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Epithelial Membrane Protein-2 (EMP2) Antibody Blockade Reduces Corneal Neovascularization in an In Vivo Model

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Purpose. Pathologic corneal neovascularization is a major cause of blindness worldwide, and treatment options are currently limited. VEGF is one of the critical mediators of corneal neovascularization but current anti-VEGF therapies have produced limited results in the cornea. Thus, additional therapeutic agents are needed to enhance the antiangiogenic arsenal. Our group previously demonstrated epithelial membrane protein-2 (EMP2) involvement in pathologic angiogenesis in multiple cancer models including breast cancer and glioblastoma. In this paper, we investigate the efficacy of anti-EMP2 immunotherapy in the prevention of corneal neovascularization.

Methods. An in vivo murine cornea alkali burn model was used to study pathologic neovascularization. A unilateral corneal burn was induced using NaOH, and subconjunctival injection of either anti-EMP2 antibody, control antibody, or sterile saline was performed after corneal burn. Neovascularization was clinically scored at 7 days postalkali burn, and eyes were enucleated for histologic analysis and immunostaining including VEGF, CD31, and CD34 expression.

Results. Anti-EMP2 antibody, compared to control antibody or vehicle, significantly reduced neovascularization as measured by clinical score and central cornea thickness, as well as by histologic reduction of neovascularization, decreased CD34 staining, and decreased CD31 staining. Incubation of corneal limbal cells in vitro with anti-EMP2 blocking antibody significantly decreased EMP2 expression, VEGF expression and secretion, and cell migration.

Conclusions. This work demonstrates the effectiveness of EMP2 as a novel target in pathologic corneal neovascularization in an animal model and supports additional investigation into EMP2 antibody blockade as a potential new therapeutic option.

Keywords: cornea, corneal neovascularization, epithelial membrane protein-2, vascular endothelial growth factor, angiogenesis

The cornea is a transparent tissue that plays a critical role in light refraction and vision. As it is in physical contact with the external environment, the cornea also acts as a mechanical barrier to provide protection against microorganisms, toxins, and injury. Under normal conditions, the cornea is clear and has several antiangiogenic factors including soluble VEGF-R1 which helps maintain its unique avascular status. However, pathologic neovascularization can occur secondary to trauma, chemical burns, immunologic disease, or infection,7 leading to an upregulation of proangiogenic factors including VEGF-A that promotes hemangiogenesis and inflammation.8 Corneal neovascularization typically presents as ingrowth of blood vessels from the limbus toward the clear center of the cornea. The resulting abnormal blood vessel growth, corneal opacity, and corneal edema reduces the transparency of the cornea, which is critical for good visual acuity.9 As such, neovascularization remains a major cause of blindness worldwide. As many as 1.4 million Americans are seen by physicians each year for vision impairment secondary to abnormal blood vessel growth,5,6 and these causes of corneal blindness present a pressing challenge to address.

Treatment options for corneal neovascularization are limited. Steroids and anti-inflammatory drugs are currently used, but efficacy can be variable and these drugs can produce significant side effects when chronically administered.2 Biologic agents, including intravitreal injections of anti-VEGF antibodies, are increasingly being used to treat a wide variety of ocular diseases involving abnormal retinal neovascularization, including age-related macular degeneration, diabetic retinopathy, and retinopathy of prematurity. Given the clinical efficacy of anti-VEGF in the treatment of these retinal diseases, some studies support efficacy in reducing corneal neovascularization in both animal models and clinical trials.7–9 These anti-VEGF agents have shown initial therapeutic success with at least partial reduction of corneal neovascularization through topical, subconjunctival, and intraocular application.10–14 Although these results are encouraging, anti-VEGF biologics have been associated with...
significant systemic toxicity and severe ocular complications, and additional therapeutics to complement these drugs would be advantageous.

Our lab has extensively studied the role of EMP2, a membrane bound tetraspan protein, in multiple models of vascular development. Prior studies demonstrated a role for EMP2 in pathologic angiogenesis in breast cancer, endometrial cancer, glioblastoma, and placental angiogenesis. EMP2 mRNA is expressed at high levels in the eye, lung, and uterus. In the eye, EMP2 is localized to the epithelial layers of the cornea, ciliary body, RPE-choroid, and stromal layers of the sclera. Within the retina, EMP2 is found on the membrane of the RPE, and appears to contribute to the pathogenesis observed in proliferative vitreoretinopathy.

