Pigment Epithelium-Derived Factor Peptide Promotes Corneal Nerve Regeneration: An In Vivo and In Vitro Study

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Purpose. To investigate the potential of a pigment epithelium-derived factor (PEDF) peptide 44-mer to promote nerve regeneration in a rabbit corneal nerve injury model to demonstrate its neurotrophic ability in cultivated mouse trigeminal neuron cells.

Methods. Subconjunctival or intrastromal injection of 44-mer on the cornea was performed in a rabbit model of corneal nerve injury created by corneal epithelial debridement. Immunocytochemical analysis (44-mer, anti-tubulin III, SMI312, CD11b, and α-SMA) and in vivo confocal microscopy were performed. Corneal sensation was estimated using a Cochet-Bonnet corneal esthesiometer. Primary cultivated mouse trigeminal neurons were used to examine the in vitro neurotrophic ability of 44-mer. The cellular morphology and the immunocytochemical staining with anti-tubulin III and SMI312 in different concentrations of 44-mer were compared, and a quantitative assessment of neurite outgrowth was performed.

Results. Immunohistochemical staining showed the retention of 44-mer in the corneal stroma for at least 7 days after a single dose of corneal intrastromal injection and promoted corneal nerve regeneration revealed by in vivo confocal microscopy. Corneal esthesiometer demonstrated gradual recovery of the corneal sensation in 44-mer-treated eyes with a lower corneal touch threshold than wounded vehicles and closer to baseline at 3 weeks after corneal injury ($P < 0.001$). In vitro studies showed a dose-dependent neurotrophic effect of 44-mer in cultivated trigeminal neuron cells.

Conclusions. The 44-mer showed in vivo and in vitro corneal neurotrophic abilities. Our results suggest that intrastromal injection of 44-mer into the corneal stroma may have a potential role in treating diseases related to corneal nerve damage.

Keywords: pigment epithelium-derived factor, 44-mer, peptide, cornea, nerve regeneration

The cornea is densely innervated by free unmyelinated nerve endings emerging from the trigeminal nerve's ophthalmic branch and forms a sub-basal plexus under the corneal epithelium. Sensory nerve terminals extend into the cornea's surface as nociceptors and transmit sensory stimuli from the central nervous system's ocular surface. The maintenance of the cornea's refractive and protective functions depends on the healthiness of its nerve fibers.[1,2] Several ocular and systemic diseases can adversely affect corneal sensory nerves resulting in neurotrophic keratopathy, hallmarked by the corneal sensitivity impairment associated with the epithelial breakdown, which leads to a deficiency in the healing process for corneal ulceration, and subsequent loss of vision. Several ophthalmologic surgical interventions, such as refractive surgery and corneal transplantation, result in the disruption of corneal nerves, thus losing the corneal sensation that might take years to recover. In some cases, it is permanently reduced.[3,4] Although there is no specific standard treatment regimen for neurotrophic keratopathy based on this condition's pathobiology, efforts have been made to promote epithelial healing with ocular lubricants, autologous serum, bandage soft contact lenses, or tarsorrhaphy. Unfortunately, few therapeutic interventions are available today that can successfully promote the

recovery of corneal sensation.[5] In dealing with ocular surface disorders associated with corneal neuropathy, developing medications with neurotrophic ability is essential to achieving corneal surface healing and restoration of epithelial integrity.

Pigment epithelium-derived factor (PEDF), a 50 kDa size with 418 amino acids in length secreted glycoprotein, has several biological effects: neurotrophic neuroprotective, antitumorigenic, and anti-angiogenic functions on a variety of cell types. The 44-mer fragment of PEDF (residues 58–101 and amino acids Val78-Thr121) determinates its neurotrophic and neuroprotective activity.[6] It binds neurons' surface receptors in the eye, brain, and spinal cord, conferring protection against neurodegenerative insults,[7–10] oxidative stress,[11] ischemia,[12,13] glutamate excitotoxicity,[8] and axotomy.[9] Recently, several studies reported PEDF and the 44-mer peptide have a mitogenic effect on numerous stem/progenitor cell populations, including neuronal progenitor cells,[14] retinal and retinal pigmented epithelium progenitor cells,[15,16] human embryonic stem cells,[17] limbal stem cells,[18] muscle satellite cells,[19] and hepatic stem cells.[20] Supported by previous studies that PEDF has neurotrophic, neuroprotective, and mitogenic effects in several types of neural and neuro-progenitor cells of adult mammals, we hypothesized that PEDF might also play a role in corneal nerve regeneration after injury. In this study, we aimed to investigate the effect of 44-mer on corneal nerve regeneration in a rabbit model of corneal nerve injury using the in vivo confocal microscopy and determine the efficacy and safety of subconjunctival or intrastromal injection of 44-mer in promoting corneal nerve regeneration.

Materials and Methods

Chemicals and Antibodies

The bovine serum albumin (BSA), goat serum, Triton X-100, dimethyl sulfoxide (DMSO), Hoechst 33258 dye, collagenase, thiamylal sodium, and formalin were all from Sigma-Aldrich (St. Louis, MO, USA). The 4% paraformaldehyde was from EMS (Hatfield, PA, USA). The Dulbecco's modified Eagle's medium (DMEM)/F-12, trypsin-EDTA, Alexa Fluor 488, and 594-conjugated antibodies (Carlsbad, CA, USA) and fluorescent dye-conjugated secondary antibodies (A11039 and A11005; Waltham, MA, USA) were purchased from Invitrogen. The anti-PEDF (ab18071; Abcam,Cambridge, MA, USA), anti-CD11b (ab8878; Abcam), and anti-Beta-III tubulin monoclonal antibody (ab41489; Abcam) were purchased from Abcam. The anti-SMI312 (837904; San Diego, CA, USA) were purchased from BioLegend. The alpha-smooth muscle actin antibody (#NBP2-32808; Centennial, CO, USA) was purchased from Novus Biologicals LLC VECTASHIELD. The antifade mounting medium for fluorescence was purchased from Vector Laboratories (Burlingame, CA, USA). The PEDF peptide 44-mer was synthesized with the acetylation of the NH_2 termini and the amidation of the COOH termini for stability and characterized by mass spectrometry (>95% purity) to order at GenScript (Piscataway, NJ, USA).

Animals

New Zealand albino rabbits (2.0–3.0 kg, 6 months old, males) were used in this study and housed separately and maintained on a 12-hour light/dark cycle at the temperature of 24 ± 2°C. Animal care was carried out in accordance with the directories of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The Committee approved the experimental procedures for Animal Research of the Mackay Memorial Hospital. All procedures were performed on animals while they were under general anesthesia induced by the intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg) and topical anesthesia with 0.5% proparacaine hydrochloride (Alcaine; Alcon, Fort Worth, TX, USA). The right eye of each animal was surgically injured, and the left eye remained uninjured and untreated. The corneal surface nerve injury model was implemented surgically in all animals in the same manner by a single investigator. After the injury, the rabbits were randomly divided into 44-mer subconjunctival ($n = 6$) or corneal intrastromal ($n = 6$) treatment groups and corresponding sham-operated wounded vehicle groups ($n = 6$).

