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Age-Related Macular Degeneration and Retinal Pigment Epithelium Wound Healing

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Abstract

Choroidal new vessel (CNV) excision may improve vision in patients with age-related macular degeneration (AMD) by eliminating the source of subretinal bleeding and scarring. Visual recovery after CNV excision is usually poor in AMD patients, probably because of removal of the associated retinal pigment epithelium (RPE), coupled with the inability of native RPE at the edge of the dissection bed to resurface the iatrogenic RPE defect. Experiments using in vitro and in vivo RPE wound-healing models have provided insight into the factors that regulate RPE wound healing *in situ.* Wound-healing studies using aged submacular human Bruch's membrane in organ culture show that resurfacing of localized RPE defects is influenced by the depth of damage to Bruch's membrane as well as factors that are intrinsic to the aged RPE at the wound edge. The Bruch's membrane organ-culture paradigm provides a surface for RPE wound healing that closely resembles the surface on which RPE must grow after CNV excision in AMD patients. An understanding of the factors that influence RPE wound healing might lead to treatments that stimulate RPE resurfacing and improve visual outcome after CNV excision.

Index Entries: Age-related macular degeneration; retinal pigment epithelium; Bruch's membrane; wound healing; organ culture; cell migration.

Introduction

The retinal pigment epithelium (RPE) is a monolayer of pigmented cuboidal cells located between the sensory retina and the choroid. The RPE is separated from the subjacent choriocapillaris, which supplies blood flow to the RPE and the outer one-third of the retina (including the photoreceptors), by Bruch's membrane. Bruch's membrane is a pentalaminar structure consisting of the RPE basement membrane, the inner collagenous layer, the elastic layer, the outer collagenous layer, and the choriocapillaris basement membrane (Fig. 1). The RPE per-

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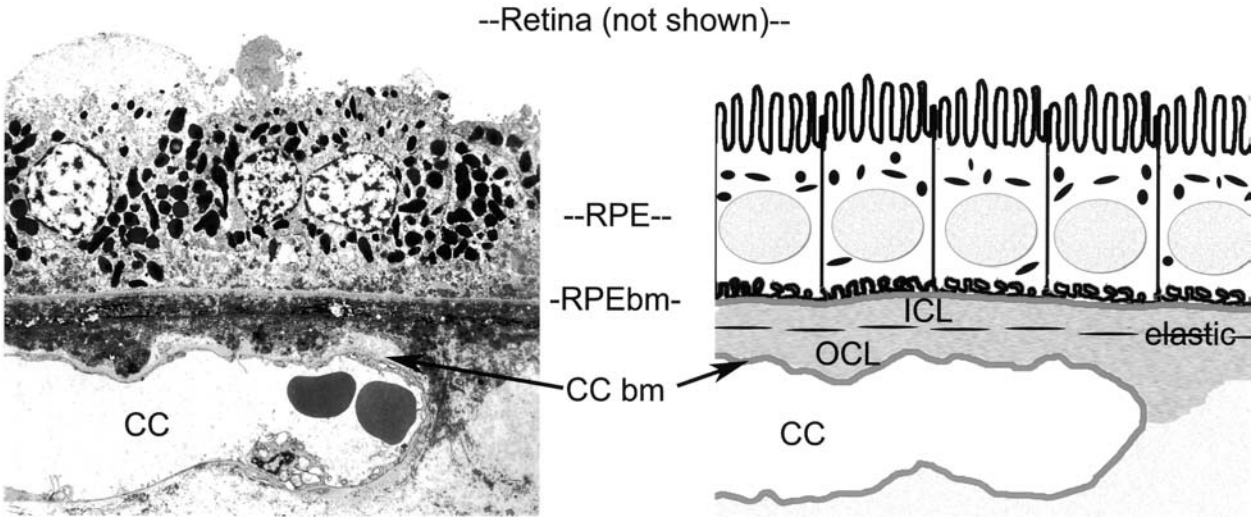

Fig. 1. The RPE is a monolayer of cells located between the retina and the choriocapillaris (cc). The basal surface of the RPE is attached to Bruch's membrane, which is composed of the RPE basement membrane (RPEbm), the inner collagenous layer (ICL), the elastic layer (elastic), the outer collagenous layer (OCL), and the choriocapillaris basement membrane (CC bm). Removal of RPE with varying degrees of damage to Bruch's membrane can occur in patients with age-related macular degeneration following choroidal neovascular membrane excision.

forms a variety of functions, including phagocytosis of shed outer segment discs, vitamin A transport from the blood to the photoreceptors, dehydration of the subretinal space, and maintenance of normal choriocapillary anatomy and physiology. RPE dysfunction or absence can lead to photoreceptor and choriocapillaris degeneration.

Age-related macular degeneration (AMD) is the major cause of legal blindness among persons older than 55 yr of age in the United States. Approximately 70% of cases of severe visual loss in AMD are caused by growth of abnormal blood vessels, known as choroidal new vessels (CNVs), under the RPE and retina with secondary exudative retinal detachment, subretinal hemorrhage and lipid exudation, and outer retinal degeneration *(1–5).* The only proven treatments for CNVs in AMD are laser photocoagulation and photodynamic therapy (PDT), but these treatments are associated with poor visual outcome, and many (~40–50%) AMD patients with CNVs are not eligible for laser or PDT treatment *(6–9).* Submacular surgery with CNV excision offers the possibility of removing large CNVs while preserving the overlying retina, thus preventing/reversing the photoreceptor damage and blindness associated with subretinal bleeding and scarring in AMD. Submacular surgery does not depend on a precise delineation of the CNV boundaries, in contrast to laser photocoagulation and PDT, and is therefore potentially applicable to a much larger proportion of all patients with AMD-associated CNVs *(10).* However, visual recovery after CNV excision is usually poor in AMD patients *(11–13)* as a result of the removal of adjacent native RPE and RPE basement membrane *(13,14)* and incomplete/aberrant RPE growth into the dissection bed *(13,15–17).* Thus, CNV excision creates a localized RPE defect with subjacent Bruch's membrane abnormalities (e.g., removal of the RPE basement membrane and, to varying degrees, portions of the inner collagenous layer of Bruch's membrane). Lack of RPE ingrowth into the dissection bed probably results in choriocapillaris and photoreceptor atrophy *(13,18).* These findings indicate that RPE transplantation or stimulation of RPE resurfacing by residual native

RPE may improve visual outcome after CNV excision in AMD patients.

