Hypoxia-Inducible Factor-Dependent Expression of Angiopoietin-Like 4 by Conjunctival Epithelial Cells Promotes the Angiogenic Phenotype of Pterygia

Qianli Meng,1,2 Yaowu Qin,1,3 Monika Deshpande,1 Fabiana Kashiwabuchi,1 Murilo Rodrigues,1 Qiaozhi Lu,4–7 Hui Ren,1,3 Jennifer H. Elisseeff,4–7 Gregg L. Semenza,8–13 Silvia V. Montaner,14–16 and Akrit Sodhi1

1Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
2Guangdong Eye Institute, Department of Ophthalmology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou Guangdong, China
3EENT Hospital, Fudan University, Shanghai, China
4Translational Tissue Engineering Center, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
5Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
6Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
7Bloomberg-Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
8Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
9Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
10Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
11Department of Radiation Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
12Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
13McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States
14Department of Oncology and Diagnostic Sciences, School of Dentistry, University of Maryland, Baltimore, Maryland, United States
15Department of Pathology, School of Medicine, University of Maryland, Baltimore, Maryland, United States
16Greenbaum Cancer Center, University of Maryland, Baltimore, Maryland, United States

Correspondence: Akrit Sodhi, Wilm er Eye Institute, Johns Hopkins School of Medicine, 400 N. Broadway Street, Smith Building, 4059, Baltimore, MD 21287, USA; asodhi1@jhmi.edu.
Silvia V. Montaner, Department of Oncology and Diagnostic Sciences, School of Dentistry, 650 W. Baltimore Street, University of Maryland, Baltimore, MD 21201, USA; smontaner@umaryland.edu.
QM and YQ contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: March 30, 2017
Accepted: July 25, 2017
Citation: Meng Q, Qin Y, Deshpande M, et al. Hypoxia-inducible factor-dependent expression of angiopoietin-like 4 by conjunctival epithelial cells promotes the angiogenic phenotype of pterygia. Invest Ophthalmol Vis Sci. 2017;58:4514–4523. DOI: 10.1167/iovs.17-21974

Purpose. Disappointing results from clinical studies assessing the efficacy of therapies targeting vascular endothelial growth factor (VEGF) for the treatment of pterygia suggest that other angiogenic mediators may also play a role in its development. We therefore explore the relative contribution of VEGF, hypoxia-inducible factor (HIF)-1α (the transcription factor that regulates VEGF expression in ocular neovascular disease), and a second HIF-regulated mediator, angiopoietin-like 4 (ANGPTL4), to the angiogenic phenotype of pterygia.

Methods. Expression of HIF-1α, VEGF, and ANGPTL4 were examined in surgically excised pterygia, and in immortalized human (ih) and primary rabbit (pr) conjunctival epithelial cells (CjECs). Endothelial cell (EC) tubule formation assays using media conditioned by ihCjECs in the presence or absence of inducers/inhibitors of HIF-1α or RNA interference (RNAi) targeting VEGF, ANGPTL4, or both were used to assess their relative contribution to the angiogenic potential of these cells.

Results. HIF-1α and VEGF expression were detected in 6/6 surgically excised pterygia and localized to CjECs. Accumulation of HIF-1α in was confirmed in ihCjECs and prCjECs, including stratified prCjECs grown on collagen vitrigel, and resulted in expression of VEGF and the promotion of EC tubule formation; the latter effect was partially blocked using RNAi targeting VEGF mRNA expression. We demonstrate expression of a second HIF-regulated angiogenic mediator, ANGPTL4, in CjECs in culture and in surgically excised pterygia. RNAi targeting ANGPTL4 inhibited EC tubule formation and was additive to RNAi targeting VEGF.

Conclusions. Our results support the development of therapies targeting both ANGPTL4 and VEGF for the treatment of patients with pterygia.

