

The Role of Inverted Internal Limiting Membrane Flap in Macular Hole Closure

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PURPOSE. To investigate the mechanism of macular hole (MH) closure following the inverted internal limiting membrane (ILM) technique.

METHODS. We performed the inverted ILM flap surgical technique as an experimental MH model in monkeys, and investigated the process of MH closure immunohistochemically. We then investigated the effects of type IV collagen, fibronectin, and laminin, which are constituent proteins of the ILM, on the proliferation and migration of cultivated Müller cells (MIO-M1). We also investigated the expression of neurotrophic factors and basic fibroblast growth factor (bFGF) in human ILM and MIO-M1 cells, and the effect of MIO-M1 migration on the expression of these factors, via immunohistochemical staining and the real-time reverse transcription polymerase chain reaction.

RESULTS. Ten days after inverted ILM flap surgery, the MH had closed and proliferating glial fibrillary acidic protein (GFAP)-positive cells surrounded the ILM. Type IV collagen, fibronectin, and laminin all enhanced the proliferation of MIO-M1 cells, and type IV collagen and fibronectin enhanced the migration of MIO-M1 cells. Neurotrophic factors and bFGF were present on the surface of the human ILM, and MIO-M1 cells produced these factors. Neurotrophic factors and bFGF were expressed to a significantly greater extent by migrating MIO-M1 cells than by these cells in their static state.

CONCLUSIONS. During MH closure, the ILM functioned as a scaffold for the proliferation and migration of Müller cells, and may promote Müller cell activation. Neurotrophic factors and bFGF produced by activated Müller cells and present on the surface of the ILM may contribute to MH closure.

Keywords: macular hole, internal limiting membrane, Müller cell

Macular hole (MH) is an anatomic opening in the retina that develops at the fovea.^{1,2} It occurs most commonly in the elderly, with a prevalence ranging from 0.2% to 0.8% in the general population.^{3,4} As the fovea is the site responsible for central vision, the development of a MH results in severe vision loss, with visual acuity frequently decreasing to 20/200 or below. Early treatment is therefore required. The standard treatment is vitrectomy with internal limiting membrane (ILM) peeling, gas tamponade, and postoperative face-down positioning, with the objective of completely eliminating all traction on the macula and thus allowing the edges of the hole to come together.^{5,6} The mechanism of MH closure in this procedure is believed to be multifactorial. In addition to the elimination of tangential traction on the fovea, ILM peeling is believed to increase the extensibility of the retina and Müller cell gliosis is associated with bridging the gap between the edges of the retinal hole.⁷⁻¹³

The MH is closed by this standard procedure in approximately 90% of cases.¹⁴ However, in some cases the standard procedure does not successfully close the MH. These MHs

include MHs with large diameters, MHs that have existed for a long time, and secondary MHs resulting from severe myopia, ocular trauma, uveitis, and proliferative vitreoretinopathy.^{15,16} Until recently, there was no effective treatment for these MHs. In 2010, however, Michalewska¹⁵ reported the inverted ILM flap technique, and our group reported the technique of autologous transplantation of the ILM for patients who have already had the ILM peeled.¹⁶ These procedures are based on the same concept of facilitating MH closure by putting the ILM into the MH, but do not involve removing the ILM. Both procedures are effective, and the closure rates of MHs are more than 80%.^{15,16} However, the details of the mechanisms by which the MH closes following these procedures are unknown.

The objective of the current study was to identify the mechanisms by which the inverted ILM flap technique and autologous transplantation of the ILM effectively close MHs, focusing in particular on the roles of the ILM and Müller cells in MH closure, using a primate model of MH as well as human ILM and cultured Müller cells.



MATERIALS AND METHODS

Antibodies and Chemicals

Anti-brain-derived neurotrophic factor (BDNF); anti-ciliary neurotrophic factor (CNTF); anti-gial cell-derived neurotrophic factor (GDNF); and anti-basic fibroblast growth factor (bFGF) primary antibodies were purchased from Abcam (Cambridge, UK). Anti-GFAP, anti-laminin, and anti-Ki67 primary antibodies were purchased from Dako (Glostrup, Denmark); Sigma-Aldrich Corp. (St. Louis, MO, USA); and BD Pharmingen (Franklin Lakes, NJ, USA), respectively. AlexaFluor 488 donkey anti-mouse IgG, AlexaFluor 546 donkey anti-goat IgG, and AlexaFluor 488 goat anti-rabbit IgG secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fibronectin and laminin purchased from Corning (Corning, NY, USA) were used for the extracellular matrix coating the dish in the relevant experiments. Type I collagen and type IV collagen were purchased from Thermo Fisher Scientific (Tokyo, Japan) and Nippi, Inc. (Tokyo, Japan), respectively.

Animals

All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by the Animal Care and Use Committee at Santen Pharmaceutical Co., Ltd. (Nara, Japan). Male cynomolgus monkeys (*Macaca fascicularis*) aged 3 to 4 years and weighing 3.0 to 5.0 kg were purchased from Eve Bioscience, Ltd. (Wakayama, Japan). The animals were housed individually in stainless steel cages (width, 47 cm; depth, 89 cm; height, 76 cm) in an animal study room where the environmental conditions were room temperature 24°C, relative humidity 60%, and illumination 12-hour lighting (7 AM to 7 PM) at 300 luxes. The animals were fed 100 g/animal/day of pellet food for monkeys (Monkey Bit; Nosan Corporation, Yokohama, Japan). Tap water from a feed-water nozzle was supplied ad libitum to the animals.

