Minireview

Retinal pigment epithelial cell proliferation

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Abstract

The human retinal pigment epithelium forms early in development and subsequently remains dormant, undergoing minimal proliferation throughout normal life. Retinal pigment epithelium proliferation, however, can be activated in disease states or by removing retinal pigment epithelial cells into culture. We review the conditions that control retinal pigment epithelial proliferation in culture, in animal models and in human disease and interpret retinal pigment epithelium proliferation in context of the recently discovered retinal pigment epithelium stem cell that is responsible for most *in vitro* retinal pigment epithelial proliferation. Retinal pigment epithelial proliferation-mediated wound repair that occurs in selected macular diseases is contrasted with retinal pigment epithelial proliferation-mediated fibroblastic scar formation that underlies proliferative vitreoretinopathy. We discuss the role of retinal pigment epithelial proliferation in age-related macular degeneration which is reparative in some cases and destructive in others. Macular retinal pigment epithelium wound repair and regression of choroidal neovascularization are more pronounced in younger than older patients. We discuss the possibility that the limited retinal pigment epithelial proliferation and latent wound repair in older age-related macular degeneration patients can be stimulated to promote disease regression in age-related macular degeneration.

Keywords: Retinal pigment epithelium, retinal pigment epithelial proliferation, wound repair, stem cell, age-related macular degeneration, age-related macular degeneration, choroidal neovascularization, choroidal neovascularization, proliferative vitreoretinopathy, proliferative vitreoretinopathy

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Introduction

The retinal pigment epithelium (RPE) is a cellular monolayer located under the neural retina.[1–4] Key functions of the RPE layer are to support overlying photoreceptor cells and regulate transport across the blood–retina barrier. In development, RPE cells undergo terminal differentiation early, starting at four to six weeks of gestation in humans.[5] The resulting early differentiated RPE cells normally remain dormant throughout life, dividing rarely or not at all. Quiescent adult RPE cells, however, can be activated to proliferate when placed into cell culture where a variety of growth factors promote their proliferation.[6,7] The *in vitro* proliferation and expansion of RPE cultures occur mainly in a subpopulation of RPE stem cells termed the retinal pigment epithelium stem cell (RPESC).[8] The self-renewing properties of RPESC allow expansion of cultures to produce large numbers of human cells that are useful for both the study of RPE cell behavior and as a source tissue for RPE cell replacement therapy.

The microenvironment in which stem cells reside, known as the niche, regulates their dormancy and manipulation of the niche is generally required to activate stem cell mitosis.[9] RPE cells reside in a highly structured niche composed of a basal Bruch's basement membrane, lateral cell–cell contact within the cuboidal RPE monolayer, and apical apposition to a layer of highly organized photoreceptor cell outer segments. Niche components such as contact with apposing tissues and exposure to soluble factors in the extracellular milieu combine with cell-intrinsic signals to control RPE cell proliferation. In our view, RPE cells are maintained dormant by their two-dimensional niche throughout life, with release from the niche at any time permissive for proliferation.

Ongoing proliferation increases the number of progeny produced by a stem cell but does not determine the type of progeny generated. Self-renewal, a defining feature of stem cells, reproduces the original stem cell type. An expanded population of stem cell progeny may remain undifferentiated in a stem cell state or differentiate into a variety of specific mature cellular phenotypes. To understand RPE cell proliferation which occurs mostly via RPESC, it is important to consider both the number and type of progeny produced. Under expansion culture conditions where

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RPESCs self-renew, the progeny produced are newly born RPESC. In contrast, differentiation culture conditions cause the RPESC to produce progeny of varied types. The RPESCs are epigenetically poised to produce RPE cells (unpublished observations), and mature RPE monolayers containing large numbers of RPE cell are readily produced. Other specific differentiation conditions, however, can drive RPESC to differentiate into mesenchymal phenotypes including bone, fat or cartilage that are not normally found in the RPE layer.[8]