Keywords: pterygia, angiogenesis, vascular endothelial growth factor, angiopoietin-like 4, hypoxia-inducible factor

A pterygium is a nonneoplastic, degenerative, fibrovascular proliferation of conjunctival tissue that extends onto the cornea that can affect up to 22% of the population in certain geographic regions. Inflammation of pterygia, resulting in conjunctival hyperemia and irritation, is not uncommon. Advanced pterygia can lead to significant visual impairment if they encroach on the central visual axis, cause the formation of a corneal delle, or promote irregular astigmatism. Current
ANGPTL4 Expression in Pterygia

cells were filtered and counted. PrCJECs were passaged in bronchial epithelial cell growth medium (BEGM; Lonza, Walkersville, MD, USA). Immortalized human (Nh) CJEC line was kindly provided by Ilene K. Gipson, PhD (Schepps Eye Research Institute, Harvard Medical School) and cultured with keratinocyte serum-free medium (KSEM, Gibco, Grand Island, NY, USA) supplemented with human recombinant epidermal growth factor (EGF; 5 ng/mL), bovine pituitary extract (50 µg/mL), and 1% penicillin/streptomycin (Corning) as previously described.29 Cells were cultured at 37°C in a 5% CO2 incubator. For hypoxia experiments, cells were exposed to 1% O2 using an Oxygen Controller Glove Box (Coy Laboratory Products, Inc., Grass Lake, MI, USA), equilibrated with a gas mixture containing 1% O2, 5% CO2, and 94% N2 at 37°C.30

Vitrigel Culture

The collagen vitrigel (CV) membrane was prepared as previously described.31,32 Briefly, type I collagen solution from bovine dermis (5 mg/mL, Cosmo Bio, Tokyo, Japan) was mixed at equal volume with DMEM (Thermo Fisher Scientific, Waltham, MA, USA) on ice, which contains 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 20 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES; Thermo Fisher Scientific). The mixture was then transferred to Transwell inserts (polyester membrane with 0.4-µm pore size, 300 µL/cm2; Corning) and incubated at 37°C for 2 hours. After gelation, the collagen gel was vitrified under 40% relative humidity (RH) at 40°C for 1 week. Before cell seeding, the gel membrane was rinsed with phosphate-buffered saline (PBS; Thermo Fisher Scientific) three times.

PrCJEC were then seeded on Transwell containing CV membranes at a density of 2 × 106 cells/cm2 and cultured at 37°C with 5% CO2. BEGM was added to both upper and lower compartments until the cells reached confluence after about 5 days. Afterwards, medium was switched to a 1:1 mixture of BEGM and DMEM/F12 to induce stratification as previously described.28 Induction medium was added to only the lower compartment for airlifting culture, allowing the cells to be exposed to the air-liquid interface. Cells were kept in air-lifting culture for 1 week before hypoxia exposure.

For histology, prCJECs cultured on CV membranes were fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA, USA) and washed with PBS. The CV/cell samples were further dehydrated through ethanol gradients, cleared in xylenes, and embedded in paraffin. Five-micrometer thick sections were used for hematoxylin and eosin (H&E; Sigma-Aldrich Corp.) staining, according to the manufacturer’s manuals. Images of stained samples were taken on the Axio Imager 2 fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

siRNA Transfection

Cells were seeded and grown to 60% to 80% confluence prior to transfection. Lipofectamine RNAiMAX reagent was diluted in Opti-MEM medium. Thirty picomoles of siRNA from stock of 10 µM was diluted in Opti-MEM medium. Diluted siRNA was added to diluted Lipofectamine RNAiMAX reagent (1:1 ratio) and incubated for 5 minutes at room temperature. siRNA-lipid complex was added to cells and incubated at 37°C for 24 hours. The medium was then washed out and cells were ready for experiments.

Western Blot Assays

Cells in culture dishes were washed with PBS and lysed using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich...
Corps) with 10% protease inhibitor cocktail (Cell Signaling, Danvers, MA, USA). Cell lysates were then solubilized in lithium dodecyl sulfate (LDS)-sample buffer (Life Technologies) and incubated for 5 minutes at 95°C. Lysates were subjected to 4% to 15% gradient SDS/PAGE (Life Technologies). After blocking the membrane with 5% milk (Bio-Rad, Hercules, CA, USA), the membrane was then incubated with mouse anti-human HIF-1α (610959; BD, Franklin Lakes, NJ, USA) or mouse anti-rabbit HIF-1α (Thermo Fisher Scientific, PAI-16601) or with mouse anti-human GAPDH monoclonal antibody (Fitzgerald, Acton, MA, USA) or mouse anti-rabbit GAPDH monoclonal antibody (NB300-328SS; Novusbio, Littleton, CO, USA) overnight at 4°C. After washing, the membrane was incubated with (HRP)-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling) for 1 hour and then visualized with ECL Super Signal West Femto (Thermo Scientific, Rockford, IL, USA). Western blot scans are representative of at least three independent experiments.