Experimental MH Models and the Inverted ILM Flap Surgical Procedure

Three monkeys were anesthetized with an intramuscular injection of ketamine hydrochloride (10 mg/kg). Topical drops of 0.5% phenylephrine hydrochloride and 0.5% tropicamide were used for mydriasis. The right eye of each animal underwent surgery. Transconjunctival 25-gauge, 3-port pars plana vitrectomies were performed with a commercially available vitrectomy machine (Accurus; Alcon Laboratories, Inc., Fort Worth, TX, USA). After core vitrectomy, MHs were created via the method described by Yamana et al.¹⁷ Briefly, 0.1 to 0.2 mL of balanced salt solution (BSS; Alcon Laboratories, Inc.) was injected into the subfoveal space with a 38-gauge subretinal infusion cannula (MedOne, Sarasota, FL, USA). After an MH had been opened in the fovea by the water pressure from the BSS injection, a back-flush needle (Dorc International, Zuidland, Netherlands) was used to enlarge the MH to approximately 0.5 of the optic disc diameter. In one eye of three created MHs, ILM peeling was performed. In other two eyes, the inverted ILM technique was performed in accordance with the methods described by Michalewska et al.¹⁵ and Hirano et al.¹⁸ Briefly, the ILM was peeled off in a circular fashion for approximately 2 disc diameters around the experimental MH. We used indocyanine green to stain the ILM. In one eye, the ILM was removed completely. In the other two eyes, the ILM was left attached to the edges of the MH. The ILM was then inverted so that it could cover the entire MH.

Then, after fluid-air exchange, the vitreous cavity was perfused with a 20% sulfur hexafluoride gas tamponade in all three eyes. The fovea was scanned via optical coherence tomography (OCT; Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany) preoperatively and on postoperative day 10. On postoperative day 11, monkeys were killed with an intravenous overdose injection of pentobarbital. The eyes were fixed with Superfix (mixture of methanol and formalin; Kurabo, Osaka, Japan) in the first 2.5 hours at room temperature, followed by 3 days at 4°C. The eyes were horizontally embedded in paraffin, and cut into 3- μ m specimens for hematoxylin and eosin staining and immunohistochemistry.

Cell Culture

MIO-M1 cells, an immortalized model of Müller glial cells, were a gift from Astrid Limb (University College London, London, UK). The cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Cell Proliferation Assay

After coating 35-mm cell culture dishes with type I collagen, type IV collagen, laminin, or fibronectin, MIO-M1 cells were seeded at a density of 3.5×10^5 cells per dish, which corresponds to approximately 60% confluence. After incubation for 72 hours, cells were harvested from each dish and the numbers of cells were counted with a hemocytometer.

Cell Migration Assay

A gap closure assay¹⁹ with slight modification was used. We used a room temperature vulcanization silicon rubber (Shin-Etsu silicone; Tokyo, Japan) as a cell migration barrier. The center area of each plate, which is the same size as the barrier (13 mm²), was coated with type I collagen, type IV collagen, laminin, or fibronectin (Figs. 1A, 1B). After attaching the cell barrier onto the coated area (Figs. 1C, 1D), the cells were seeded onto plates at 80% confluence and cultured until 100% confluence was reached (Fig. 1E). The barrier was then removed, allowing the cells to migrate to the cell-free area (Fig. 1F). The cells were observed under a phase contrast microscope (CKX-41; Olympus Corp., Tokyo, Japan), and photographs were taken at the time of barrier removal and 72 hours thereafter (Figs. 1G–J). The size of the cell-free area was measured using ImageJ software (<http://imagej.nih.gov/ij/>); provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Human ILM Removal During Vitrectomy for the Treatment of Idiopathic MH

ILMs were obtained from 10 eyes of 10 patients who underwent vitrectomy for idiopathic MH from April 2015 to July 2015. The ILMs were stained with 0.25 mg/mL Brilliant Blue G solution (Coomassie BBG 250; Sigma-Aldrich Corp.) and removed using end-gripping forceps (Revolution DSP 25Ga ILM forceps; Alcon Laboratories, Inc.). Removed ILM specimens were fixed in 4% paraformaldehyde and used for immunohistochemical analysis. The study protocol was approved by the Ethics Committee of Okayama University Hospital and was performed in accordance with the tenets of the Declaration of

