Molecular Vision 2016; 22:189-202 <http://www.molvis.org/molvis/v22/189>
Received 10 December 2015 | Accepted 25 February 2016 | Published 27 February 2016

© 2016 Molecular Vision

Regulation of signaling events involved in the pathophysiology of neovascular AMD

Haibo Wang, M. Elizabeth Hartnett

The John A. Moran Eye Center, University of Utah, Salt Lake City, UT

Neovascular age-related macular degeneration (AMD) is a complex disease in which an individual's genetic predisposition is affected by aging and environmental stresses, which trigger signaling pathways involving inflammation, oxidation, and/or angiogenesis in the RPE cells and choroidal endothelial cells (CECs), to lead to vision loss from choroidal neovascularization. Antiangiogenic therapies have greatly improved clinical outcomes in the last decade; however, vision improves in less than half of patients treated for neovascular AMD, and treatments remain inadequate for atrophic AMD. Many studies focus on genetic predisposition or the association of outcomes in trials of human neovascular AMD but are unable to evaluate the effects between different cell types involved in AMD and the signaling events that take place to cause pathologic biologic events. This manuscript complements other reviews in that it describes what is known generally in human AMD studies and clinical trials testing methods to inhibit vascular endothelial growth factor (VEGF inhibitors) and presents pathologic signaling events that develop in two important cell types, the RPE cells and the CECs, when stimulated by stresses or placed into conditions similar to what is currently understood to occur in neovascular AMD. This manuscript complements other reviews by discussing signaling events that are activated by cell–cell or cell–matrix interactions. These considerations are particularly important when considering growth factors, such as VEGF, which are important in physiologic and pathologic processes, or GTPases that are present but active only if GTP bound. In either case, it is essential to understand the role of signaling activation to distinguish what is pathologic from what is physiologic. Particularly important is the essential role of activated Rac1 in CEC transmigration of the RPE monolayer, an important step in blindness associated with neovascular AMD. Other concepts discussed include the importance of feed-forward loops that overwhelm mechanisms that seek to restore homeostasis in cells and the importance of regulating, instead of abolishing, signaling events in a chronic, complex disease, such as neovascular AMD. These concepts are important as we move to the next stages in developing treatments for neovascular AMD. A novel therapeutic strategy that will be discussed is activating an isoform of the GTPase, Rap1, which can regulate downstream signaling and a pathologic feed-forward loop leading to Rac1 activation and migration of CECs.

Concepts and importance of causal analysis with limitations of models of human disease as related to AMD: Age-related macular degeneration (AMD) is a leading cause of blindness in the elderly throughout the world [1]. AMD has historically been characterized as "dry" or "wet" AMD. Dry AMD makes up approximately 90% of all forms of AMD identified and includes clinical/pathologic features of pigmentary changes, basal laminar and linear deposits, drusen, and the advanced form of dry AMD, geographic atrophy [2]. Wet AMD is the less common form but accounts for most of the cases of legal blindness that occur from choroidal neovascularization (CNV), sometimes polypoidal choroidal vasculopathy [3] (although there is controversy that this may represent a different pathophysiology) [4], retinal angiomatous proliferation [5], or retinal pigment epithelial detachment [6]. The current nomenclature of AMD has evolved somewhat. Now, the appearance of drusen and pigmentary changes without neovascularization or geographic atrophy is sometimes referred to as "early" AMD, and wet, or neovascular AMD, and geographic atrophy fit into the category of "advanced" AMD [7]. This change in nomenclature occurred partly because early AMD can lead to either advanced form (geographic atrophy or neovascular AMD), and both advanced forms can coexist in the same eye [8,9].

Early AMD often is asymptomatic and includes the clinically identified features of drusen and pigmentary changes. From histopathologic and proteomic analyses, there is increased deposition of many compounds (for example, enzymes (TIMP3 and SerpinA3), vitronectin [10], bisretinoid fluorophores, [11] complement, [12, 13] oxidized proteins [14], lipoprotein derived debris [15], cholesterol esters, 7-ketocholesterol [16], advanced glycation end products [17], and linoleic hydroperoxide [18]) beneath the retinal pigment epithelial (RPE) cells and within Bruch's membrane. Recognition of drusen and RPE defects, believed to represent stressed and/or dying RPE cells, can be accomplished with slit-lamp biomicroscopy on clinical examination, but the

Correspondence to: M. Elizabeth Hartnett, 65 N. Mario Capecchi Drive, Salt Lake City, UT, 84132; Phone: (801) 213-4044; FAX: (801) 581-3357; email: ME.Hartnett@hsc.utah.edu

thickened extracellular matrix that makes up basal linear or laminar deposits is difficult to detect even on retinal imaging studies, such as optical coherence tomography (OCT), infrared imaging, and fluorescein angiography (FA) [19].

Advanced forms of AMD include atrophic AMD and neovascular AMD. Both forms are often symptomatic with loss of contrast sensitivity, the presence of scotomata or blind spots, and distortion, for example. In the advanced dry form of AMD, i.e., atrophic AMD, or geography atrophy, there is atrophy of the RPE cells and choriocapillaris with later photoreceptor loss [20,21]. In neovascular AMD, endothelial cells from the choriocapillaris of the choroid migrate to and across the RPE monolayer and into the sensory retina. The choroidal endothelial cells (CECs) proliferate and develop into CNV at any location between the choriocapillaris and neural retina. Neovascular AMD is associated with the loss of choriocapillaris that is hypothesized to create a hypoxic stimulus for the overlying RPE cells [21] and initiate the development of later CNV. CNV that may remain beneath the RPE monolayer but does not invade the photoreceptors and neural retina is known as occult or type 1 CNV, whereas CNV that proliferate within the sensory retina is termed classic or type 2 CNV [22,23]. Other forms of neovascular AMD include fluid beneath RPE cells, called RPE detachments, or vascular lesions arising from the retinal vasculature in the deep retina, currently known as retinal angiomatous proliferation (RAP) [19,24], or type 3 neovascularization. Historically, CNV has been classified by its location and associated fluid determined with OCT and leakage on FA. With the advent of OCT angiography (OCTA), characterization of CNV based on location and flow may be possible [25] and provide information about how active or aggressive a CNV lesion is.

The most important risk factor of AMD is advanced age [26], and some studies suggest that the longer humans live, the greater the likelihood of developing advanced AMD. Increasingly, genetic variants have been found in association with increased risk of AMD, suggesting that these variants may increase the predisposition to advanced AMD [27]. Several genetic variants have been identified, including on chromosome 10, 10q26, *ARMS2* (Gene ID: 387715; OMIM number: 611313)*/HTRA1* (Gene ID: 5654; OMIM number: 602194), and those affecting factors in the complement system. The initial reports of the Y402H variant of complement factor H [13,28-30] led to several hypotheses surrounding the complement system in AMD, but recent reports suggest more complex and previously unsuspected mechanisms involving lipoprotein turnover in Bruch's membrane and will be discussed in the following section [28]. Additional studies reported rare variant alleles with high impact [29]. In addition to the role genetics plays in AMD risk, environmental and/or external stresses further increase the risk of AMD in individual patients [30]. Despite greater understanding of gene and environment associations with increased risk of AMD, the pathophysiologic mechanistic links are still incompletely understood in AMD.