Quantitative Real-Time RT-PCR
mRNA was isolated from cultured cells with PureLink RNA Mini Kit (Life Technologies), and cDNA was prepared with MuLV Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) and StepOnePlus Real-Time PCR Detection System (Life Technologies). β-actin and β2-microglobulin were used for normalization of human cell lines or for rabbit cells, respectively. Primers of human cell lines for quantitative PCR (qPCR) include: VEGF, forward–GGGCAGAATCATCAGAAGT and reverse–TCACCACTTCGTGGGGTTTATT; ANGPTL4, forward–GGACACGGTGATGTTGGACTCCTCA; and reverse–GGCCATTTTGGGGAGCCTCTT; ANGPTL4, forward–GAGCCAGGTTGGTCTCCTGA and reverse–AGCACTGTGTTGGCGTACAG. Primers of rabbit cell lines for qPCR include: VEGF, forward–GCTCTACCTCCACGGCCTATAGCCTG and reverse–CTCTTGGCGCAACCGCTC; ANGPTL4, forward–GGACACGGTGATGTTGGACTCCTCA; and reverse–GGCCATTTTGGGGAGCCTCTT; ANGPTL4, forward–GGACACGGTGATGTTGGACTCCTCA; and reverse–GGCCATTTTGGGGAGCCTCTT; β-actin and β2-microglobulin, forward–GAGCCAGGTTGGTCTCCTCA and reverse–CAGTTCACCCCGCTTTCAC and reverse–CAGTTCACCCCGCTTTCAC and reverse–CAGTTCACCCCGCTTTCAC and reverse–CAGTTCACCCCGCTTTCAC

Enzyme-Linked Immunosorbent Assay
Conditioned medium diluted 1:1 were analyzed for ANGPTL4 and VEGF with ELISAs performed according to the manufacturer’s protocols (R&D Systems).

Endothelial Cell (EC) Tubule Formation Assay
EC tubule formation assay was performed using growth factor-reduced Matrigel (Corning; 356231). Fifty microliters of Matrigel was added into a prechilled 96-well plate and placed in a 37°C CO2 incubator for 30 minutes. Human dermal microvascular endothelial cells (HMVECs) were then counted and plated at 2 × 104 cells/well on the Matrigel in a 96-well plate. Eighteen hours later, images were captured and analyzed using ImageJ software to measure total tube length.55 Tubule formation assay with conditioned medium from ihCjECs was performed with an addition of 100 μL/well of conditioned medium to the cell suspension prior to adding into the Matrigel-coated wells.

Immunohistochemistry
An ABC system (Dako, Glostrup, Denmark) was performed in paraffin-embedded human tissue as previously described.25-27,34 Primary antibodies used include: CD34 (Cova-
digoxin (Fig. 2F; Supplementary Fig. S1B). Similarly, tubule formation was markedly increased in HMVECs treated with media conditioned by ihCjECs exposed to DFO or DMOG as compared to media conditioned by ihCjECs exposed to 20% O₂ (Fig. 2G; Supplementary Fig. S1C). The effect of DFO was inhibited by digoxin (Fig. 2H; Supplementary Fig. S1D).