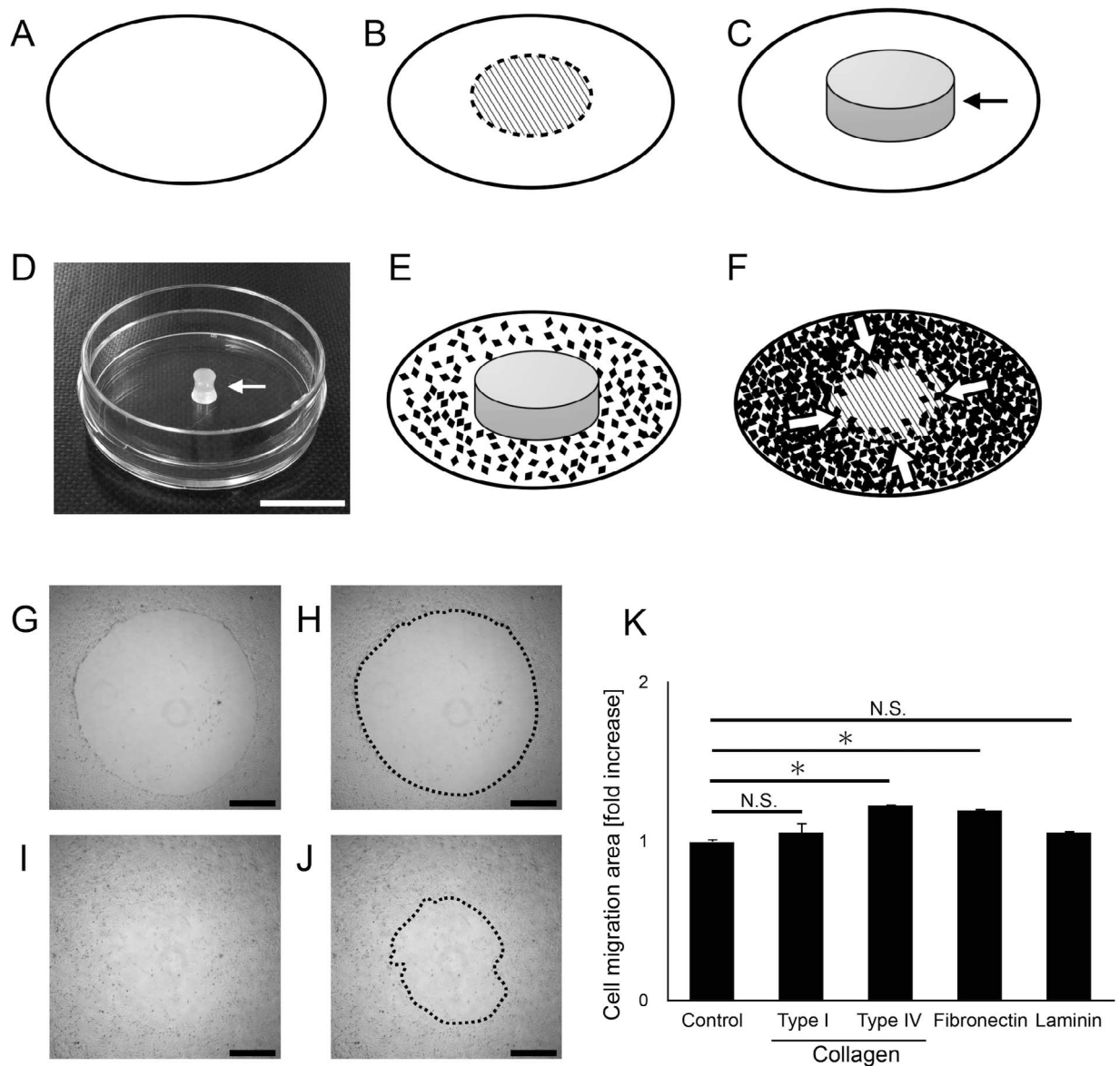


FIGURE 1. Effect of coating with constituents of the ILM on the migration of MIO-M1 cells. (A-E) The method used to conduct the cell migration assay. Arrows in (C) and (D) indicate silicone barriers. Arrows in (F) indicate migrating cells. (G-J) Representative photographs at the start of migration (G) and 72 hours later (I). The cell-free-areas in (G) and (I) are indicated by a broken line in (H) and (J), respectively. Type IV collagen and fibronectin promoted significant migration of MIO-M1 cells in comparison with uncoated plates, but type I collagen and laminin did not (K). The *Scale bar* in (D) is 10 mm, and in (G) through (J), the *scale bars* are 1 mm. Results are expressed as the mean \pm SEM. *Statistically significant difference compared to uncoated controls at $P < 0.05$. NS, not statistically significant.

Helsinki. Written informed consent for the research was obtained from all participants.

Immunohistochemistry

Immunofluorescein staining of paraffinized sections of the retina, cultured cells, and human ILMs were performed in accordance with standard protocols.²⁰⁻²² After incubation with primary antibodies overnight at 4°C, secondary antibodies were incubated for 2 hours at room temperature. The cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich Corp.). We obtained DAB (DAB Universal Kit; Ventana Medical Systems, Tucson, AZ, USA) and fluorescence images using a light microscope (Olympus Corp.), a fluorescence microscope (FSX100; Olympus Corp.), or a confocal laser scanning microscope (LSM780; Carl Zeiss Meditec AG, Jena, Germany).

Real-Time Reverse Transcription Polymerase Chain Reaction

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) (StepOne; Thermo Fisher Scientific) was conducted in accordance with the manufacturer's instructions.²³ The following Taqman (Thermo Fisher Scientific) gene expression assay primers (Gene/ABI Assay #) were used: BDNF/Hs02718934_s1, CNTF/Hs04194755_s1, GDNF/Hs01931883_s1, and FGF2/Hs00266645_m1.

Statistical Analysis

Cell proliferation and cell migration assays were repeated a minimum of three times, and mean \pm standard error of the mean were calculated for all data. Statistical differences