Environmental and external stresses most known to affect the risk and progression of AMD include smoking, obesity, and reduced dietary antioxidants [27]. Since causal analyses are difficult in human studies, experimental models often use mice modified to express or knock out genetic variants, and that are exposed to external stresses known to increase AMD risk, such as smoking [31,32] or fat-feeding [28,33], to seek causal roles. Studies are fraught with the lack of cigarette a robust animal model of AMD [33]. Rodents lack maculae, and drusen or changes in the RPE monolayer and extracellular matrix rarely occur in mice until after 1 or 2 years of age. Even though rodents do not have maculae, these animals are useful in various models to address specific hypotheses. The laser-induced CNV model in mice is a robust model of injury and inflammatory-induced angiogenesis and has similarities to human neovascular AMD [34]. Growth factors, particularly vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), are involved, as well as inflammatory and oxidative compounds [35,36]. The laser-induced CNV model also involves wound-healing, which is a recognized process involved in human neovascular AMD [37]. The model has been criticized for being acute and short-term, because AMD is a chronic condition, but the short-term nature of the model provides the ability to address focused hypotheses through the use of genetic models and external stresses. Human studies are essential but provide limited mechanistic insight into complex events in AMD. Even exploratory studies of genetic variants, mRNA sequencing, and proteomic associations may not provide causal information of biologic events in AMD, because they do not distinguish whether the gene, mRNA, or protein is expressed and activated to affect a signaling pathway or what cells are involved in the pathophysiology. Therefore, it is important to integrate data from mechanistic studies, often using animal models and cell culture, with knowledge gained from human tissue and experiments involving human cells, and from genetic, transcriptional and proteomic studies to piece together hypotheses that related causal events in AMD.

Therefore, to study the mechanisms that surround AMD pathophysiology (Figure 1), it is helpful to interpret data from several different types of experimental designs, including analysis of human clinical and pathologic specimens

[10,12,20,38,39], imaging and genetic studies to develop hypotheses related to biologic events in AMD followed by a combination of culture techniques, including relevant coculture models, and genetically modified mice exposed to external stresses to model outcomes representative of forms of AMD. As information regarding outcomes from interventions in clinical trials accrues, it is also helpful to use this information to refine hypotheses to explore, for example, why differences between treatment regimens and various antiangiogenic agents are seen. When interpreting human studies, it is important to take into account that clinical trials require a specific hypothesis and that recruitment criteria may be too narrow to address the hypothesis; thus, the outcomes are not generalizable to all groups of patients with a complex disease, such as AMD.

Myriad processes involved in AMD: In addition to the roles of genetics and complement, abnormal cell events have been experimentally studied in relationship to AMD and include endoplasmic reticulum stress and the unfolded protein response [40], dysregulated autophagy [41], and effects through the mammalian target of rapamycin (mTOR)/Akt pathways [42], effects from oxidized compounds including oxidized lipids [43] and other forms of oxidative stress [44,45] on inflammatory cells and angiogenesis [46]. Inflammatory processes involve macrophages [47] and microglia [46] with release of inflammatory cytokines, including tumor necrosis factor alpha (TNF-α) [48], that trigger signaling in other cell types. Many ligand and receptor pairs and signaling compounds have also been implicated in neovascular AMD, including hepatocyte growth factor (HGF)/c-Met receptor, Wnt/LRP6 [49,50], platelet-derived growth factor (PDGF)/PDGFR [35,51], transforming growth factor β (TGF-β)/TGF-β receptor [37,52], fibroblast growth factors (FGFs), TNF-α/TNFR, and eotaxin/CCR3 [53], as examples [54].

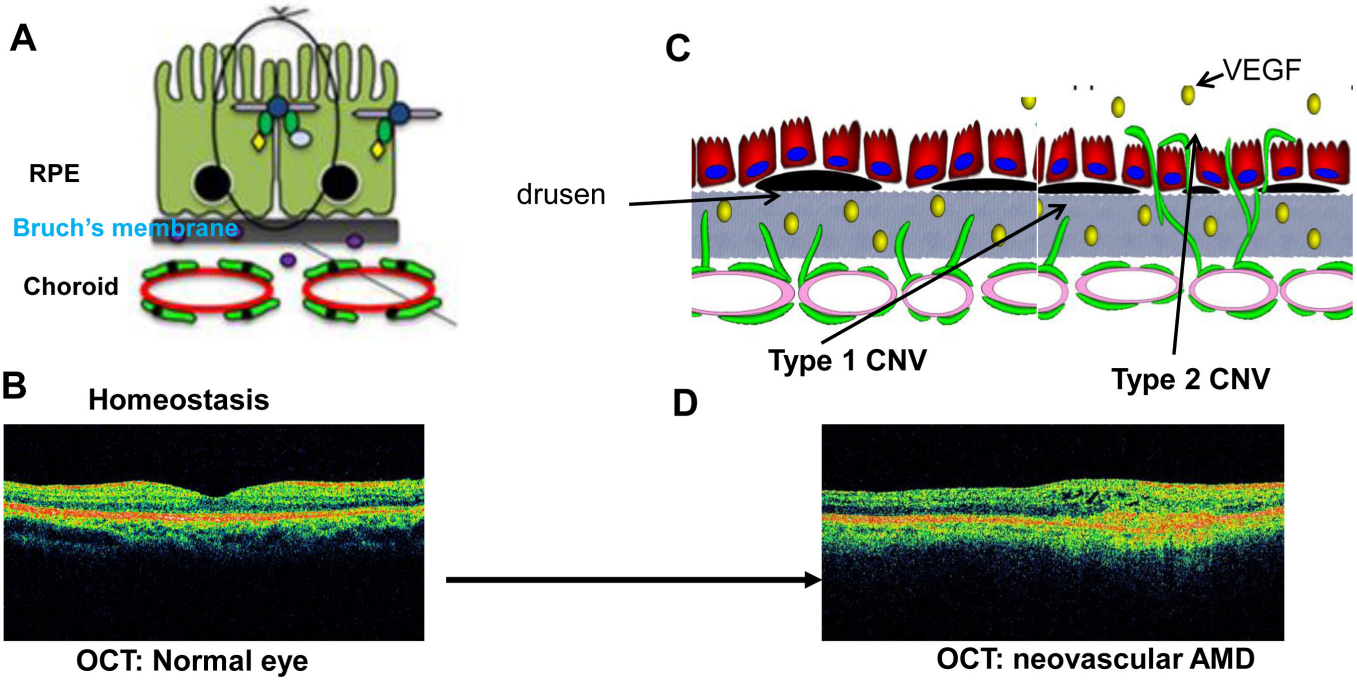

Figure 1. Pathologic events in CNV. **A**: In healthy cells, RPE cells have strong junctional complexes and form a monolayer on Bruch's membrane separate from the choroidal endothelial cells (CECs). **B**: Optical coherence tomography (OCT) of a human eye without age-related macular degeneration (AMD). Note the normal architecture of retinal layers, lack of drusen, and good foveal contour. **C**: With increasing age and related to genetic predisposition, diet, smoking, oxidative stress, and inflammation, Bruch's membrane and the RPE extracellular matrix change in appearance with the formation of drusen and in composition, with deposition of oxidized lipoprotein, debris, complement, and many other factors that incite inflammatory, oxidative, and angiogenic signaling. Microglia are activated and release cytokines (e.g., tumor necrosis factor alpha, TNF-α) that stimulate RPE cells to express vascular endothelial growth factor (VEGF). Concurrently, CECs activated to migrate toward the RPE monolayer and proliferate into type 1 choroidal neovascularization (CNV). RPE cell–CEC contact initiates events that lead to RPE barrier compromise, which permits cells and growth factors to move from basal to apical aspects. CECs are attracted to migrate across the RPE monolayer and into the sensory retina toward a VEGF gradient to proliferate into type 2 CNV. **D**: OCT of a human eye with neovascular AMD, showing loss of the architecture of the retina and the RPE monolayer, loss of the foveal depression, and cysts within the inner retinal layers.

