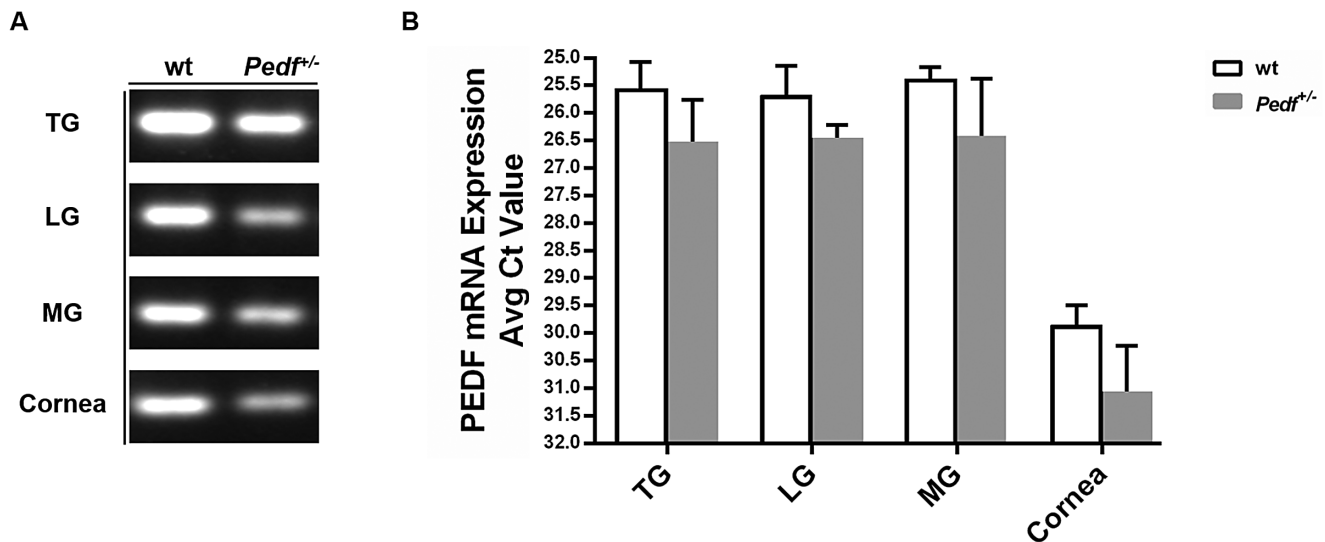
A

B

FIGURE 4. PEDF mRNA expression levels in the TG, LG, MG, and cornea tissues of C57BL6 wildtype and *Pedf*^{+/−} mice. (**A**) The PCR gel shows expression of PEDF in all four tissues and their relative amounts in the PEDF hemizygous mice. (**B**) The quantitative data were calculated from the average ct values of triplicate analyses per animal with one ct value difference representing a twofold change (n = 3 mice/tissue/group; two weeks old). Data are expressed as the mean ± SD.

in *Pedf* [+/−] and *Pedf*^{−/−} were reduced by 27.0% (p ≤ 0.05) and 39.3% (p ≤ 0.01), respectively compared to wildtype controls (Fig. 3E).

Expression of Corneal trophic Support Decreases in the Absence of PEDF

We used tissues at the two-week time point as representative of corneal nerve pathology, seen at all stages between two to 12 weeks in PEDF deficient mice, to study expression of trophic support to the cornea from the TG, LG, and MG and their expression of the PEDF receptors. The LG and MG are both innervated by the TG and support the ocular surface and its physiological functions. Loss of PEDF may interfere with the cornea trophic support function of these glands.

PEDF mRNA was expressed in the normal mouse TG, LG, MG and cornea with comparatively lower levels in the wildtype cornea and in the corresponding *Pedf*^{+/−} tissues as expected (Fig. 4). In the cornea and its supporting glands, PEDF mRNA levels were reduced by approximately one qPCR cycle representing a twofold decrease (∼50%) in expression with a loss of one PEDF allele (*Pedf*^{+/−}) compared to wildtype mice.

Given the neuroprotective function of PEDF, we next assessed how a loss of this factor influences expression of other trophic epithelial support to the cornea including NGF, BDNF, NT-3, GDNF and EGF from the TG, LG, and MG. In the TG, NGF and NT-3 were the most negatively affected and notably reduced to near depletion when both PEDF alleles were absent (*P* ≤ 0.01). *Pedf*^{+/−} mice showed similar trends as well, although to a lesser extent. BDNF was decreased by 2.36- and 2.82-fold (*P* ≤ 0.05) in *Pedf*^{+/−} and *Pedf*^{−/−}, respectively compared to wildtype while GDNF and EGF showed no significant expression changes to wildtype in the TG (Fig. 5A).

In LG samples, NGF, BDNF, and GDNF were all significantly reduced in expression levels in both *Pedf*^{+/−} and *Pedf*^{−/−} samples, whereas NT-3 showed significantly increased levels in both compared to wildtype (Fig. 5B). BDNF expression was most severely affected in the LG in both PEDF genotypes but in all cases, these changes were more pronounced in the homozygous PEDF KO. There was no significant expression level changes in EGF in this tissue.

Similarly, levels of four neurotrophic factors were decreased in the *Pedf*^{−/−} MG with the most acute change in expression detected in GDNF compared to wildtype (Fig. 5C). Changes in EGF expression in both PEDF genotypes were unremarkable and modulations in NGF and GDNF levels in *Pedf*^{+/−} were not significant.

In the cornea, expression levels of all trophic factors examined except BDNF were significantly decreased in both PEDF genotypes with NT-3 undetectable. The most severely downregulated trophic factor was BDNF in the *Pedf*^{−/−} mouse corneas compared to wildtype (Fig. 5D). The only significantly detectable difference in expression of EGF among the tissues relative to wildtype controls was observed in the corneas by ∼1.2-fold (*P* ≤ 0.05) decrease in both PEDF genotypes.