VEGF Secretion Is Not Sufficient to Explain the Angiogenic Potential of Cultured CjECs

To determine whether expression of the potent HIF-regulated angiogenic factor VEGF is responsible for the increased HIF-dependent angiogenic phenotype of CjECs, we next examined
the expression of VEGF by CjECs in response to HIF-1α accumulation. Exposure of ihCjECs to hypoxia or HIF inducer (DMOG or DFO) resulted in an increase in VEGF mRNA expression (Fig. 3A). Similar results were observed in prCjECs (Fig. 3B). In turn, this resulted in a HIF-dependent increase in VEGF protein secretion by CjECs (Fig. 3C). However, while RNA interference (RNAi) targeting VEGF effectively inhibited VEGF mRNA expression (D) and protein secretion (E) compared by ihCjECs transfected with RNAi targeting VEGF and exposed to 1% O₂ for 24 hours. Mock transfected (Mock) or scrambled RNAi (Scr) transfected cells were used as a control. (F G) EC tubule formation by HMVECs treated with conditioned media from ihCjECs transfected with VEGF RNAi and exposed to 1% O₂ for 24 hours reported as fold induction (F) or percent reduction (G).

**ANGPTL4 Expression Is Increased in Response to HIF-1α Accumulation in Cultured CjECs**

The observation that blocking VEGF expression only partially inhibits the angiogenic potential of CjECs is consistent with prior observations from clinical studies that antiangiogenic therapies targeting VEGF are not sufficient to prevent the growth or pterygia in treated patients. One explanation for these observations is that other angiogenic mediators may contribute to the prominent fibrovascular component of pterygia. We therefore set out to assess whether another HIF-regulated angiogenic factor contributed to the ability of CjECs to promote angiogenesis. To this end, we examined the expression of ANGPTL4 in CjECs in response to HIF-1α accumulation. Exposure of ihCjECs to hypoxia or HIF inducer (DMOG or DFO) resulted in an increase in ANGPTL4 mRNA expression (Fig. 4A). Similar results were observed in prCjECs (Fig. 4B). In turn, this resulted in a HIF-dependent increase in ANGPTL4 protein secretion by CjECs (Fig. 4C). Pharmacologic inhibition of HIF-1 with digoxin effectively blocked ANGPTL4 mRNA expression and protein secretion (Figs. 4D, 4E). Similar results were observed in prCjECs (Fig. 4E).

**ANGPTL4 Expression Is Detected in Surgically Excised Pterygia and Localizes to the Conjunctival Epithelium**

We next examined the expression of ANGPTL4 in surgically excised pterygia. Immunohistochemical staining demonstrated expression of ANGPTL4 in the conjunctival epithelium and, to a lesser extent, the underlying fibrovascular stroma in 6/6 pterygia (Fig. 5A). The pattern of expression for ANGPTL4 was similar to that observed for VEGF. By contrast, expression of ANGPTL4 was not readily detected in normal conjunctival epithelium (Fig. 5B).

**Inhibition of ANGPTL4 Expression by CjEC Reduces Their Angiogenic Potential**

To evaluate the contribution of ANGPTL4 to the angiogenic potential of CjECs, we used RNAi to knock down expression of ANGPTL4 in ihCjECs exposed to hypoxia. RNAi targeting ANGPTL4 inhibited hypoxia-induced ANGPTL4 mRNA expression and protein secretion (Figs. 6A, 6B), but did not influence VEGF mRNA expression (Fig. 6C). Similar results were observed for DFO-induced promotion of ANGPTL4 mRNA and protein expression (Figs. 6D, 6E). Conditioned media from ihCjECs exposed to hypoxia or DFO and treated with RNAi resulted in a marked reduction in the promotion of EC tubule formation (Figs. 6F, 6G; Supplementary Fig. S3), similar to targeting VEGF (Fig. 3).

**Inhibition of Both ANGPTL4 and VEGF Expression by CjEC Abolishes Their Angiogenic Potential**

To evaluate the therapeutic potential of combined therapy targeting both ANGPTL4 and VEGF to inhibit the angiogenic
potential of CjECs, we used RNAi to knock down expression of ANGPTL4, VEGF, or both in ihCjECs exposed to hypoxia. RNAi targeting ANGPTL4 inhibited hypoxia-induced ANGPTL4 mRNA expression and protein secretion (Figs. 7A, 7B), but did not influence VEGF mRNA expression or protein secretion (Figs. 7C, 7D). Similarly, RNAi targeting VEGF inhibited hypoxia-induced VEGF mRNA expression and protein secretion (Figs. 7C, 7D), but did not influence ANGPTL4 mRNA expression or protein secretion (Figs. 7A, 7B). RNAi targeting both ANGPTL4 and VEGF inhibited mRNA expression and protein secretion for both (Figs. 7A–D). Conditioned media from ihCjECs exposed to hypoxia and treated with RNAi targeting either ANGPTL4 or VEGF partially inhibited the promotion of EC tubule formation, while RNAi targeting both ANGPTL4 and VEGF completely abolished the ability of CjECs to promote EC tubule formation (Figs. 7E, 7F; Supplementary Fig. S4).