The Wnt signaling pathway has been implicated in neovascular AMD and in models of laser-induced CNV [49,50]. Platelet-derived growth factor modulates pericytes and stabilizes developing blood vessels [35]. PDGF antagonists are currently being studied with anti-VEGF agents in neovascular AMD [51]. The TGF-β signaling pathway is involved in wound healing, which is implicated in neovascular AMD [37]. Inhibition of TGF-β has been found to maintain RPE cells in an epithelial phenotype and reduce the transition from epithelial to mesenchymal types of cells [52]. Thrombospondin-1 is a potent antiangiogenic and anti-inflammatory molecule that has been found to play an important role in CEC phenotype. *TSP1*$^{-/-}$ mice were susceptible to CNV following laser, in part through recruitment of macrophages. Choroidal endothelial cells from *TSP1*$^{-/-}$ mice had reduced capillary morphogenesis and greater generation of nitric oxide, suggesting a role of thrombospondin-1 in angiogenesis, inflammation, and oxidation [55].

Although not the focus of this review, the formation of the inflammasome has been implicated in atrophic AMD through many pathways [56,57], including related to increased expression of VEGF [58].

In many of these studies, the effects of ligand and receptor pairs have been based on studies in solo cultures, animal models, and increased expression of the factor or receptor in human specimens. In this review, we focus on the effect of cell–cell interactions on downstream signaling mechanisms, particularly related to VEGF, because anti-VEGF agents are the standard of care for CNV in human AMD. We also focus on two GTPases, Rac1 and Rap1, which can be involved in signaling cascades related to many of the factors described. GTPases are activated by guanine nucleotide exchange factors to be GTP-bound and are inactivated by GTPase activating proteins to be GDP-bound. In this way, GTPases act as biologic switches and represent a potential mechanism to regulate pathologic processes in complex, chronic diseases, like AMD.

Types 1 and 2 CNV will be discussed in neovascular AMD. With the availability of agents that interfere with the bioactivity of VEGF, improvement in visual acuity is now possible for this once blinding disease. However, anti-VEGF treatments are effective in only about 40% of patients, and there is a concern that broad inhibition of pathologic and beneficial effects of VEGF may reduce the health of the neuronal and glial retina [59,60]. In addition, VEGF is essential to the health of the choriocapillaris, photoreceptors, and RPE cells [61-63]. Several experimental studies in which VEGF released from RPE cells was reduced either by poisoning the RPE monolayer or by specific inhibition showed loss of the choriocapillaris [64,65]. Loss of RPE cells has been associated with geographic atrophy [20,21]. Therefore, recent studies have evaluated whether anti-VEGF agents affect the progression of geographic atrophy, in which RPE cells and choriocapillaris become atrophic.

Need to regulate but not abolish angiogenesis in human neovascular AMD: Unlike in cancer, chronic diseases may be managed best by regulating, instead of inhibiting, angiogenic signaling or biologic events. In neovascular AMD, strong inhibition of angiogenesis may affect physiologic choroidal vasculature. VEGF has beneficial effects on the choriocapillaris and on the neural retina, including in adults [61-63]. In addition, the course of the disease may vary in the individual patient, and there is no way to know how much neutralization of VEGF is ideal at a specific time for a given diseased eye. Therefore, it is unknown whether the dose needed changes over the course of the disease in the individual patient. Currently, we rely first on information from clinical trials that determines mean changes in parameters from many patients for guidance about patient management and ocular and systemic safety. Imaging of the individual patient's macula is used to assess intraretinal and subretinal fluid and determine how frequently to treat a patient. There are now anti-VEGF agents with different potencies (e.g., ranibizumab and bevacizumab that bind VEGF compared to more potent aflibercept that can bind VEGF and another family member, placental growth factor). The clinician, therefore, can choose another agent if efficacy is not found following one treatment regimen. Other agents can be used in combination, including various steroid formulations [66], and photodynamic therapy [67].

Knowledge gained from clinical trials: Treatment is initiated with monthly injections of an anti-VEGF agent. The decision to treat monthly or at longer intervals is most often based on the visual acuity, the presence of subretinal or intraretinal fluid on OCT and the clinical appearance of features like hemorrhage or leakage determined with FA [68]. Different management strategies include the decision to treat if features of subretinal or intraretinal fluid are present or wait until a future visit, or by a "treat and extend" approach in which treatment is given even if no features are found on OCT, but the duration until the next visit is increased before retreatment. Several agents have been studied (ranibizumab, bevacizumab, and aflibercept), and all agents have been found to improve outcomes in neovascular AMD [69]. Pegaptanib, an aptamer to $VEGF_{165}$ [70], was the first anti-VEGF agent and has less effect than the three other agents.

The effect of anti-VEGF agents on geographic atrophy is difficult to assess because the end stage of neovascular AMD

is the formation of a fibrovascular scar with loss of the architecture of the RPE monolayer and the outer retina [22,23]. The Inhibit VEGF in Age-related Choroidal Neovascularisation (IVAN) study reported that new geographic atrophy was more common in eyes that received monthly anti-VEGF treatment compared to treatment based on features of disease regardless of the anti-VEGF agent used (ranibizumab versus bevacizumab) [71]. The Seven-Year Observational Update of Macular Degeneration Patients Post-MARINA/ANCHOR and HORIZON Trials (SEVEN-UP) analyzed 14 studies of subjects treated with ranibizumab followed for a mean of 7.3 years and found an increase in geographic atrophy after treatment with anti-VEGF [72]. The Comparative of AMD Treatment Trials (CATT) study, which reported equivalent outcomes following treatment with ranibizumab or bevacizumab for AMD [73], reviewed eyes treated with anti-VEGF agents and compared the outcomes of geographic atrophy in those that were successfully treated with anti-VEGF to eyes that did not respond and reported that geographic atrophy occurred in both groups with no difference in progression noted between treatment responses [74]. In a later study, the CATT group reported that ranibizumab use might be more often associated with geographic atrophy than bevacizumab [75]. These studies were retrospective evaluations of clinical trial outcomes, and limitations associated with retrospective studies must be considered. Additional study is needed to assess whether there is a difference in geographic atrophy from anti-VEGF use, and a prospective design may provide greater insight. Other safety concerns with anti-VEGF agents include increased intraocular pressure, but studies do not agree on the presence of this or on possible causes [76]. Thus, anti-VEGF agents reduce vision loss from AMD, but safety concerns exist. Other treatments are needed to improve efficacy and safety.