Corneal Epithelial Wound Healing and Nerve Regeneration in a Rabbit Model

The corneal sub-basal nerve plexus of the experimental eye was injured by removing an 8-mm diameter central epithelium of the cornea using a corneal rust ring remover (Algerbrush II 0.5-mm burr; Alger Equipment, Lago Vista, TX, USA).[21,22] We performed in vivo confocal microscopy after the experimental eye was injured to ensure that the corneal sub-basal nerve plexus was adequately removed. No nerve terminals were detectable throughout the central corneal confocal scanning (data not shown). The area of the corneal epithelial wound was determined by fluorescein staining, observed under the operating microscope (OPMI Pico I; Carl Zeiss Meditec, Jena, Germany) at 0, 7, 14, 21, and 28 days and measured by Image J software (Wayne Rasband, Bethesda, MD, USA). External eye photography, in vivo confocal microscopy, and corneal esthesiometry were performed twice a week for 3 weeks. All experiments were repeated six times.

Treatments

The 44-mer was reconstituted in DMSO as stock (5 mM) and diluted in PBS to a concentration of 100 µM.[18] In the treatment groups, the experimental eyes were wounded with central corneal epithelial removal and then treated with a single dose subconjunctival or central corneal intrastromal injection (Ultra-Fine, 29G insulin needle; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) of 44-mer (100 µM) in 200 µl PBS, once a week for 3 weeks. Wounded vehicle animals were treated with DMSO injection after surgery, correspondingly. Clinical, histological, and cell study characteristics of the corneal surface nerve recovery from 44-mer treated eyes and wounded vehicle controls were compared with untreated eyes from an unused cohort of rabbits (unwounded controls).

Immunohistochemical and Immunocytochemical Analysis

For immunohistochemistry, rabbits were euthanized with an intravenous injection of 240 mg/kg thiamylal sodium. Rabbit

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eyes were cut into 8-µm sections after cryopreservation, air-dried, and fixed in 4% paraformaldehyde for 10 minutes. Triton X-100 was used at 0.4% to permeabilize these sections and was blocked with BSA and goat serum. For visualization of 44-mer in the cornea after injection, sections were incubated with a primary antibody against PEDF at room temperature for 2 hours, followed by incubation with fluorescein isothiocyanate (FITC)–conjugated secondary antibody for 1 hour at 37°C. The staining pattern of the tissue sections was observed by conventional fluorescence microscopy using an Eclipse E800 Nikon Microscope with a VFM epi-fluorescence attachment (Nikon, Melville, NY, USA) that was equipped with a Spot Digital Camera and Spot version 1.1 CE software (Diagnostic Instruments, Sterling Heights, MI, USA). All experiments were repeated six times to ensure consistent results.

For immunocytochemical studies, we assessed the expression of beta-III tubulin (a component of the microtubule network in neurons that serves as an early mitotic neuronal marker, which correlates with ongoing neurogenesis[23]), SMI312 (a pan-axonal neurofilament marker that is selectively used against highly phosphorylated mature axons[24]), CD11b (a pan-macrophage and microglia marker[25]), and alpha-smooth muscle actin (α-SMA, a marker for myofibroblasts, which are essential effector cells of tissue fibrogenesis). Primary cultivated trigeminal ganglion cells from mice in different conditions were washed, fixed in 4% paraformaldehyde, and permeabilized with Triton X-100. After washing, cells were incubated in 2% goat serum, followed by incubating with respective primary antibodies at 4°C overnight, including anti-beta-III tubulin and SMI312 antibodies at 4°C overnight. Following incubation with the primary antibodies, cells were incubated with secondary antibodies that were conjugated to Alexa Fluor 488 and 594, counter-stained with a nuclear stain Hoechst 33258, and mounted with a VECTA SHIELD mounting medium. The cells' staining pattern was observed by conventional fluorescence microscopy using an Olympus BX51 Microscope with a U-RFLT-Fluorescence attachment (Olympus, Shinjuku, Tokyo, Japan) that was equipped with Olympus DP2-BSW software. All experiments were repeated six times to ensure consistent results.

Confocal Imaging of the Regenerated Corneal Nerve Plexus

By definition, the localization of the sub-basal nerve plexus is between the basal epithelial and Bowman layers. We used HRT3 in vivo confocal microscope (Heidelberg Engineering GmbH, Heidelberg, Germany) to evaluate the corneal epithelium and the sub-basal corneal nerve plexus with image dimensions of 400 × 400 µm2 and transverse resolution of 1 µm. After corneas were topically anesthetized, we used carbomer gel (Vidisic; Bausch & Lomb, Rochester, NY, USA) as a coupling medium between the applanation lens and the cornea. Rabbit corneas were examined on day 7, day 14, and day 21, after weekly 44-mer treatment. For each cornea, at least 300 good-quality images of all corneal layers were obtained. The selection of the sub-basal nerve plexus images was based on the best focus and contrast. ImageJ software (Wayne Rasband, Bethesda, MD, USA) was used to calculate the total length of corneal sub-basal nerve plexus. We repeated all experiments six times to ensure consistency of the results.

Corneal Touch Threshold

We used a Cochet-Bonnet aesthesiometer (Luneau Ophtalmologie, Chartres Cedex, France) to evaluate the corneal sensitivity. The monofilament tip was used to touch the cornea with a 5 mm increment decrease of the fiber length until a blink reflex. Corneal touch threshold (CTT) was defined as the stimulus that educed a corneal blinking reflex more than 50% of the time (at least 3 or more times out of 5 attempts).[26,27] Once the monofilament's length achieved the threshold (maximal length), we increased the number of testing attempts from 5 to 10 to enhance its accuracy. If no blink reflex could be evoked at a monofilament length of 0.5 cm, the CTT was registered as null. The baseline CTT was determined using the animal's contralateral untouched eye and compared with the study eye. One single examiner, blinded to the treatment, performed all measurements.

Culture of Mouse Trigeminal Ganglion Cells

We adopted a protocol for mouse trigeminal ganglion cells' primary culture from a published method.[28–30] Trigeminal ganglia were retrieved from Bltw: ICR mouse (BioLASCO, Taipei, Taiwan) and washed with ice-cold Dulbecco's modified Eagle's medium (DMEM)/F-12. These ganglia were first digested with 5 mg/mL collagenase II (Gibco, Grand Island, NY, USA), 0.25 mg/mL trypsin, and 0.2 mg/mL DNAse I (Sigma Aldrich) in DMEM/F-12 solution for 20 minutes at 37°C then treated. The digested ganglia were washed and then ground with fire-polishing pipettes. The cell suspension was centrifuged at 1500 rpm for 5 minutes, and cells were resuspended in DMEM/F-12 with 10% fetal bovine serum (FBS). The culture medium was then changed to DMEM/F-12 with different concentrations of PEDF 44-mer (0, 500, 1000, 1500, and 2000 µM) and replaced every 2 days.

The neurite outgrowth and the branching number in each region were calculated using the "Neurite Outgrowth" feature in MetaMorph Offline. Statistical analyses were performed using Sigmaplot version 10.0. Data were presented as mean ± SD. The significance was analyzed with two independent sample *t*-tests.