Current obstacles to successful RPE transplantation in patients who are undergoing CNV excision include graft survival and, in the case of allogeneic transplants, immune rejection. RPE transplants in AMD patients who have undergone CNV excision have failed with poor vision and, in patients who are not immune-suppressed, subretinal fibrosis and chronic fluid leakage in the dissection bed *(19–23).* Graft failure may be the underlying cause of poor visual results in an immune-suppressed patient who underwent CNV excision and transplantation using uncultured adult RPE *(24).* Histopathology has shown that the RPE cells were not organized as a monolayer; large areas of Bruch's membrane lacking RPE were present, and there was photoreceptor atrophy over the dissection bed, including over the transplant *(24).* Transplanted fresh adult human RPE show very limited adherence to aged submacular human Bruch's membrane in vitro *(25).* Preliminary data indicate that cultured fetal human RPE can adhere to the inner collagenous layer of aged human submacular Bruch's membrane, but survival and differentiation may be impaired *(26).* If RPE cells cannot adhere to their basement membrane (or comparable surface) within 24 h, they undergo apoptosis *(27).* All previous demonstrations of successful RPE transplants in laboratory animals have involved transplantation onto normal Bruch's membrane (or onto native RPE) (*see* refs. *28–43*). In AMD, Bruch's membrane is itself abnormal (*see* refs. *44–47*).

An alternative to RPE transplantation is to stimulate RPE ingrowth from the edge of the dissection bed. Histology of excised CNVs *(13,14,48),* histopathology of eyes following CNV excision *(16,17),* and postoperative clinical findings *(13,49–51)* all suggest that the CNV dissection exposes both the superficial and deeper layers of the inner collagenous layer, which will constitute much of the surface that native RPE must repopulate. In vivo and in vitro studies of RPE wound healing will improve our understanding of the ability of RPE to resurface defects similar in size to those created by CNV excision. This research could lead to the development of treatments to alter the denuded Bruch's membrane surface and/or stimulate the RPE cells so that resurfacing of the iatrogenic RPE defect occurs *in situ,* thus obviating the need for RPE transplantation.

RPE Wound Healing in Cell Culture

Resurfacing of wounds made in established RPE-cell monolayers in culture is characterized by ingrowth in a regulated fashion that is reproducible from wound to wound with a predictable rate of wound closure. In general, following a latent period after wounding (within 12 h for early-passage bovine RPE *[52]*), cells close to the wound edge lose their polygonal shape and become spindle-shaped *(52,53)* or spread toward the wound center *(54,55).* Next, the cells proliferate and migrate into the wound to resurface the defect in a uniform manner. When bovine RPE resurface wounds that measure 6 mm in diameter, wound closure is linear for the first 4 d, followed by a hyperbolic phase of closure. Cell density increases linearly with time until wound closure is complete *(52).* Initial closure of the wound occurs centrally by enlarged cells *(53).* Cells distal to the wound remain unchanged *(52).* RPE can migrate as single cells or as sheets, depending on the amount of time the cells have been confluent in culture *(54).* Time at confluence also affects rate of wound closure *(54).* Kaida and colleagues speculate that cells at confluence for months may never reacquire their original morphology following resurfacing of a defect *(54).* Rat RPE begin to deposit extracellular matrix (ECM) at ~50% wound closure (e.g., at 24 h) with the initial appearance of fibronectin followed by laminin and collagen at 48 h. Accumulation of these ECM constituents in the entire wound follows complete wound closure *(56).* Cell proliferation usually occurs at the leading edge of ingrowth *(56).*

Because of the predictable rate of closure, wound-healing models using cultured cells are useful for evaluating pharmacological responses. The amount of serum in the culture

medium *(53,57,58),* the presence of Vitamin A, human platelet concentration *(59),* and various pharmacological agents such as cytokines and growth factors *(56,57,60)* affect the rate of wound closure. RPE wound healing can be inhibited by polyamine blockers *(58),* colchicine, 5-fluorouracil, cytochalasin B *(52),* or vitamin E succinate *(61).* Since bovine RPE migration was found to be regulated by protein kinase C (PKC), stimulation or inhibition of this enzyme may affect migration *(62).*

The study of wound healing in culture also can characterize events induced within RPE cells after wounding. Human RPE at the wound edge show increased expression of platelet-derived growth factor (PDGF) and PDGF-β receptors as well as proliferating cell nuclear antigen (PCNA) labeling, which indicates that growth stimulation is important in wound repair *(63).* TGF-β2 may be important in RPE regeneration, since production is increased substantially after laser photocoagulation of cultured human fetal RPE *(64)* Gene array analysis of ARPE-19 cells after wounding of confluent cultures shows increased expression of molecules involved in wound repair *(65).* Actin cytoskeleton reorganization occurs in bovine RPE cells at the wound edge, with fibers oriented parallel to the movement into the center. These cells express α-smooth-muscle actin (not expressed in highly differentiated cultures) and show decreased cytokeratin-18 expression, a differentiation marker for RPE *(55).*

RPE Wound Healing in Chick Organ Culture

Cell-substratum interactions are very important for cell migration and proliferation. Chick embryo organ cultures have been used to characterize RPE wound healing on native RPE basement membrane in choroid explants *(66–69).* As in wound healing on artificial substrates, RPE wound healing on RPE basement membrane shows a latent phase, followed by cell spreading along the wound edge with reorientation of actin fibers parallel to the direction of migration. However, cell involvement extends further from the wound edge, as far as 10–15 rows away from the wound edge. Cells at the edge of the wound lead with their basal surfaces, and cells further away from the wound edge show apical surfaces leaning toward the wound center. RPE cells at the wound edge form stress fibers and vinculin-positive focal contacts along their ventral surface at the ends of stress fibers *(68,69).* Cell morphology indicates that RPE cells generate the force for migration along their basal regions while being restrained by junctions at their apical ends. In the zone of apical and basal slanting, cells are separated by intercellular gaps. Tractional forces are evidenced by folds along the basement membrane near focal contacts, by cell orientation, and by the presence of prominent stress fibers, especially in areas in which opposite wound edges are in close proximity *(68).*