Transition from type 1 to type 2 CNV when CECs transmigrate the RPE monolayer: Neovascular AMD can be distinguished between occult or type 1 CNV, classic or type 2 CNV, and RAP, or type 3 neovascularization. Early studies found that occult CNV could be asymptomatic and associated with good vision [77], whereas vision loss often occurs in type 2 CNV [78]. Type 1 or occult CNV refers to CNV that remains beneath the RPE monolayer. In contrast, type 2 or classic CNV refers to CNV that has invaded the neural retina. Infrared imaging of the macula in humans can identify features of type 1 CNV even before "ill-defined leakage of undetermined origin that is associated with retinal thickening" manifests on FA [19]. Type 2 CNV presents as lacy hyperfluorescence on early frames of a fluorescein angiogram followed by late leakage of fluorescein dye. Now, OCTA can distinguish CNV lesions [79] by the location of the optical segment where particle motion is detected [19]. OCTA avoids dye injection but requires particle motion to detect CNV [80].

Type 1 CNV can be associated with stable visual acuity. It has been reported that more than 50% of the time, severe vision loss occurs when activated CECs migrate through Bruch's membrane to contact the RPE cells and then migrate across the RPE monolayer into the sensory retina and become type 2 CNV [81]. Vision loss can also occur with RPE barrier compromise identified as leakage of fluorescein dye on angiography in type 1 CNV. These findings and reports suggest that CNV that remains contained beneath the sensory retina and without compromise of the RPE barrier integrity may not be detrimental. An extension is that CNV that is quiescent so as not to induce a fibrovascular response may potentially be beneficial by providing oxygenation and a means for removing accumulated debris. Retinal angiomatous proliferation vasculature for (RAP or type 3 neovascularization) can be more difficult to identify. It is seen on videoangiography with indocyanine green or fluorescein dyes as having a feeding retinal arteriole to an intraretinal angiomatous formation and draining venule [82,83]. OCT and OCTA can also detect RAP [84]. RAP was postulated to be associated with extensive outer retinal debris and degeneration [82] and has currently been shown to be associated with reticular pseudodrusen [85], which are associated with choroidal atrophy, thinning of the choroidal layers [86], and geographic atrophy [87]. RAP lesions have been shown to be associated with geographic atrophy in several clinical studies [84].

Importance of cell–cell interactions and signaling events in RPE cells and CECs: One way to study signaling events surrounding CEC activation and migration across the RPE monolayer is to use cocultures of human CECs and RPE cells. Solo cultures are limited, because cells change phenotype over time and do not always behave in culture as in their tissue microenvironment. Cocultures may be more representative by virtue of cell–cell interactions [35,88] and permit the ability to assess effects of one cell type on the other. It is important to use cells of low passage that retain cell markers and characteristics when in their tissue microenvironments.

To study the effects of CEC interactions with RPE cells, we developed a coculture model [89] that represents the step when CECs make contact with RPE cells after having migrated through Bruch's membrane. Human adult RPE cells are grown on an inverted Transwell insert overnight until the RPE cells attach. The insert is then turned over, and the RPE cells are allowed to grow in culture until they form tight barrier properties determined by high transepithelial electrical resistance (>100 ohms/cm^2). Human CECs are then

grown within the insert. By varying the width of the pore size of the insert, migration of CECs into the RPE monolayer is controlled. In the coculture assay, CECs extend processes through pores to contact RPE cells but are restricted from migrating [89]. Contact between RPE cells and CECs triggers the activation of signaling pathways within CECs or RPE cells. The individual cell types can be distinguished with vital dyes and separated by scraping cells off either side of the insert. In addition, it is possible to isolate CECs by using CD31 coated-magnetic beads to attract CECs or by flow cytometry. Activation of signaling pathways in each cell type can be determined. This model allows one to compare the role of contact between RPE cells and CECs to the effects of coculture without contact (i.e., non-contacting coculture), in which RPE cells and CECs are grown separately without contact but share the same media. In each case, the conditioned media or each cell type, RPE cells or CECs, can be analyzed. The non-contacting coculture may represent the physiologic situation between RPE cells and CECs, in which semiporous Bruch's membrane lies between RPE cells and CECs. However, neither coculture model insert aims to represent Bruch's membrane. Once activated signaling effectors are identified in either cell type, mutations or shRNAs can be introduced into the individual cell type, and the effect on CEC transmigration of the RPE monolayer is determined in the transmigration assay. The transmigration assay has an insert with wider pores that permit CECs to migrate across the RPE monolayer. In the transmigration assay, CECs, vitally labeled to distinguish them from RPE cells, are counted.

Using the coculture model, there was an increase in the fold expression of $VEGF_{189}$, a cell-associated splice variant of VEGFA, in RPE cells grown in contacting coculture with CECs. There was little change in the expression level of other splice variants of VEGF in the contacting coculture or of any splice variant of VEGF in RPE cells grown in non-contacting or solo culture. There was also no significant change in VEGF splice variant expression level in CECs grown in solo or either coculture condition [90]. However, increased age of donor eyes or exposure to hydrogen peroxide caused VEGF splice variant expression levels to be increased in RPE cells. In addition, stresses that increased $VEGF_{189}$ in RPE cells also increased phosphorylation of VEGFR2, but not of VEGFR1, in cocultured CECs. Knockdown of $VEGF_{189}$ in human RPE cells reduced phosphorylation of VEGFR2 in CECs and inhibited, but did not abolish, CEC transmigration across the RPE monolayer by approximately 40%, similar to reported outcomes in human clinical studies with anti-VEGF agents [52]. CECs grown in contact with RPE cells had activation of the rhoGTPase, Rac1. In contrast to $VEGF_{189}$ knockdown, inhibition of Rac1 by expression of the Rac binding domain of Rac1 effector, POSH, abolished the increased CEC transmigration across the RPE monolayer [91]. These data provide strong evidence that Rac1 activation is necessary for CEC transmigration of the RPE monolayer.

Rac1 GTPase is a common effector of several signaling events. In addition to the role of VEGF-induced VEGFR2 activation, activation of Rac1 has also been proposed through the guanine nucleotide exchange factor (GEF), VaV2 [92]. We found activation of Rac1 in CECs stimulated with the chemokine/receptor, CCL11/CCR3 [93], or by activation of phospho-inositol 3 kinase (PI-3 kinase) [91]. PI-3 kinase was either downstream or parallel to VEGFR2-induced Rac1 activation. Others have reported that thrombospondin-1 regulation is affected by activation of Rac1 [94]. Inflammatory cytokines, such as interleukin-17, induce angiogenesis in CECs through activation of Rac1 [95]. Thus, Rac1, an essential component in CEC transmigration and activation, is downstream of multiple different pathways, with some evidence of crosstalk (Figure 2) and might be an effective target for neovascular AMD. However, knockout of Rac1 is lethal, and Rac1 inhibitors have been studied in cancer and angiogenesis but have not proven as effective or safe [53,54], as hoped [96]. Therefore, other ways to inhibit Rac1-mediated CEC transmigration may be important for neovascular AMD. It is also important to recognize the need to target a pathway at several junctures to reduce the effect of crosstalk with other signaling pathways, and this strategy may be safer and more effective than attempts to abolish upstream signaling.