EMP2 controls VEGF expression in the RPE cell line ARPE-19. Given the protein’s role in development of vasculature in multiple disease contexts, and its expression in several discrete subanatomic locations in the eye including corneal epithelium, we sought to investigate its involvement in pathologic neovascularization of the cornea. In this paper, we characterize the expression of EMP2 in the human cornea and localize it to the corneal epithelium. Moreover, we show that EMP2 contributes to corneal neovascularization in a murine corneal burn model in vivo and VEGF expression in vitro in a corneal limbal derived cell line.

**Materials and Methods**

**Animals**

We obtained 6- to 8-week-old female Balb/c mice from Jackson Laboratories (Bar Harbor, ME, USA), and housed at the University of California, Los Angeles, in the Division of Laboratory Animal Medicine facility. All animal experiments were reviewed and approved by the University of California, Los Angeles, Chancellor’s Animal Research Committee, in adherence to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

**Corneal Neovascularization Induction and Treatment**

Corneal neovascularization was induced in 6- to 8-week-old female Balb/c mice using an alkali burn method. Animals were anesthetized with 100 µL of ketamine (50 mg/kg) and xylazine (5 mg/kg) solution delivered via intraperitoneal route. The right eye (OD) of each animal was exposed to a 2-mm diameter filter paper soaked with sterile 1 M NaOH for 45 seconds, filter paper was removed using sterile forceps, and the eye was immediately rinsed twice with 10 mL of sterile saline. The left eye (OS) of each animal remained unburned as an untreated internal control. Following the alkali burn, the animals were treated once with either saline, 175 µg of control antibody (human IgG antibody, Sigma-Aldrich Corp., St. Louis, MO, USA), or 175 µg of human anti-EMP2 antibody (purified humanized monoclonal IgG1) via one subconjunctival injection in the temporal region of the conjunctiva. A total of eight mice were used per treatment group. We administered 100 µL of buprenorphine (0.1 mg/kg) via subcutaneous route in the shoulder area prior to alkali burn to aid with pain management. Erythromycin ointment was applied daily for 3 days and monitored daily. The experiment was terminated using euthanasia at day 7 postburn and both eyes were enucleated for additional evaluation.

**Clinical Vascular Evaluation**

Assessment of corneal neovascularization was performed in a masked fashion using a surgical microscope (Leica S6D; Leica Microsystems; Buffalo Grove, IL, USA). Neovascularization scoring was based on a scale of 0 to 3, as previously described at 7 days post-alkali burn, and prior to enucleation.

Areas of neovascularization in the corneas exposed to alkali burn were traced using the Simple Neurite Tracer plugin via NIH ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Images were automatically converted to 32-bit grayscale images by the Simple Neurite Tracer plugin.

**Cell Culture**

Human corneal limbal epithelial (HCLE; HCLE cells were provided by Ilene Gipsen, PhD, at Schepens Eye Research Institute, Harvard Medical School) cells were cultured using keratinocyte serum-free medium, per manufacturer’s formulation (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C, in a humidified 5% CO2 incubator. HCLE cells were grown to 80% confluence and exposed to 60 µg/mL of anti-EMP2 antibody for 6, 12, 24, or 48 hours. Human umbilical vein endothelial cells (HUVECs; ATCC, Manassas, VA, USA) were cultured in VEC MCDB-131 complete media (VEC Technologies, Rensselaer, NY, USA) at 37°C in a humidified 5% CO2 incubator. HUVECs for in vitro assays were used at passage number 3.