Studies of cell proliferation in this organ-culture system show a delayed onset of proliferation compared to cells seeded onto glass cover slips. PCNA expression is initially observed in some cells around the wound edge 48 h after wounding, but ceases by 14 d after wounding, even in large defects showing bare RPE basement membrane. Total resurfacing is not observed in 1 mm-wide defects. Hergott and colleagues hypothesized that the delay in and cessation of proliferation may be caused by the negative influence of the underlying substratum *(66).* Cells cultured on glass show rapid onset of proliferation and continued proliferation for at least 28 d. In contrast, Matrigel®—a solubilized basement membrane preparation composed primarily of laminin with collagen IV, heparan sulfate proteoglycan, and entactin/nidogen—causes growth inhibition in RPE cells. Proliferative vitreoretinopathy which is associated with loss of RPE contact with substratum, is characterized by a lack of RPE growth inhibition *(66).* Wounds that are 125 μm and smaller on RPE basement membrane are resurfaced by cell spreading only. Hergott and colleagues noted that 125 μm corresponds to slightly more than the diameter of two maximally spread RPE cells (or four stationary RPE-cell widths), and they theorized that spreading alone is not sufficient to induce cell prolifera-

tion, and that active migration of the RPE cells is required *(66,69).*

Hergott and colleagues found that embryonic chick RPE focal adhesions contain β1 integrin subunits *(67).* Antibodies to β1 partially inhibit wound closure. The partial wound closure observed in the presence of anti-β1 antibodies is probably the result of cell spreading, which indicates that different mechanisms are used for spreading and migration. Hergott and colleagues found expression of PCNA staining in RPE at the wound edge was inhibited by very high concentrations of anti-β1 antibody—higher concentrations than those required to inhibit migration *(69).*

RPE Wound Healing In Vivo

To study RPE ingrowth in vivo on an undamaged surface, in vivo RPE wound-healing models use relatively gentle methods of RPE removal such as hydraulic RPE debridement *(70–73),* grasping the RPE with forceps *(74),* or gently brushing the RPE with a silicone brush *(75,76)* or silicone-tipped cannula *(77),* which creates little or no damage to subjacent Bruch's membrane. In vivo wound healing following these gentle RPE removal methods shows complete, rapid healing of defects ranging from 1.5 mm *(76)* to over 3 mm *(75)* in width. Healing in these studies occurs on the native RPE basement membrane, even in animals that showed foci of disruption or detachment of the RPE basement membrane *(70).* For example, the RPE and choriocapillaris basement membranes are normally fused in the cat, and after hydraulic RPE debridement, areas of separation of these two basement membranes are evident. However, RPE cells can migrate over such areas *(73).* Following wounding, RPE at the leading edge of the debridement become flattened and elongated with their long axis pointed in the direction of migration. The RPE cells also exhibit altered apical-basal polarity and undergo cytoskeletal reorganization to that of a migrating phenotype *(70).* Following initial wound closure, cells in the defect are of variable morphology, ranging from elongated to flattened. At later times following closure, the cells appear more like *in situ* RPE—e.g., smaller and more rounded. Eventually, the cells become morphologically homogeneous. In pigmented animals, RPE resurfacing the defect is hypopigmented *(70,71,77),* and can remain so even after 9 mo *(74).* Focal areas of RPE bi- and multilayers can be seen in studies with intact overlying retina *(70,77).* After hydraulic RPE debridement in cats, for example, foci of RPE multilayers are localized mostly to sites of retinal folds, but healing of some RPE defects shows more extensive multilayering throughout the debridement zone *(72).* In rabbit RPE studies retaining intact overlying retina, tubuloacinus formation is observed in wounds 2–3 mm in diameter by 7–14 d with increasing frequency at longer time-points (e.g., 28–56 d) *(70).* In these rabbit studies, Bruch's membrane appears to be normal, even in areas of RPE multilayering. No reduplication or excess basement membrane deposition is observed *(70).* In contrast, Ozaki and colleagues rarely found RPE multilayers and acinus formation in animals with intact overlying retina. Animals in which RPE debridement was combined with retinectomy showed RPE resurfacing with RPE multilayer and tubuloacinus formation. Wound closure occurred by four days after surgery whether the overlying retina was intact or was removed surgically *(76).* Rabbits with large RPE debridements (3.5-disc diameters) and retinectomy showed similar RPE wound-healing responses. The authors observed focal thickening of Bruch's membrane by increased collagen and elastic layer thickening *(75).* Focal thickening of Bruch's membrane is also observed in cats following hydraulic RPE debridement *(73).*

Further damage to Bruch's membrane can be caused at the time of RPE removal by using a silicone-tipped metal cannula to debride the RPE cells. This debridement technique allows study of RPE wound healing on a surface more damaged than that seen following gentler debridements *(71–73,77,78).* In an experiment in which the overlying retina was removed, Heriot and Machemer made 9×6 mm^2 RPE

defects in rabbits using a silicone-tipped blunt needle. Their debridement method left fragments of RPE basement membrane on the surface with the remainder of Bruch's membrane intact, although the elastin layer was more discontinuous than normal. Cells at the edge of the defect at d 3 were pleomorphic, flattened with pseudopods extending between adjacent cells, which were small and round or elongated. Isolated pigmented cells were seen occasionally in the defect. By d 7, the defects were fully resurfaced, with flattened cells centrally. As with hydraulically debrided defects, some acinus formation was present in the area of resurfacing. Tritiated-thymidine incorporation showed cell proliferation at the edge of the defect and in cells within the defect. Similarly, debridements in cats show RPE growth over surfaces in which Bruch's membrane is disorganized, missing, or split. In areas of the RPE debridement that RPE does not resurface, primary damage to the retina and/or choriocapillaris may contribute to incomplete wound healing *(73).* Furthermore, there is apparent regression of RPE resurfacing at four weeks compared to 1 wk after RPE debridement. This result may be caused by the inability of the degenerate choriocapillaris to support newly ingrown RPE *(73).*

In vivo studies of RPE wound healing allow an evaluation of the efficacy and toxicity of pharmacological agents, to the RPE as well as to other parts of the eye. One such study by Kimizuka and colleagues tested the effectiveness of basic fibroblast growth factor on RPE wound healing and its effect on the overlying retina *(79).* Small, linear wounds were made in the RPE monolayer (0.8×3.5 mm^2) with a silicone brush. After quantifying the number of cells that grew into the wounded area by light microscopy, they found no change in the number of cells resurfacing the denuded area at d 28 in treated and untreated wounds. These small wounds showed overgrowth of RPE at approx $4 \times$ the normal number seen in situ at d 28 in both conditions. The authors did not comment on any histological changes in the overlying retina.