Need for a feed-forward loop to overwhelm homeostasis: Effects found in experimental models may not translate to human neovascular AMD for several reasons: There are species differences; limitations of models, as discussed earlier; difficulty in translating the effects of nutrition and activities of daily life to the cell microenvironment; and the possibility that experimental outcomes in models can be overcome in the human body through mechanisms to restore homeostasis. Generally, compounds that broadly inhibit a pathway (e.g., anti-VEGF agents or corticosteroids) and affect multiple downstream pathways may be effective but are also likely to have more side effects than a more targeted downstream inhibitor. However, the safer targeted inhibitor may not be as effective if upstream signaling pathways have crosstalk with other signaling pathways involved in the pathophysiology. In cancer treatment, broad therapy may be necessary to increase a patient's survival against death, and the time for intervention is short. However, in chronic diseases, regulation instead of inhibition, is important, and many stresses that induce signaling must also be considered. It is important to address feed-forward loops that occur and

multiple pathways involved based on also crosstalk between pathways.

Oxidation, inflammation, and angiogenesis in CECs and RPE cells: In addition to its role in angiogenesis, Rac1 is also a subunit for some NOX isoforms that aggregate to activate NADPH oxidase, a leading generator of superoxide and reactive oxygen species. It is now appreciated that reactive oxygen species (ROS) can be important signaling effectors in addition to being damaging to cell membranes. (NADPH oxidase is also a leading means by which leukocytes fight pathogens, which is essential to the health of the individual. Therefore, this represents another potential reason why inhibiting Rac1 may present safety concerns.) NADPH oxidase is a leading generator of ROS in endothelial cells, and oxidative signaling can cause angiogenesis [97,98]. Furthermore, antioxidants and zinc given to patients with early AMD slow the progression to neovascular AMD [99,100]. In CECs, VEGF activated Rac1 and increased CEC migration. Activated Rac1 also led to ROS generation in CECs, as measured by increased 2',7'-dichlorofluorescein diacetate (DCFDA) [101] (Figure 2), and ROS were inhibited by NADPH oxidase inhibitor, diphenyleneiodonium, or antioxidant, apocynin, or n-acetyl cysteine. These agents all reduced partially, but significantly, VEGF-induced CEC migration. Together, these findings suggest that Rac1 works in at least two known ways to increase CEC migration, directly by increasing actin cytoskeletal events for cell migration and through NADPH oxidase–generated ROS. Furthermore, inhibiting NOX2-induced NADPH oxidase through the use of apocynin or p47phox knockout mice led to significant reduction in CNV induced by laser.

Inflammation is recognized as important in the pathophysiology of neovascular AMD. One concept proposed in human AMD is that of parainflammation [102], which is a tissue adaptive response to toxins or cellular dysfunction. Parainflammation can occur from stimuli, including oxidative stress, dead cells, and potential changes in the extracellular matrix. A characteristic of parainflammation is the activation of microglia. Activated microglia have been proposed in neovascular AMD and angiogenesis to release angiogenic and inflammatory cytokines, including TNF-α [46,103]. One potential event that activates microglia is the

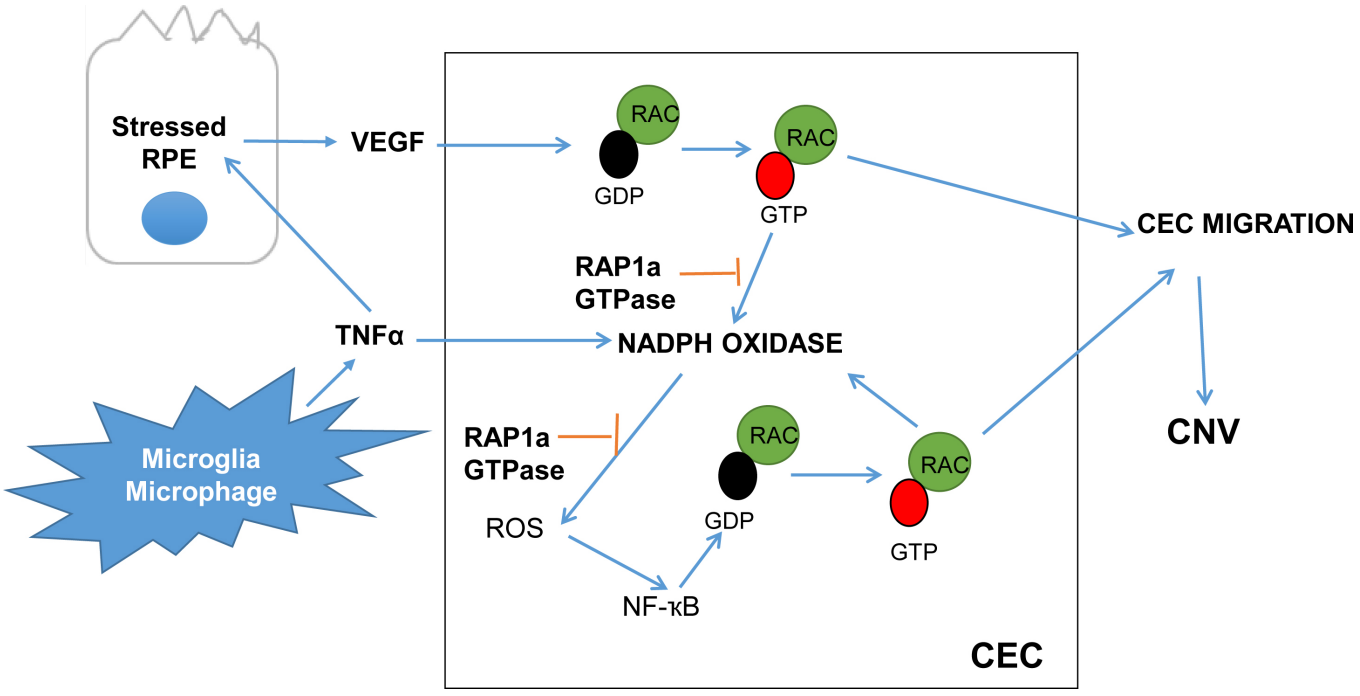

Figure 2. Signaling pathways in a feed-forward loop involve inflammation, oxidative stress, and angiogenesis. Tumor necrosis factor alpha (TNF-α) released by activated microglia causes RPE cells to overexpress vascular endothelial growth factor (VEGF). VEGF attracts and activates choroidal endothelial cells (CECs) through activation of Rac1, which aggregates with other subunits to activate NADPH oxidase. NADPH oxidase–generated reactive oxygen species (ROS) trigger Rac1 activation through nuclear factor-kappa B (NF-κB) signaling, which can further activate NADPH oxidase, thus setting up a feed-forward loop. Activated CECs migrate and proliferate to form choroidal neovascularization (CNV). Activated Rap1a safely inhibits CNV by reducing NADPH oxidase–generated ROS. Permission was granted from Elsevier to use parts of Figure 18 from Prog Retin Eye Res. 2008 Jul;27(4):331-71, namely the OCT images used in panel B and D.

age-related and diet-affected deposition of oxidized lipoproteins within Bruch's membrane, which trigger activation of microglia to release TNF-α. Therefore, we studied the effects of TNF-α on CECs and RPE cells. In CECs, TNF-α led to increased generation of ROS by NADPH oxidase, as shown by inhibition with apocynin or knockdown of subunit, p22phox [104]. TNF-α increased CEC migration to a similar degree as did VEGF. Apocynin also inhibited TNF-α-induced CEC migration and Rac1 activation. Knockdown of Rac1 inhibited TNF-α-induced CEC migration. TNF-α induced the nuclear factor-kappa B (NF-κB) subunit, p-p65, and this was reduced by apocynin but not by knockdown of Rac1. Together, these data provide evidence that TNF-α mediated Rac1 activation via ROS triggered NF-κB [104]. Activated Rac1 then caused CEC migration. Intravitreal administration of a TNF-α antibody inhibited CNV in the laser-induced model and reduced labeling of ROS with E06 and of activated Rac1 in the mouse. These data point to a feed-forward loop in which TNF-α induced NADPH oxidase–generated ROS that activated Rac1, which could then activate NADPH oxidase. It also shows crosstalk with VEGF, which activates Rac1 through the guanine nucleotide exchange factor, Vav2 [92], and leads to NADPH oxidase activation (Figure 2).