PEDF Receptors are Downregulated With PEDF Gene Deletion

We also investigated how deletion of the PEDF gene impacts expression of five putative PEDF-Rs, namely ATGL, LRP6, PLXDC1, PLXDC2, and LR,[18,19,22,23] which are important to the neurotrophic or antiangiogenic functions of PEDF. Our results showed that all five PEDF-R were expressed and transcribed at varying levels in the TG, LG, MG, and cornea. Expression of all were significantly decreased in these tissues with loss of one or both PEDF alleles except for in the cornea. In the TG of both PEDF genotypes, levels of LRP6 and PLXDC1 were barely detected whereas ATGL, PLXDC2, and LR, although decreased, were the most highly expressed in the PEDF-deficient mice compared to wildtype (Fig. 6A).
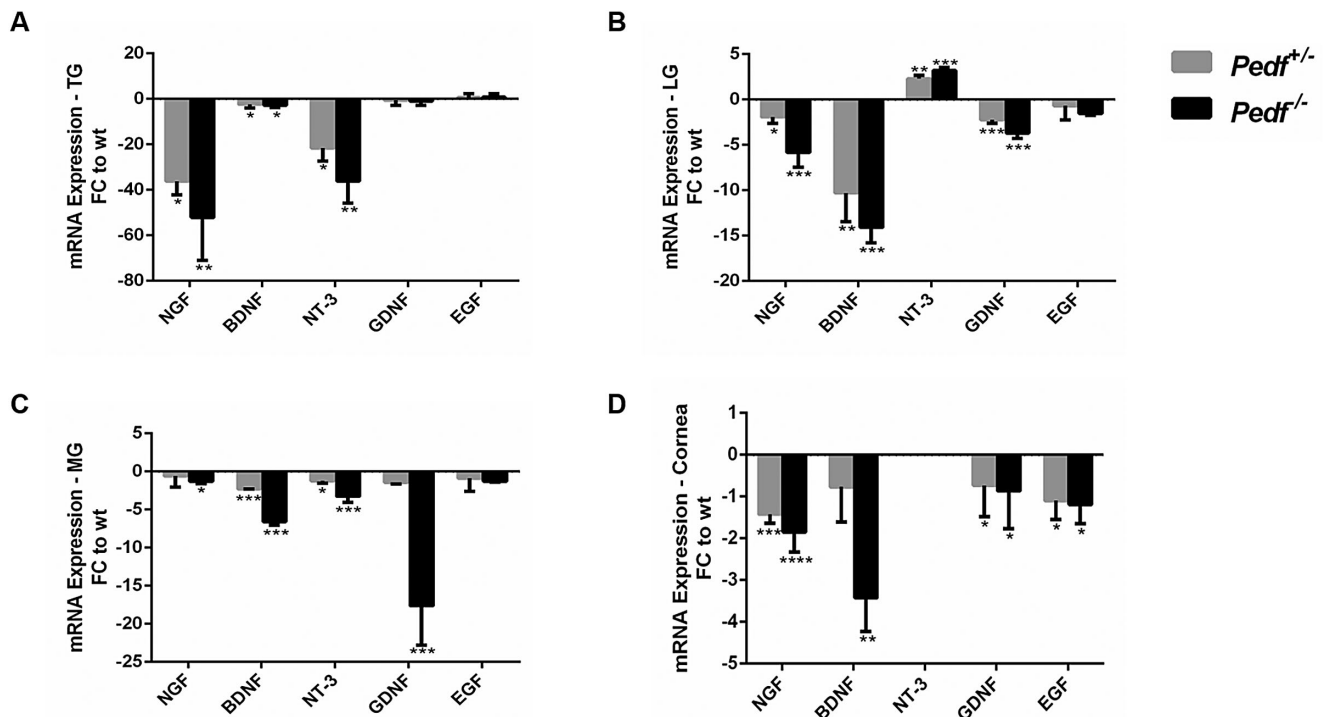
FIGURE 5. Expression of the cornea supporting factors NGF, BDNF, NT-3, GDNF, and EGF in (**A**) TG, (**B**) LG, (**C**) MG, and (**D**) cornea in PEDF-deficient mice compared to wildtype controls. NGF, BDNF, and GDNF were near depletion in one or more of the tissues. A significant decrease in EGF was detected in the corneas of PEDF deficient mice. NT3 expression levels were increased in the LG only and undetectable in the cornea. The magnitude of expression level changes in all trophic factors was greater when both PEDF alleles were deleted. **Y-axis:** FC to wt: Fold change to wildtype (n = 3 mice/tissue/group; age: 2 weeks). Data are expressed as the mean ± SD. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

Similar trends of decreased receptor transcription occurred in the LG where levels of all five receptors were downregulated in both PEDF genotypes relative to wildtype and the most severe decrease seen for PLXDC2 expression (Fig. 6B). In the MG, expression of ATGL and PLXDC1 were at their lowest levels with 4.40- and 5.4-fold differences ($P \leq 0.001$), respectively in $Pedf^{-/-}$, whereas ATGL showed ~twofold ($P \leq 0.05$) decreased expression in $Pedf^{+/-}$ mice compared to wildtype (Fig. 6C). LRP6, PLXDC2 and LR transcription levels were also significantly downregulated but to a lesser extent with loss of either one or both PEDF alleles. Interestingly, in the cornea levels of ATGL, LRP6 and LR were all significantly upregulated in the $Pedf^{-/-}$ mice, whereas PLXDC1 and PLXDC2 were undetectable (Fig. 6D). LR was the only receptor that showed a significant upregulation in expression in both PEDF genotypes compared to wildtype. Thus all five receptors appeared to be targets of regulation in the constitutive absence of PEDF expression.