**DISCUSSION**

Pterygia are a common cause of cosmetic disfigurement within the equatorial zone and can lead to significant vision loss when...
**Figure 6.** Inhibition of ANGPTL4 expression by CjECs reduces their angiogenic potential. (A, B) ANGPTL4 mRNA expression (A) and protein secretion (B) in ihCjECs transfected with RNAi targeting ANGPTL4 and exposed to 1% O2 for 24 hours. (C) VEGF mRNA expression in ihCjECs transfected with RNAi targeting ANGPTL4 and exposed to 1% O2 for 24 hours. (D, E) ANGPTL4 mRNA expression (D) and protein secretion (E) in ihCjECs transfected with RNAi targeting ANGPTL4 and exposed to 100 μM DFO for 24 hours. (F, G) EC tubule formation by HMVECs treated with conditioned media from ihCjECs pretreated with RNAi targeting ANGPTL4 and exposed to 1% O2 or 100 μM DFO for 24 hours reported as fold induction (F) or percent reduction (G).

**Figure 7.** Combined inhibition of both VEGF and ANGPTL4 expression by CjECs abolishes their angiogenic potential. (A–D) ANGPTL4 (A, C) and VEGF (B, D) mRNA expression (A, B) and protein secretion (C, D) in ihCjECs transfected with RNAi targeting ANGPTL4, VEGF, or both and exposed to 1% O2 for 24 hours. (E, F) EC tubule formation by HMVECs treated with conditioned media from ihCjECs pretreated with RNAi targeting ANGPTL4, VEGF, or both and exposed to 1% O2 for 24 hours reported as fold induction (E) or percent reduction (F).
left untreated. There are currently no reliable medical treatments to prevent or even reduce the progression of pterygia. Consequently, aside from avoiding environmental risk factors (e.g., ultraviolet and infrared radiation from sunlight, human papillomavirus infection, trauma, inflammation, and exposure to irritants including sand and wind) in vulnerable populations, little else can be offered to patients to help prevent the development or progression of pterygia. Topical treatments (e.g., artificial tears, decongestants, nonsteroidal anti-inflammatory drugs, and steroids) may provide comfort and symptomatic relief for irritation and inflammation in some patients. However, definitive treatment requires surgery.2,58 Even with surgery prevention of recurrence remains a challenge. The recurrence rates for simple surgical excision is variable, but high, ranging from 50% to over 90%; this rate has been reduced to less than 10% in some studies with the introduction of amniotic membranes or conjunctival autografts.59 More advanced surgical techniques and the addition of adjuvant therapies (e.g., β-radiation, mitomycin C, and 5-fluorouracil) has further reduced this high rate of recurrence to under 5% to 10%.59 With the growing appreciation for the roles of inflammation and angiogenesis on recurrence rates, conjunctival grafting and/or the use of amniotic membranes, in combination with antiproliferative, antifibrotic, and/or anti-inflammatory approaches are becoming the standard of care to help reduce the rate of recurrences. Nonetheless, preventing recurrences remains a challenge, and adjuvant medications can themselves be associated with an increased risk for significant morbidity as a result of their adverse effects.39,40

Recent reports demonstrating the expression of VEGF in pterygium tissue have encouraged clinicians to extend the use of anti-VEGF therapy to the treatment of pterygia with the goal of inducing regression of the fibrovascular component of the pterygium, thereby reducing or preventing growth.19 While preliminary evidence suggested that local treatment with anti-VEGF therapy may be effective in the treatment of ocular surface neovascularization in preclinical models, the impact of this approach in the clinic have been less impressive; most patients have only a partial response and the effects are often transient.15 In patients with pterygia, the use of anti-VEGF therapy has similarly met with unimpressive results.19 It remains unclear whether the limited efficacy of anti-VEGF therapy for the treatment of pterygia is because antiangiogenic therapies are simply not an effective approach for the treatment of pterygia, or if therapies targeting only VEGF are not sufficient to adequately prevent the angiogenic phenotype of pterygia. If the latter is true, then identifying and targeting additional angiogenic factors may be required to provide an effective antiangiogenic approach for the treatment of pterygia.

