HGF regulation of RPE proliferation in an IL-1β/retinal hole-induced rabbit model of PVR

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Purpose: To understand molecular events that lead to retinal pigment epithelial (RPE) cell proliferation and migration during the early phases of proliferative vitreoretinopathy (PVR) in a rabbit model.

Methods: Retinal holes were created and interleukin-1β (IL-1β) was injected intravitreally. Eyes were examined by indirect ophthalmoscopy and eyecup pieces containing retinal holes were analyzed at different times after the surgery up to 4 weeks. RPE proliferation and migration were examined by immunohistochemistry. Tyrosine phosphorylation of extracellular signal regulated kinase (ERK) and hepatocyte growth factor receptor (HGFR or c-met) was determined by immunoprecipitation and western blot analysis. Tyrosine phosphorylation of c-met and morphological studies were performed on vitreous treated ARPE-19 cells. Expression of c-jun was determined by Northern blot analysis. Matrix metalloproteinase (MMP) content in vitreous was assessed by zymography.

Results: Indirect ophthalmoscopy identified formation of epiretinal membrane and immunohistochemistry identified proliferative and migratory RPE and other cells in the posterior segment containing retinal holes at 4 weeks post-surgery. Tyrosine phosphorylation of ERK and c-met occurred in this segment within 30 min of surgery. ARPE-19 cells treated with vitreous from the 24 h post-surgical eyes, but not with control vitreous or IL-1β, showed morphological changes and tyrosine phosphorylation of c-met. Northern blot analysis in this segment identified upregulation of c-jun within 30 min of surgery and the expression peaked at 72 h. Zymographic analysis of vitreous identified MMP-9 in 12-72 h post-surgery.

Conclusions: These data suggest that the presence of retinal holes and IL-1β may lead to activation of HGF, mitogen activated protein kinases (MAPK), c-jun and extracellular matrix remodeling, resulting in proliferative and migratory cells in the wounded retina.

Morphologically and functionally differentiated retinal pigment epithelial (RPE) cells in the normal eye retain the ability to migrate and proliferate in response to a wide range of pathological insults [1]. In proliferative vitreoretinopathy (PVR), a common cause of retinal detachment after successful surgical repair, epiretinal membranes arise from dispersed vitreal RPE cells, glial cells, inflammatory cells, and extracellular matrix, and may cause traction retinal detachment [2]. Even when PVR does not develop, failure of the RPE to revert from the migratory and proliferative phenotype to its normal phenotype may be a cause of poor return of vision after retinal reattachment [3]. Since the RPE plays an active role in PVR, a better understanding of the mechanism through which RPE growth control is achieved would provide important information that could have clinical implications. Toward this end, recent studies have suggested that several autocrine or paracrine loops involving cytokines, growth factors and their tyrosine kinase receptors may be critical as pathological insults to RPE cells. In particular, interleukin-1-beta (IL-1β) has been shown to be produced by RPE cells [4] and has been found in ocular fluids during inflammation, trauma and PVR [5,6]. Hepatocyte growth factor (HGF) and its receptor, c-met, may also have a role in the migration and proliferation of RPE cells in PVR [7-9]. In addition, the wound healing process of PVR involves the unbalanced action of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) in the degradation and contraction of the extracellular matrix [10,11].

To study molecular mechanisms involved in and potential therapy for PVR, various in vivo models of PVR have been designed. Most of these models rely on the vitreal introduction of exogenous cells that are found in PVR patients, such as RPE cells [12] or fibroblasts [13,14], and the procedure often includes other manipulations such as lensectomy, vitrectomy, or both. The time course of membrane formation and retinal detachment depends on the type and number of cells introduced. Other models include the introduction of platelets combined with additional components. A noncellular model of PVR involving the introduction of PDGF and fibronectin also included vitreous extrusion, a potential source of traction on the retina [15]. Another noncellular model without lensectomy or vitrectomy involved intraocular injection of dispase, whose enzymatic activity dissociates local tissues and degrades extracellular matrix, also generated similar result [16]. These models show membranes composed mostly of fibrous tissues that cause retinal detachment. However, RPE
cells have not been identified in these membranes [15,16] and the retinas appear intact without cellular invasion from the RPE layer [14]. A recent model of PVR has been designed that relied on the actions of endogenous cells and without lensectomy or vitrectomy. In this model, intraocular injection of IL-1β and formation of retinal holes triggered a cascade of events including RPE hyperplasia, epiretinal membrane formation, and MMP induction that lead to PVR [17]. However, RPE proliferation and the molecular events that lead to the proliferation were not characterized. We re-established this model in order to study these molecular events.