ERK and Akt Activation is Reduced When the PEDF Gene is Deleted

Considering the close relationship between the TG and the corneal nerves, we examined activation of two signaling molecules, ERK and Akt, previously shown to transmit PEDF neuroprotective signals in the retina and other tissues.[11,14,55] In the TG, ERK and Akt proteins appeared as isoform doublets at ~42/44 kDa and ~54/60 kDa, respectively. Phosphorylation of both ERK1(p-ERK; 44kDa) and ERK2(p-ERK; 42kDa) was decreased in the TG by 57.2% ($P \leq 0.01$) and 65.4% ($P \leq 0.01$), respectively, and that for Akt (p-Akt; 60 kDa) by 75.2% (p ≤ 0.001) in $Pedf^{-/-}$ mice compared to wildtype. Phosphorylation of the lower Akt isoform (p-Akt; 54 kDa) was relatively undetectable in the PEDF knockout TGs. Although ERK and Akt activation was dramatically decreased in the PEDF KO compared to wt, the reverse was seen for their protein levels. ERK1/2 protein levels for both isoforms were increased by 37.3%/41.2% ($P \leq 0.05$) and Akt 60kDa isoform levels by 18.9% ($P \leq 0.05$) in the $Pedf^{-/-}$ mice relative to age-matched wildtype controls (Figs. 7A, 7B).

In the cornea, phosphorylation of ERK1 and ERK2 was decreased by 51.8% ($P \leq 0.001$) and 47.8% ($P \leq 0.05$), respectively, with PEDF gene deletion. Similar to the TG, ERK1 protein levels were increased by 23.3% with no significant change in ERK2 protein levels in $Pedf^{-/-}$ relative to wildtype controls (Fig. 7C). Only the 60kDa Akt isoform was detected in the cornea and both its activation and protein levels in this tissue remained unchanged between the wildtype and $Pedf^{-/-}$ mice (Fig. 7D). In summary, in the PEDF KO mice there was a significant increase in ERK protein levels but a decrease in its activation in both the TG and cornea. The same was true for Akt in the TG but not in the cornea with PEDF gene deletion.

DISCUSSION

The cornea and its adjacent supporting glands are densely innervated by fibers of the trigeminal ganglion. The development and maintenance of this innervation is critical to
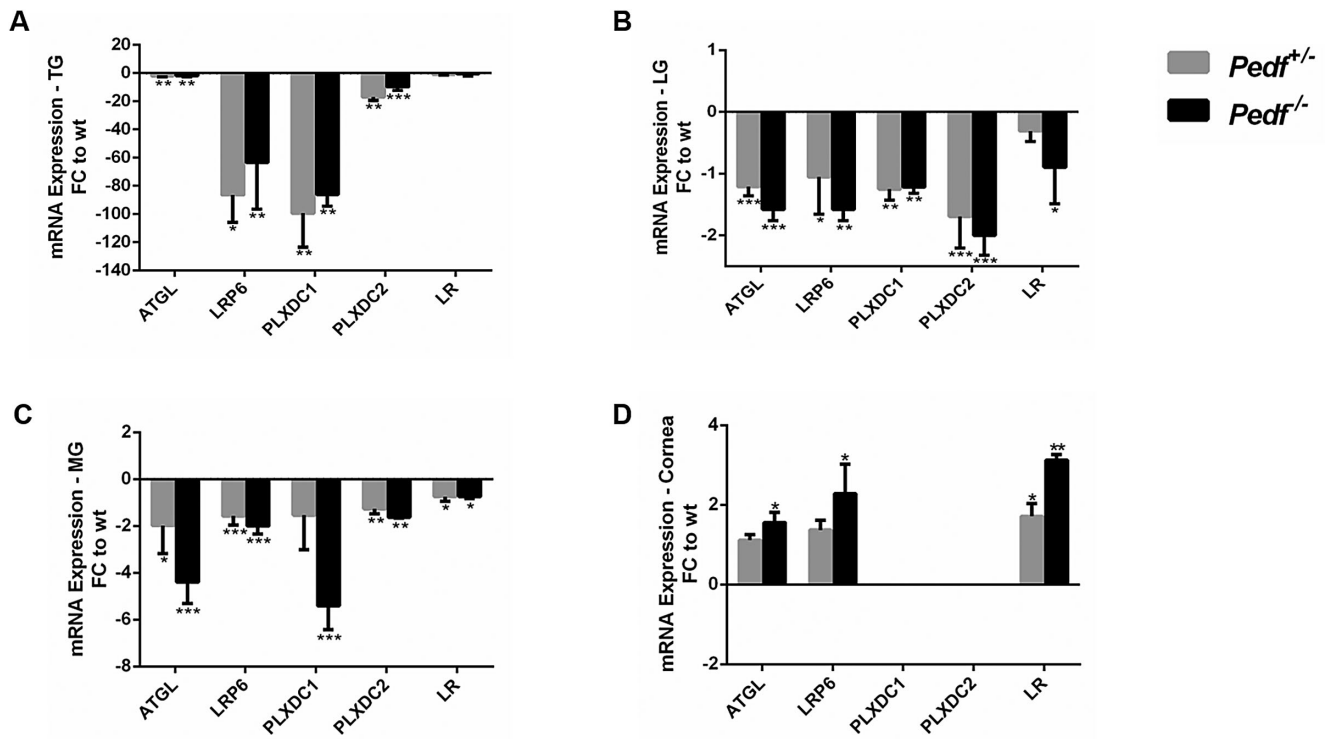
Figure 6. Expression of PEDF-R in (**A**) TG, (**B**) LG, (**C**) MG, and (**D**) cornea in PEDF-deficient mice compared to controls. The five receptors all showed varying levels of downregulation in the TG, LG, and MG of both PEDF genotypes. Three of the five receptors, ATGL, LRP6, and LR, were upregulated in the cornea in PEDF deficient mice with loss of both PEDF alleles having greater effects on their upregulation. **Y-axis:** FC to wt: Fold change to wildtype (n = 3 mice/tissue/group; age: two weeks old). Data are expressed as the mean ± SD. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
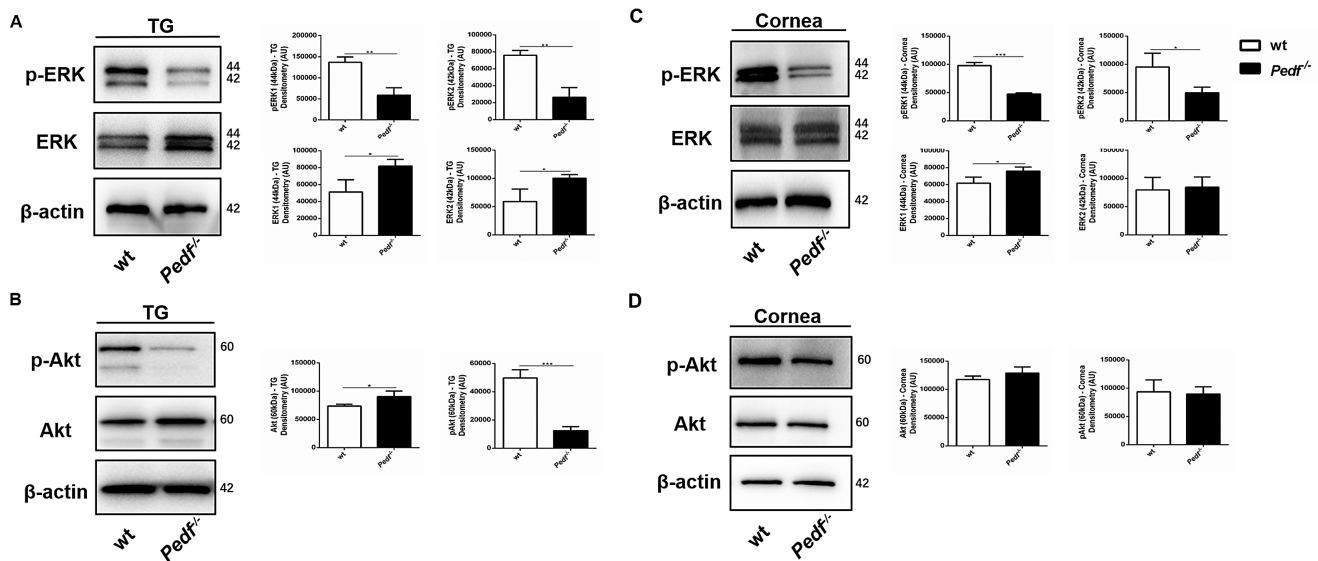