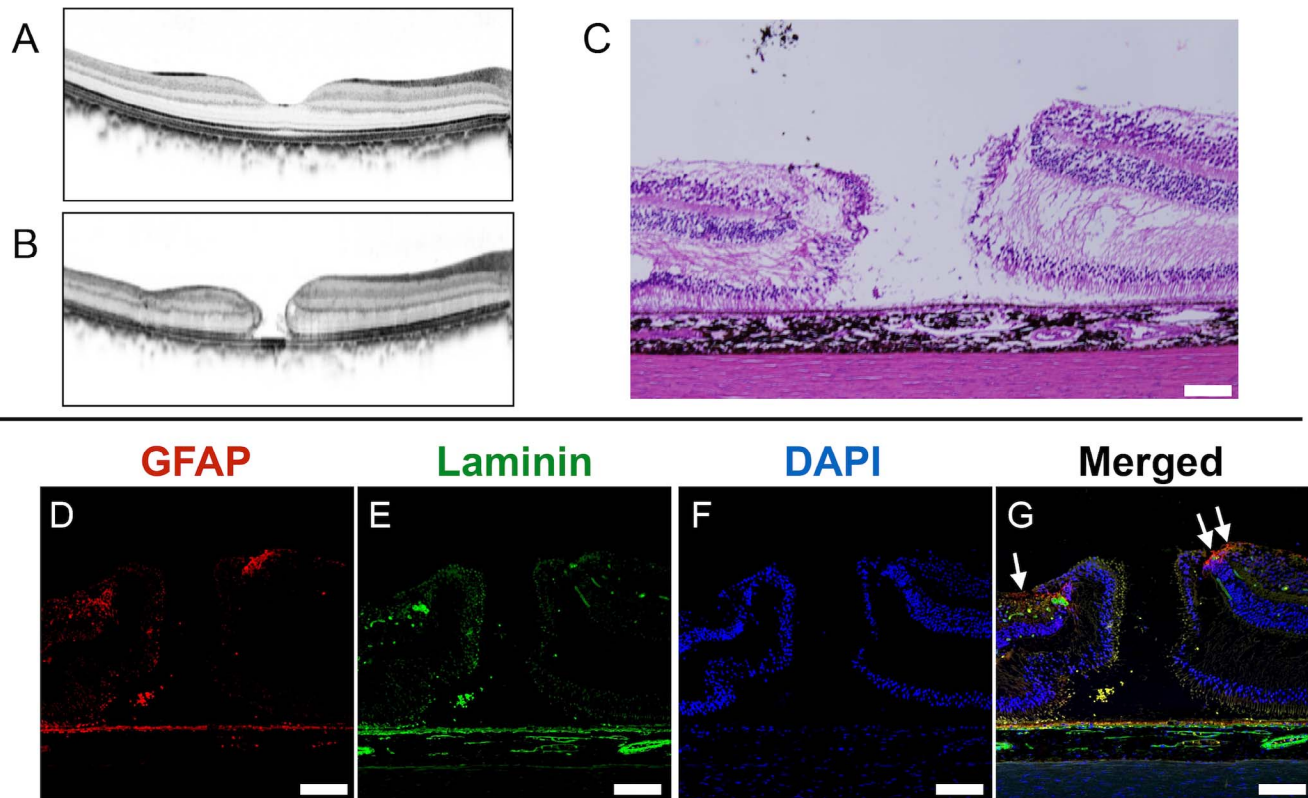


FIGURE 2. OCT images and histological analysis of an experimental MH after ILM peeling. Preoperative OCT showing a normal macular structure in a monkey eye (A). OCT 10 days after surgery, showing the unclosed MH (B). Hematoxylin-eosin staining showed that the unclosed MH and the ILM surrounding it was peeled and removed (C). (D–G) Immunostaining micrographs showing a few GFAP-positive cells (arrows in [G]) at the retinal surface where ILM was peeled. Scale bars: 100 μ m.

between two groups were analyzed via the unpaired Student's *t*-test. Multiple group comparison was performed via 1-way ANOVA with Bonferroni's test. Differences were considered significant at $P < 0.05$.

RESULTS

Proliferating GFAP-Positive Cells Surrounding the Inverted ILM in Monkey Eyes

In the eye with ILM peeling, OCT scans and optical microscopy showed that the MH was not closed on postoperative day 10 (Figs. 2A–C). Optical microscopy showed that the ILM surrounding the MH was peeled and removed (Fig. 2C). Immunohistochemical analysis revealed a few GFAP-positive cells at the retinal surface where the ILM was peeled. (Figs. 2D–G). On the other hand, in two eyes with inverted ILM flap technique, OCT scans showed that the MHs were closed and the inverted ILM flap was visible on the surface of the retina on postoperative day 10 (Figs. 3A, 3B). At this time-point, optical microscopy showed that the inverted ILM flap covered the surface of the macula, with part of it intruding into the MH (Fig. 3C). Immunohistochemical analysis revealed numerous GFAP-positive cells in contact with and surrounding the inverted ILM flap (Figs. 3D–G). Some of these GFAP-positive cells were also positive for Ki-67, which is a marker of cell proliferation (Figs. 3H–K).

Factors Stimulating Müller Cell Proliferation and Migration

The ILM is the basement membrane for Müller cells, and is mainly composed of type IV collagen, laminin, and fibronectin.^{24,25} Based on the results of experiments on a monkey MH model, we carried out *in vitro* investigations to identify which of the constituent proteins of the ILM are important for enhancing the proliferation and migration of Müller cells. As shown in Figure 4, coating the dishes with any one of the above-mentioned constituents significantly enhanced proliferation compared with the uncoated control dishes. We then conducted cell migration assays to investigate their effects on the migration of Müller cells. After a 72-hour incubation, migrated areas in dishes coated with type IV collagen or fibronectin were significantly larger than those of uncoated dishes (Fig. 1K). However, in dishes coated with laminin, there was no significant enhancement of cell migration.