METHODS

Reagents and antibodies: The rabbit polyclonal antibodies for rat ERK 1 (p44, also reactive with ERK 2 [p42]), and mouse monoclonal antibodies for pan-cytokeratin (IgG1), proliferating cell nuclear antigen (PCNA; IgG1), mouse c-met, blocking peptide for c-met, human p-ERK, phosphorytrosine, as well as Protein A/G Plus Agarose used in immunoprecipitation and western blot or for immunohistochemical analyses were all purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The normal mouse IgG was from Calbiochem (San Diego, CA). The Oregon Green or Texas Red labeled goat anti-rabbit and goat anti-mouse subtype specific IgG antibodies were purchased from Molecular Probes (Eugene, Or). P42/p44 MAP Kinase Assay Kit was purchased from Cell Signaling/New England Biolabs (Beverly, MA). Peroxidase labeled anti-rat or anti-mouse IgG and Enhanced Chemiluminescence (ECL) reagent was obtained from Amersham (Piscataway, NJ). Dubelcco’s phosphate buffered saline (PBS), and protease inhibitor cocktail were obtained from Sigma (St. Louis, MO). DNase, oligo-dT, RNase inhibitor, MMLV reverse transcriptase, D-MEM/F-12 with L-glutamine, Ham’s F10 and other cell culture reagents including ITS were obtained from Life Technologies (Grand Island, NY). Nitrocellulose membranes were obtained from Schleicher & Schuell (Keene, NH). Cell transfection reagent, Cytofectectant, standard protein assay or detergent compatible protein assay kits, and nonfat dry milk were obtained from Bio-Rad (Hercules, CA).

Animals, surgical, and clinical procedures: Thirty two pigmented rabbits weighing between 4 and 6 pounds were obtained from Myrtle’s Rabbit Farm (Thompson Station, TN), maintained on a 12 h light: 12 h dark lighting cycle and were fed the rabbit chow diet (Teklad Global High Fiber Rabbit Diet, Madison, WI). Care and use of the animals adhered to the principles set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbit model of PVR was prepared according to a modified method using intravitreal injection of IL-1β in eyes containing preexisting retinal holes [17]. Briefly, rabbits were anesthetized, pupils dilated, and 15 retinal holes, half disc size in size, were created in a single eye with a 27 gauge needle inferiorly to the medullated nerve fiber layer. Two hundred-fifty units of human IL-1β in 0.1 ml balanced saline solution (BSS) were then injected to the injured eye intravitreally over the optic nerve. The contralateral eye was used as a control and received 0.1 ml of BSS alone. The development of PVR was determined by clinical examinations, including fundus photographs taken with Kowa portable fundus camera once per week for 4 weeks. The rabbits were then used for various analyses in time course studies. Clinical observations were made regarding anterior segment inflammation, clarity of the cornea and lens, pupillary dilation, vascular tortuosity and dilation, hemorrhage, distortion of medullary wings, presence of intravitreal haze and membranes, and formation of retinal breaks, traction, and detachment. The methods of anesthesia, using the intramuscular injections of ketamine and xylazine, and euthanasia, using an intravenous 300 mg overdose of sodium pentobarbital, are consistent with the Panel on Euthanasia of the American Veterinary Medical Association. Photographs were made using a Nikon camera.

Tissue processing: After overdose with sodium pentobarbital, whole eyes were enucleated and anterior chambers containing the lenses were removed. After the removal of the vitreous, the remaining eyecups were further dissected with a dermatology circular cutter (AcuPunch, Acuderm Inc., Ft. Lauderdale, FL) to collect 10 mm diameter pieces that contain all the retinal holes. These eyecup pieces were frozen in OCT for at least 24 h and sectioned at 10 µm. For immunoprecipitation and western blot analyses, the retina and RPE were collected with a dissecting spatula and homogenized in 500 µl of an ice cold protease and phosphatase inhibiting lysis buffer.