Figure 7. Expression and phosphorylation of ERK and Akt proteins in the trigeminal ganglion and cornea of *Pedf*^{−/−} and C57BL6 wildtype mice. (**A, B**) Representative Western blots and densitometry measurements of ERK and Akt protein levels and their activation in the TG of wildtype and *Pedf*^{−/−} mice. (**C, D**) Representative Western blots and densitometry analysis of ERK and Akt protein and their phosphorylation in the cornea. The β-actin was used as an internal reference for loading efficiency and densitometry analyses. ERK protein levels were increased while its activation was reduced in both the TG and cornea of the *Pedf*^{−/−} mice relative to controls. Akt protein and phosphorylation levels showed a similar profile in the TG but showed no detectable differences in the cornea (n = 3 mice/tissue/group; age: two weeks old). Data are expressed as the mean ± SD of triplicate measurements. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

the function of the cornea and are under the regulation of several neurotrophic factors. One of the key neurotrophic factors regulating the ocular surface is PEDF. Originally described as a neuronal differentiation factor and neurite promoting agent for retina tumors, PEDF is shown to have neurotrophic activity in the retina and in several other tissues where it protects neurons in neurodegenerative conditions and promotes their differentiation, thereby allowing them to extend neurites and axons.[11,55–57] PEDF is active in the CNS, where it promotes survival and axon growth in spinal motor neurons as well as survival of cerebellar granules cells and retinal neurons in degenerative conditions. It is also active in the PNS where it promotes dorsal column axon regeneration and cell survival in dorsal root ganglion neurons after injury, as well as corneal nerve regeneration.[11,26,58–61]

This protein is highly expressed in the limbus,[11] and in this study we also showed that PEDF is expressed in the TG, the LG, and MG and to a lesser extent in the cornea. Previous studies showed that treatment with PEDF, or its bioactive 44-mer N-terminal peptide, promotes functional regeneration of damaged corneal nerves in several animal models. For example, in rabbits, exogenous PEDF induced a threefold increase in subepithelial corneal nerves after corneal stromal dissection and recovered corneal sensitivity by ~75%.[30,60] Our studies complement the regeneration studies and show that in the absence of PEDF there are severe consequences to corneal nerve development in both hemizygous and homozygous PEDF knockout mice, with the homozygous showing more severe effects.

In addition to the anatomical changes in corneal innervation, PEDF deletion has multiple functional consequences to the cornea including a failure to show the normal developmental increase in corneal sensitivity. Although this effect was more acute in the PEDF homozygous knockout, it was still pronounced after deletion of a single PEDF allele. A strong correlation between corneal sensitivity and PEDF is supported by other published studies. For example, in diabetic keratopathy exogenous treatment with PEDF and docosahexaenoic acid after diabetic eyes were wounded resulted in a significant recovery of corneal sensitivity, tear production, corneal epithelial nerve regeneration, and acceleration of corneal wound healing.[26]

In both the hemizygous and homozygous PEDF knockout mice, we observed a significant level of corneal surface injury. Evidence for a role of PEDF in supporting epithelial wound healing was previously reported after experimental nerve damage in rabbits.[62] In that study,[26] PEDF was given after nerve damage for eight weeks and in addition to promoting nerve regeneration and recovery of corneal sensitivity, there was accelerated corneal wound healing indicating a strong correlation between PEDF expression and corneal wound healing. Our study highlights a critical role for PEDF in reducing corneal surface injury, given that the levels of EGF, a key corneal wound healing factor,[63] was not significantly modulated in the corneal supporting glands and was downregulated in corneas of the PEDF knockout mice relative to controls.