In this mini-review, we survey RPE layer proliferation under a variety of conditions that disrupt the niche both *in vitro* and in disease states. We discuss the role that RPE cell proliferation may have in proliferative vitreoretinopathy (PVR) and age-related macular degeneration (AMD). PVR is a feared result of retinal detachment in which RPE cells are released to proliferate massively into fibrotic scar tissue. AMD is a common condition of older patients. The initial steps in AMD pathogenesis form extracellular aggregates of protein, lipid, and carbohydrate termed drusen deposited at the RPE- Bruch's membrane interface. Drusen formation is accompanied by progressive atrophy of the RPE layer and resulting dysfunction of the overlying photoreceptor cells. A gradual loss of central vision occurs in this non-exudative, or dry, form of AMD. About 10% of dry AMD patients progress to exudative, or wet, AMD where choroidal neovascular vessels (CNV) grow through the damaged Bruch's membrane into the RPE layer and neurosensory retina to cause more rapid loss of central vision.

Our perspective is that proliferation in the eye, like RPE cell proliferation in the culture dish, is mediated by activation of RPESCs that are poised to produce normal RPE cell progeny but may also, under certain conditions, produce abnormal progeny having mesenchymal or abnormal pathologic phenotypes. In this context, we review proliferation in RPE monolayer cultures, in animal models and in patients, and discuss the potential roles of RPE proliferation in retinal wound-repair and disease progression.

RPE proliferation *in vitro*

Proliferative cultures of RPE cells have been developed over the past several decades.[10,11] The early culture techniques achieved RPE proliferation but with limited control of differentiation upon expansion which resulted in epithelial to mesenchymal transition (EMT) to produce mixed cultures of RPE cells and fibrous mesenchymal progeny. The mesenchymal progeny was proliferative and overgrew the cultures resulting in fibroblastic rather than RPE phenotypes.[12–14] Since these early pioneering experiments, culture conditions have been defined as those that avoid EMT to allow multi-passage expansions to produce large quantities of RPE cell progeny in the absence of mesenchymal contamination. Under appropriate conditions, modern RPE cell cultures express surface markers, physiologic function, and a transcriptome closely resembling native RPE.[8,15–19] It is now possible to routinely produce stable, homogenous cultures containing large quantities of highly differentiated human RPE cells useful both to study RPE cell behavior and as source tissue for RPE cell transplantation replacement therapy.

In our recent work, we found that the human RPE layer contains a subpopulation of RPE stem cells which after removal from the niche into cell culture media containing growth factors such as FGF2, activate into a self-renewing stem cell state, the RPESC, that can be expanded extensively.[8] The RPESC self-renews, defining it as a stem cell, and routinely produces homogenous cultures of more than 5×10^8 RPE cell progeny. RPESC progeny readily differentiate into RPE cells after removal of the growth factor (FGF2) that promotes self-renewal. In addition, when expanded, RPESC cultures are exposed to culture conditions developed to differentiate pluripotent stem cells such as embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) into mesenchymal fates, corresponding mesenchymal progenies are produced by the RPESC. Endodermal differentiation conditions designed to differentiate pluripotent ESC and iPSC along endodermal lineages, however, do not drive RPESC to produce endodermal progeny. Thus, RPESC are multi-potent rather than pluripotent, and are poised to differentiate into RPE cells and also able to differentiate along mesenchymal lineages, as illustrated in Figure 1.

Molecules that signal RPE cell proliferation include extrinsic growth factor levels, cell–cell [20] and cell–substrate contact-mediated interactions, and cell intrinsic signals.

Soluble growth factor control of RPESC proliferation signal transduction involves mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways that are common to a variety of stem cell types.[21] In this signal transduction pathway, growth factor receptor activates ras GTPase which leads to MAPK/ERK phosphorylation. Phosphorylated MAPK/ERK signals nuclear cell cycle transcripts and transcription factor expression in RPE[22] that include myc, Pax6, klf4, and MITF.[23] Specific mitogens to stimulate human RPESC proliferation include FGF, EGF, HGF, and PDGF[7] and perhaps additional growth factors, and factor combinations, yet to be identified.