Immunohistochemistry: Immunohistochemical analysis was used to localize cytokeratins and PCNA in intact eyes from rabbits. The 10 µm thick cryosections of rabbit eyes were fixed with ice cold acetone and blocked with 4% normal goat serum. Samples were incubated with one of the monoclonal antibodies. Tissue sections were incubated for 3 h at room temperature at a dilution of 1: 100 followed by an overnight incubation at 4 °C. Incubation with normal mouse serum served as negative control. After rinsing, all samples were incubated overnight at 4 °C with an Oregon Green conjugated goat anti-mouse IgG at a dilution of 1:500. Cryosections were optically sectioned (z series) using a Bio-Rad MRC-600 Laser Scanning Confocal Imaging System.

Immunoprecipitation and western blot analyses: For immunoprecipitation and western blot analyses, rabbit retina/RPE tissues were lysed in ice cold lysis buffer containing 10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% NP-40, 1 mM Na3 VO4, 50 mM NaF, and 10 µM protease inhibitor cocktail containing AEBSF, bestatin, aprotime, E-64, pepstatin-A, and leupeptin. The lysates were centrifuged for 15 min at 14,000 rpm in a microcentrifuge. Protein content of the resultant supernatants was measured by Bio-Rad detergent compatible protein assay. The supernatants containing 500 µg proteins were then incubated for 2 h or overnight at 4 °C with 10 µg (2 µg) Protein A/G Plus agarose beads conjugated to anti-phosphotyrosine antibodies. Immune complexes were collected and washed 4 x with the lysis buffer. Immunoprecipitates, tissue or cell lysates were resolved on 10% sodium
dodecyl sulfate-polyacrylamide gel under reducing conditions and electroblotted to nitrocellulose membrane. The membranes were blocked for 1 h at room temperature in 5% nonfat dry milk in 1 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 (TTBS), and probed with antibodies to c-met, ERK or pERK (1: 500 in 0.5% nonfat dry milk in TTBS) for 1-2 h at room temperature. The membranes were then washed 3 times with TTBS, incubated with horseradish peroxidase labeled secondary rat anti-mouse IgG (1: 3,000) in 0.5% nonfat dry milk in TTBS, and washed 3 times in TTBS. The membranes were then developed with ECL reagent.

Cell culture: ARPE-19 (ATCC, passage number 22; [18]) cell line was maintained in Dulbecco’s Modified Eagle Media (D-MEM)/F-12 with L-glutamine and supplemented with 10% fetal calf serum (unless otherwise mentioned), 0.1 mg/ml streptomycin and 100 U/ml penicillin. All cells were maintained at 37 °C under 5% CO₂. Vitreous humor samples from normal rabbit eyes or from eyes at different times after surgical treatment were diluted with serum free medium to a final concentration of 25% vitreous, and filtered through a 0.45 µm filter. Subconfluent cells (1x10⁴/cm²) cultured in chamber slides were starved in serum free medium for 18 h and were treated with fresh serum-free medium with IL-1β at the level of 250 U/ml or with 25% vitreous for 1, 4, or 48 h. Cells were screened daily, contaminated cultures were removed immediately.

RNA extraction and Northern blot analysis: RNA was extracted from sand-homogenized rabbit eyecup pieces using total RNA isolation reagent kit, RNAwiz, which is based on the use of detergent, chaotropic salts and phenolic extraction to denature the RNase and solubilize the total RNA. In some applications, RNA was also extracted by RNeasy, which is based on the selective binding properties of silica gel based membrane with the microspin technique. For Northern blot analysis, 10 µg of total RNA was resolved on 1% formaldehyde-agarose gel and transferred to nitrocellulose membranes, baked and hybridized with RNA probes according to a formaldehyde-based system from Ambion as described by the manufacturer. The antisense RNA probes for c-jun and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were transcribed and labeled with ³²P-UTP (800 Ci/mm, Amersham). G3PDH was used as an internal standard.