RPE Wound Healing on Aged Human Submacular Bruch's Membrane Explants

Human pathological studies indicate that RPE ingrowth following CNV excision in AMD patients is incomplete and aberrant *(13,16,80,81).* RPE at the edge of the CNV dissection bed must grow over residual native RPE basement membrane and the superficial and deeper portions of the inner collagenous layer of Bruch's membrane to resurface the iatrogenic RPE defect *(13,14,48,82).* The behavior of RPE ingrowth on surfaces similar to that present in patients after CNV excision has been studied using submacular Bruch's membrane. Berger and colleagues found that RPE wound healing in organ culture did not begin until 48–72 h after wounding in contrast to wound healing of passaged RPE cells on tissue-culture plastic. BRDU staining indicated that in organ culture, cell division begins after migration, whereas in culture dishes, there is rapid onset of proliferation and more limited cell migration *(83).* We have studied RPE wound healing on aged human submacular Bruch's membrane in organ culture *(84).* RPE are debrided mechanically to create ~3-mm-diameter RPE defects of different depths, thus exposing the RPE basement membrane (RPEbm[+]), the inner collagenous layer surface immediately below the basement membrane (superficial inner collagenous layer, SICL), or the deep inner collagenous layer (DICL). Mechanical debridement mimics the iatrogenic changes in Bruch's membrane created by CNV excision and therefore creates surfaces on which aged adult RPE cells must migrate following CNV excision. Histopathology of patients who have undergone CNV excision indicates that postoperative resurfacing of the dissection bed by native RPE is incomplete *(15,16).*

Onset of ingrowth on all three surfaces occurs between d 2–4 following wounding. Cells migrating on the native RPE basement membrane show the resurfacing pattern most similar to those seen in vivo and in vitro (Fig. 2). At d 10 following wounding, resurfacing on deeper layers of Bruch's membrane (DICL) is

Table 1
Average Wound Size, Area Resurfaced, and Percentage of Resurfacing in Different Types of RPE Defects

	RPEbm (+)	vs	SICL	RPEbm (+)	vs	DICL
Wound Area (mm^2)	4.02 ± 0.65 (n = 4)		4.63 ± 0.46 (n = 4)	4.69 ± 0.69 (n = 7)		5.19 ± 0.66 (n = 7)
Area Resurfaced (mm^2)	2.67 ± 0.67 (n = 4)		2.86 ± 0.67 (n = 4)	3.94 ± 0.83 (n = 7)		2.78 ± 0.74 (n = 7)
% of Resurfacing	67.35 ± 18.82 (n = 4)		64.26 ± 16.07 (n = 4)	84.07 ± 15.35 (n = 7)		54.00 ± 14.54 (n = 7)

No significant difference is present between healing in the presence of native RPE basement membrane and SICL in terms of the wound size, area resurfaced, and percentage of resurfacing (student's paired t test, $p > 0.05$). The area resurfaced and the percentage of resurfacing in RPEbm(+) defects and DICL defects are significantly different (t test, $p < 0.05$), whereas the difference in the wound area between the two surfaces is not significant (t test, $p > 0.05$). (From Wang et al. (2003), Retinal pigment epithelium wound healing on human Bruch's membrane explants. *Invest. Ophthal. Vis. Sci.*, **44**, 2199–2210.)

incomplete. Resurfacing on the RPE basement membrane and superficial portion of the inner collagenous layer (subjacent to the RPE basement membrane) shows more resurfacing (Table 1, Fig. 2–4). In contrast to resurfacing in chick organ culture *(66)*, some explants show close to 100% resurfacing of these large defects. Proliferation studies of cells resurfacing RPEbm(+) and DICL defects at d 10 shows cell proliferation in a zone including the original wound edge and the leading edge of cells migrating into the RPE defect. The greatest number of proliferating cells was observed in a zone between the original wound edge and leading edge (e.g., in the transition zone) (Fig. 5) *(84)*. The morphology of resurfacing cells at d 10 appears to depend on which surface is exposed to the migrating cells.

Cell morphology on all three surfaces is similar at the original wound edge. The cells are elongated with their axis pointing in the direction of migration. Cells in the transition zone are present as continuous sheets of flattened or elongated cells when resurfacing the RPE basement membrane and the superficial inner collagenous layer (Fig. 2,3). However, cells on the superficial inner collagenous layer, do not form as continuous a monolayer, showing gaps between adjacent cells (Fig. 3B). Cell morphology in the transition zone growing on the DICL is variable, ranging from isolated patches of flattened cells and spindle-shaped cells to morphology similar to that seen on the superficial inner collagenous layer (Fig. 4B). At the leading edge, cells growing on the RPE basement membrane appear as flattened sheets or elongated cells, with lamelipodia and filopodia extending towards the bare surface or onto neighboring cells. Most cells are in contact with neighboring cells (Fig. 2B). Few single, flattened cells are seen. RPE growing on the superficial inner collagenous layer is polymorphic at the leading edge with some flattened cells maintaining contact with cells at the front, and other flattened or spindle-shaped cells are present singly or as patches. In addition, some cells are observed with their long axis parallel to the original wound edge (Fig. 3B). In all DICL defects, RPE cells at the leading edge are mostly thick and elongated or spindle-shaped, and appear singly. Many cells are oriented parallel to the wound edge (Fig. 4B).

In addition to the surface affecting RPE migration, RPE at the edge of the defect appears to differ with regard to the ability to resurface the RPE basement membrane, the superficial inner collagenous layer, and the deep inner collagenous layer. In other words, within a given Bruch's membrane explant, all cells resurfacing a given Bruch's membrane sublamina do not behave similarly. These observations, which are different from those reported in the in vivo and in vitro studies discussed in previous sections, have led us to hypothesize that aging changes in the Bruch's membrane sublaminae and/or RPE cells may affect resurfacing of the CNV dissection bed in AMD patients.