Neurotrophic Factors and bFGF in Human ILM

Extracellular matrix is present on the surface of the ILM.²⁶ In general, the extracellular matrix functions to retain growth factors, trophic factors, and biologically active substances, and it is known to affect the function of neighboring cells.²⁷ It is unclear, however, what factors are retained on the surface of the ILM. Factors BDNF, CNTF, GDNF, and bFGF are known to inhibit apoptosis and promote the survival of retinal neurons and photoreceptor cells,^{28–32} and we therefore investigated the presence of these factors in samples of

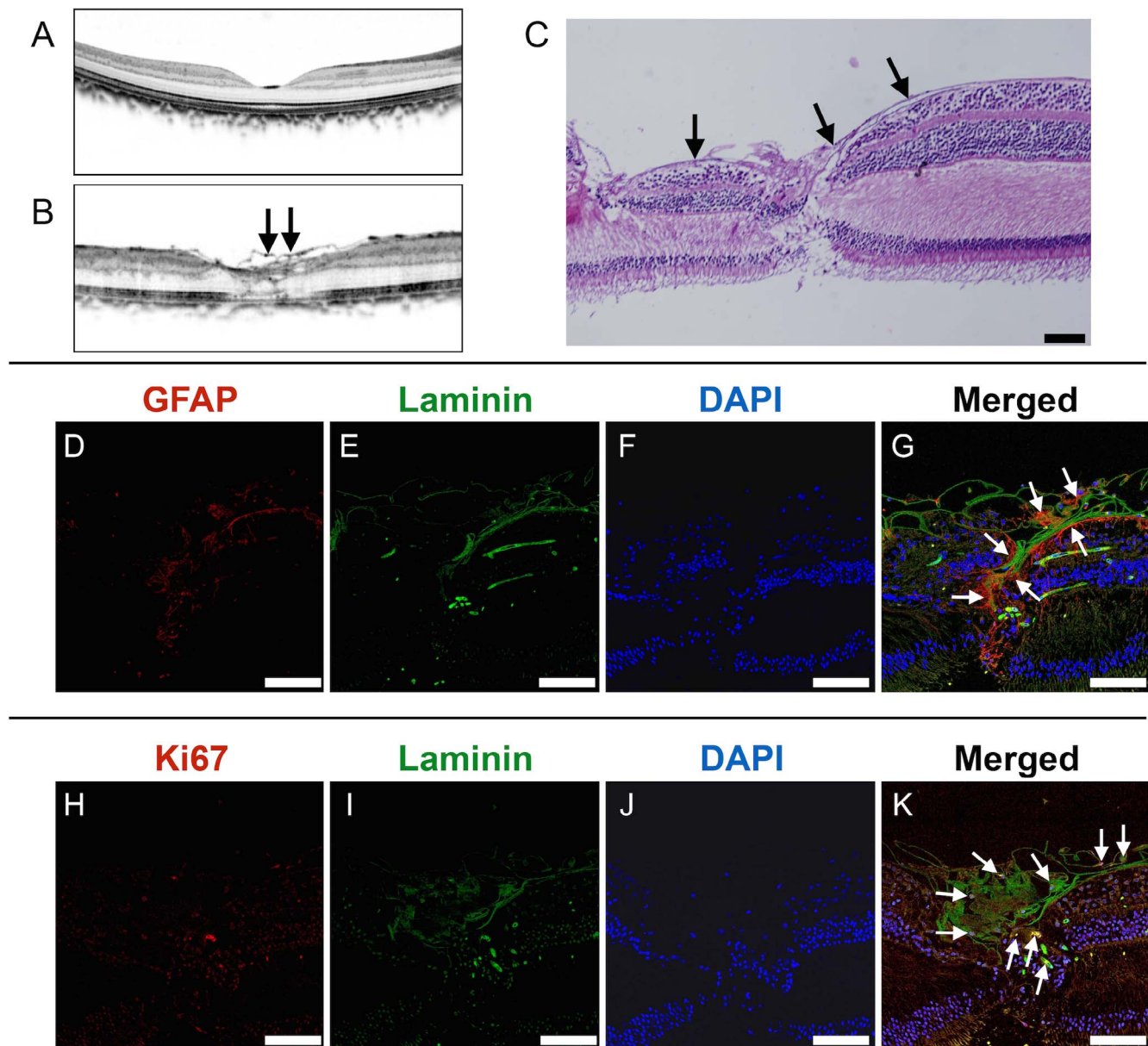


FIGURE 3. OCT images and histologic analysis of an experimental MH after inverted ILM flap technique. Preoperative OCT showing a normal macular structure in a monkey eye (A). OCT 10 days after surgery, showing the closure of the MH and the presence of an inverted ILM flap (arrow [B]). Hematoxylin-eosin staining showing the presence of the ILM flap (arrows [C]). (D–K): Immunostaining micrographs showing GFAP-positive cells (arrows in [G]) and Ki67-positive cells (arrows in [K]) in contact with and surrounding the ILM flap (laminin in [E, I]). Scale bars: 100 μ m.

human ILM. We collected ILM from 10 eyes of patients with MH, and conducted immunostaining for the target factors. As shown in Figure 5, all the factors were found to be present on the ILM.

Migrating Müller Cells and Expression of Neurotrophic Factors and bFGF

Activated glial cells proliferate and migrate to the site of nerve injury, and contribute to nerve healing by producing trophic factors and growth factors.^{33–36} We therefore investigated whether trophic factors and growth factors were produced by Müller cells, and whether there was any association between Müller cell activation and the amount of trophic factors and growth factors produced. As shown in Figures 1A through 1F,

we encouraged the migration of Müller cells and used immunostaining to investigate the expression of various factors. We found that BDNF, CNTF, GDNF, and bFGF were all expressed by migrating Müller cells. Furthermore, migrating Müller cells exhibited higher immunoreactivity compared with static nonmigrating Müller cells (Figs. 6A–P). To confirm this, we took separate samples of migrating cells (cells at the forefront of migration to the center of the dish) and static nonmigrating cells (cells located at the periphery of the dish) from the same dish, and compared their expression of BDNF, CNTF, GDNF, and bFGF mRNA. We found that for all the factors, mRNA expression of migrating cells was significantly higher than that of nonmigrating cells, which was consistent with the results of immunostaining (Figs. 6Q–T).