Metalloproteinase activity of vitreous: Rabbit vitreous humor samples of approximately 1 ml were homogenized by vigorous shaking at 4 °C and centrifuged at 16,000x g for 15 min. The supernatant fraction containing soluble proteins were collected, aliquoted and stored at -70 °C until use. For gelatinolytic zymogram, the vitreous samples of equal amount of protein, determined by Bio-Rad Protein Assay, were mixed with SDS-PAGE sample buffer in the absence of reducing agent (50 mM Tris-HCl, pH 7.5, 1% SDS, 10% glycerol, and 0.1% bromophenol blue). Samples were electrophoresed in 9% polyacrylamide gel containing 0.1% (w/v) gelatin. Electrophoresis was performed at 4 °C with 100 volts for 15 min.

Figure 1. Fundus photographs of normal rabbit eyes and eyes with retinal holes and inflammation. Fundus photographs of normal rabbit eyes and eyes containing retinal holes produced by surgery followed by IL-1β injections. A: Normal eye. B: Surgically treated eyes (4 weeks post-surgery); membranes have formed over the holes and extended into the vitreous body, forming adhesions with other portions of the retina.

Figure 2. Proliferative RPE cells around the retinal holes with inflammation. Hematoxylin and eosin stained and anti-pan-cytokeratin (Oregon Green)/anti-PCNA (Texas Red) double stained sections of posterior segments in normal eyes and eyes with retinal holes produced by surgery followed by IL-1β injections. A: Normal eye, Hematoxin & Eosin stained; B: Normal eye, anti-pan-cytokeratin/anti-PCNA double-stained; C: Post-surgically treated eye (72 h), anti-pan-cytokeratin/anti-PCNA double-stained. D,E: Post-surgically treated eye (4 weeks), anti-pan-cytokeratin/anti-PCNA double stained. Arrows indicate proliferating RPE cells.
and with 155 volts for 2 h. After electrophoresis, SDS in the gel was removed by incubation with 2.5% Triton X-100 in 50 mM Tris.HCl, pH 7.5. Gelatinolytic activities were developed in buffer containing 10 mM CaCl₂ in 50 mM Tris.HCl, pH 7.5 for 16 h at 37°C. The gelatinolytic activities were visualized by staining with Coomassie Blue G-250 and destained with water.

RESULTS

Clinical observations: During the first 24 h after retinal surgery, eyes showed an intense inflammatory response in the anterior segment while the controls showed little or no reaction. This included signs of engorgement of the perlimbal blood vessels, which peaked at 24 h, after which it slowly decreased and finally resolved by the end of the second week (data not shown, similar results were reported previously [17]). By the end of 4 weeks after retinal surgery, extensive membranes developed in 6 of 8 eyes (Figure 1). Membranes originated over a retinal hole, extended into the vitreous body, and formed adherions to other portions of the retina. In these eyes, retinas mostly remained attached. However, prominent surface wrinkling retinopathy and star-fold-like configurations were observed.

Proliferative RPE cells in the injured retina with inflammation: The retinal and RPE cells in the surgically treated eyes and control eyes were examined by PCNA and pan-cytokeratin immunohistochemically. PCNA, a highly conserved nuclear protein, was used as a marker for proliferating cells because of its prominent appearance in the nucleoli in the late S phase of the cell cycle. As shown in Figure 2, the cytokeratin-positive RPE, along with other cell types, become proliferative and migrate through defects within the retinal layer to the vitreoretinal interface and eventually form epiretinal membranes (data not shown).

Phosphorylation of p42/p44 MAPK (pERK): The effect of retinal injury and injected IL-1β on the activity of p42/p44 MAP kinase (ERK) was analyzed in the rabbit model. Immunoblotting with anti-pERK on retinal and RPE cell homogenates isolated from normal eyecup pieces or those containing retinal holes suggested that ERK phosphorylation was low in normal retina/RPE (Figure 3, upper panel). Phosphorylation of ERK occurred within 30 min of surgery in the rabbit. Repplotting the blots with an antibody to ERK indicated even loading of the gels (Figure 3, lower panel).

Tyrosine phosphorylation of c-met: Tyrosine phosphorylation of HGF receptor c-met was determined in the rabbit model of PVR. Immunoprecipitating tyrosine-phosphorylated proteins from rabbit retinal/RPE homogenates from normal eyecup pieces or from those containing retinal holes, followed by Western blotting for c-met showed weak c-met phosphorylation at approximately 145 kDa in the normal, control retinas (Figure 4). Increased immunoreactivity was observed in injured retina/RPE within 30 mins of surgery in the rabbit.