Data Evaluation and Statistical Methods

Statistical analyses were performed using Prism 8 (GraphPad Software, San Diego, CA, USA). The nonparametric test, the Kruskal-Wallis test, followed by Dunn's post hoc test, was used for paired comparison. All data were expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

Results

Immunolocalization of PEDF 44-mer in the Cornea After Intrastromal and Subconjunctival Injection

The 44-mer distribution in the central and peripheral cornea was examined after 1 and 7 days of subconjunctival or corneal intrastromal injection and compared with wounded vehicle controls. PEDF immunostaining was intense in the central anterior corneal stroma adjacent to the injection site 1 day after the intrastromal injection. It was also detected in the peripheral cornea at a lower intensity. These findings persisted with a decrease in density one week after treatment (Fig. 1A). In the eyes treated with subconjunctival

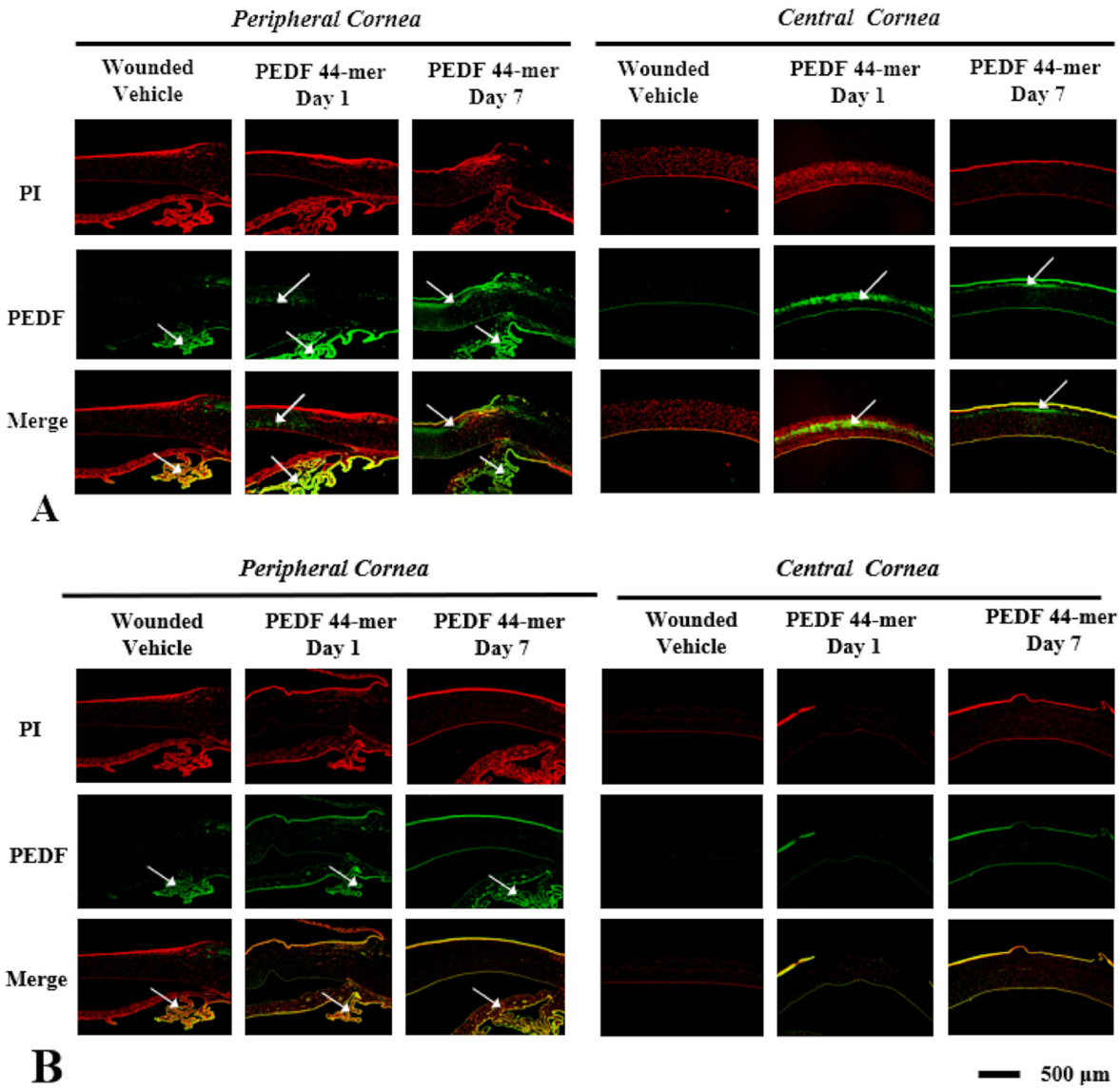

Figure 1. Immunofluorescence analysis of the distribution of PEDF in the wounded vehicle control and 44-mer treated eyes 1 and 7 days after (**A**) corneal intrastromal or (**B**) subconjunctival injection. The corneal sub-basal nerve plexus of the experimental eye was injured by removing an 8-mm diameter central epithelium of the cornea. PEDF 44-mer stained with anti-PEDF primary antibodies followed by highly absorbed-Alexa 488 (PEDF, *green*) secondary antibodies. *Arrows* denote PEDF immunostaining in the corneal stroma adjacent to the intrastromal injection site and ciliary pigmented epithelium. Nuclei were visualized with propidium iodide (PI, *red*) staining. Scale bar: 200 µm. Original magnification: × 40. Data represent two independent experiments and six rabbits per group.

injection, neither central nor peripheral cornea was stained for PEDF (Fig. 1B). Although PEDF immunostaining was not detected in the wounded vehicle corneas, it was observed in the ciliary pigmented epithelium in both the controls and the treated eyes during the entire period of observation. These findings demonstrated that the anti-PEDF antibody used in our study targeted both the native PEDF from ciliary pigment epithelia and the injected 44-mer in the cornea.

Safety of the 44-mer Intrastromal Injection

We evaluated the 44-mer intrastromal injection safety by assessing corneal epithelial wound healing, stromal haze, and neovascularization at 7, 14, 21, and 28 days after weekly intrastromal injection and compared it with wounded vehicle controls. The experimental eyes were injured with 8 mm central corneal epithelial debridement using a corneal rust ring remover. The epithelial defect area stained by fluorescein was comparable in the vehicle controls and the 44-mer–treated group with complete healing within 7 days. Neither stromal opacification nor corneal neovascularization was observed in the wounded vehicle and 44-mer–treated eyes during the follow-up period of 1 month (Fig. 2). The corneal epithelial integrity and stromal clarity remained intact in the central cornea at the intrastromal injection site in both wounded vehicle and 44-mer–treated eyes.

The Effects of 44-mer in Corneal Nerve Regeneration

In vivo confocal microscopy images of the sub-basal epithelial layer of the central cornea (at the site above where the injections were made) and peripheral cornea