Rap1aGTP, a potential regulator of RPE cell junctions and Rac1-mediated CEC events: The RasGTPase, Rap1, is important in cell junctions and motility [105,106]. Rap1 has two isoforms, which are 94% similar but are transcribed on different chromosomes. Rap1a is important in barrier properties [105,107], and Rap1b in angiogenesis [108]. As a GTPase, Rap1 also acts as a biologic switch, being activated by GEFs, which causes Rap1 to be GTP bound, and inactivated by GTPase-activating proteins (GAPs) that lead to the inactive GDP-bound Rap1. Activation of Rap1 pharmacologically with 8CPT-2Me-cAMP or with an adenoviral vector that introduced constitutively active Rap1aGTP into cultured CECs reduced TNF-α-induced ROS and Rac1-mediated CEC migration. Activation of Rap1 by chemical 8CPT reduced CNV and ROS generation in RPE cell and choroidal lysates from laser-injured mice, commensurate with the inhibition that occurred from intravitreal TNF-α antibody. One mechanism in which active Rap1a worked was by binding to the NADPH oxidase subunit, p22phox, to reduce the generation of ROS [107,109] (Figure 2).

TNF-α can mediate VEGF expression in RPE cells through NOX4/NADPH oxidase activation. TNF-α-induced VEGF was not inhibited by NF-κB inhibitor, Bay117082, but was inhibited by β-catenin inhibitor, XAV939. Either apocynin or XAV939 reduced the interaction of β-catenin with nuclear tissue cell factor-1 (TCF-1)/LEF. Together, these data support the hypothesis that TNF-α leads to β-catenin activation, which translocates to the nucleus to bind with TCF1/LEF and increases VEGF transcription [110]. These findings suggest an interaction between inflammatory mediator, TNF-α, and VEGF expression in RPE cells, mediated through NADPH oxidase. Since VEGF has soluble as well as cell-associated properties, it can access CECs and activate Rac1. VEGF also can act as a chemoattractant for migrating CECs (Figure 2).

In addition to activation of CECs, another important step in type 2 CNV is the loss of integrity of the RPE barrier. The RPE monolayer provides the outer blood retinal barrier that regulates what substances have access to its apical and basal aspects. The concept of tight junctions has evolved from a static barrier to a dynamic situation in which junctions constantly break down and remodel under physiologic stresses. However, pathologic stresses that overwhelm homeostatic mechanisms lead to barrier dysfunction with movement of fluid and substances across the barrier to regions that are usually protected. In the case of AMD, cells and substances, such as VEGF, can access the photoreceptors in the sensory retina. Rap1a is important in baseline RPE barrier properties [105], and Rap1b may be important in reformation of RPE cell junctions following a stress [107], such as breakdown of RPE cell junctions following calcium EGTA. However, because of Rap1b's role in angiogenesis, Rap1b may be a less favorable therapeutic target than Rap1a [108].

ROS have been shown to reduce RPE barrier integrity by phosphorylation of cadherin-β-catenin complexes. Activation of Rap1a reduced ROS-mediated RPE barrier compromise [109]. With reduced RPE barrier integrity, VEGF can access the neural retina and potentially attract activated CECs to migrate and form CNV. Mice given intravitreal Rap1 activator, 8CPT, had significantly reduced laser-induced CNV [107]. Rap1b knockout mice treated with 8CPT have activated Rap1a only and also showed significant reduction in laser-induced CNV [109].

Role of extracellular matrix: It is essential to consider the role of the extracellular matrix (ECM) in cell migration. The ECM is the matrix onto which cells attach and engage during migration. Many studies have reported changes in the ECM and Bruch's membrane that occur with aging [38], cigarette smoking [111], and fat-feeding [41]. Using a murine model of *Cfh−/−* mice aged 2 years and then fat-fed, Bowes-Rickman et al. found that CFH played a role in lipoprotein turnover in Bruch's membrane [28]. This finding aligned with clinicohistopathologic evidence by Curcio et al. [15,16] that increased deposition of cholesterol esters and oxidized cholesterol occurs in AMD. In addition to dietary sources for cholesterol

to the choriocapillaris, there is evidence that cholesterol can be produced in the retina [112]. One predominant component of oxidized lipoprotein debris that accumulates in Bruch's membrane and the extracellular matrix in neovascular AMD is 7-ketocholesterol, which can cause angiogenesis [113]. Oxidized lipoproteins activate microglia, which release cytokines, including TNF-α.

Taken together, one scenario for the complexity of events in neovascular AMD is proposed. With increasing age and potentially through diet, oxidized lipoprotein debris and 7-ketocholesterol accumulate in ECM/Bruch's membrane in AMD, and inflammation is increased as 7-ketocholesterol activates microglia to release cytokines. This event may occur earlier in life in patients with genetic predisposition, such as with complement factor H (Y402H) or *ARMS2/HTRA1*. TNF-α mediates β-catenin-induced expression of VEGF in RPE cells, which is released preferentially from the basal aspects [114] of the RPE monolayer. Secreted VEGF can affect CEC migration, potentially by permitting engagement with integrins in ECM components [115]. Secreted VEGF also activates CEC Rac1 and initiates CEC activation and migration. CECs that contact RPE cells are further activated via RPE cell-associated $VEGF_{189}$. VEGF and TNF-α crosstalk leads to further activation of CEC Rac1 in a feed-forward loop. Generated ROS also reduce RPE barrier integrity and permit secreted VEGF to enter the neural retina where it attracts CECs to migrate, proliferate, and form CNV (Figure 2). In addition, age-related increases in CCL11 in RPE cells and CCR3 in CECs exacerbate Rac1 activation in CECs [93].

Additional questions exist. The events that must occur within CECs to migrate require coordination of multiple intracellular signaling cascades. Likewise, numerous events within RPE cells must be coordinated. CECs must engage the extracellular matrix and be affected by changes in its composition over the course of the development of AMD.