To verify the bands as HGFR, the same immunoprecipitation was repeated. Western blotting for phosphorylated c-met was carried out using anti-c-met antibody (2 mg) preincubated with lysis buffer or with HGFR blocking peptide (5 mg) at 4°C overnight. The bands were detected by the lysis buffer treated antibody, but not by the blocking peptide treated antibody (data not shown).

Morphology change and c-met tyrosine phosphorylation of ARPE-19 cells: Morphology of serum starved subconfluent ARPE-19 cells treated with vitreous humor samples was studied. These samples were from eyes at 24 h after surgery or from normal eyes. As a control, cells were also treated with IL-1β. As shown in Figure 5, serum starved cells or cells in the presence of 25% normal vitreous or with IL-1β were with epitheloid morphology. However, cells treated with 25% vitreous from surgically treated eyes showed marked morphologic changes to “spindle-shaped” cells.

Tyrosine phosphorylation of c-met was determined in serum starved subconfluent ARPE-19 cells treated with the vitreous humors from control or the 24 h post-surgical eyes for 1 or 4 h. Immunoprecipitating tyrosine phosphorylated proteins from the cell lysates, followed by Western blotting for c-met showed only faint c-met phosphorylation in cells.
treated with the normal vitreous or treated 1 h with vitreous from injured eyes (Figure 6). Increased c-met phosphorylation was observed in cells treated for 4 h with vitreous from injured eyes.

**C-jun expression:** The effect of retinal injury and injected IL-1β on the gene activity of c-jun was analyzed by Northern blot analysis. Hybridization with c-jun probe on retinal and RPE RNA isolated from normal eyecup pieces suggested that c-jun expression was low in normal retina/RPE (Figure 7, upper panel). Upregulation of c-jun occurred within 30 min of surgery in the rabbit and further increase of expression continued until 72 h. Reprobing the blots with a probe for G3PDH indicated even loading of the gels (Figure 7, lower panel).

**Matrix metaloproteinases in the vitreous:** The active components formation of epiretinal membranes in the vitreous of our rabbit model must require synthesis and degradation of extracellular matrix components, particularly collagen. Primary among the enzymes involved in such processes is a series of MMPs. We used zymography to determine the effect of intravitreal injection of IL-1β and retinal holes on the collagen degrading MMPs of the vitreous humor (Figure 8). Condition media from TPA treated HT-1080 cells that express both MMP-2 and MMP-9 was used for identification of these two MMPs [19]. Normal vitreous humor contains only MMP-2, whereas increased MMP-2 and MMP-9 were identified in vitreous humor 12 h after the surgery and maximal levels identified at 24 h. After 24 h both MMPs were reduced.

**DISCUSSION**

Data presented in this study suggest that in the injured retina, cells in or near the retinal holes respond immediately to injury and inflammation. The rapid phosphorylation or activation of MAPK is likely due to IL-1β induction [20-22]. Activation of MAPK cascade is one of the most rapid cellular responses to different stimuli, including IL-1β [20-22] and HGF [23-25]. The activated MAPK is known to phosphorylate transcription factors that induce expression of c-fos, c-jun and other early

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Figure 5. Morphological changes of ARPE-19 cells treated with rabbit vitreous. Subconfluent ARPE-19 cells that were starved in serum free medium were treated for 48 h with IL-1β (250 U/ml) or vitreous humor samples (25%) from normal eyes or from eyes at different times after surgery.

Figure 6. Tyrosine phosphorylation of HGFR in ARPE-19 cells treated with rabbit vitreous. Serum starved subconfluent ARPE-19 cells were treated for 1 or 4 h with vitreous humor from eyes 24 h after surgery or with vitreous from normal eyes. Protein equivalent aliquots of cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and then analyzed by western blot using anti-HGFR antibody (top) or directly analyzed by western blot using anti-HGFR antibody (bottom). The positions and the molecular weight (kD) of protein markers are indicated.