**Western Blot Analysis**

Cell lysates were prepared and lysed in Laemmli buffer. Proteins were separated on 4% to 20% SDS-PAGE gels (Thermo Fisher Scientific) under reducing conditions, transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA), and blocked with 10% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20. Membranes were probed with the following primary antibodies: anti-VEGF (1 µg/mL; Santa Cruz Biotechnology, Dallas, TX, USA); anti-EMP2 (1:2000; polyclonal antibody; created in our lab and previously published); anti-β-actin (0.5 µg/mL; US Biological, Salem, MA, USA). Protein bands were detected using horseradish peroxidase (HRP)-labeled secondary antibodies (Southern Biotechnology, Birmingham, AL, USA), followed by chemiluminescence (ECL; EMD Millipore, Burlington, MA, USA). Densitometric analysis of the protein bands was performed using NIH ImageJ software. Loading variability between samples was normalized to β-actin loading controls. At least three independent experiments were performed, and statistical significance was evaluated using Student’s t-test.

**ELISA**

Conditioned media were collected from HCLE cells incubated with media only, or with 60 µg/mL of anti-EMP2 antibody for 6, 12, 24, or 48 hours. Secreted VEGF from conditioned media was detected and analyzed per manufacturer’s instructions (Human VEGF Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA). Each condition was assayed in duplicate and VEGF standards were provided by the manufacturer for quantitation. Optical density was determined using a microplate reader (SpectraMax; Molecular Devices, Sunnyvale, CA, USA). Protein bands were detected using horseradish peroxidase (HRP)-labeled secondary antibodies (Southern Biotechnology, Birmingham, AL, USA), followed by chemiluminescence (ECL; EMD Millipore, Burlington, MA, USA). Densitometric analysis of the protein bands was performed using NIH ImageJ software. Loading variability between samples was normalized to β-actin loading controls. At least three independent experiments were performed, and statistical significance was evaluated using Student’s t-test.
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**Cell Migration Assay**

HUVECs were seeded into the upper compartment of transwell inserts of Boyden chambers (24-well plate; Corning, Corning, NY, USA). Conditioned media from HCLE cells were prepared as described above. Bevacizumab (Avastin; Genentech Bio-Oncology, San Francisco, CA, USA) at 60 μg/mL was added to untreated HCLE media as a negative control for the migration assay. We added 500 μL of HCLE conditioned media into the lower compartment of the Boyden chamber. After 5 hours of incubation at 37°C in a humidified 5% CO₂ incubator with the conditioned media, the transwell inserts were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. The inserts were carefully washed with distilled water and left to dry overnight at room temperature. The inserts were imaged and the migrated cells were quantified to determine number of migrated HUVEC cells. At least three independent experiments were performed, and statistical significance was evaluated using Student’s t-test.

**Immunostaining and Immunofluorescent Microscopy**

We fixed 5 μm OCT frozen sections from unfixed human corneal limbus samples (gift from Sophie Deng, MD, PhD, Stein Eye Institute, University of California, Los Angeles, CA, USA) were fixed with 4% paraformaldehyde for 10 minutes, blocked with 1% normal goat serum for 1 hour, and incubated overnight at 4°C with anti-EMP2 (1:1500; polyclonal antibody). Visualization of the immunostaining was performed using an anti-rabbit kit (IgG ABC Vector kit; Vector Laboratories, Burlingame, CA, USA), according to manufacturer’s instructions. Alcian blue/methyl green counterstain was used, and slides were mounted for brightfield microscopy (BX51; Olympus, Waltham, MA, USA).

HCLE cells were cultured on glass cover slips for 24 to 48 hours at 37°C, in a humidified 5% CO₂ incubator. Cells were fixed with 4% paraformaldehyde for 10 minutes, and stained with primary antibody, anti-EMP2 (1:500; polyclonal antibody) overnight at 4°C. Goat anti-rabbit IgG AlexaFluor 594 (2 μg/mL; Thermo Fisher Scientific) was used for secondary detection. Coverslips were counterstained with DAPI and mounted. Images were obtained at ×400 magnification by immunofluorescence microscopy (Olympus).