The identification of additional angiogenic mediators in proliferative retinopathies has been successful, in part, due to early recognition that VEGF expression is increased in ischemic retinal disease due to increased accumulation of the transcription factor, HIF-1α. However, hypoxia is not known to play a major role in the pathogenesis of pterygia. Rather, inflammation, oxidative stress, and DNA damage are believed to be driving forces for pterygia development.55 Interestingly, these same factors have also been shown to lead to the accumulation of HIF-1α.7,10 Motivated by these observations, we set out to determine whether HIF-1, and in turn other HIF-regulated angiogenic mediators, contribute to the angiogenic phenotype of pterygia.

Here we demonstrate that HIF-1α expression is increased in conjunctival epithelium from surgically excised pterygia compared to normal controls, in a similar pattern as the expression of VEGF. This observation supports a role for HIF in pterygia development. Interestingly, these major role in the pathogenesis of pterygia. Rather, inflammation factor, HIF-1α, contributes to the angiogenic phenotype of pterygia. These observations provide an explanation for why anti-VEGF monotherapy has not been sufficient as an antiangiogenic approach for the treatment of patients with pterygia. Instead, our findings suggest that pharmacotherapy independently targeting VEGF and ANGPTL4, or targeting HIF-1 to inhibit both, may be a more effective antiangiogenic treatment for patients with pterygia.

**Acknowledgments**

Supported by the National Eye Institute, National Institutes of Health Grant K08-EY021189 (AS), an unrestricted grant from Research to Prevent Blindness (AS), and a grant from the National Natural Science Foundation of China (81571031; QM). Akrit Sodhi gratefully acknowledges the support he receives as a Special Scholar Award recipient from Research to Prevent Blindness, Inc.

Disclosure: Q. Meng, None; Y. Qin, None; M. Deshpande, None; F. Kashiwabuchi, None; M. Rodrigues, None; Q. Lu, None; H. L.

**ANGPTL4 Expression in Pterygia**

**Joint** September 2017 | Vol. 58 | No. 11 | 4521
References

1. Hilgers JH. Pterygium: its incidence, heredity and etiology. 

2. Janson BJ, Sikder S. Surgical management of pterygium. 

3. Bradley JC, Yang W, Bradley RH, Reid TW, Schwab IR. 

4. Liu T, Liu Y, Xie L, He X, Bai J. Progress in the pathogenesis of 

5. Aspiotis M, Tsanou E, Gorezis S, et al. Angiogenesis in 
   pterygium: study of microvessel density, vascular endothelial 
   growth factor, and thrombospondin-1. Eye (Lond). 2007;21: 
   1095–1101.

   expression of vascular endothelial growth factor implies the 
   1030.

7. Lee DH, Cho HJ, Kim JT, Choi JS, Joo CK. Expression of 
   vascular endothelial growth factor and inducible nitric oxide 

   pterygium: morphometric and immunohistochemical study. 

9. Semenza GL. HIF-1: using two hands to flip the angiogenic 

10. Semenza GL, Agani F, Feldser D, et al. Hypoxia, HIF-1, and 
     the pathophysiology of common human diseases. 

11. Kim KW, Ha HS, Kim JC. Ischemic tissue injury and progenitor 
     cell tropism: significant contributors to the pathogenesis of 

12. Pagoulatos D, Pharmakakis N, Lakoumentas J, Assimakopoulos 
     M, Etaypoxia-inducible factor-lalpha, von Hippel-Lindau 
     protein, and heat shock protein expression in ophthalmic 
     pterygium and normal conjunctiva. Mol Vis. 2014;20:441– 
     457.

