Materials and Methods

Reagents

Reagents and antibodies, which we used in the present study, are summarized in Supplement I and II.

Animals

Male Balb/c or C57BL/6 mice (6- to 8-week-old) were purchased from Taconic Farms (Germantown, NY). Animals were anesthetized with intraperitoneal injections of ketamine (120 mg/kg) and xylazine (20 mg/kg). All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee of the Schepens Eye Research Institute.

Corneal micropocket angiogenesis model

The corneal micropocket angiogenesis model was performed as previously described.[1] Briefly, micropellets with 160 μg VEGF-A were implanted into Balb/c mouse corneas and the corneas were examined by slit-lamp biomicroscopy for corneal neovascularization. Using a grid system, neovascularization was graded between 0 and 3 in increments of 0.5.

Immunofluorescence staining of corneal flat mounts

At day 2, 7, and 11 after micropellet insertion five mice from each group were sacrificed, the eyeballs were enucleated, and whole-mount corneal flaps were prepared for staining of blood vessels (BV) and $PTK7^{+}$ cells. All stained corneas were imaged under an inverted epifluorescence microscope (Nikon, Eclipse TE2000 instrument equipped with a Nikon digital camera, model DXM 1200, NY, USA) and/or a confocal microscope (Eclipse C1, Nikon, NY). The length of the BVs, number of spouting vessels, and infiltrating cells were analyzed by Image Grabbor (Version 1.4, Scion Corp., Frederick, MA).

Preparation of single-cell suspensions

Seventy-two hours after the insertion of a micropellet neovascularized corneas were harvested (pooled 10 corneas/group) and digested with collagenase D (Sigma-Aldrich, St. Louis, MO) at 37℃ for 1 hour. The suspension was passed through steel mesh and all cells were collected. Additionally, total bone marrow (BM) and peripheral blood cells were obtained at day 0, 3, and 7 after micropellet insertion. PBMCs and BM monoculear cells (BMMCs) were separated by Histopaque 1083 (Sigma-Aldrich) density gradient centrifugation as previously described.[2, 3]

Flow Cytometry

Single-cell suspensions from each sample were incubated with anti-FcR monoclonal antibody (Biolegend, San Diego, CA) for 15 minutes at 4℃ to prevent unspecific binding of fluorochrome-conjugated antibodies. Next, cells were incubated with fluorochrome-conjugated antibodies (see supplement 1) for 60 minutes at 4℃, and then analyzed using a FACSCalibur™ or sorted using a FACSAria III™ (BD Biosciences, Billerica, MA, USA).

Quantitative real-time Polymerase Chain Reaction (PCR)

Total cellular RNA was purified (Trizol, Molecular Research Center, Cincinnati, OH) and reverse-transcribed into cDNA using SuperscriptII enzyme (Invitrogen, Carlsbad, CA) and random hexamer primers. Quantitative real-time PCR was performed using SYBR Green master mix (Roche, Indianapolis, IN) with the primers listed in Supplement **2** and the

StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). The results were analyzed by the comparative threshold cycle method and normalized to GAPDH as an internal control.

Western blot analysis
Total protein concentrations of the supernatant were determined using the bicinchoninic acid (BCA) protein assay (BioRad Laboratories, Hercules, CA). Equal amounts of protein were boiled in equal volumes of 2× sodium dodecyl sulfate (SDS) Laemmli sample buffer, and resolved on 8% (w/v) or 10% (w/v) SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes, probed overnight with indicated primary antibodies (see Supplement 1) at 4 °C. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies and visualized by the enhanced chemiluminescence technique.

Coculture of PTK7$^+$CD11b$^+$CD45$^+$ cells with vascular endothelium on matrigel
Mouse peripheral blood or corneal PTK7$^+$CD11b$^+$CD45$^+$ or PTK7$^-$CD11b$^+$CD45$^+$ cells were isolated using a FACSAria Cell sorter. MS1 cells (a mouse pancreatic endothelial cell line) were trypsinized, counted, and resuspended in serum-free Dulbecco's Modified Eagle Medium (DMEM). The matrigel basement membrane (Chemicon, Billerica, MA) was re-suspended in dilution buffer without growth factors or heparin, placed into each well of a flat-bottomed, 48-well tissue culture plate (150 µl/well), and incubated for 1-2 hours at 37°C until adequate polymerization. Next, 1×10^4 cells/ml mononuclear cells (PTK7$^+$CD11b$^+$CD45$^+$ or PTK7$^-$CD11b$^+$CD45$^+$ cells) and 5×10^4 cells/ml VECs (MS1) were cocultured (100 µl/well) on the matrigel surface, and incubated at 37°C. Tube formation and cell migration were observed for two weeks using an inverted microscope, as described earlier.[1]
To determine Tie-2 phosphorylation of PTK7$^+$CD11b$^+$ cells, 500 µl of MS1 and PTK7$^+$CD11b$^+$ cells were cocultured using a 6-well plate. For the Boyden chamber assay, a pore size of 4.0 µm and 6-well plates (#3450, Corning, Tewksbury, MA) were used. PTK7$^+$CD11b$^+$ cells were plated in the upper and VECs (MS1) were plated in the lower chamber. After treatment with VEGF-A 30 ng/ml for 24 hours, cells were analyzed for Tie-2 phosphorylation.

Enzyme linked immunosorbent assay (ELISA)
ELISA assay was used to determine the protein concentration of ANG-1 and ANG-2 in the supernatant from the VEC and PTK7$^+$CD11b$^+$ cell coculture using the Boyden chamber (BC) or a mixed co-culture (MC) following manufacturer's instruction (Millipore, Darmstadt, Germany).

Transient downregulation of PTK7 using small interfering RNA
To knock-down PTK7, cells were transfected with small interfering (si)RNA directed against *PTK7* mRNA (siPTK7), or a control siRNA (siCon), as previously described.[1] In brief, 20 pmol of siRNA was mixed with 1 µL Lipofectamine 2000 (Invitrogen, Carlsbad, CA) to form a transduction complex that was then added to culture medium of low serum concentration. After 4 hours of transduction, the medium was changed to normal medium. siRNA transduction effectiveness, were confirmed by real-time PCR and Western blot.

Transfection and luciferase assays
To test for NF-κB-dependent transcriptional activity, PTK7$^+$CD11b$^+$ cells were transfected with PTK7 siRNA or control siRNA, and a NF- B luciferase reporter plasmid using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA) for 12 hours. Cells were stimulated

with or without VEGF-A for the indicated time. Luciferase assays were performed using a luciferase kit (Promega, Madison, WI) following the manufacturer's protocol, and luciferase activity was detected using a luminometer.

Statistics

All data are expressed as mean±standard deviation (SD). Differences between groups were examined by multivariate analyses using the Newman-Keuls test or analysis of variance, followed by the Bonferroni post-test using SPSS 21.0 (Chicago, IL). Values of $p<0.05$ were considered to be statistically significant.

References

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