The depletion of NGF in the TG, LG, and cornea and the decreased expression of EGF after PEDF gene deletion, as well as the relatively lowered PEDF levels in the cornea are likely to be a set of key coordinating mechanisms to the overall lack of corneal epithelial repair in the PEDF deficient mice. Although several have shown that exogenous treatment with PEDF supports corneal nerve regeneration and wound healing, our study shows that deletion of the PEDF gene disrupts the corneal nerve innervation and surface integrity, emphasizing the importance of this gene to ocular surface health and function.

Although it could be argued that PEDF deficiency has a direct effect on trigeminal ocular neuron differentiation and nerve extension, an alternate consideration is that the disruption of corneal nerve innervation is augmented by the decreasing expression of other important corneal supporting factors such as NGF and NT-3 in the trigeminal ganglion as a response to PEDF gene deletion. Besides, several of the known PEDF receptors also show a decrease in transcriptional regulation in corneal supporting glands with deletion of the PEDF gene implying that multiple upstream events requiring neurotrophic-receptor support coordinate corneal nerve growth and function.

Similarly, we cannot at present distinguish the direct effects of PEDF loss on corneal sensitivity and injury from those resulting from the subsequent decrease in expression of other trophic support and wound healing factors normally present on the corneal surface from lacrimal and meibomian gland secretions. Nonetheless, studies showing that PEDF treatment restores several corneal pathologies argue for a direct PEDF coordination of signals promoting corneal health.

With a loss of corneal innervation in PEDF deficient mice, there was also a loss of corneal stromal thickness. The tissues in our study were paraffin embedded and therefore dehydrated with a graded series of ethanol, which would have removed any potential swelling of the cornea caused by the surface barrier injury noted in the PEDF-deficient mice. Thus the decrease in stromal thickness is most likely due to loss of innervation and stromal cells, as well as loss of PEDF. The PEDF protein is well known to have collagen binding sites[64] and especially strong affinity to type 1 collagen in the corneal stroma.[65] Collagen type 1 is predominant in the corneal stroma and forms collagen fibrils with type III and V. Type VI collagen occurs as fine filaments in the interfibrillary matrix of the stroma.[47] Loss of PEDF is then likely to interfere with collagen 1 interactions leading to a decrease in stromal thickness. The decrease in thickness noted may reflect a loss in stromal cells, innervation, PEDF, and the overall trophic support.

Given the deleterious effects of PEDF deletion on corneal health, we expected to see a decrease in tear production in the PEDF knockout mice; however, PEDF deficiency had the opposite effect. PEDF is normally found in measurable levels in human tear fluid where its role is linked to maintaining ocular surface health.[66] The concentration of this protein, however, increases in the tears of dry eye patients where it was shown to suppress the expression of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL17A.[67] The increase in tear production in the PEDF knockout mice in our study could potentially be due to a compensatory attempt by the LG, in the absence of PEDF, to increase levels of ocular surface support, such as NT3, a trophic factor that showed increased expression in the LG of PEDF knockout mice compared to controls. Increased tears in the PEDF-deficient mice are also a potentially important mechanism to dampen down inflammation at the corneal surface. The increased expression of three of the PEDF receptors in the cornea, ATGL, LRP6, and LR, are also likely changes that compensate for the loss of PEDF by increasing the corneal response to glandular secretions of trophic and anti-inflammatory support in ways that would improve corneal function and surface environment in the absence of PEDF.
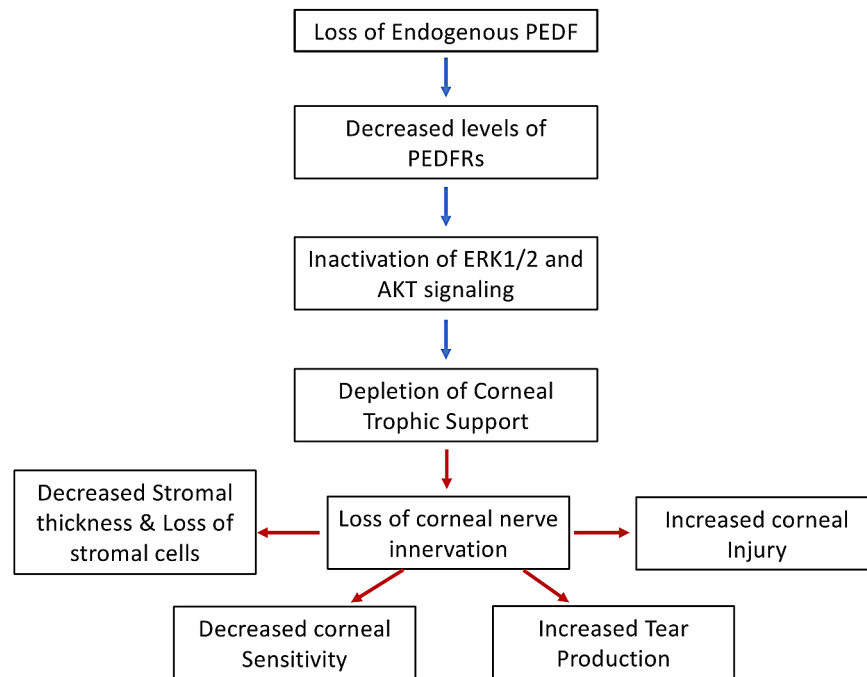
FIGURE 8. A Schematic summarizing our findings and effects of PEDF gene deletion on the structure and function of the cornea in mice.