Unburned and alkali-burned eyes were enucleated, formalin-fixed, and sectioned at 4 μm. Some sections were stained using hematoxylin and eosin (H&E). Additional sections were deparaffinized and incubated at 95°C for 25 minutes in 0.1 M citrate, pH 6.0, for antigen retrieval and use in immunohistochemical studies. The following primary antibodies were used: anti-CD34 (5 μg/mL; Abcam, Cambridge, MA, USA), anti-CD31 (10 μg/mL; Dianova, Hamburg, Germany), anti-LYVE-1 (0.2 μg/mL; R&D Systems) and anti-VEGF (4 μg/mL, clone A-20; Santa Cruz Biotechnology). Detection of the immunostaining was executed using anti-rat IgG and anti-rabbit kit (Vector Laboratories), according to manufacturer’s instructions. Sections were counterstained and mounted. Detection of bound anti-CD31 or the anti-LYVE-1 antibodies was performed using anti-rat IgG AlexaFluor 594 and anti-goat IgG AlexaFluor 594 (Thermo Fisher Scientific) and immunofluorescent evaluation. Sections were counterstained with DAPI, and mounted with Prolong (Thermo Fisher Scientific). Images were obtained at ×400 magnification by brightfield microscopy (Olympus). NIH Image J software was used to quantitate CD31 and LYVE-1 fluorescent staining. Images were converted to 8-bit grayscale images and positively stained areas were quantitated.

**RESULTS**

**EMP2 is Expressed in Human Corneal Epithelium and HCLE Cells**

Sections of human cornea were stained for EMP2 expression to confirm EMP2 localization in human cornea. EMP2 was strongly expressed in corneal epithelium (Fig. 1A). EMP2 expression was also independently confirmed in human corneal-limbal epithelial (HCLE) cells, a frequently used immortalized human corneal epithelial cell line (Fig. 1B). The HCLE cells demonstrated strong punctate staining of EMP2, which interestingly appeared to be localized to sites of direct cell-cell contact, as observed in other cell types.²⁸,³⁶,³⁷

We next sought to explore whether EMP2 expression plays a pathologic role in a murine model of aberrant corneal neovascularization. To study corneal neovascularization, we utilized an in vivo murine corneal burn model as described in the methods section.⁵²

**Blockade of EMP2 Results in Clinically Decreased Corneal Neovascularization in an In Vivo Mouse Burn Model**

Alkali burn resulted in significant opacification and neovascularization of the cornea in all burned eyes compared to internal unburned control (data not shown). No cases of corneal perforations or infections were observed at any point in these studies. All eyes were used in subsequent analyses. Surgical microscopy images at 7 days postcorneal burn showed significantly less neovascularization in the anti-EMP2 treated eyes compared to saline control and control antibody treated eyes (Fig. 2). Vasculature was manually traced to highlight the degree of neovascularization (Fig. 2A, lower panel). A clinical scoring protocol was used to quantitate neovascularization based on a previously published clinical assessment protocol.⁵² Corneal neovascularization was clinically scored by masked observers using day 7 corneal images comparing burned eye with internal control eye, with 0 = no neovascularization; 1 = minor neovascularization at the corneal limbus; 2 = moderate neovascularization at the corneal limbus with few vessels approaching corneal center; 3 = extensive neovascularization at the corneal limbus and moderate to many vessels spanning the corneal center.⁵⁵ The mean clinical neovascularization score from 8 animals per group revealed significantly less corneal neovascularization in the anti-EMP2 antibody treated group compared to controls (Fig. 2B).

**Anti-EMP2–Treated Animals Have Reduced Central Cornea Thickness and Decreased CD34, VEGF, and CD31 Staining in Cornea**