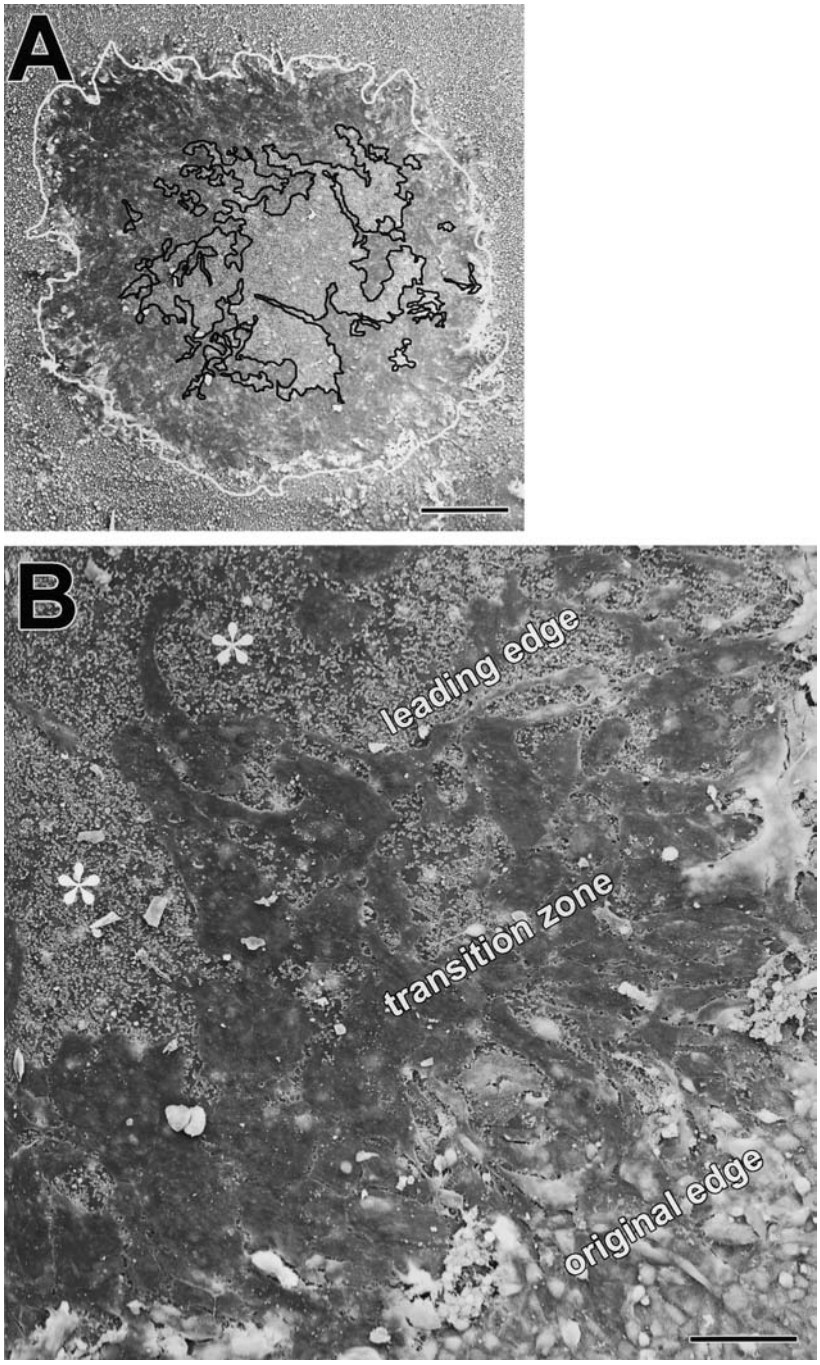

Fig. 2. Scanning electron micrograph of RPE resurfacing on the RPE basement membrane of aged Bruch's membrane at d 10 (donor age: 56 yr). **(A)** RPE cells have covered 73.3% of the defect. The white line defines the original wound edge. Black lines enclose the unresurfaced areas of the defect. Scale bar = 400 μm. **(B)** At the original edge, cells are elongated, with their long axis pointing toward the defect. Cells in front of them ("the transition zone") are flattened and form a monolayer or multilayer covering the defect. At the resurfacing leading edge, most flattened cells are in contact with one another, comprising the migration front. Uncovered areas show RPE basement membrane covered with debris (asterisks). Scale bar = 100 μm. (From Wang, et al. (2003), Retinal pigment epithelium wound healing on human Bruch's membrane explants. *Investig. Ophthal. Vis. Sci.* **44**, 2199–2210.)