One mechanism by which PEDF exerts its biological activity is through activation of the MAPK and AKT pathways.[11] We have shown previously that exogeneous PEDF treatment activates members of the MAPK and AKT pathways to mediate its neuroprotective activity in diabetic retinas.[15] In the present study, PEDF deficiency had the reverse effect on these signaling pathways and their cognate activating kinases resulting in increased translation of ERK1/2 in the TG and cornea but an acute decrease in its phosphorylation in both tissues. ERK activation is critically linked to the cell's translation and transcription machineries through many downstream transcription factors that regulate cell cycle, cell differentiation, and cell survival genes[68–70] and was recently implicated as essential in promoting corneal wound healing.[71–73] It could be argued that the observed reduction in ERK phosphorylation when PEDF is absent may be a direct effect of PEDF deficiency and the subsequent downregulation of its receptors. On the other hand, however, because ERK1/2 can also be activated by NGF and other neurotrophic factors, its inactivation could be a result of PEDF-induced decreases in levels of other neurotrophic molecules and receptors or because of a concerted effect from loss of PEDF and decreased levels of these factors. Regardless of whether its effect is direct or indirect, this study implicates both PEDF and the MAPK pathway in mechanisms that control corneal nerve growth and function. Lack of PEDF similarly increased AKT protein levels and decreased its activation in the TG, but these changes were not evident in the cornea. This may well be because the cornea receives other AKT stimulating factors via increased tear levels from the lacrimal gland.

From our data, our proposed model suggests that with a loss of endogenous PEDF ligand, levels of PEDF-Rs are decreased, resulting in the inactivation of key PEDF downstream signals via ERK1/2 and AKT, which might lead to a cascade of transcriptional events that has a negative effect on many corneal trophic supporting factors and proteins involved in corneal function. This, in turn, could result in the disruption of corneal nerve innervation, decreased corneal sensitivity, thinning of the corneal stroma, loss of stromal cells and a compensatory increase in nutrient and trophic laden tear volume to mitigate these events (Fig. 8).

Overall our results show that PEDF, PEDF receptors, and several neurotrophic factors are expressed in the corneal supporting tissues of the TG, LG and the MG and that PEDF is an essential molecule for the development and maintenance of corneal nerve growth and the health and function of the ocular surface. The pleiotropic effects of its removal on multiple factors, receptors and signal pathways suggest that it serves as a master integrator of many factors important for maintaining this tissue and can be exploited as a potential therapeutic option for corneal nerve pathology and other ocular surface diseases.

References

1. Eghrari AO, Riazuddin SA, Gottsch JD. Overview of the cornea: structure, function, and development. *Prog Mol Biol Transl Sci.* 2015;134:7–23.

2. DelMonte DW, Kim T. Anatomy and physiology of the cornea. *J Cataract Refract Surg*. 2011;37:588–598.

3. Zhang X, Qu Y, He X, et al. Dry eye management: targeting the ocular surface microenvironment. *Int J Mol Sci*. 2017 Jun 29;18:1398.

4. Muller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. *Exp Eye Res*. 2003;76:521–542.

5. Labetoulle M, Baudouin C, Calonge M, et al. Role of corneal nerves in ocular surface homeostasis and disease. *Acta Ophthalmol*. 2019;97:137–145.

6. Marfurt CF, Cox J, Deek S, Dvorscak L. Anatomy of the human corneal innervation. *Exp Eye Res*. 2010;90:478–492.

7. He J, Bazan HE. Neuroanatomy and neurochemistry of mouse cornea. *Invest Ophthalmol Vis Sci*. 2016;57:664–674.

8. Al-Aqaba MA, Dhillon VK, Mohammed I, Said DG, Dua HS. Corneal nerves in health and disease. *Prog Retin Eye Res*. 2019;73:100762.

9. Gonzalez-Coto AF, Alonso-Ron C, Alcalde I, et al. Expression of cholecystokinin, gastrin, and their receptors in the mouse cornea. *Invest Ophthalmol Vis Sci*. 2014;55:1965–1975.

10. Murata Y, Masuko S. Peripheral and central distribution of TRPV1, substance P and CGRP of rat corneal neurons. *Brain Res*. 2006;1085:87–94.

11. Tombran-Tink J, Barnstable CJ. PEDF: a multifaceted neurotrophic factor. *Nat Rev Neurosci*. 2003;4:628–636.

12. Tombran-Tink J, Johnson LV. Neuronal differentiation of retinoblastoma cells induced by medium conditioned by human RPE cells. *Invest Ophthalmol Vis Sci*. 1989;30:1700–1707.

13. Tombran-Tink J. The neuroprotective and angiogenesis inhibitory serpin, PEDF: new insights into phylogeny, function, and signaling. *Front Biosci*. 2005;10:2131–2149.

14. Tombran-Tink J, Barnstable CJ. Therapeutic prospects for PEDF: more than a promising angiogenesis inhibitor. *Trends Mol Med*. 2003;9(6):244–250.

15. Liu Y, Leo LF, McGregor C, Grivitishvili A, Barnstable CJ, Tombran-Tink J. Pigment epithelium-derived factor (PEDF) peptide eye drops reduce inflammation, cell death and vascular leakage in diabetic retinopathy in Ins2(Akita) mice. *Mol Med*. 2012;18:1387–1401.

16. Huang WT, Chong IW, Chen HL, et al. Pigment epithelium-derived factor inhibits lung cancer migration and invasion by upregulating exosomal thrombospondin 1. *Cancer Lett*. 2019;442:287–298.

17. He X, Cheng R, Benyajati S, Ma JX. PEDF and its roles in physiological and pathological conditions: implication in diabetic and hypoxia-induced angiogenic diseases. *Clin Sci (Lond)*. 2015;128:805–823.

18. Karakousis PC, John SK, Behling KC, et al. Localization of pigment epithelium derived factor (PEDF) in developing and adult human ocular tissues. *Mol Vis*. 2001;7:154–163.