The foregoing results suggested that antibody-targeting of EMP2, when given at the time of injury, decreased development of pathologic corneal neovascularization. To further characterize this finding, alkali burned eyes from the anti-EMP2 antibody, control antibody, and saline groups were further compared histologically and immunohistochemically for the markers CD34, VEGF, and CD31 (Fig. 3A). Notably, anti-EMP2-treated corneas have significantly reduced corneal thickness as compared to saline treated eyes (Fig. 3B). This may be clinically important because corneal edema independently contributes to loss of visual acuity even in the absence
of neovascularization, as in the case of a variety of diseases involving corneal endothelial dysfunction. Burned eyes demonstrate reduced thickness of the corneal epithelial layer, however when all of the eyes were evaluated the average epithelial thickness is unchanged regardless of treatment (Fig. 3C). Expression of CD34, a marker for hematopoietic progenitor cells and a probe for neovascularization in multiple tumor models, was identified using immunohistochemistry. Corneas from animals that received anti-EMP2 antibody showed significantly decreased CD34 staining compared to controls (Fig. 3A, second row), quantified by immunohistologic staining intensity (Fig. 3D). CD34 staining was more strongly observed in the corneal epithelium and the areas adjacent to the corneal endothelium with minimal expression in the corneal stroma. Little to no CD34 expression is observed in control unburned corneas. Expression of an endothelial cell specific marker, CD31, was also used as an independent validation to detect neovascularization, via immunofluorescent staining (Fig. 3A, bottom row). Quantitation of positively stained CD31 areas of immunofluorescent staining showed that anti-EMP2 antibody significantly decreased CD31 staining compared to vehicle and IgG controls (Fig. 3E). Conical staining with CD34 and CD31 were concordant, in that anti-EMP2 antibody treated animals showed reduced neovascularization compared to control groups. Anti-EMP2–treated eyes also showed a similar reduction in VEGF expression compared to saline and control antibody treated eyes (Fig. 3A, third row), with minimal to no expression of VEGF in anti-EMP2 antibody treated eyes. Additional staining using a marker for lymphatics (LYVE-1) was used and did not show significant staining in the central cornea under any of the tested conditions (Supplementary Fig. 1A, top panel). LYVE-1 staining was observed in the peripheral cornea of all 3 tested conditions (Supplementary Fig. 1A, bottom panel); however, upon quantitation of the stained areas, no significant differences across the three groups were observed (Supplementary Fig. 1B). We proceeded to further characterize the relationship between EMP2 and VEGF in vitro.

**Incubation With Anti-EMP2 Antibody Results in Decreased EMP2 Expression and Decreased VEGF Expression In Vitro in HCLE Cells**

Our lab had previously demonstrated that EMP2 controls VEGF expression in the retinal cell line ARPE-19. To
investigate the relationship between EMP2 expression and VEGF expression in cells derived from the corneal epithelium, we used the human HCLE cell line exposed to anti-EMP2 antibody and quantified expression of EMP2 and VEGF by western blot (Fig. 4A). Anti-EMP2 treatment appears to decrease both EMP2 and VEGF expression in a time-dependent fashion. Expression of EMP2 protein reached a nadir after 12 to 24 hours of anti-EMP2 blockade (Fig. 4B), while expression of VEGF reached a nadir at 12 hours of anti-EMP2 blockade (Fig. 4C). This demonstrated that the expression of EMP2 and VEGF is correlated, and both can be downregulated in a time-dependent fashion by EMP2 antibody blockade.

**Supernatants From HCLE Cells Previously Incubated With Anti-EMP2 Antibody Show Reduction in Secreted VEGF and Reduced Cell Migration in a Transwell Migration Assay**

Supernatants from HCLE cells treated with EMP2 antibody were further tested for presence of secreted VEGF and its functional capacity in a transwell migration assay. Secreted VEGF-A was directly measured by ELISA and demonstrated a time-dependent reduction in VEGF secretion with EMP2 blockade (Fig. 5A). The supernatants were also used to test function in an HUVEC transwell migration assay. Notably, there is a statistically significant reduction in number of cells migrated at 12 and 24 hours using supernatants from HCLE cells exposed to EMP2 blockade (Fig. 5B). This reduced cell migration is likely secondary to reduction in secreted growth factors including VEGF following exposure to the anti-EMP2 antibody.

**DISCUSSION**

In this paper, an interaction between EMP2 and VEGF in the murine alkali burn model of corneal neovascularization is demonstrated, which is concordant to other studies that link EMP2 to control of VEGF in tumor models and in the retinal epithelial cell line ARPE-19. EMP2 blockade using anti-EMP2 antibodies reduce pathologic neovascularization and corneal edema following corneal burn. This suggests EMP2 as a potentially new target for prevention of corneal neovascularization.

In corneal epithelium and model HCLE cells, we observed that EMP2 was localized to sites of direct cell-cell contact. EMP2 is a member of the GAS-3 protein family, whose cell biology includes interaction with integrins and caveolin-1, mediation of protein trafficking including MHC class I to lipid raft microdomains, a role in apical membrane recycling, and in cell-cell and cell-extracellular matrix interactions. These biologic precedents are consistent with localization of EMP2 in sites of cell-cell contact in corneal epithelium, and suggest an analogous functional cell biology in this cell type.