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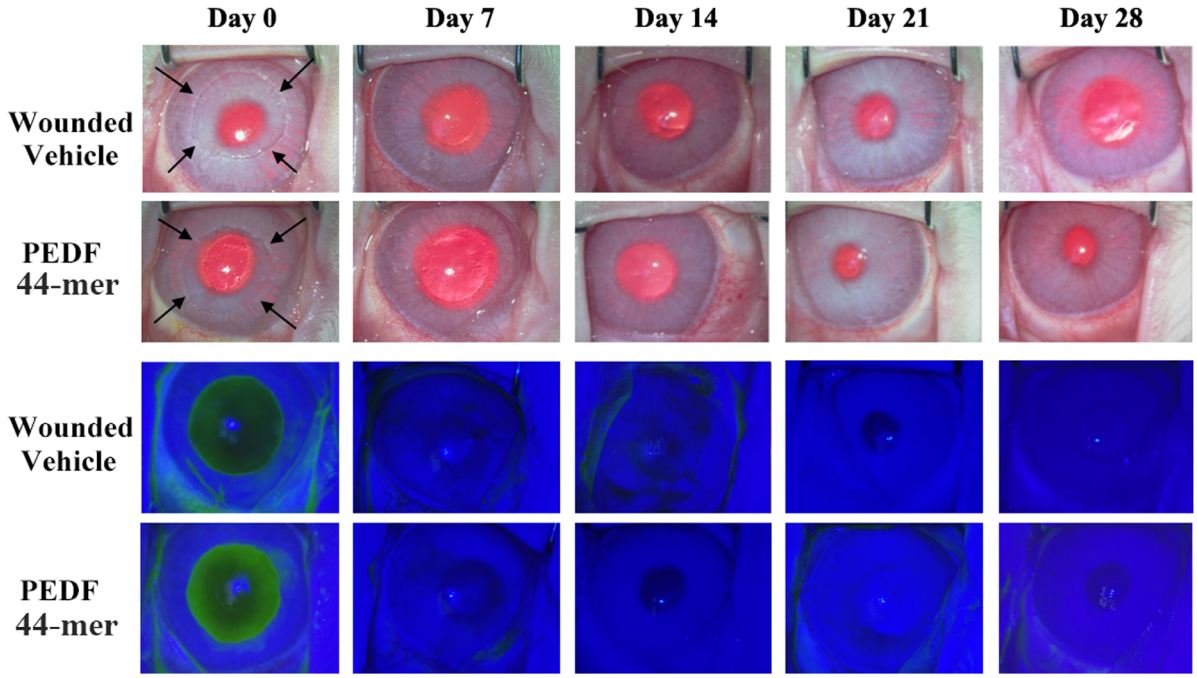

Figure 2. External photographs of the corneal wound healing pattern in the 44-mer and wounded vehicle groups. Corneal epithelial defect stained with topical fluorescein revealed complete wound healing in both 44-mer and wounded vehicle eyes after 1 month of PEDF treatment by weekly intrastromal injection. The corneal epithelial integrity and stromal clarity remained intact at the injection site in both wounded vehicle and 44-mer-treated corneas. Corneal neovascularization was not detected in either group. Representative images were from six independent experiments. *Arrows* indicate the corneal epithelial wound's margin.

(outside the wounded area) were obtained after 7, 14, and 21 days of intrastromal injection to compare the corneal nerve regeneration in unwounded control, wounded vehicle, and 44-mer–treated corneas. There were no evident morphological changes of the corneal epithelium among the treatment and control groups (data not shown). In the 44-mer-treated corneas, budding of sub-basal corneal nerve terminals occurred as soon as 1 week after treatment. In the wounded vehicle group, it was not detectable until 3 weeks of intrastromal injection. Regeneration of nerves with numerous beading and sprouting of corneal nerve terminals progressed in length and density throughout the follow-up weeks in 44-mer-treated corneas, showing significant difference compared with the wounded vehicle controls (Fig. 3). Quantification of the regenerating nerves was not provided due to the scarcity of nerve buddings in the wounded vehicle corneas to be reliably measured by conventional manual or automated cornea confocal nerve fiber metrics.

Double immunofluorescence staining of axonal neurofilament (SMI312) and dendritic cell (CD11b) markers on day 7 and day 21 after weekly PEDF intrastromal injection was performed. Evidence of SMI312 positive regenerating nerves was observed in the central cornea at the subbasal epithelial layer of PEDF treated eyes comparable to unwounded controls. Neither CD11b positive dendritic cells nor α-SMA positive myofibroblast was demonstrated in the central cornea of PEDF treated eyes. SMI312 positive nerve buddings and CD11b positive dendritic cells were visualized only in the epithelium and stroma of the peripheral cornea and limbus of both unwounded and PEDF treated corneas. These results showed that PEDF 44-mer intrastromal injection induced corneal nerve regeneration without scar formation and excluded the possibility that the nerve buddings seen via in vivo confocal microscopy might be dendritic cells (Fig. 4).

The Effects of 44-mer in Corneal Sensation

We determined the CTT on days 7, 14, and 21 after treatment to evaluate the corneal tactile sensitivity. Six rabbits were assessed per group and time point in three independent experiments. CTT measurement was performed at the previously injured central area of the cornea. The mean CTT was calculated and compared among the groups. The lower CTT values indicate a higher sensitivity, and the higher CTT values indicate a lower sensitivity. The mean CTT was 17.71 ± 2.48 mg/S (S: sectional area of the filament = 0.0113 mm2) at baseline (unwounded controls), 60.70 ± 12.21 mg/S in the wounded vehicle group, and 39.75 ± 12.27 mg/S in the 44-mer group at first week after treatment. CTT decreased progressively to 57.09 ± 10.75 mg/S and 34.43 ± 11.64 mg/S, in the wounded vehicle and 44-mer groups, respectively, in the second week. After three weeks of treatment, the mean CTT was 52.04 ± 11.07 mg/S, and 30.63 ± 6.80 mg/S in the wounded vehicle and 44-mer treated corneas, respectively. The corneas treated with 44-mer had lower CTT (higher sensitivity) that were closer to unwounded controls at 3 weeks after corneal abrasion than wounded vehicles ($P < 0.001$; Fig. 5).

The Dose-Dependent Neurotrophic Effect of 44-mer on Cultivated Mouse Trigeminal Neuron Cells

We demonstrated dose-dependent neurotrophic effects of 44-mer on cultivated mouse trigeminal neuron cells when

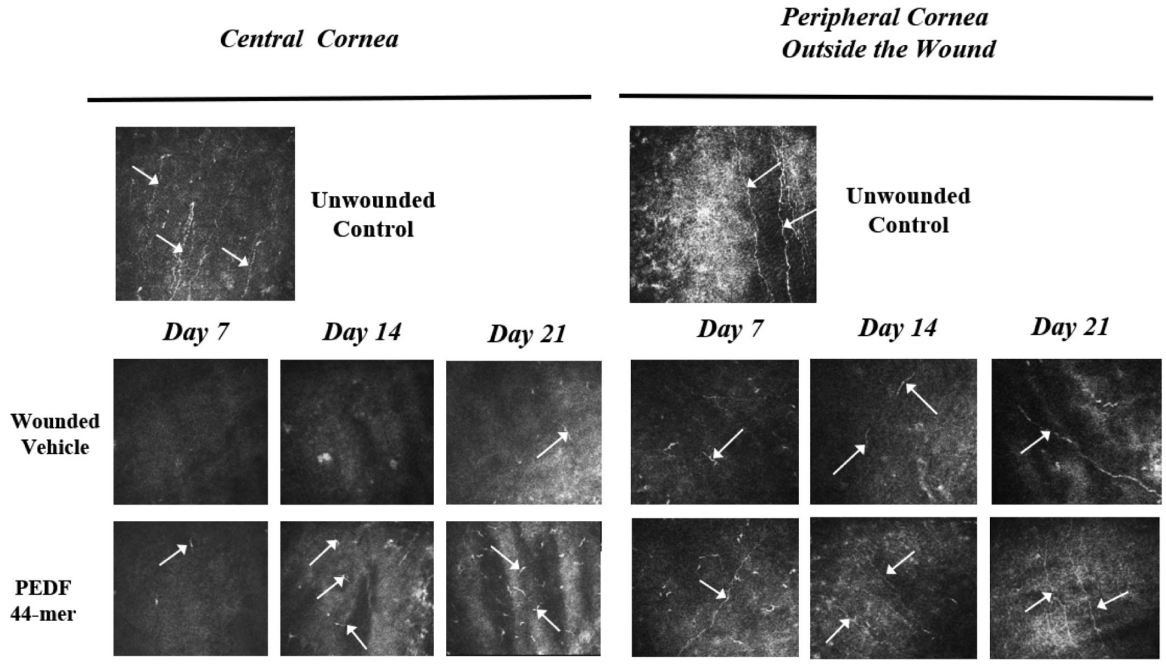

Figure 3. Confocal microscopy images of the sub-basal epithelial layer were obtained from the central cornea (above the intrastromal injection site) and peripheral cornea (outside the wounded area) of unwounded control, wounded vehicle, and 44-mer-treated eyes. Sub-basal corneal nerve terminal buddings (*white arrows*) occurred 1 week after 44-mer treatment, with a gradual increase in length and density at 2 and 3 weeks of treatment compared with unwounded controls. In the wounded vehicle group, scarce nerve budding was detectable 3 weeks after intrastromal injection. Representative images were from six independent experiments.