In addition to soluble growth factors, RPE cell proliferation is influenced by cell density dependent mechanisms, including cell–cell and cell–substrate contact that can effect proliferation via regulation of soluble factor receptors or by direct soluble factor-independent intracellular signaling. Tight junction proteins such as claudins and occludins and gap junctional connexins are known to have a role in RPE cell–cell contact inhibition.[12,20,24] The substrate on which RPE cells are grown is another determinant of proliferation where integrins[25] and other cell surface adhesion molecules mediate RPE cell–substrate adhesion and intracellular signaling. Understanding RPE cell–cell and cell–substrate adhesion is an area of active research. Proliferating RPE maintain phenotype, cobblestone morphology, and E-cadherin expression when grown on amniotic membranes.[26] Several degradable and non-degradable synthetic substrates such as PLGA, collagen, PCL and others have been developed[27] to grow RPE cells both for study and for use as a RPE cell carrier scaffold for transplantation.

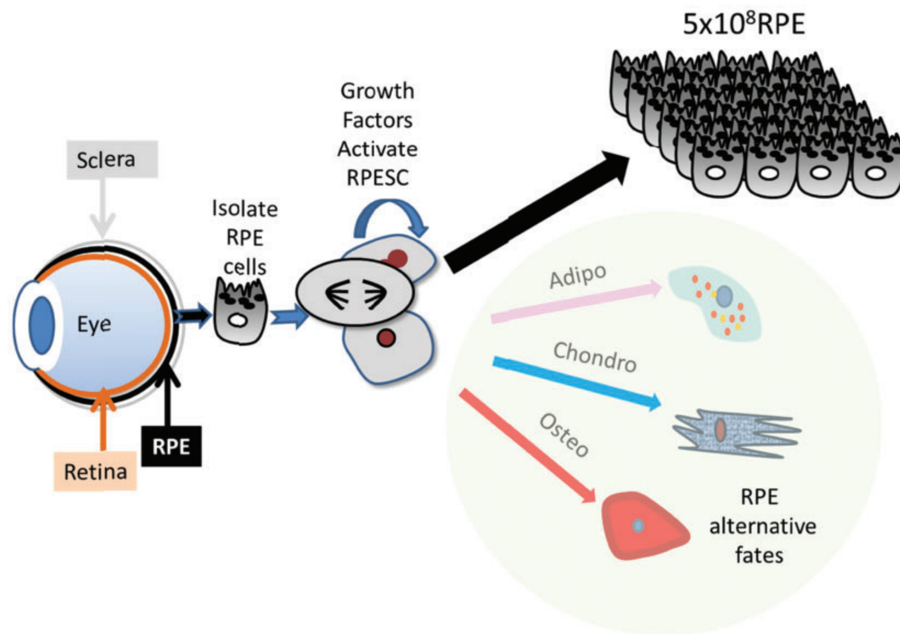

Figure 1 **RPESC.** Human RPE cells isolated from the eye are induced to proliferate by culture in the presence of growth factors. Growth factors activate adult RPE cells into a stem cell state, the RPESC, that self-renews and expands extensively. Differentiation conditions based on those developed for pluripotent stem cells cause the expanded RPESC cultures to differentiate into RPE or mesenchymal progeny; figure modified from Salero et al.[8]

Cobblestone RPE cells can be grown on native Bruch's basement membranes that have been removed from donor eyes.[24] Proliferation of cobblestone monolayers is reduced, however, for RPE grown on older Bruch's membranes from donors with AMD.[28] Manipulation of the RPE cell-Bruch's membrane interaction by chemical resurfacing of older membranes with fibronectin, collagen, and vitronectin promotes RPE attachment, migration, and growth.[29–31] RPE cells will themselves partially resurface damaged Bruch's membrane[32] and a promising recent finding is that media conditioned by bovine corneal endothelium contains a factor highly effective at promoting resurfacing of aged Bruch's membrane for RPE cell attachment and growth.[33] The *in vitro* use of Bruch's membrane is a useful model to understand the role of RPE–substrate contact in regulating RPE cell proliferation, influencing AMD pathogenesis and controlling the stability of transplanted RPE progeny.[34]