19. Notari L, Baladron V, Aroca-Aguilar JD, et al. Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor. *J Biol Chem*. 2006;281:38022–38037.

20. Protiva P, Gong J, Sreekumar B, et al. Pigment epithelium-derived factor (PEDF) inhibits Wnt/beta-catenin signaling in the liver. *Cell Mol Gastroenterol Hepatol*. 2015;1:535–549.e14.

21. Cheng G, Zhong M, Kawaguchi R, et al. Identification of PLXDC1 and PLXDC2 as the transmembrane receptors for the multifunctional factor PEDF. *Elife*. 2014;3:e05401.

22. He T, Hu J, Yan G, et al. Pigment epitheliumderived factor regulates microvascular permeability through adipose triglyceride lipase in sepsis. *Clin Sci (Lond)*. 2015;129:49–61.

23. Bürger S, Meng J, Zwanzig A, et al. Pigment epithelium-derived factor (PEDF) receptors are involved in survival of retinal neurons. *Int J Mol Sci*. 2020;22:369.

24. Ma L, Tang SM, Rong SS, et al. Association of PEDF polymorphisms with age-related macular degeneration and polypoidal choroidal vasculopathy: a systematic review and metaanalysis. *Sci Rep*. 2015;5:9497.

25. Lee SJ, Duncan DS, Echevarria FD, McLaughlin WM, Hatcher JB, Sappington RM. Pressure-induced alterations in PEDF and PEDF-R expression: implications for neuroprotective signaling in glaucoma. *J Clin Exp Ophthalmol*. 2015;6:491.

26. He J, Pham TL, Kakazu A, Bazan HEP. Recovery of corneal sensitivity and increase in nerve density and wound healing in diabetic mice after PEDF plus DHA treatment. *Diabetes*. 2017;66:2511–2520.

27. Fan NW, Ho TC, Wu CW, Tsao YP. Pigment epithelium-derived factor peptide promotes limbal stem cell proliferation through hedgehog pathway. *J Cell Mol Med*. 2019;23:4759–4769.

28. Amaral J, Becerra SP. Effects of human recombinant PEDF protein and PEDF-derived peptide 34-mer on choroidal neovascularization. *Invest Ophthalmol Vis Sci*. 2010;51:1318–1326.

29. Tian X, Wang T, Zhang S, et al. PEDF reduces the severity of herpetic simplex keratitis in mice. *Invest Ophthalmol Vis Sci*. 2018;59:2923–2931.

30. He J, Cortina MS, Kakazu A, Bazan HE. The PEDF neuroprotective domain plus DHA induces corneal nerve regeneration after experimental surgery. *Invest Ophthalmol Vis Sci*. 2015;56:3505–3513.

31. Boyd JG, Gordon T. Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. *Mol Neurobiol*. 2003;27:277–324.

32. Markus A, Patel TD, Snider WD. Neurotrophic factors and axonal growth. *Curr Opin Neurobiol*. 2002;12:523–531.

33. Skaper SD. The neurotrophin family of neurotrophic factors: an overview. *Methods Mol Biol*. 2012;846:1–12.

34. Roux PP, Barker PA. Neurotrophin signaling through the p75 neurotrophin receptor. *Prog Neurobiol*. 2002;67:203–233.

35. Qi H, Chuang EY, Yoon KC, et al. Patterned expression of neurotrophic factors and receptors in human limbal and corneal regions. *Mol Vis*. 2007;13:1934–1941.

36. Di G, Qi X, Zhao X, Zhang S, Danielson P, Zhou Q. Corneal epithelium-derived neurotrophic factors promote nerve regeneration. *Invest Ophthalmol Vis Sci*. 2017;58:4695–4702.

37. Chaudhary S, Namavari A, Yco L, et al. Neurotrophins and nerve regeneration-associated genes are expressed in the cornea after lamellar flap surgery. *Cornea*. 2012;31:1460–1467.

38. Esquenazi S, Bazan HE, Bui V, He J, Kim DB, Bazan NG. Topical combination of NGF and DHA increases rabbit corneal nerve regeneration after photorefractive keratectomy. *Invest Ophthalmol Vis Sci*. 2005;46:3121–3127.

39. Bonini S, Lambiase A, Rama P, Caprioglio G, Aloe L. Topical treatment with nerve growth factor for neurotrophic keratitis. *Ophthalmology*. 2000;107:1347–1351; discussion 1351-1352.

40. Lambiase A, Mantelli F, Bonini S. Nerve growth factor eye drops to treat glaucoma. *Drug News Perspect*. 2010;23:361–367.

41. Keefe KM, Sheikh IS, Smith GM. Targeting neurotrophins to specific populations of neurons: NGF, BDNF, and NT-3 and their relevance for treatment of spinal cord injury. *Int J Mol Sci*. 2017;18:548.

42. Iulita MF, Bistue Millon MB, Pentz R, et al. Differential deregulation of NGF and BDNF neurotrophins in a transgenic rat

model of Alzheimer's disease. *Neurobiol Dis*. 2017;108:307–323.

43. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science*. 1993;260(5111):1130–1132.

44. Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci*. 2002;3:383–394.

45. Wang H, Yang Y, Yang M, et al. Pigment epithelial-derived factor deficiency accelerates atherosclerosis development via promoting endothelial fatty acid uptake in mice with hyperlipidemia. *J Am Heart Assoc*. 2019;8(22):e013028.

46. Pham TL, Kakazu A, He J, Bazan HEP. Mouse strains and sexual divergence in corneal innervation and nerve regeneration. *FASEB J*. 2019;33:4598–4609.

47. Chen Z, You J, Liu X, et al. Biomaterials for corneal bioengineering. *Biomed Mater*. 2018;13(3):032002.

48. Huang Q, Wang S, Sorenson CM, Sheibani N. PEDF-deficient mice exhibit an enhanced rate of retinal vascular expansion and are more sensitive to hyperoxia-mediated vessel obliteration. *Exp Eye Res*. 2008;87:226–241.