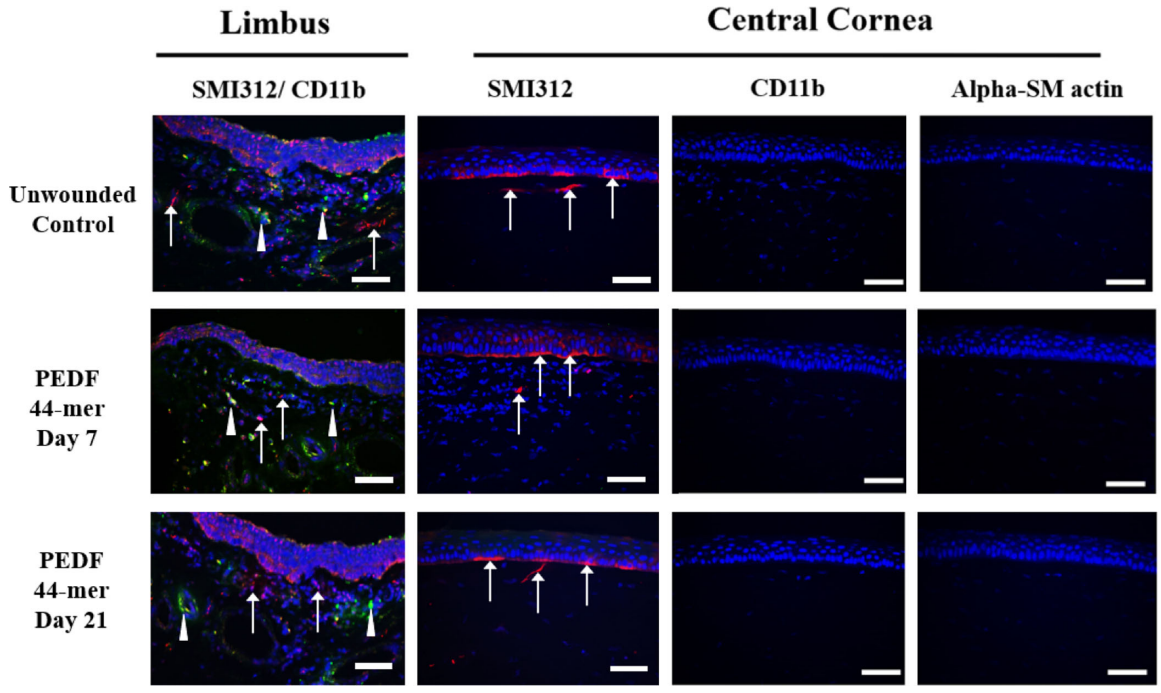

Figure 4. Immunofluorescence analysis of PEDF treated corneas after weekly intrastromal injection. Double immunofluorescence staining of axonal neurofilament (SMI312, *red*) and dendritic cell (CD11b, *green*) markers on day 7 and day 21 after PEDF treatment showed the presence of regenerated nerves (*arrows*) in the sub-basal epithelial layer of the previously injured central area of the cornea comparable to unwounded controls. CD11b-positive cells (*arrowheads*) were observed in the peripheral cornea and limbus, but not in the central cornea of both unwounded and PEDF-treated eyes. Alpha smooth muscle actin (α-SMA, *green*), a fibrogenic transformation marker, was not detected in the central cornea where consecutive intrastromal injections of PEDF were applied. Nuclei were visualized with Hoechst 33258 staining. Scale bar: 200 µm. Original magnification: × 40. Data represent two independent experiments and six rabbits per group.

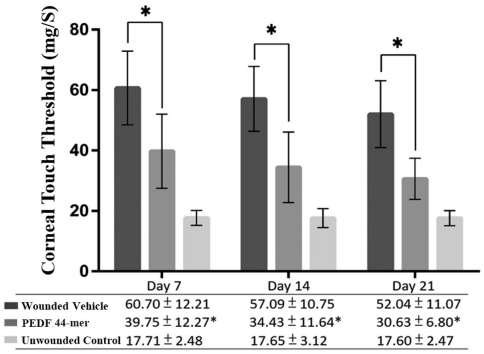

Figure 5. Comparison of the neurotrophic effect of PEDF 44-mer intrastromal treatment in the central corneal tactile sensation. Histogram showed a progressive decrease of CTT values (higher sensitivity) throughout the treatment weeks in 44-mer-treated corneas than wounded vehicle controls. Data are presented as mean ± SD when applicable. * Significant difference between PEDF 44-mer and vehicle ($P < 0.001$). CTT: corneal touch threshold = mean value of pressures (mg/S). S: 0.0113 mm^2 sectional area of the filament. Unwounded controls (baseline) were untreated eyes from a nonoperative group of rabbits. Data represent three independent experiments and six rabbits per group and time point.

added in culture media. The percentage of cells with staining for beta-III tubulin and SMI312 showed a dose-dependent increase when cultured with 1000 nM to 1500 nM of PEDF 44-mer. Higher concentrations of 44-mer at 2000 nM had adverse effects on neuron growth and differentiation. Our results demonstrated that 1000 nM was the best at promoting the cellular expression of beta-III tubulin and SMI312, followed by 1500 nM 44-mer (Fig. 6). The neurite outgrowth and the branching number in each region were calculated using the "Neurite Outgrowth" feature in MetaMorph Offline. The neurons treated with 1000 nM and 1500 nM PEDF 44-mer induced more neurite outgrowth and branching than the 0 nM PEDF 44-mer group ($P < 0.01$). In 2000 nM PEDF 44-mer treated neurons, the increase in neurite outgrowth ($P < 0.05$) and branching ($P < 0.01$) were lesser but still statistically significant as compared to 0 nM PEDF 44-mer group (Fig. 7).