RPE cell culture has produced useful models of wound healing by the RPE layer. When a defect is created by debridement in a confluent RPE cell monolayer, proliferation and migration of the surrounding RPE cells are stimulated to repair the defect.[20,24,35] Wound repair in RPE monolayers occurs over the few days following local debridement. Wound repair initiates with migration of neighboring RPE cells into the denuded area where they proliferate to reform the monolayer. In some cases of *in vitro* wound repair, however, RPE cells undergo EMT resulting in over-proliferation of fibroblastic mesenchymal progeny resembling that found in proliferative vitreoretinopathy (PVR).[12]

Cellular stress can alter the type of progeny produced by monolayer RPE cultures to model fibro-proliferative diseases such as PVR and also degenerative diseases such as dry AMD. Oxidative stress can drive RPESC cultures to differentiate into pathologic progenyy that overproduce the drusen proteins[36] found in dry AMD patients.[37] It is fascinating to consider the possibility that pathologic RPESC lineages lead to mis-differentiation of RPESC that have been inappropriately released from their niche. Such pathologic RPESC progeny may contribute to dry AMD pathogenesis through overexpression of drusen proteins.[36,37]

Understanding the role of soluble factors, cell–cell and cell–substrate contact in regulating the balance between regenerating RPE progeny and EMT is an area of intensive research.[12,13,19] The progeny produced when RPESCs undergo EMT closely resemble the cells found in surgically excised PVR membranes which are the major cause of retinal detachment repair surgery failure. In addition to regulation of the ERK/MAPK signal transduction pathway and cell-adhesion, cytokines can drive EMT. Cytokines known to promote EMT in RPE include TGF-beta[12] and TNF-alpha[38] and we have recently developed an *in vitro* model of RPESC EMT using a combination of TNF and TGF (Blenkinsop, personal communication).

Human RPESC cultures provide valuable disease models for both PVR and AMD. Culture conditions that promote EMT provide a PVR model useful to screen for potential therapeutic drug candidates.[19] Similarly, culture conditions that promote drusen protein overproduction by RPESC are useful to screen for drug candidates to treat dry AMD.[36] Fortunately, conditions that maintain the normal RPE cell phenotype are available to produce large numbers of RPE cells, useful for study and for replacement therapy treating atrophic diseases of the RPE layer such as AMD.[17–19,39–42]

RPE cell proliferation *in vivo*

In normal development, the RPE divides and then undergoes terminal differentiation very early in embryogenesis. Subsequently, the post-mitotic RPE are dormant but can be activated to proliferate under a variety of circumstances. *In vivo* RPE proliferation has been reported to be activated after RPE atrophy. A simple animal model for RPE atrophy is mechanical debridement of the RPE layer. Debridement results in an immediate loss of RPE cells followed by rapid repopulation of the RPE layer within weeks. This *in situ* wound-repair involves migration and proliferation of neighboring RPE cells to fill in the defect[43,44] in a process that mirrors *in vitro* self-repair of the RPE monolayer.

Although human RPE has a limited proliferative response to injury, it has been long known that some lower animals such as amphibians have extraordinary regenerative capacity. In several animals, RPE layer damage elicits a proliferative response that completely repairs the RPE layer and, in some cases, even has the capacity to regenerate the neurosensory retina.[45–47] In humans, by contrast, robust proliferation of the RPE is uncommon and when it does occur is generally accompanied by pathologic EMT, resulting in fibroblastic mesenchymal progeny such as those found in surgically excised human PVR[12,48] rather than regeneration of the RPE layer or neurosensory retina.

The lack of regenerative capacity in mammalian central nervous system tissues such as the RPE is an important reason to develop stem cell-based replacement therapies for atrophic diseases like dry AMD. Several clinical studies to replace RPE cells are underway or planned.[42,49–53] One early report suggests proof of principal evidence that increasing RPE cell number by transplantation can effectively treat atrophic diseases of the RPE including AMD.[54] The hope that RPE cell transplantation will benefit the great unmet medical need posed by dry AMD has significantly accelerated progress in the field of RPE cell biology.

In dry AMD patients, unfortunately, RPE proliferation is limited and a gradual overall loss of RPE cells is the dominant feature. Interspersed areas of pigment hypotrophy and hypertrophy common in all forms of AMD suggest limited RPE proliferation and remodeling does occur[55] but that these foci of proliferation are not sufficient to counteract the ongoing, overall loss of RPE cells.