13. Bahar I, Yeung SN, Sella R, Slomovic A. Anterior segment uses 

     endothelial growth factor in the management of pterygium. 

15. Mauro J, Foster CS. Pterygia: pathogenesis and the role of 
     subconjunctival bevacizumab in treatment. Semin Ophthal 

16. Enkvetchakul O, Thanathanee O, Rangsri R, Lekhanont K, 
     Suwan-Apichon O. A randomized controlled trial of intrale 
     rnal administration of subconjunctival bevacizumab injection 
     on primary pterygium: preliminary results. Cornea. 2011;30: 
     1213–1218.

17. Fallah MR, Khosravi K, Hashemian MN, Beheshtnezhad AH, 
     Rajabi MT, Gohari M. Efficacy of topical bevacizumab for 
     inhibiting growth of impending recurrent pterygium. 

18. Fallah Tafiri MR, Khorasvifard K, Mohammadpour M, Hash 
     emian MN, Kiarudi MY. Efficacy of intralensal bevacizumab 
     injection in decreasing pterygium size. Cornea. 2011;30:127– 
     129.

19. Hu Q, Qiao Y, Nie X, Cheng X, Ma Y. Bevacizumab in the 
     treatment of pterygium: a meta-analysis. Cornea. 2014;33: 
     154–160.

     O, Hanutsaha P. Randomized controlled trial of subconjunct 
     ival bevacizumab injection in impending recurrent pterygium: 

     Demirok A. Topical application of bevacizumab as an 
     adjunct to recurrent pterygium surgery. Cornea. 2013;32: 
     855–858.

     Preliminary results of subconjunctival primary pterygium 

     ari RE. Subconjunctival bevacizumab immediately after excision 
     of primary pterygium: the first clinical trial. Cornea. 2011;30: 
     1219–1222.

24. Teng CC, Patel NN, Jacobson L. Effect of subconjunctival 
     bevacizumab on primary pterygium. Cornea. 2009;28:468– 
     470.

     like 4 is a potent angiogenic factor and a novel therapeutic 
     target for patients with proliferative diabetic retinopathy. 

     up-regulates Angiopoietin-like 4 promoting angiogenesis and 
     vascular permeability in Kaposi’s sarcoma. Proc Natl Acad Sci 

     cells promote vascular permeability by HIF-1-dependent up-
     regulation of angiopoietin-like 4. Proc Natl Acad Sci USA. 

     945:31–43.

29. Gipson IK, Spurr-Michaeld S, Argueso P, Tisdale A, Ng TF, 
     Russol C. Mucin gene expression in immortalized human 
     corneal-limbal and conjunctival epithelial cell lines. Invest 

30. Semenza GL. Oxygen homeostasis. Wiley Interdiscip Rev Syst 

     properties of collagen vitrigel membranes for ocular repair 
     and regeneration applications. Biomaterials. 2012;33:8286– 
     8295.

     conjunctival equivalent for ocular surface reconstruction. 

     cell tube formation assay for the in vitro study of angiogen 

34. Sodhi A, Montaner S. Angiopoietin-like 4 as an emerging 
     therapeutic target for diabetic eye disease. JAMA Ophthal 
     mol. 2015;133:1375–1376.

     of an immortalized human microvascular endothelial cell line. 

36. Zhang H, Qian DZ, Tan YS, et al. Digoxin and other cardiac 
     glycosides inhibit HIF-lalpha synthesis and block tumor 

37. Cardenas-Cantu E, Zavala J, Valenzuela J, Valdez-Garcia JE. 
     Molecular basis of pterygium development. Semin Ophthal 

38. Hacioglu D, Erdol H. Developments and current approaches 
     in the treatment of pterygium. Int Ophthalmol. 2017;37: 
     1073–1081.

39. Kaufman SC, Jacobs DS, Lee WB, Deng SX, Rosenblatt MI, 
     Shtein RM. Options and adjuvants in surgery for pterygium: 
     a report by the American Academy of Ophthalmology. Oph 

40. Fan Gaskin JC, Nguyen DQ, Soon Ang G, O’Connor J, 
     Crowston JG. Wound healing modulation in glaucoma 
     filtration surgery-conventional practices and new perspec-