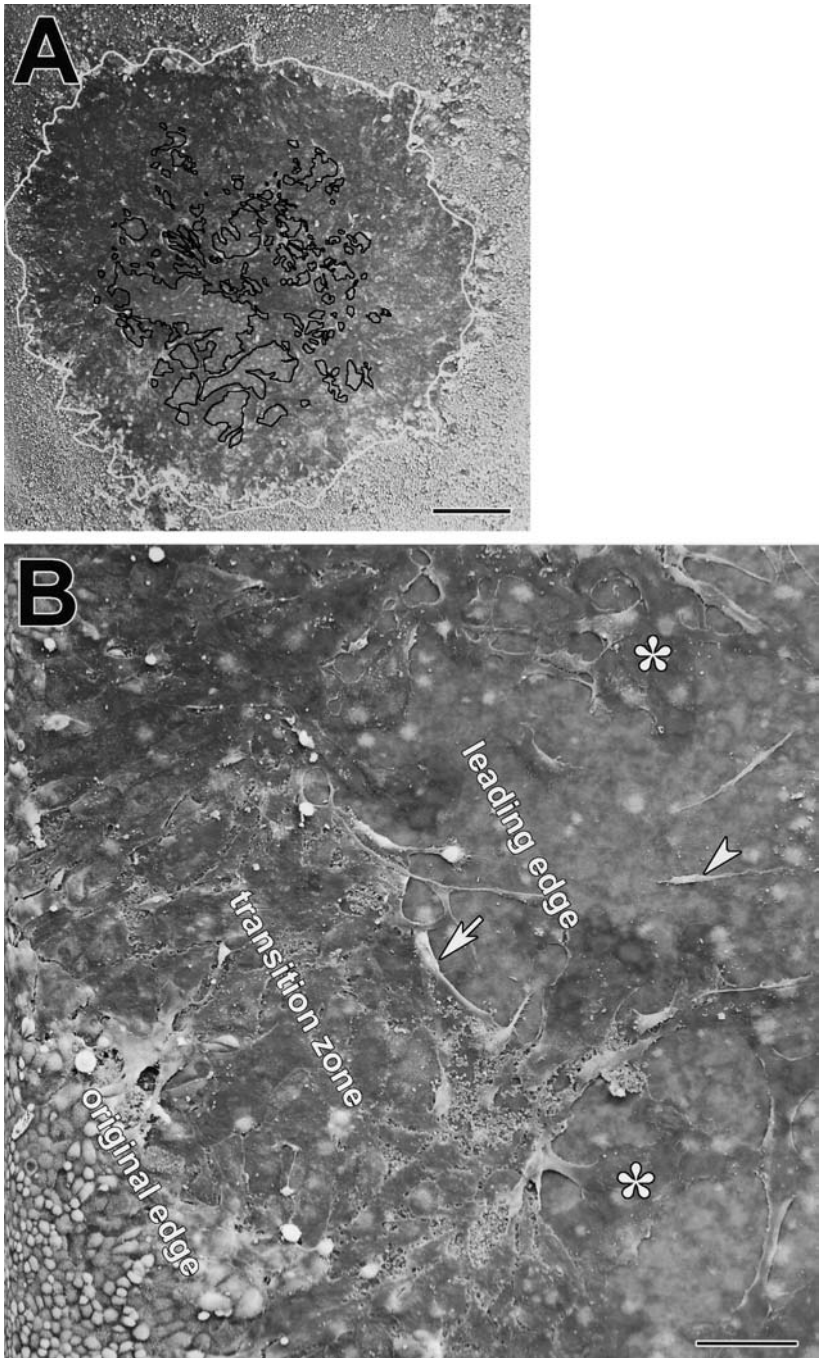

Fig. 3. Scanning electron micrograph of RPE resurfacing on the superficial inner collagenous layer of aged Bruch's membrane at d 10 (donor age: 56 yr). **(A)** RPE cells have covered 82.1% of the defect. The white line defines the original wound edge. The black lines outline the areas not resurfaced by RPE. Scale bar = 400 µm. **(B)** Cells at the original wound edge and in the transition zone appear similar to that on the RPE basement membrane defect (Fig. 2). However, at the leading edge, not only flattened cells are seen (*)—some spindle-shaped cells are also present, with their long axis pointing toward the defect (arrowhead) or elongated parallel to the leading edge (arrow). Scale bar = 100 µm. (From Wang, et al. (2003), Retinal pigment epithelium wound healing on human Bruch's membrane explants. *Investig. Ophthal. Vis. Sci.* **44**, 2199–2210.)

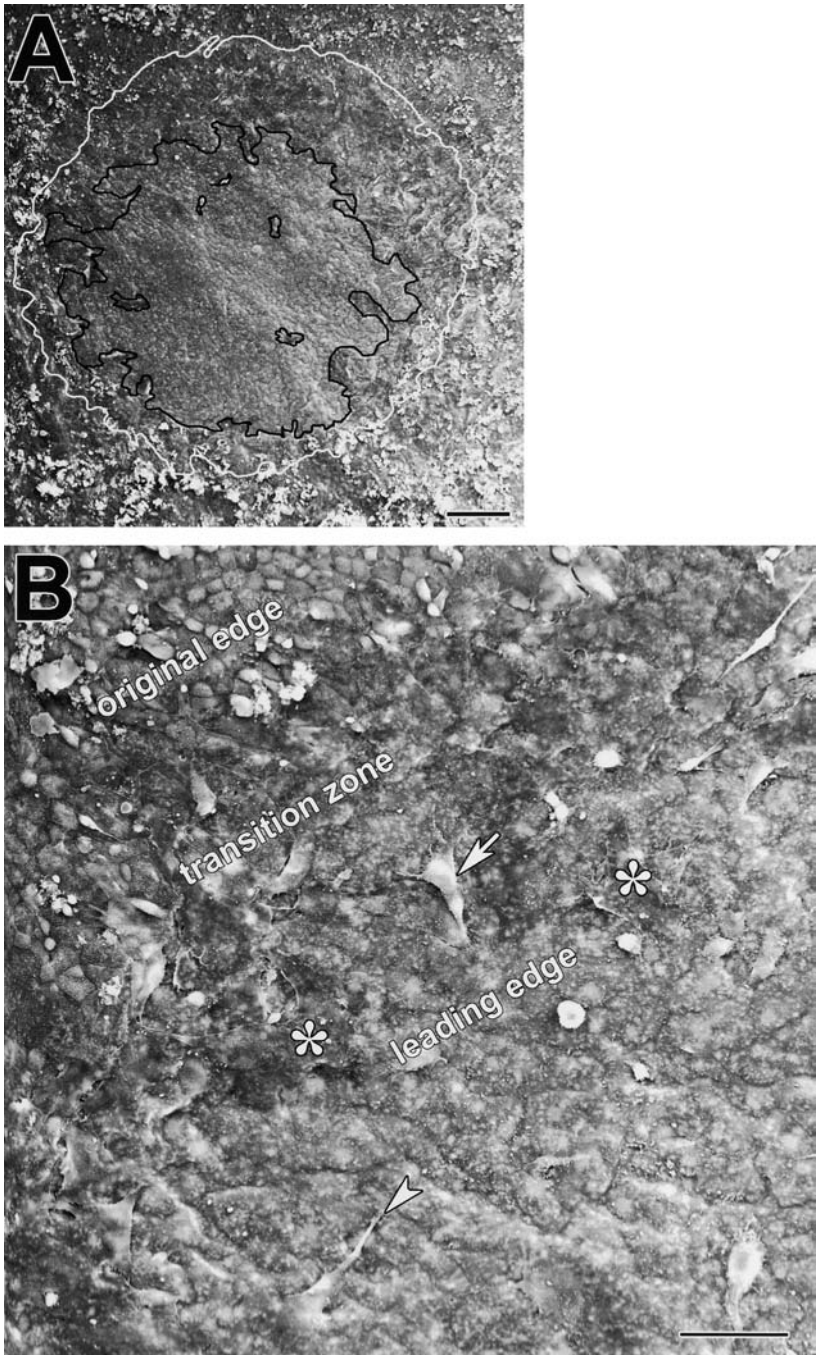

Fig. 4. Scanning electron micrograph of RPE resurfacing on the DICL of aged Bruch's membrane at d 10 (donor age: 77 yr). **(A)** RPE cells have covered 43.0% of the defect. The white line defines the original wound edge. The outer black line defines the new edge of the healed defect. Inner polygons outline islands of cells that have migrated into the center of the defect, discontinuous with the leading edge of resurfacing cells. Scale-bar, 400 µm. **(B)** At the original wound edge, cells appear similar to that on the RPE basement membrane (Fig. 2), and on the superficial inner collagenous layer (Fig. 2). In the transition zone, patches of flattened cells are seen. At the leading edge, cells appear thick (arrow), elongated, or spindle-shaped (arrowhead). Some flattened cells are also observed (asterisk). Scale bar = 100 µm. (From Wang, et al. (2003), Retinal pigment epithelium wound healing on human Bruch's membrane explants. *Investig. Ophthal. Vis. Sci.* **44**, 2199–2210.)