Conclusions: Neovascular AMD remains a leading cause of vision loss worldwide despite the use of antiangiogenic agents in AMD. Recent advances in diagnosis and treatment include new imaging modalities, new anti-VEGF agents, and greater understanding of the roles of feed-forward signaling loops and of cell–cell and cell–extracellular matrix interaction. Future therapeutic directions may involve regulating instead of abolishing pathologic signaling causing biologic events. Examples include "quieting" CECs activated to migrate, strengthening the barrier integrity of the RPE monolayer, and addressing the accumulation of debris within the extracellular matrix in the RPE monolayer and Bruch's membrane. Clinical imaging modalities require additional study as a means of evaluating the efficacy of anti-VEGF treatment and to address ways to reduce geographic atrophy.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health P30EY014800, R01EY015130, and R01EY017011 to M.E.H., a grant from the March of Dimes 6-FY13–75 to M.E.H, and an Unrestricted Grant from Research to Prevent Blindness, Inc., New York, NY, to the Department of Ophthalmology & Visual Sciences, University of Utah. Elsevier to use parts of Figure 18 from Prog Retin Eye Res. 2008 Jul;27(4):331-71, namely the OCT images used in Figure 1B,D.

REFERENCES

1. Bird AC. Therapeutic targets in age-related macular disease. J Clin Invest 2010; 120:3033-41. [PMID: 20811159].
2. Green WR, Enger C. Age-related macular degeneration histopathologic studies. The 1992 Lorenz E. Zimmerman Lecture. Ophthalmology 1993; 100:1519-35. [PMID: 7692366].
3. Yannuzzi LA, Sorenson J, Spaide RF, Lipson B. Idiopathic polypoidal choroidal vasculopathy (IPCV). Retina 1990; 10:1-8. [PMID: 1693009].
4. Dansingani KK, Balaratnasingam C, Naysan J, Freund KB. En face imaging of pachychoroid spectrum disorders with swept-source optical coherence tomography. Retina 2015; [PMID: 26335436].
5. Hartnett ME, Weiter JJ, Garsd A, Jalkh AE. Classification of retinal pigment epithelial detachments associated with drusen. Graefes Arch Clin Exp Ophthalmol 1992; 230:11-9. [PMID: 1547961].
6. Poliner LS, Olk RJ, Burgess D, Gordon ME. Natural history of retinal pigment epithelial detachments in age-related macular degeneration. Ophthalmology 1986; 93:543-51. [PMID: 2425324].
7. Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, Yannuzzi LA, Willett W. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Eye Disease Case-Control Study Group. JAMA 1994; 272:1413-20. [PMID: 7933422].
8. Green WR, Key SN 3rd. Senile macular degeneration: a histopathologic study. Trans Am Ophthalmol Soc 1977; 75:180-254. [PMID: 613523].
9. Sunness JS, Gonzalez-Baron J, Bressler NM, Hawkins B, Applegate CA. The development of choroidal neovascularization in eyes with the geographic atrophy form of age-related macular degeneration Ophthalmology 1999; 106:910-9. [PMID: 10328389].
10. Sohn EH, Khanna A, Tucker BA, Abramoff MD, Stone EM, Mullins RF. Structural and biochemical analyses of choroidal thickness in human donor eyes. Invest Ophthalmol Vis Sci 2014; 55:1352-60. [PMID: 24519422].

11. Sparrow JR, Ueda K, Zhou J. Complement dysregulation in AMD: RPE-Bruch's membrane-choroid. Mol Aspects Med 2012; 33:436-45. [PMID: 22504022].

12. Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Retin Eye Res 2001; 20:705-32. [PMID: 11587915].

13. Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, Hageman JL, Stockman HA, Borchardt JD, Gehrs KM, Smith RJ, Silvestri G, Russell SR, Klaver CC, Barbazetto I, Chang S, Yannuzzi LA, Barile GR, Merriam JC, Smith RT, Olsh AK, Bergeron J, Zernant J, Merriam JE, Gold B, Dean M, Allikmets R. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci USA 2005; 102:7227-32. [PMID: 15870199].

14. Crabb JW, Miyagi M, Gu X, Shadrach K, West KA, Sakaguchi H, Kamei M, Hasan A, Yan L, Rayborn ME, Salomon RG, Hollyfield JG. Drusen proteome analysis: An approach to the etiology of age-related macular degeneration. Proceedings of the National Academy of Sciences 2002:222551899.

15. Curcio CA, Millican CL, Bailey T, Kruth HS. Accumulation of cholesterol with age in human Bruch's membrane. Invest Ophthalmol Vis Sci 2001; 42:265-74. [PMID: 11133878].

16. Rodriguez IR, Clark ME, Lee JW, Curcio CA. 7-ketocholesterol accumulates in ocular tissues as a consequence of aging and is present in high levels in drusen. Exp Eye Res 2014; 128:151-5. [PMID: 25261634].

17. Handa JT, Verzijl N, Matsunaga H, Aotaki-Keen A, Lutty GA, te Koppele JM, Miyata T, Hjelmeland LM. Increase in the advanced glycation end product pentosidine in Bruch's membrane with age. Invest Ophthalmol Vis Sci 1999; 40:775-9. [PMID: 10067983].

18. Spaide RF, Armstrong D, Browne R. Continuing medical education review: choroidal neovascularization in age-related macular degeneration–what is the cause? Retina 2003; 23:595-614. [PMID: 14574243].

19. Hartnett ME, Elsner AE. Characteristics of exudative age-related macular degeneration determined in vivo with confocal and indirect infrared imaging. Ophthalmology 1996; 103:58-71. [PMID: 8628562].

20. Bhutto I, Lutty G. Understanding age-related macular degeneration (AMD): relationships between the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex. Mol Aspects Med 2012; 33:295-317. [PMID: 22542780].

21. McLeod DS, Grebe R, Bhutto I, Merges C, Baba T, Lutty GA. Relationship between RPE and choriocapillaris in age-related macular degeneration. Invest Ophthalmol Vis Sci 2009; 50:4982-91. [PMID: 19357355].

22. Grossniklaus HE, Gass JD. Clinicopathologic correlations of surgically excised type 1 and type 2 submacular choroidal neovascular membranes. Am J Ophthalmol 1998; 126:59-69. [PMID: 9683150].

23. Grossniklaus HE, Green WR. Choroidal neovascularization. Am J Ophthalmol 2004; 137:496-503. [PMID: 15013874].

24. Yannuzzi LA, Negrao S, Iida T, Carvalho C, Rodriguez-Coleman H, Slakter J, Freund KB, Sorenson J, Orlock D, Borodoker N. Retinal angiomatous proliferation in age-related macular degeneration. Retina 2001; 21:416-34. [PMID: 11642370].

25. Choi W, Moult EM, Waheed NK, Adhi M, Lee B, Lu CD, de Carlo TE, Jayaraman V, Rosenfeld PJ, Duker JS, Fujimoto JG. Ultrahigh-Speed, Swept-Source Optical Coherence Tomography Angiography in Nonexudative Age-Related Macular Degeneration with Geographic Atrophy. Ophthalmology 2015; 122:2532-44. [PMID: 26481819].

26. Rudnicka AR, Jarrar Z, Wormald R, Cook DG, Fletcher A, Owen CG. Age and gender variations in age-related macular degeneration prevalence in populations of European ancestry: a meta-analysis. Ophthalmology 2012; 119:571-80. [PMID: 22176800].

27. Sobrin L, Seddon JM. Nature and nurture- genes and environment- predict onset and progression of macular degeneration. Prog Retin Eye Res 2014; 40:1-15. [PMID: 24374240].