Pathologic RPE layer pigment hypotrophy intermixed with areas of pigment hypertrophy is an early clinical finding in several retinal diseases including AMD. Our perspective is that the balance between RPE cell loss and proliferation favors RPE atrophy in dry AMD, and that ongoing loss of RPE cells leads to reduced metabolic support of photoreceptor cell function with a corresponding loss of central vision. Although the ophthalmoscopically visible areas of focal RPE hypertrophy commonly observed as dry AMD progresses suggest that foci of limited RPE proliferation occur, little is known about the extent of RPE proliferation in dry AMD. Limited foci of RPE layer hyperplasia also occur in other outer retinal diseases, such as retinitis pigmentosa (RP) where direct evidence of RPE proliferation is available. The characteristic pigment spicules in RP arise from chains and aggregates of RPE cells that traverse the retina.[56] Histological studies indicate that the sporadic pigment occurring in RP is due to cells containing pigment detached from Bruch's membrane.[57] RPE proliferation has also been documented in another retinal condition, RPE rips, which involve rapid loss of the RPE layer. After a RPE rip occurs, RPE proliferation is activated that can repair the initial RPE layer defect.[58]

In contrast to atrophic retinal diseases such as dry AMD which have limited RPE proliferation, more robust RPE proliferation occurs in proliferative retinal disease. For example, after retinal detachment, massive proliferation of the RPE can occur with associated EMT and growth of epiretinal membranes.[59,60] RPE proliferation in human patients has also been reported to occur over choroidal tumors,[61] and in rare metaplastic or dysplastic tumors of the RPE.[62]

A very common retinal disease with vascular proliferation is the neovascular form of AMD. Wet AMD occurs in about 10% of dry AMD patients in whom RPE layer atrophy is followed by defects in Bruch's membrane through which choroidal neovascularization (CNV) grows to invade the RPE layer and retina. Growth of CNV in wet AMD is characterized by rapid initial expansion followed by late involution over a period of months.[63,64] In advanced cases, uncontrolled growth of the CNV may progress to form a large fibrovascular disciform scar.[65,66] Much of the proliferative tissue in the disciform lesion is of vascular origin, mixed with significant tissue arising from activation of RPE proliferation that results in clusters of RPE cells as well as mesenchymal progeny.[66]

CNV has been modeled in animals using a laser to create focal defects in Bruch's membrane through which CNV grows.[67–69] In these models, robust RPE proliferation occurs around CNV accompanied by CNV regression. RPE proliferation generally progresses over a few weeks coincident with CNV regression and repair of the initial Bruch's membrane defect.[70] Although RPE proliferation associated with CNV regression and Bruch's membrane repair is common in mammalian models of CNV, such repair, unfortunately, is rare in untreated exudative AMD patients.[65]

Although RPE wound healing is limited in the older human wet AMD patient population, histology indicates significant RPE cell proliferation over the surface of CNV.[71,72] The proliferative RPE response to CNV, however, rarely results in CNV regression in AMD patients. This is in contrast to several non-AMD diseases that affect younger patients who develop a defect in Bruch's membrane that weakens the barrier between the choroid and retina. CNV in younger patients is positively associated with a proliferative response of the RPE and accompanying CNV regression. Examples include spontaneous CNV,[73] myopic CNV,[74] POHS-associated CNV,[75] or viral infection-associated CNV.[76] In these young patients with CNV, pigment hypertrophy surrounds the CNV which then regresses. We recently reported a similar phenomenon in older wet AMD patients treated with anti-VEGF drugs that slow CNV growth. In a small subset of AMD patients, a ring of pigment hypertrophy forms at the level of the RPE that is associated with CNV regression.[77] Two clinical cases of such