Discussion

The corneal nerve integrity is essential in maintaining a healthy ocular surface for their protective role from irritants and preserving the cornea homeostasis from their trophic properties.[1,31–33] Previous studies maintained that corneal denervation due to trigeminal nerve injury resulted in corneal epithelium abnormalities, including increased permeability,[34] decreased cell proliferation,[35] phenotypic changes,[34,36,37] and delayed healing.[34,36] Clinically, corneal nerve disruption may lead to neurotrophic corneal diseases, such as neurotrophic keratopathy (NK),[38,39] dry eye disease (DED)-related neuropathic pain,[40,41] post-corneal surgery-induced neural damage,[42] and post-herpetic corneal anesthesia.[38] The corneal nerve releases neurotrophic factors, such as substance P, calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), and neuropeptide 3 (NPY),[38] which aid corneal epithelial turnover. The corneal epithelium and keratocyte also release neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF), which have been shown to influence nerve survival.[43] These studies suggested a close

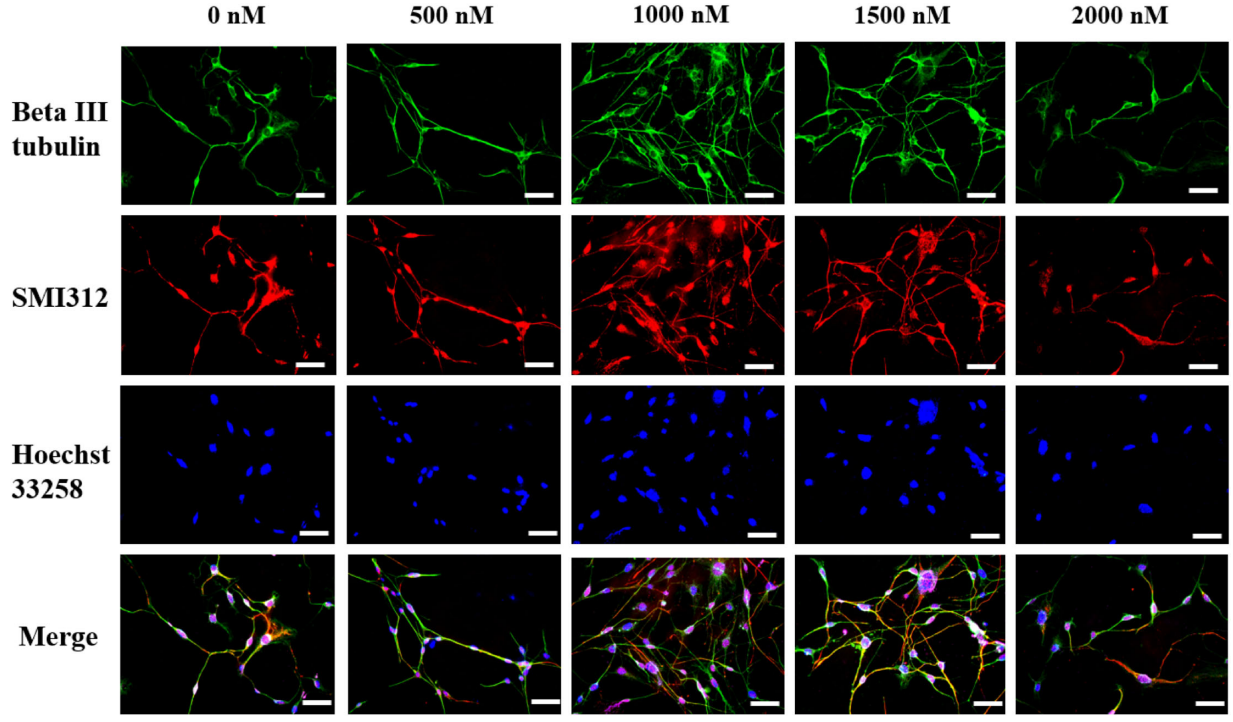

Figure 6. The representative immunofluorescence staining results of beta-III tubulin (*green*) and SMI312 (*red*) expression in trigeminal ganglion cells were cultivated in different concentrations of PEDF 44-mer in culture media. Concentrations of 1000 nM to 1500 nM of 44-mer were better at promoting neuron growth and differentiation than higher concentrations of 2000 nM. The blue fluorescent dye Hoechst 33258 was used to stain the nucleus. Scale bar: 50 µm. Representative results from six separate experiments are shown.

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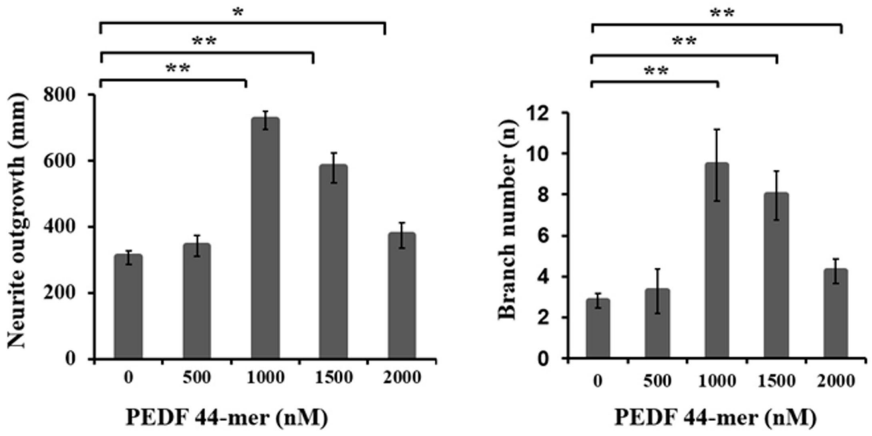

Figure 7. Quantitative analysis of in vitro neurite outgrowth and branching after PEDF 44-mer treatment. The neurons treated with 1000 nM and 1500 nM PEDF 44-mer induced more neurite outgrowth and branching than 0 nM PEDF 44-mer group; $^{**}P < 0.01$. In 2000 nM PEDF 44-mer–treated neurons, the increase in neurite outgrowth ($^{*}P < 0.05$) and branching ($^{**}P < 0.01$) were lesser but still statistically significant as compared to 0 nM PEDF 44-mer group. Quantitative and statistical analyses were performed by using MetaMorph and Sigmaplot version 10.0., respectively. Data were presented as mean ± SD. The significance was analyzed with two independent samples *t*-tests.

interaction between the corneal innervation and its epithelium, which is decisive in controlling epithelial healing and ocular surface homeostasis.

Neurotrophic factors' potential ability to promote corneal epithelial health and corneal nerve regeneration has long been studied. Neuropeptides like substance P and insulin-like growth factor-1 (IGF-1) have been tested in animals' neurotrophic corneas with delayed wound healing and, after photorefractive keratectomy, rapid corneal epithelium resurfacing.[44–46] Daniele et al. used topical mouse-derived epidermal growth factor (EGF) and reported successfully eliminated NK.[47] The REPARO study group recently reported on the efficacy and safety of human recombinant NGF in NK treatment regarding nonhealing corneal defects in a multicenter, randomized, vehicle-controlled pivotal trial.[48] Currently, NK is one of the few corneal diseases that are difficult to cure medically or surgically. Therefore, developing efficient, assessable, and affordable neurotrophic drugs is necessary for treating these patients long term.

PEDF plus docosahexaenoic acid (DHA) was first reported by He et al. to modulate inflammation and induce the regeneration of damaged corneal nerves in rabbit cornea following herpes simplex virus (HSV)-1 infection. A topical application of a 72-hour collagen shield soaked in PEDF plus DHA was used to deliver PEDF. This combined treatment also promoted corneal sensitivity recovery, increased nerve density, and enhanced wound healing in diabetic mice.[49] He et al. also reported that 44-mer plus DHA could induce corneal nerve regeneration after an experimental corneal stromal dissection.[50] Pham et al. defined the mechanistic link of PEDF/PEDF peptide to DHA for its neurotrophic effect. DHA is an omega-3 family of fatty acids found in the brain and retina in synapses and cellular membranes. During postnatal development, the accumulation of DHA in the brain correlates to synaptogenesis, dendrite formation, and photoreceptor biogenesis. As such, it is involved in aging, memory formation, synaptic membrane function, and neuroprotection.[51,52] In addition, DHA-derived lipid mediators may play a role in the anti-inflammatory and neuroprotective actions of NGF and PEDF.[53,54] To the best of our knowledge, all previously published reports combined PEDF with DHA to promote nerve regeneration.