Figure 7. C-jun expression induced by the retinal holes and inflammation. Northern blot analysis of c-jun in retina and RPE from rabbit eyes with retinal holes and with IL-1β injection. RNA equivalent aliquots from eyes at different post-surgical time points were analyzed by Northern blot using c-jun and G3PDH probes. The positions of 28S and 18S ribosomal RNA are indicated.
growth response genes [26-28]. Data presented here also suggest a relatively slow c-met phosphorylation in response to retinal injury. In addition, these data have also identified MMP-2 and MMP-9 in the vitreous, as shown previously [17]. MMP-2 has been identified in the vitreous in normal eyes. On the other hand, MMP-9 has been identified in the vitreous in PVR patients [10]. Interestingly, maximal MMP-9 is detected at the same time, as c-met phosphorylation reaches maximal levels.

The morphological changes and c-met phosphorylation observed in ARPE-19 cells treated with vitreous from the 24 h post-surgical eyes, but not from the normal eyes, are possibly due to at least MMP-9 and HGF. Similar cellular morphological changes were observed when 10-25% subretinal fluid or vitreous from patients with retinal detachment or PVR was added to the culture medium of human RPE cells [29]. These observations in the rabbit model of PVR or patients with PVR suggest the initial breakdown of blood-retinal barriers, which allows circulating components including HGF and MMPs to enter the intraocular milieu. Active MMP-9 may provide a mechanism for RPE cell dissociation from their basement membrane. The RPE could then migrate through the retina and facilitate remodeling events at the inner and outer limiting membranes of the detached retina, leading to epiretinal membrane formation. MMP-9 can be synergistically induced in response to a combination of growth factors and cytokines [30]. Pro-MMPs are activated by plasmin, which can initiate a cascade of MMP activation at the cell surface [31]. Plasmin is activated by urokinase or tissue plasminogen activator, which is activated by HGF [32].

Our data implicate HGF and c-met as candidate molecules for initiating reactive cellular changes. HGF, also known as scatter factor, is a pleiotropic factor that functions as a mitogen, a motogen and a morphogen for a variety of cells, particularly epithelial cells, bearing c-met [33-35]. HGF is secreted as an inactive precursor form, and proteolytic activation of the precursor form in the extracellular milieu is a critical limiting step in the HGF induced signaling pathway [36]. This may explain the relatively slower c-met activation as compared to MAPK activation in the injured retina of our rabbit model. HGF may have a role in the migration and proliferation of the RPE in PVR because c-met is expressed in normal RPE cells [9], and in RPE cells in the epiretinal membranes removed from patients with PVR [7]. Moreover, active HGF, measured by its scatter activity, was detected in the early phase of PVR [7] during which RPE proliferation and migration occur [37,38]. These findings are consistent with our data presented here and are also consistent with the hypothesis that HGF may play a role in the RPE mesenchymal transformation that typifies PVR [29].

There are many growth factors in addition to HGF that could play a role in proliferative disorders that result in epiretinal membrane formation. In particular, an autocrine loop that involves platelet derived growth factor (PDGF) has been described in cultured human RPE cells, and a similar autocrine or paracrine loop has been suggested to contribute to PVR [39,40]. On the other hand, RPE cells produced many growth factors and when RPE conditioned medium was run on a PDGF antibody affinity column, most but not all of its chemotactic and mitogenic activities were removed [41], suggesting that other factors also contribute to these activities. In addition, there are many situations in which other growth factors exert their proliferative effects indirectly through HGF, such as the upregulation of HGF by cytokines, PDGF, and aFGF and bFGF [42]. The identification of HGF as a contributor to epiretinal membrane formation has potential clinical implications. Recently, a human HGF variant, NK4, has been shown to suppress HGF induced receptor tyrosine phosphorylation and associated mitogenic, motogenic and morphogenic activities in cultured cells and tumor growth and invasion in vivo [43]. NK4 acts in a dose dependent manner, but does not produce its own biological effects [43]. Moreover, NK4 inhibits endothelial growth induced by HGF, bFGF, or VEGF in vitro, inhibits angiogenesis in chick chorioallantoic membranes and rabbit corneal neovascularization induced by bFGF, and suppresses tumor angiogenesis, growth, and metastasis in mice [44]. Work is in progress to determine the effect of NK4 in the epiretinal membrane formation in our rabbit PVR model.

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