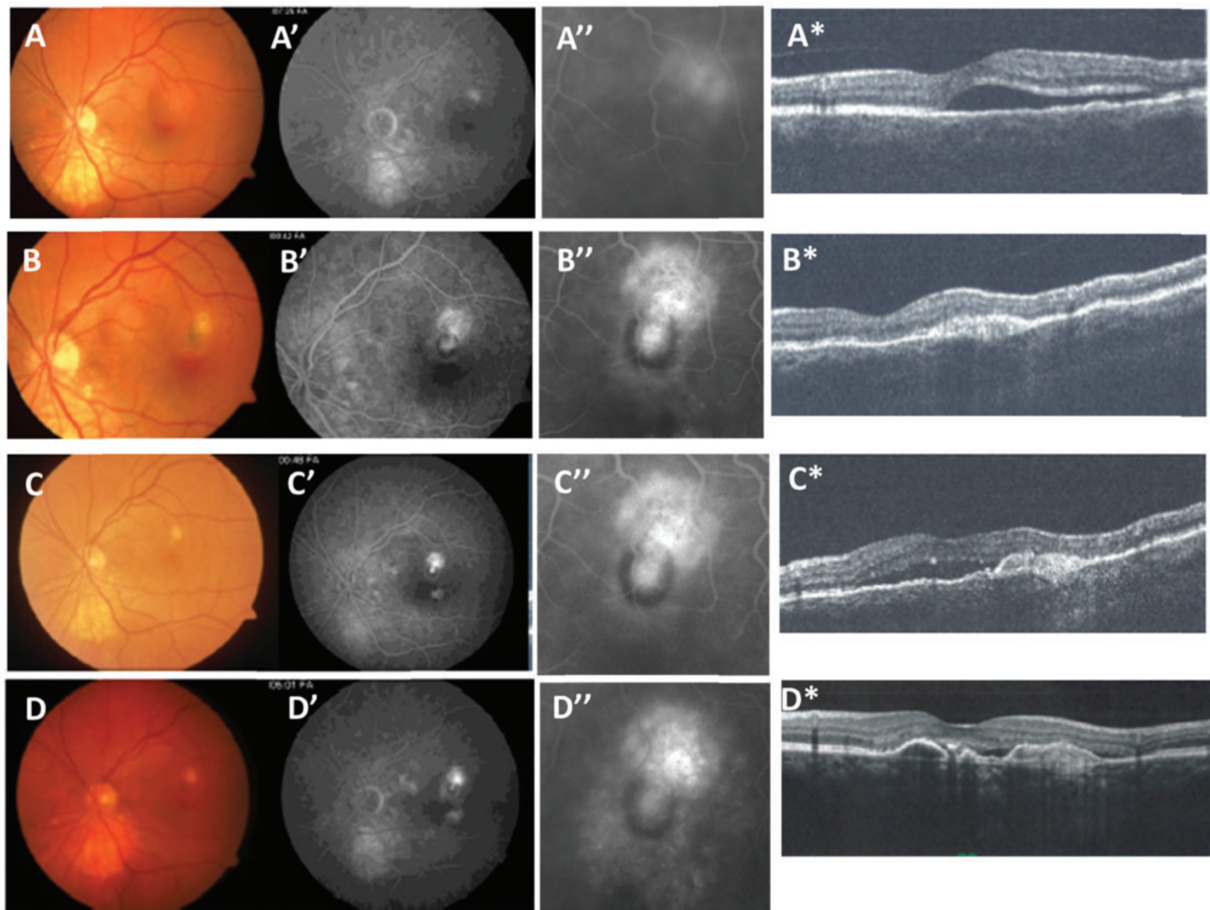

Figure 2 CNV blocked only in the direction of RPE pigment hypertrophy. Fundus photograph (a–d), fluorescein angiogram (FA) mid-phase (a'–c'), magnified FA (a″–d″), and OCT (a*–d*) images from a 57-year-old female patient presenting with worsening visual acuity to 20/30 and metamorphopsia. (a, a*): Images taken prior to treatment showing foveal fluorescein leakage with serous detachment due to subfoveal CNV. The patient was treated with thermal laser to ablate the CNV lesion. (b, b*): After six months, CNV recurred in the supero-temporal aspect of the laser scar. The recurrence was treated with combined therapy of two PDT and four bevacizumab injections applied over a six-month period. (c, c*): After remaining stable for nine months without treatment, the CNV again recurred in the direction of the thermal laser scar but not in the direction of the partial pigment ring. This recurrence was treated with serial anti-VEGF antibody injections and then remained stable after 42 months with anti-VEGF therapy as shown in (d, d*). (Data from Stern et al.[77])

RPE level pigment hypertrophy associated with CNV regression in AMD patients are described in the following section.