In normal and neoplastic cell types, EMP2 previously has been shown to promote autonomous VEGF expression, a phenotype that could be reversed by molecular inhibition of EMP2 expression or antibody blockade with anti-EMP2 antibody. These precedents, and EMP2 expression in corneal epithelium, formed the rationale for testing whether EMP2 blockade could block corneal neovascularization.

We demonstrated that blocking EMP2 signaling results in a time-dependent decrease in EMP2 expression as well as VEGF expression. Our experiments showed that after anti-EMP2 blockade, expression of EMP2 protein reached a nadir after 12
Figure 3. Anti-EMP2-treated animals have reduced central cornea thickness and decreased CD34, VEGF and CD31 staining in cornea. (A, top row) H&E-stained sections were obtained, and the average of three measurements in the central region of each burned cornea were taken. (A, second row) Immunohistochemical staining of CD34 expression in the treated and untreated corneas show an increase in CD34-positive staining in the saline and control antibody–treated animals, with markedly reduced CD34 staining in anti-EMP2 treated animals. Little to no CD34 expression is observed in the control unburned cornea. (A, third row) Immunohistochemical staining of VEGF expression shows a similar increase in VEGF expression of saline and control antibody treated animals, with markedly reduced VEGF staining in anti-EMP2 treated animals. (A, bottom row) Staining via immunofluorescence with anti-CD31, an endothelial cell specific marker, shows increased expression, as predicted, in the control burns.
to 24 hours. Correspondingly, VEGF secretion was decreased between 6 and 24 hours, with a nadir around 12 hours. This is consistent with our hypothesis that VEGF expression decreases in response to an EMP2 decrease, with the caveat that use of the anti-EMP2 blocking antibody does not characterize the relative production of EMP2 and VEGF protein in a time dependent manner. However, previous experiments have shown that siRNA knock-down of EMP2 reduces total VEGF expression by 57%, and conversely EMP2 overexpression increased VEGF expression by 1.5-fold.

The expression of the growth factor VEGF is complex and regulated by many factors at the transcription, translation, and secretion levels. Rapid release is possible due to preexisting pools of protein regulated at the secretion level, with inducible secretion seen as rapidly as within 30 min in human vascular smooth muscle cells, platelets, and neutrophils, as well as neuroblastoma tumor cells. Additionally, certain ocular cell models (retinal pigment epithelium/choroid in ex vivo organ culture and the human retinal pigment epithelium cell line ARPE-19) constitutively express and secrete VEGF, with their constitutive secretion further modifiable by external factors. Furthermore, low levels of constitutive expression of VEGF-A has also been reported in human corneal epithelial cells and human corneal stromal fibroblasts, corroborating evidence here that VEGF and EMP2 play integral roles in the corneal vascular state.

Anti-VEGF antibody therapy is an FDA approved and clinically successful treatment for multiple conditions involving abnormal angiogenesis, including retinal neovascularization. Several direct VEGF pathway inhibitors are currently FDA approved, including various classes of biologicals and orally available small molecule downstream tyrosine kinase inhibitors. In addition to therapeutic efficacy in several human cancers, anti-VEGF immunotherapy has an established role in ophthalmology, including age-related macular degeneration and proliferative diabetic retinopathy. Additionally, anti-VEGF therapy has evidence of efficacy in other neovascular diseases, including diabetic macular edema, neovascular glaucoma, branch retinal vein occlusion, and central retinal vein occlusion. Furthermore, low levels of constitutive expression of VEGF-A have been used with ARPE-19 cells in the successful treatment of a rabbit model of experimental proliferative vitreoretinop-
athy with no evidence of toxicity to the eye by histology or by electroretinography (Telander D, unpublished observations, 2010). In summary, anti-EMP2 immunotherapy has efficacy in prevention of corneal neovascularization in the murine burn model. Additional studies are warranted to explore the possibility of this target in human ocular neovascular disease.

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