This study showed that 44-mer alone has a strong neurotrophic effect in vivo after a corneal intrastromal injection and the in vitro dose-dependent neurotrophic effect in cultivated trigeminal neuron cells. A possible explanation is the high concentration of 44-mer after intrastromal injection. In our study, the immunostaining of intrastromal 44-mer persisted in the central cornea for at least 7 days after a single dose injection. The concomitant staining of the peripheral cornea evidenced the spread of the medication toward the entire corneal stroma, which might have facilitated corneal nerve regeneration because the nerve trunks emerged from the peripheral cornea. These effects were not observed after subconjunctival injection, where immunostaining of 44-mer was not detectable in the corneal stroma.

Moreover, the in vivo confocal microscopy demonstrated the neurotrophic effect of 44-mer with the regeneration of sub-basal corneal nerve terminals and the corneal tactile sensation's gradual recovery. Our treatment strategy is simple, clinically effective, is easy to apply, and has long-lasting effects. This treatment could avoid the drawbacks of current corneal neurotrophic factors, including the high cost of the recombinant human nerve growth factor (rhNGF),[48,55] production and storage difficulties of blood derivative topical agents,[56–60] and the inconvenience of the frequent application of topical drugs and medical costs.

There are a few limitations to our study. First, the corneal nerve regenerative animal model in this study can only represent mild NK caused by nerve terminal damage.[27] Other mouse models of NK, such as those formed by the trigeminal nerve's electrolysis, may provide a more suitable animal model for studying the therapeutic effects of 44-mer on moderate to severe NK, mainly caused by the damage to the trigeminal nerve trunk. Second, there were some observational errors in measuring rabbit corneal sensation via the Cochet–Bonnet esthesiometer despite repeated testing. Last, we did not measure the effect of other application methods, such as the topical application of 44-mer or the possible additional effect with DHA. Further experiments evaluating the effect of combined treatment with DHA and comparing the commercialized rhNGF are now underway.

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In conclusion, our study demonstrates that intrastromal injection of 44-mer has the neurotrophic ability in vivo in a rabbit model of corneal wound healing. Our results suggest that 44-mer may be a potential treatment modality for ocular surface diseases associated with corneal nerve injury.

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References

1. Muller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents, and function. *Exp Eye Res*. 2003;76:521–542.
2. He J, Bazan HE. Neuroanatomy and neurochemistry of mouse cornea. *Invest Ophthalmol Vis Sci*. 2016;57:664–674.
3. Lee BH, McLaren JW, Erie JC, Hodge DO, Bourne WM. Reinnervation in the cornea after LASIK. *Invest Ophthalmol Vis Sci*. 2002;43:3660–3664.
4. Patel SV, Erie JC, McLaren JW, Bourne WM. Keratocyte density and recovery of subbasal nerves after penetrating keratoplasty and in late endothelial failure. *Arch Ophthalmol*. 2007;125:1693–1698.
5. Lambiase A, Rama P, Aloe L, Bonini S. Management of neurotrophic keratopathy. *Curr Opin Ophthalmol*. 1999;10:270–276.
6. Filleur S, Volz K, Nelius T, et al. Two functional epitopes of pigment epithelial-derived factor block angiogenesis and induce differentiation in prostate cancer. *Cancer Res*. 2005;65:5144–5152.
7. Cao W, Tombran-Tink J, Chen W, Mrazek D, Elias R, McGinnis JF. Pigment epithelium-derived factor protects cultured retinal neurons against hydrogen peroxide-induced cell death. *J Neurosci Res*. 1999;57:789–800.
8. DeCoster MA, Schabelman E, Tombran-Tink J, Bazan NG. Neuroprotection by pigment epithelial-derived factor against glutamate toxicity in developing primary hippocampal neurons. *J Neurosci Res*. 1999;56:604–610.
9. Houenou LJ, D'Costa AP, Li L, et al. Pigment epithelium-derived factor promotes the survival and differentiation of developing spinal motor neurons. *J Comp Neurol*. 1999;412:506–514.
10. Bilak MM, Becerra SP, Vincent AM, Moss BH, Aymerich MS, Kuncl RW. Identification of the neuroprotective molecular region of pigment epithelium-derived factor and its binding sites on motor neurons. *J Neurosci*. 2002;22:9378–9386.
11. He Y, Leung KW, Ren Y, Pei J, Ge J, Tombran-Tink J. PEDF improves mitochondrial function in RPE cells during oxidative stress. *Invest Ophthalmol Vis Sci*. 2014;55:6742–6755.
12. Ogata N, Wang L, Jo N, et al. Pigment epithelium derived factor as a neuroprotective agent against ischemic retinal injury. *Curr Eye Res*. 2001;22:245–252.
13. Stellmach V, Crawford SE, Zhou W, Bouck N. Prevention of ischemia-induced retinopathy by the natural ocular antiangiogenic agent pigment epithelium-derived factor. *Proc Natl Acad Sci USA*. 2001;98:2593–2597.
14. Ramirez-Castillejo C, Sanchez-Sanchez F, Andreu-Agullo C, et al. Pigment epithelium-derived factor is a niche signal for neural stem cell renewal. *Nat Neurosci*. 2006;9:331–339.
15. De Marzo A, Aruta C, Marigo V. PEDF promotes retinal neurosphere formation and expansion in vitro. *Adv Exp Med Biol*. 2010;664:621–630.
16. Zhu D, Deng X, Spee C, et al. Polarized secretion of PEDF from human embryonic stem cell-derived RPE promotes retinal progenitor cell survival. *Invest Ophthalmol Vis Sci*. 2011;52:1573–1585.
17. Silva Dos Santos D, Coelho de Oliveira VC, Asensi KD, et al. Human menstrual blood-derived mesenchymal cells as new human feeder layer system for human embryonic stem cells. *Cell Med*. 2014;7:25–35.
18. Ho TC, Chen SL, Wu JY, et al. PEDF promotes self-renewal of limbal stem cell and accelerates corneal epithelial wound healing. *Stem Cells*. 2013;31:1775–1784.
19. Ho TC, Chiang YP, Chuang CK, et al. PEDF-derived peptide promotes skeletal muscle regeneration through its mitogenic effect on muscle progenitor cells. *Am J Physiol Cell Physiol*. 2015;309:C159–C168.
20. Shih SC, Ho TC, Chen SL, Tsao YP. Pigment epithelium-derived factor (PEDF) peptide promotes the expansion of hepatic stem/progenitor cells via ERK and STAT3-dependent signaling. *Am J Transl Res*. 2017;9:1114–1126.
21. Kakazu A, He J, Kenchegowda S, Bazan HE. Lipoxin A(4) inhibits platelet-activating factor inflammatory response and stimulates corneal wound healing of injuries that compromise the stroma. *Exp Eye Res*. 2012;103:9–16.
22. Huang C-T, Chu H-S, Hung K-C, et al. The effect of human platelet lysate on corneal nerve regeneration [published online ahead of print Nov 20, 2019]. *Br J Ophthalmol*, https://doi.org/bjophthalmol-2019-314408.
23. Ferrucci M, Biagioni F, Lenzi P, et al. Rapamycin promotes differentiation increasing betaIII-tubulin, NeuN, and NeuroD while suppressing nestin expression in glioblastoma cells. *Oncotarget*. 2017;8:29574–29599.
24. Ulfig N, Nickel J, Bohl J. Monoclonal antibodies SMI 311 and SMI 312 as tools to investigate the maturation of nerve cells and axonal patterns in the human fetal brain. *Cell Tissue Res*. 1998;291:433–443.
25. Mayer JU, Demiri M, Agace WW, MacDonald AS, Svensson-Frej M, Milling SW. Different populations of CD11b(+) dendritic cells drive Th2 responses in the small intestine and colon. *Nat Commun*. 2017;8:15820.
26. Blocker T, Van Der Woerdt A. A comparison of corneal sensitivity between brachycephalic and domestic short-haired cats. *Vet Ophthalmol*. 2001;4:127–130.
27. Huang CT, Chu HS, Hung KC, et al. The effect of human platelet lysate on corneal nerve regeneration [published online ahead of print Nov 20, 2019]. *Br J Ophthalmol*, https://doi.org/bjophthalmol-2019-314408.
28. Katzenell S, Cabrera JR, North BJ, Leib DA. Isolation, purification, and culture of primary murine sensory neurons. *Methods Mol Biol*. 2017;1656:229–251.
29. Simonetti M, Fabbro A, D'Arco M, et al. Comparison of P2X and TRPV1 receptors in ganglia or primary culture of trigeminal neurons and their modulation by NGF or serotonin. *Mol Pain*. 2006;2:11.
30. Okada Y, Sumioka T, Ichikawa K, et al. Sensory nerve supports epithelial stem cell function in healing of corneal epithelium in mice: the role of trigeminal nerve transient receptor potential vanilloid 4. *Lab Invest*. 2019;99:210–230.
31. Al-Aqaba MA, Fares U, Suleman H, Lowe J, Dua HS. Architecture and distribution of human corneal nerves. *Br J Ophthalmol*. 2010;94:784–789.
32. Marfurt CF, Cox J, Deek S, Dvorscak L. Anatomy of the human corneal innervation. *Exp Eye Res*. 2010;90:478–492.