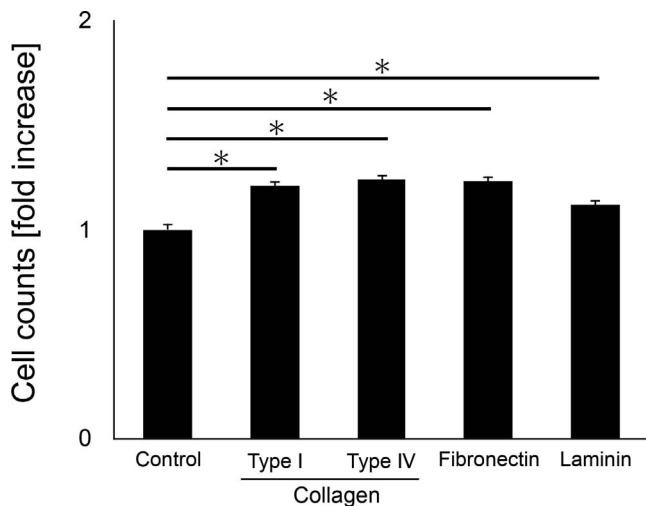


FIGURE 4. Effect of coating with constituents of the ILM on the proliferation of MIO-M1 cells. The numbers of MIO-M1 cells 72 hours after plating were significantly higher in dishes coated with either type I collagen (3 mg/mL); type IV collagen (500 µg/mL); fibronectin (50 µg/mL); or laminin (200 µg/mL) compared with uncoated control dishes. Results are normalized to control and expressed as mean ± SE. *Statistically significant difference compared to uncoated controls at $P < 0.05$.

DISCUSSION

The results of the current study suggest the following: (1) the ILM may function as a scaffold for the proliferation and migration of Müller cells (Figs. 1–4); (2) neurotrophic factors and bFGF may be produced by activated Müller cells (Fig. 6); and (3) neurotrophic factors and bFGF may also be present on the ILM itself (Fig. 5), and that the ILM placed in the MH may supply the hole with neurotrophic factors and bFGF. These findings suggest that Müller cell gliosis induced by inverted or

transplanted ILM flap, as well as humoral factors such as neurotrophic factors and bFGF, may contribute to the closure of MHs (Fig. 7). Studies of idiopathic MH have found that the migration and gliosis of Müller cells are induced in environments where a scaffold such as the ILM is present, or when rather than being surrounded by vitreous fluid they are in a semidried environment, which can be achieved when the fluid inside the eye is replaced with gas.^{8,37,38} To induce more intense and longer-term gliosis of Müller cells, it is important that patients be kept in the prone position for a long period after inversion or transplantation of the ILM.

Although gliosis by Müller cells is effective in closing MHs, excessive gliosis has cytotoxic effects on retinal neurons, and may indicate a worse visual prognosis.^{12,39} One problem with the inverted ILM flap technique and autologous transplantation of the ILM is the inability to control gliosis by Müller cells, entailing the risk of excessive gliosis. Michalewska et al.¹⁵ reported that OCT after the inverted ILM flap technique revealed foveal hyperreflective lesions suggestive of excessive gliosis in the fovea. In our experience too, foveal hyperreflective lesions are apparent after autologous transplantation of the ILM, and the ellipsoid zone at this site is disrupted (data not shown). Although these foveal hyperreflective lesions usually disappear within a few months postoperatively, the persistent activation of glial cells may contribute to tissue damage.⁴⁰ Should it result in scar formation, this may limit the recovery of the ellipsoid zone.⁴¹ Further studies are required to identify a means of regulating the degree of gliosis, and to develop a method of preventing excessive gliosis.

The problems with the inverted ILM flap technique and autologous transplantation of the ILM involve the difficulty of the surgical procedure with regard to inverting or transplanting the ILM, and the risk of damaging the retina associated with peeling the ILM.^{42,43} In the current study, three of the primary constituents of ILM, type IV collagen, fibronectin, and laminin, were found to enhance the proliferation of Müller cells, and type IV collagen and fibronectin were found to enhance their migration. This finding suggests that it may be possible to use