RPE hypertrophy in treated exudative AMD patients

Our recently published case series reports that a ring of pigment hypertrophy around CNV can be associated with CNV regression in AMD patients who have received treatment to slow CNV growth.[77] The observed pigment hypertrophy in wet AMD patients may have arisen due to the recent introduction of treatments that slow CNV growth to alter the balance between RPE proliferation and CNV progression thereby unmasking an underlying RPE wound healing response in exudative AMD.

In our retrospective clinical study, 33 of 955 exudative AMD patients developed ophthalmoscopically visible pigment hypertrophy at the level of the RPE associated with a CNV lesion and six patients developed a ring of pigment surrounding the CNV. The following data from two AMD patients with a pigment ring illustrate the association of RPE hypertrophy with slowed CNV progression. Figure 2(a) and (a*) shows a patient presenting with perifoveal CNV. The CNV was initially ablated using thermal laser but recurred six months later. The CNV recurrence was treated with a combination of two photodynamic laser treatments and four anti-VEGF (bevacizumab) injections over a six-month period during which pigment developed to surround approximately 270° of the CNV lesion (Figure 2(b) and (b*)). Note that the fluorescein dye leakage was contained where pigment surrounded the CNV but was not contained in the area lacking the increased pigment. This patient remained stable for nine months without treatment. The CNV again recurred toward the laser scar but not in the direction of the pigment ring (Figure 2(c) and (c*)), suggesting that the pigment retarded CNV growth. This second CNV recurrence was treated with anti-VEGF injections every four to six weeks for a nine-month period during which leakage remained blocked in the direction of