Investigative Ophthalmology & Visual Science

33. Shaheen BS, Bakir M, Jain S. Corneal nerves in health and disease. *Surv Ophthalmol*. 2014;59:263–285.
34. Beuerman RW, Schimmelpfennig B. Sensory denervation of the rabbit cornea affects epithelial properties. *Exp Neurol*. 1980;69:196–201.
35. Sigelman S, Friedenwald JS. Mitotic and wound-healing activities of the corneal epithelium; effect of sensory denervation. *AMA Arch Ophthalmol*. 1954;52:46–57.
36. Araki K, Ohashi Y, Kinoshita S, Hayashi K, Kuwayama Y, Tano Y. Epithelial wound healing in the denervated cornea. *Curr Eye Res*. 1994;13:203–211.
37. Gilbard JP, Rossi SR. Tear film and ocular surface changes in a rabbit model of neurotrophic keratitis. *Ophthalmology*. 1990;97:308–312.
38. Dua HS, Said DG, Messmer EM, et al. Neurotrophic keratopathy. *Prog Retin Eye Res*. 2018;66:107–131.
39. Mastropasqua L, Massaro-Giordano G, Nubile M, Sacchetti M. Understanding the pathogenesis of neurotrophic keratitis: the role of corneal nerves. *J Cell Physiol*. 2017;232:717–724.
40. Villani E, Galimberti D, Viola F, Mapelli C, Ratiglia R. The cornea in Sjogren's syndrome: an in vivo confocal study. *Invest Ophthalmol Vis Sci*. 2007;48:2017–2022.
41. Benitez-Del-Castillo JM, Acosta MC, Wassfi MA, et al. Relation between corneal innervation with confocal microscopy and corneal sensitivity with noncontact esthesiometry in patients with dry eye. *Invest Ophthalmol Vis Sci*. 2007;48:173–181.
42. Wilson SE, Ambrosio R. Laser in situ keratomileusis-induced neurotrophic epitheliopathy. *Am J Ophthalmol*. 2001;132:405–406.
43. You L, Kruse FE, Volcker HE. Neurotrophic factors in the human cornea. *Invest Ophthalmol Vis Sci*. 2000;41:692–702.
44. Nagano T, Nakamura M, Nakata K, et al. Effects of substance P and IGF-1 in corneal epithelial barrier function and wound healing in a rat model of neurotrophic keratopathy. *Invest Ophthalmol Vis Sci*. 2003;44:3810–3815.
45. Nishida T. Translational research in corneal epithelial wound healing. *Eye Contact Lens*. 2010;36:300–304.
46. Yamada N, Matsuda R, Morishige N, et al. Open clinical study of eye-drops containing tetrapeptides derived from substance P and insulin-like growth factor-1 for treatment of persistent corneal epithelial defects associated with neurotrophic keratopathy. *Br J Ophthalmol*. 2008;92:896–900.
47. Daniele S, Gilbard JP, Schepens CL. Treatment of persistent epithelial defects in neurotrophic keratitis with epidermal growth factor: a preliminary open study. *Graefes Arch Clin Exp Ophthalmol*. 1992;230:314–317.
48. Bonini S, Lambiase A, Rama P, et al. Phase II randomized, double-masked, vehicle-controlled trial of recombinant human nerve growth factor for neurotrophic keratitis. *Ophthalmology*. 2018;125:1332–1343.
49. He J, Neumann D, Kakazu A, et al. PEDF plus DHA modulate inflammation and stimulate nerve regeneration after HSV-1 infection. *Exp Eye Res*. 2017;161:153–162.
50. 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.
51. Bazan NG. Cell survival matters: docosahexaenoic acid signaling, neuroprotection and photoreceptors. *Trends Neurosci*. 2006;29:263–271.
52. Pham TL, He J, Kakazu AH, Jun B, Bazan NG, Bazan HEP. Defining a mechanistic link between pigment epithelium-derived factor, docosahexaenoic acid, and corneal nerve regeneration. *J Biol Chem*. 2017;292:18486–18499.
53. 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.
54. 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.
55. Bremond-Gignac D, Daruich A, Robert MP, Chiambaretta F. Recent innovations with drugs in clinical trials for neurotrophic keratitis and refractory corneal ulcers. *Expert Opin Investig Drugs*. 2019;28:1013–1020.
56. Chen LW, Huang CJ, Tu WH, et al. The corneal epitheliotrophic abilities of lyophilized powder form human platelet lysates. *PLoS One*. 2018;13:e0194345.
57. Huang CJ, Sun YC, Christopher K, et al. comparison of corneal epitheliotrophic capacities among human platelet lysates and other blood derivatives. *PLoS One*. 2017;12:e0171008.
58. Lee YK, Lin YC, Tsai SH, Chen WL, Chen YM. Therapeutic outcomes of combined topical autologous serum eye drops with silicone-hydrogel soft contact lenses in the treatment of corneal persistent epithelial defects: a preliminary study. *Cont Lens Anterior Eye*. 2016;39:425–430.
59. Shen EP, Hu FR, Lo SC, et al. Comparison of corneal epitheliotrophic capacity among different human blood-derived preparations. *Cornea*. 2011;30:208–214.
60. Chen YM, Hu FR, Huang JY, Shen EP, Tsai TY, Chen WL. The effect of topical autologous serum on graft re-epithelialization after penetrating keratoplasty. *Am J Ophthalmol*. 2010;150:352–359.e2.

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