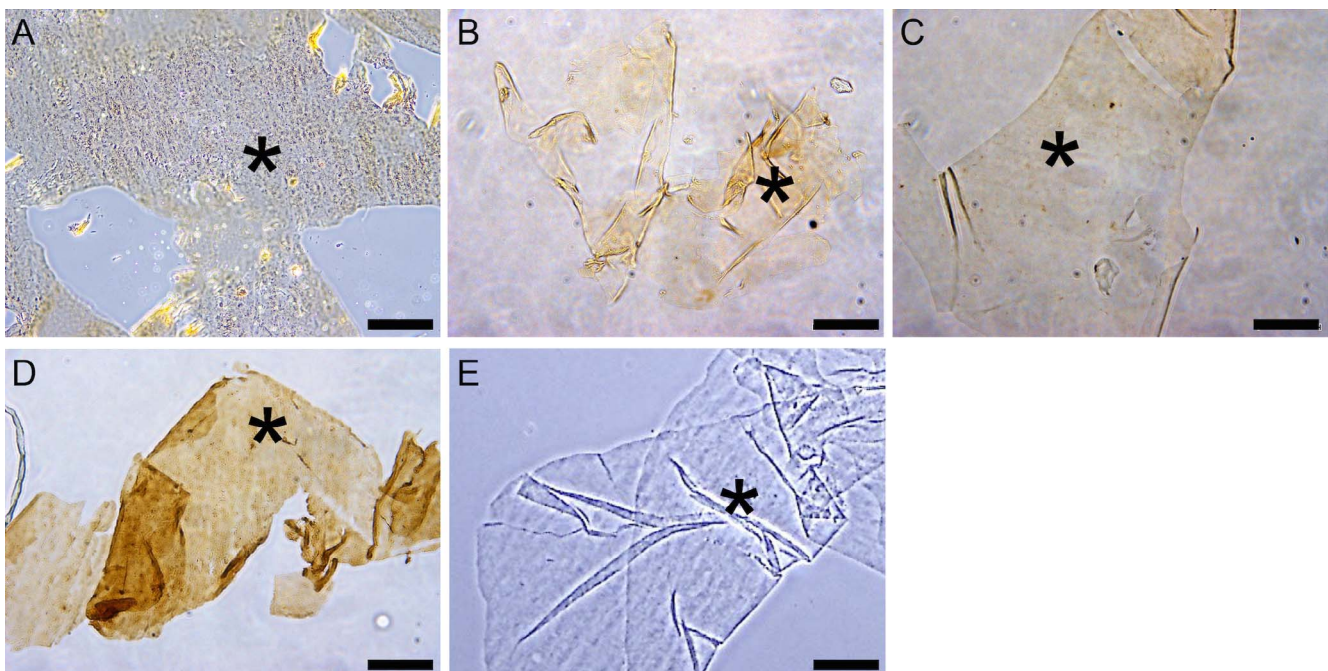


FIGURE 5. Presence of neurotrophic factors and bFGF in human ILM. Immunostaining of ILMs from patients with MH. ILMs were stained for BDNF (A), CNTF (B), GDNF (C), bFGF (D), and a negative control (E). Asterisks indicate the ILM, and the scale bars are 100 µm.