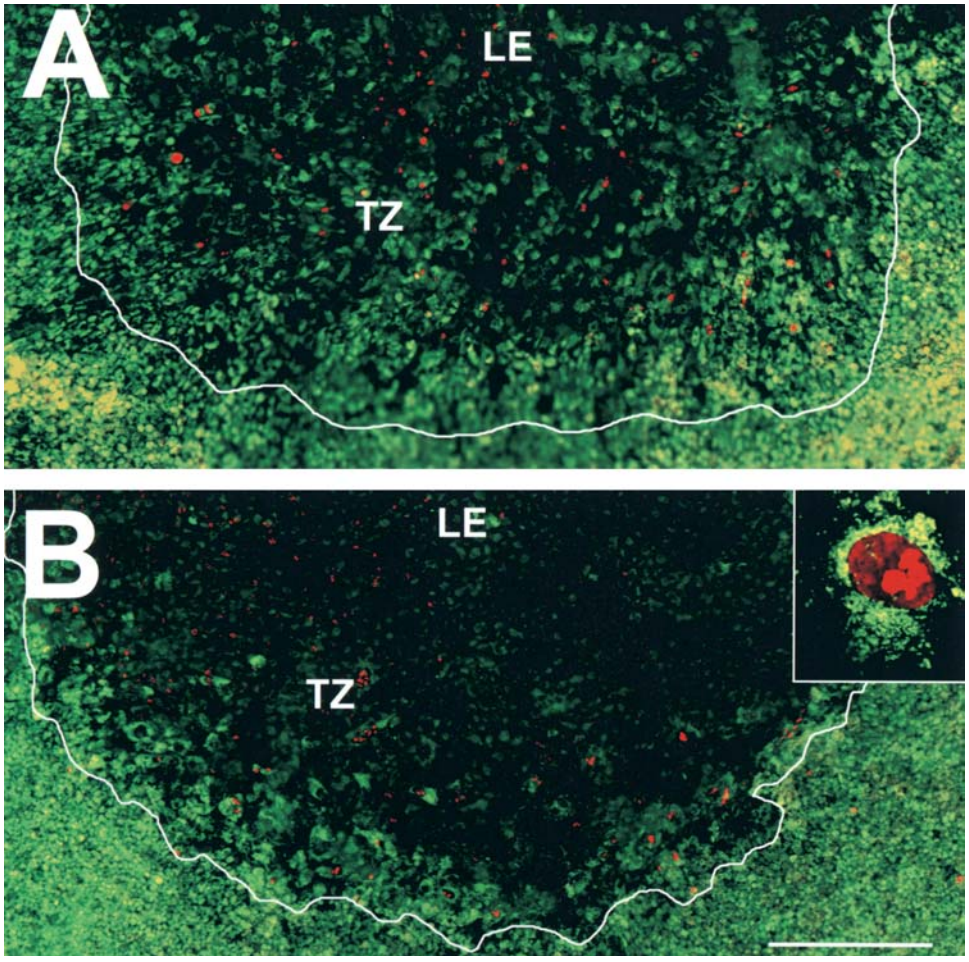

Fig. 5. Contribution of proliferation to RPE wound healing at d 10. Ki-67 labeling (red) on RPE basement membrane **(A)** and the DICL **(B)**. In both cases, Ki-67-positive cells were present at the original edge (the white line) and in the transitional zone (TZ), as well as at the leading edge (LE). However, most positive cells on both surfaces, were seen in the transitional zone. Inset: a Ki-67-positive RPE cell. The green background represents autofluorescence of RPE seen with fluorescein isothiocyarate (FITC) filters. Scale bar = 400 m*M*. Donor age of paired explant: 74 yr.

It is not clear why RPE resurfacing is better on the RPE basement membrane and on the superficial inner collagenous layer than on the DICL. Preliminary (unpublished) studies performed in this laboratory indicate that aged human RPE growth on artificial surfaces composed of collagen 1 or fibronectin is more limited than on surfaces composed of bovine corneal endothelial cell ECM, or laminin-poly-D-lysine. Migrating aged RPE cells express α1, α3, α5, β1, and β4 integrin subunits, depending on the surface on which they grow. Although several different groups of cell-substrate receptors exist, integrins constitute the dominant group and are the main receptor type used by cells for adhesion to the ECM. In many cell types, the occurrence and speed of migration are influenced by integrin-ECM ligand interactions, including ligand levels, integrin levels, and integrin-ligand binding affinities. Combinations of the β1 integrin subunit with various α subunits form most of the receptors for ECM

molecules: α1β1, α2β1, α3β1, and α6β1 are receptors for laminin and/or collagens, and α5β1, α4β1, and αvβ6 are the major receptors for fibronectin *(85–87).* Presumably, the presence of different ECM ligands in the RPE basement membrane and superficial inner collagenous layer underlies the greater ability of RPE to resurface RPEbm(+) and SICL defects *(88).* Also, age-related Bruch's membrane changes might alter ECM ligand availability, which in turn may affect the ability of aged RPE to resurface Bruch's membrane. Other AMD-associated changes in Bruch's membrane might be inhibitory to RPE cell resurfacing *(89).* The morphological differences among RPE cells resurfacing RPEbm(+), SICL, and DICL defects may reflect different cell-substratum interactions in these conditions.