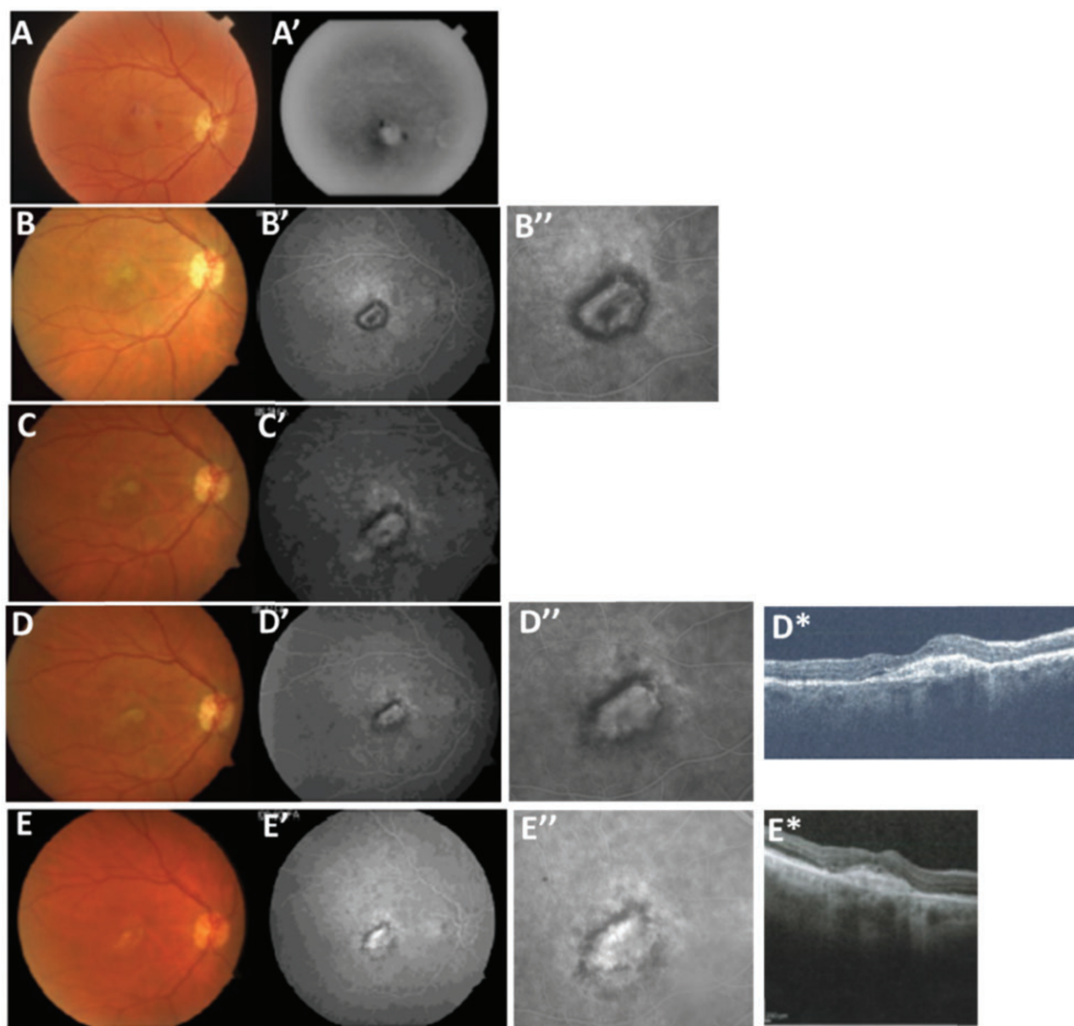

Figure 3 Time course for recurrent pigment capping. 3(a, a'): A 66-year-old male patient presented with decreasing vision. Fundus photography and angiography indicating perifoveal CNV with a small amount of hemorrhage. (b, b″): After four anti-VEGF treatments over a five-month period, a complete ring of pigment formed, surrounding the CNV. Note reduced fluorescein leakage. (c, c'): The treatment interval was extended to 20 months after which symptoms and a small infero-temproal area of fluorescein leakage recurred (d, d*). Anti-VEGF treatments were re-initiated and after five treatments over a nine-month period, a pigment ring re-formed around the CNV with elimination of the infero-temporal leakage. The treatment interval was again extended and (e) and (e*) show a stable CNV lesion with a complete pigment ring after a 16-month interval without treatment. (Data from Stern et al.[77])

the pigment ring and continued in the direction of the laser scar (Figure 2(d) and (d*)). After 42 months of anti-VEGF therapy, leakage has remained contained by the pigment ring and active toward the laser scar, with acuity at 20/40. These images show that fluorescein leakage indicative of CNV is blocked only in the direction of the increased pigment.

A time course for development of hyperpigmentation after anti-VEGF therapy is illustrated in Figure 3. Minimal pigment surrounded the CNV lesion at the time of presentation (Figure 3(a) and (a″)). A subretinal pigment ring subsequently formed during 20 weeks of anti-VEGF therapy (Figure 3(b)), after which the patient remained stable for a 20-month period without treatment. A recurrence of CNV then arose, with fluorescein leakage at the infero-temporal edge of the ring (Figure 3(c)), note reduced leakage in the direction of the pigment ring compared to the area of CNV breakthrough. After nine months of anti-VEGF therapy, the pigment ring reformed and leakage resolved (Figure 3(d)). The treatment interval was again extended with stable vision, fluorescein leakage and OCT observed 16 months after the last treatment (Figure 3(e)).

Conclusion

Wound repair of human central nervous system tissue such as the RPE and neural retina is limited. Nevertheless, RPE wound repair has been described after rips of the RPE, in animal models of AMD where RPE proliferation seals laser-induced CNV lesions, *in vitro* where RPE proliferates to repair areas of RPE loss, in younger patients with CNV and, recently, in older AMD patients treated to slow CNV lesion growth. Our understanding is that the RPE, normally dormant throughout life, can be activated to proliferate upon release from the niche that normally maintains a quiescent state. RPE proliferation can be destructive, as occurs

when RPE cells escape the niche to cause massive proliferation and EMT leading to PVR or disciform scar formation. Alternatively, the proliferative response of the RPE can be reparative in dry AMD to partially counter RPE cell atrophy, in animal models where a proliferative response in the RPE layer results in CNV regression, in younger patients with CNV and less commonly in older wet AMD patients treated to slow CNV growth. Our research focus is to explore strategies to enhance RPE proliferation-mediated wound repair for retina patient benefit.

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