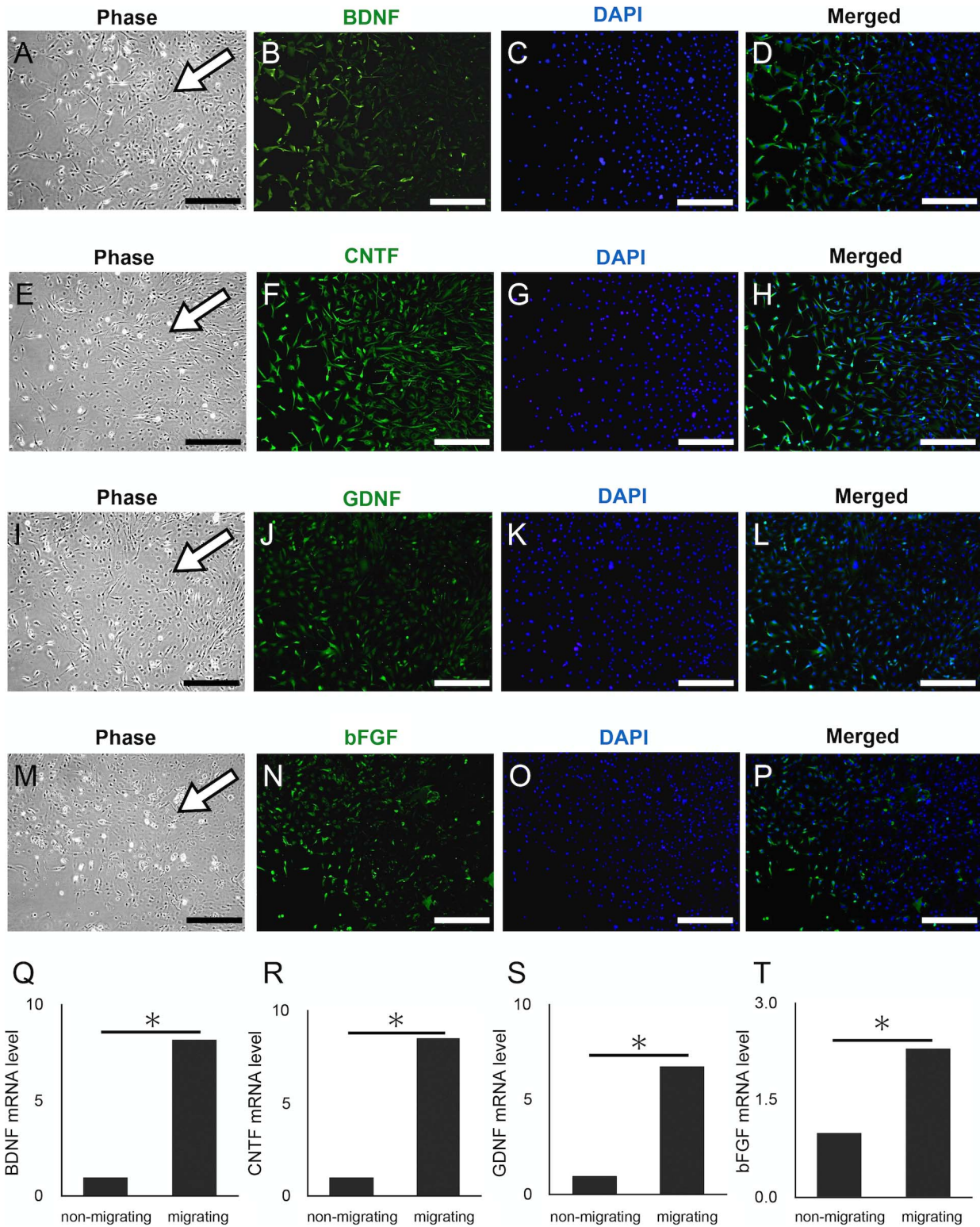


FIGURE 6. Production of neurotrophic factors and bFGF by MIO-M1 cells, and the effect of cell migration on the production of these factors. MIO-M1 cells produced BDNF (B), CNTF (F), GDNF (J), and bFGF (N). Migrating cells exhibited higher expression of these factors than static nonmigrating cells (B, F, J, N, Q-T). Arrows indicate the direction of MIO-M1 cell migration (A, E, I, M). The reactivity of cells exhibiting the most migration was stronger than that of stable cells, for all four factors (C, D, G, H, K, L, O, P). Scale bars: 500 μ m. *Statistically significant difference compared to non-migrating controls at $P < 0.05$.

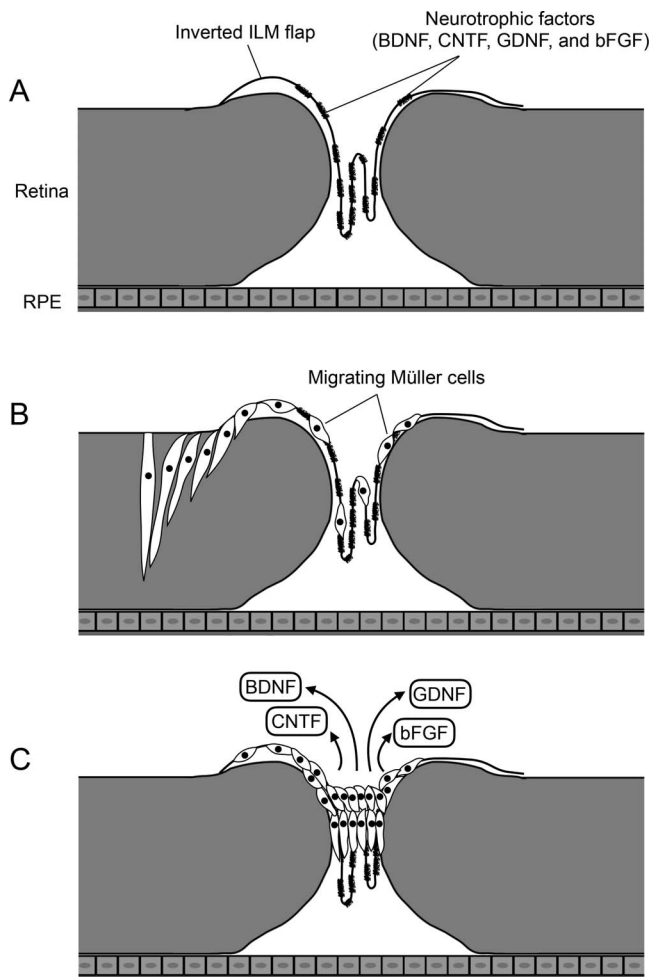


FIGURE 7. A possible mechanism of MH closure after the inverted ILM flap technique. (A) Neurotrophic factors and growth factors are retained on the surface of the ILM flap (see Fig. 5). (B) The ILM plays a role as a scaffold for Müller cell proliferation and migration (see Figs. 1–4). (C) Migrating Müller cells secrete neurotrophic factors and growth factors that may promote the survival of retinal neurons and photoreceptor cells (see Fig. 6).

these substances as adjuvants instead of ILM. Previous studies have investigated the efficacy of transforming growth factor beta 2 and autologous serum as adjuvants, but neither were found to have a significant effect.^{44,45} With regard to the use of autologous blood, it has been reported that blood dramatically reduces retinal function if it flows into the subretinal space.⁴⁶ The identification of adjuvants to use instead of ILM would enable the safer and more reliable closure of MHs compared with the inverted ILM flap technique and autologous ILM transplantation. Further *in vivo* studies are required to investigate the effects of type IV collagen, fibronectin, and laminin on MH closure.

The current study had a number of limitations. One was that the experimental MH model in monkeys does not have the same pathology as MHs in humans. However, in view of the requirements for a fovea as well as an eyeball large enough to perform the inverted ILM flap technique on, the monkey MH model used in this study is currently the best available *in vivo* model. Another limitation was that although we investigated the short-term process of hole closure after the inverted ILM flap technique, the long-term mechanism of hole closure remains unknown. In particular, we did not investigate the long-term course of the ILM flap in the MH, nor the

mechanism of recovery of the ellipsoid zone, which is important for visual function after hole closure. We have previously reported cases in which ellipsoid zones that were disrupted preoperatively were restored within 14 days to 3 months after autologous transplantation of the ILM.¹⁶ In rats and mice, glial cells have been implicated in the regeneration of retinal neurons and photoreceptor cells,^{47–49} and *in vitro* research has shown that adult human Müller cells can be a source of photoreceptors.⁵⁰ Further studies are required to elucidate the role of Müller cells in the recovery of the ellipsoid zone in the human eye. In summary, in this study we found that the mechanism whereby inversion or transplantation of the ILM facilitates the closure of MHs may involve promotion of the repair of the hole by the ILM placed within it via mediation by Müller cells.

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