In addition to biochemical changes in Bruch's membrane, intrinsic properties of native RPE cells may also play a role in the abnormal repopulation of the dissection bed after CNV excision in AMD patients. For example, RPE cells from aged donors show a longer lag period before onset of proliferation and grow much more slowly than cells from fetal donors after initial attachment onto Bruch's membrane *(90).* Wound healing on RPEbm(+) defects varies among different donors and is independent of the donor age or freshness of the tissue. In addition, some—but not all—RPE cells within a single donor are able to repopulate the DICL defects. At the resurfacing leading edge on DICL defects, where the surface shows the most morphological damage, cell morphology is variable. Some cells are flattened and spread like sheets, similar to the cells repopulating RPEbm(+) defects, and some are elongated and spindle-shaped, exhibiting the morphology of migrating cells. The polymorphic cell types at the leading edge on DICL defects may arise from subtle differences in the underlying surface and/or may reflect phenotypic heterogeneity of RPE cells *in situ (91).* The poor repopulation of RPEbm(+) defects in some donors indicates that therapy to stimulate RPE resurfacing may be necessary to promote repopulation of the dissection bed in some cases, even if the RPE basement membrane is intact.

Potential mechanisms involved in resurfacing localized RPE defects include cell spreading, cell migration, and cell proliferation. We believe that cell spreading and migration are the principal initial mechanisms of RPE resurfacing in aged human submacular Bruch's membrane organ culture, as has been noted in other in vitro systems *(52,53,66–69,83,92–94).* This hypothesis is supported by the gradual, progressive enlargement and relatively uniform distribution of the cells from the wound edge to the transitional zone seen with fluorescence microscopy and with scanning electron microscopy, as well as by Ki-67 staining of the cells during wound healing on aged submacular human Bruch's membrane.

Stimulated RPE Resurfacing As an Adjunct to CNV Excision

Current data indicate that resurfacing of ~65–85% of a ~3-mm-diameter circular RPE defect in aged submacular human Bruch's membrane occurs by approx 10 d if RPE basement membrane or the superficial inner collagenous layer is present *(84).* What must the time-course of resurfacing of the iatrogenic RPE defect be if preservation of significant numbers of photoreceptors is to be achieved after CNV excision?

Human clinical studies indicate that periods of macular detachment up to 2 wk are compatible with recovery of visual acuity of 20/50 or better in a substantial number of patients *(95).* Experiments with monkeys and cats indicate that many photoreceptors survive during retinal detachment periods of several weeks in duration, although some photoreceptors definitely die *(96,97).* Approximately 80% of the cat outer nuclear layer (ONL) survives during 3 d of detachment *(98).* The numbers of photoreceptor nuclei in detached cat retina do not begin to decline significantly (e.g., >20% decline in density) until detachment periods of more than 13 d *(99).* The cat retina, which is holangiotic, is rod-dominated. Data from cat retinal detachment studies indicate that 14-d detachments followed by 30-d reattachment is

associated with rod and cone outer segment length similar to that observed after 5-d detachments *(97).* In contrast, preliminary data regarding cone survival indicate that cones may be more prone to apoptosis with detachment (vs rods), and that 44% of cones die during a 3-d detachment in cats *(100).* (It is not uncertain whether all cones in detached retina were identified in the latter study as a result of downregulation of cone-identifying molecules [e.g., calbindin D] after detachment.) Additional experiments in the cone-dominated ground squirrel confirm these impressions *(100).* Although published experimental data do not indicate clearly what the exact survival of cones is after 2-wk periods of retinal detachment, a reasonable estimate is that 40–60% survive in the otherwise healthy retina. In addition to duration of detachment, the height of detachment influences photoreceptor survival. Macular detachments that would arise from CNV excision are quite shallow (< 2–3-mm height), which also favors photoreceptor survival during a 2-wk RPE resurfacing period. Since clinical data indicate that relatively small numbers of preserved cones are needed to support visual acuity of 20/30 *(101),* it appears that enough photoreceptors could survive combined CNV excision and RPE resurfacing to support reading vision, provided that a properly functioning RPE monolayer can be re-established within 2 wk of surgery. Thus, we believe that stimulation of RPE resurfacing as an adjunct to CNV excision is feasible, despite its possible association with photoreceptor death during the resurfacing process.

Limitations and Advantages of Studying RPE Wound Healing Using Human Bruch's Membrane in Organ Culture

There is presently no animal model of AMD. In vivo and in vitro RPE wound-healing models exist, but the relevance to AMD patients undergoing CNV excision is unclear. In vivo RPE wound-healing models (e.g., rabbit, pig, monkey) are associated with complete resurfacing of the RPE defect *(70,71,77–79)* or involve administration of compounds that complicate further experimental study (e.g., mitomycin C). *(72–74,102).* In these models, resurfacing generally is complete in RPE debridements, damaging only the RPE basement membrane *(73,78)* although larger (~1 mm wide) wounds in chick explants show limited resurfacing on RPE basement membrane *(66).* Animal models showing substantial incomplete resurfacing use harsher debridements methods, most likely causing damage to Bruch's membrane and subjacent tissues *(73,77).* In some of these studies, incomplete wound healing is observed at the retinotomy site only *(74,77).* Although studies in animals are useful for determining toxicities of wound-healing treatments, they are not ideal for predicting responses in aged human patients. Most previously described in vitro RPE wound-healing models are associated with complete wound resurfacing, in contrast to the situation in AMD patients undergoing CNV excision *(52,53,63,66–69,76,93,94,103–105).*

We have used an in vitro model to study RPE wound healing. The situation encountered *in situ* after CNV excision was modeled by studying RPE proliferation and migration on aged human Bruch's membrane, from which native RPE and—depending on the debridement depth—native RPE basement membrane has been surgically removed *(84,106).* The system uses submacular Bruch's membrane because RPE resurfacing in patients with AMD must occur under the macula, the site of most CNV ingrowth. This in vitro system permits one to study alterations in the cells as well as alterations in the RPE-denuded surface. Limitations of this in vitro organ-culture system include: i) inability to study the effects the overlying retina may have on RPE cell migration, proliferation, and/or differentiation *(66,76);* ii) inability to study the effects of various substances added to stimulate RPE migration, proliferation, and/or differentiation on the retina or choriocapillaris; iii) inability to study the effect of the immune system on retina-RPE-choriocapillaris-cell survival; and iv) inability to study the effect of the inflammatory response on retina-RPE-

choriocapillaris-cell survival. Nonetheless, in vitro resurfacing studies using this system predict poor RPE resurfacing of iatrogenic RPE defects lacking RPE basement membrane *(84,106)*, which appears to be the case in AMD patients undergoing CNV excision *(15,16)*. Thus, the model may have clinical relevance.

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