49. De Silva MEH, Hill LJ, Downie LE, Chinnery HR. The Effects of aging on corneal and ocular surface homeostasis in mice. *Invest Ophthalmol Vis Sci*.2019;60:2705–2715.

50. Murphy PJ, Lawrenson JG, Patel S, Marshall J. Reliability of the non-contact corneal aesthesiometer and its comparison with the Cochet-Bonnet aesthesiometer. *Ophthalmic Physiol Opt*. 1998;18:532–539.

51. Chucair-Elliott AJ, Zheng M, Carr DJ. Degeneration and regeneration of corneal nerves in response to HSV-1 infection. *Invest Ophthalmol Vis Sci*. 2015;56:1097–1107.

52. Reins RY, Lema C, Courson J, Kunnen CME, Redfern RL. MyD88 deficiency protects against dry eye-induced damage. *Invest Ophthalmol Vis Sci*. 2018;59:2967–2976.

53. Amparo F, Wang H, Yin J, Marmalidou A, Dana R. Evaluating corneal fluorescein staining using a novel automated method. *Invest Ophthalmol Vis Sci*. 2017;58(6):BIO168–BIO173.

54. Musayeva A, Manicam C, Steege A, et al. Role of α1-adrenoceptor subtypes on corneal epithelial thickness and cell proliferation in mice. *Am J Physiol Cell Physiol*. 2018;315:C757–C765.

55. Li H, Tran VV, Hu Y, Mark Saltzman W, Barnstable CJ, Tombran-Tink J. A PEDF N-terminal peptide protects the retina from ischemic injury when delivered in PLGA nanospheres. *Exp Eye Res*. 2006;83:824–833.

56. Steele FR, Chader GJ, Johnson LV, Tombran-Tink J. Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor gene family. *Proc Natl Acad Sci USA* 1993;90:1526–1530.

57. Tombran-Tink J, Pawar H, Swaroop A, Rodriguez I, Chader GJ. Localization of the gene for pigment epithelium-derived factor (PEDF) to chromosome 17p13.1 and expression in cultured human retinoblastoma cells. *Genomics*. 1994;19:266–272.

58. Taniwaki T, Becerra SP, Chader GJ, Schwartz JP. Pigment epithelium-derived factor is a survival factor for cerebellar granule cells in culture. *J Neurochem*. 1995;64:2509–2517.

59. Bilak MM, Corse AM, Bilak SR, Lehar M, Tombran-Tink J, Kuncl RW. Pigment epithelium-derived factor (PEDF) protects motor neurons from chronic glutamate-mediated. *J Neuropathol Exp Neurol*. 1999;58:719–728.

60. Cortina MS, He J, Li N, Bazan NG, Bazan HE. Neuroprotectin D1 synthesis and corneal nerve regeneration after experimental surgery and treatment with PEDF plus DHA. *Invest Ophthalmol Vis Sci*. 2010;51:804–810.

61. Stevens AR, Ahmed U, Vigneswara V, Ahmed Z. Pigment epithelium-derived factor promotes axon regeneration and functional recovery after spinal cord injury. *Mol Neurobiol*. 2019;56:7490–7407.

62. Cortina MS, He J, Li N, Bazan NG, Bazan HE. Recovery of corneal sensitivity, calcitonin gene-related peptide-positive nerves, and increased wound healing induced by pigment epithelial-derived factor plus docosahexaenoic acid after experimental surgery. *Arch Ophthalmol*. 2012;130:76–83.

63. Yoshioka R, Shiraishi A, Kobayashi T, et al. Corneal epithelial wound healing impaired in keratinocyte-specific HB-EGF–deficient mice in vivo and in vitro. *Invest Ophthalmol Vis Sci*. 2010;51:5630–5639.

64. Simonovic M, Gettins PG, Volz K. Crystal structure of human PEDF, a potent antiangiogenic and neurite growth-promoting factor. *Proc Natl Acad Sci USA*. 2001;98(20):11131–11135.

65. Meyer C, Notari L, Becerra SP. Mapping the type I collagen-binding site on pigment epithelium-derived factor. Implications for its antiangiogenic activity. *J Biol Chem*. 2002;277(47):45400–45407.

66. Abdiu O, Van Setten G. Antiangiogenic activity in tears: presence of pigment epithelium-derived factor. New insights and preliminary results. *Ophthalmic Res*. 2008;40:16–18.

67. Liu X, Liu H, Lu X, Tombran-Tink J, Zhao S. PEDF attenuates ocular surface damage in diabetic mice model through its antioxidant properties. *Curr Eye Res*. 2021;46:302–308.

68. Pende M, Um SH, Mieulet V, et al. S6K1(−/−)/S6K2(−/−) mice exhibit perinatal lethality and rapamycin-sensitive 5′-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol*. 2004;24:3112–3124.

69. Meloche S, Pouysségur J. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1-to S-phase transition. *Oncogene*. 2007;26:3227–3239.

70. Chambard JC, Lefloch R, Pouysségur J, Lenormand P. ERK implication in cell cycle regulation. *Biochim Biophys Acta*. 2007 Aug;1773:1299–1310.

71. Nagai N, Fukuoka Y, Ishii M, et al. Instillation of sericin enhances corneal wound healing through the ERK pathway in rat debrided corneal epithelium. *Int J Mol Sci*. 2018 Apr; 19(4):1123.

72. Chen WL, Lin CT, Li JW, Hu FR, Chen CC. ERK1/2 activation regulates the wound healing process of rabbit corneal endothelial cells. *Curr Eye Res*. 2009;34:103–111.

73. Leiper LJ, Walczysko P, Kucerova R, et al. The roles of calcium signaling and ERK1/2 phosphorylation in a Pax6+/− mouse model of epithelial wound-healing delay. *BMC Biol*. 2006;4:27.