28. Toomey CB, Kelly U, Saban DR, Bowes Rickman C. Regulation of age-related macular degeneration-like pathology by complement factor H. Proc Natl Acad Sci USA 2015; 112:E3040-9. [PMID: 25991857].

29. Fritsche LG, Fariss RN, Stambolian D, Abecasis GR, Curcio CA, Swaroop A. Age-related macular degeneration: genetics and biology coming together. Annu Rev Genomics Hum Genet 2014; 15:151-71. [PMID: 24773320].

30. Seddon JM, Silver RE, Kwong M, Rosner B. Risk Prediction for Progression of Macular Degeneration: 10 Common and Rare Genetic Variants, Demographic, Environmental, and Macular Covariates. Invest Ophthalmol Vis Sci 2015; 56:2192-202. [PMID: 25655794].

31. Fujihara M, Nagai N, Sussan TE, Biswal S, Handa JT. Chronic Cigarette Smoke Causes Oxidative Damage and Apoptosis to Retinal Pigmented Epithelial Cells in Mice. PLoS One 2008; 3:e3119-[PMID: 18769672].

32. Espinosa-Heidmann DG, Suner IJ, Catanuto P, Hernandez EP, Marin-Castano ME, Cousins SW. Cigarette smoke-related oxidants and the development of sub-RPE deposits in an experimental animal model of dry AMD. Invest Ophthalmol Vis Sci 2006; 47:729-37. [PMID: 16431974].

33. Ramkumar HL, Zhang J, Chan CC. Retinal ultrastructure of murine models of dry age-related macular degeneration (AMD). Prog Retin Eye Res 2010; 29:169-90. [PMID: 20206286].

34. Ambati J. Age-related macular degeneration and the other double helix. The Cogan Lecture. Invest Ophthalmol Vis Sci 2011; 52:2165-9. [PMID: 21471430].

35. Hirschi KK, Rohovsky SA, Beck LH, Smith SR, D'Amore PA. Endothelial cells modulate the proliferation of mural

cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. Circ Res 1999; 84:298-305. [PMID: 10024303].

36. Blasiak J, Petrovski G, Vereb Z, Facsko A, Kaarniranta K. Oxidative stress, hypoxia, and autophagy in the neovascular processes of age-related macular degeneration. BioMed Res Int 2014; 2014:768026-[PMID: 24707498].

37. Nussenblatt RB, Ferris F 3rd. Age-related macular degeneration and the immune response: implications for therapy. Am J Ophthalmol 2007; 144:618-26. [PMID: 17698021].

38. Mullins RF, Russell SR, Anderson DH, Hageman GS. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. FASEB J 2000; 14:835-46. [PMID: 10783137].

39. Edwards MM, McLeod DS, Bhutto IA, Villalonga MB, Seddon JM, Lutty GA. Idiopathic preretinal glia in aging and age-related macular degeneration. Exp Eye Res 2015; [PMID: 26220834].

40. Zhang SX, Ma JH, Bhatta M, Fliesler SJ, Wang JJ. The unfolded protein response in retinal vascular diseases: implications and therapeutic potential beyond protein folding. Prog Retin Eye Res 2015; 45:111-31. [PMID: 25529848].

41. Mitter SK, Song C, Qi X, Mao H, Rao H, Akin D, Lewin A, Grant M, Dunn W Jr, Ding J, Bowes Rickman C, Boulton M. Dysregulated autophagy in the RPE is associated with increased susceptibility to oxidative stress and AMD. Autophagy 2014; 10:1989-2005. [PMID: 25484094].

42. Lin CH, Li CH, Liao PL, Tse LS, Huang WK, Cheng HW, Cheng YW. Silibinin inhibits VEGF secretion and age-related macular degeneration in a hypoxia-dependent manner through the PI-3 kinase/Akt/mTOR pathway. Br J Pharmacol 2013; 168:920-31. [PMID: 23004355].

43. Rodriguez IR, Larrayoz IM. Cholesterol oxidation in the retina: implications of 7KCh formation in chronic inflammation and age-related macular degeneration. J Lipid Res 2010; 51:2847-62. [PMID: 20567027].

44. Klettner A. Oxidative stress induced cellular signaling in RPE cells. Front Biosci (Schol Ed) 2012; 4:392-411. [PMID: 22202067].

45. Cai X, McGinnis JF. Oxidative stress: the achilles' heel of neurodegenerative diseases of the retina. Front Biosci (Landmark Ed) 2012; 17:1976-95. [PMID: 22201850].

46. Indaram M, Ma W, Zhao L, Fariss RN, Rodriguez IR, Wong WT. 7-Ketocholesterol increases retinal microglial migration, activation, and angiogenicity: a potential pathogenic mechanism underlying age-related macular degeneration. Sci Rep 2015; 5:9144-[PMID: 25775051].

47. Sakurai E, Anand A, Ambati BK, van Rooijen N, Ambati J. Macrophage depletion inhibits experimental choroidal neovascularization. Invest Ophthalmol Vis Sci 2003; 44:3578-85. [PMID: 12882810].

48. Terasaki H, Kase S, Shirasawa M, Otsuka H, Hisatomi T, Sonoda S, Ishida S, Ishibashi T, Sakamoto T. TNF-alpha decreases VEGF secretion in highly polarized RPE cells but increases it in non-polarized RPE cells related to crosstalk between JNK and NF-kappaB pathways. PLoS One 2013; 8:e69994-[PMID: 23922887].

49. Tuo J, Wang Y, Cheng R, Li Y, Chen M, Qiu F, Qian H, Shen D, Penalva R, Xu H, Ma JX, Chan CC. Wnt signaling in age-related macular degeneration: human macular tissue and mouse model. J Transl Med 2015; 13:330-[PMID: 26476672].

50. Hu Y, Chen Y, Lin M, Lee K, Mott RA, Ma JX. Pathogenic role of the Wnt signaling pathway activation in laser-induced choroidal neovascularization. Invest Ophthalmol Vis Sci 2013; 54:141-54. [PMID: 23211829].

51. Jaffe GJ, Eliott D, Wells JA, Prenner JL, Papp A, Patel S. A Phase 1 Study of Intravitreous E10030 in Combination with Ranibizumab in Neovascular Age-Related Macular Degeneration. Ophthalmology 2016; 123:78-85. [PMID: 26499921].

52. Radeke MJ, Radeke CM, Shih YH, Hu J, Bok D, Johnson LV, Coffey PJ. Restoration of mesenchymal retinal pigmented epithelial cells by TGFbeta pathway inhibitors: implications for age-related macular degeneration. Genome Med 2015; 7:58-[PMID: 26150894].

53. Takeda A, Baffi JZ, Kleinman ME, Cho WG, Nozaki M, Yamada K, Kaneko H, Albuquerque RJC, Dridi S, Saito K, Raisler BJ, Budd SJ, Geisen P, Munitz A, Ambati BK, Green MG, Ishibashi T, Wright JD, Humbles AA, Gerard CJ, Ogura Y, Pan Y, Smith JR, Grisanti S, Hartnett ME, Rothenberg ME, Ambati J. CCR3 is a target for age-related macular degeneration diagnosis and therapy. Nature 2009; 460:225-30. [PMID: 19525930].

54. de Oliveira Dias JR, Rodrigues EB, Maia M, Magalhaes O Jr, Penha FM, Farah ME. Cytokines in neovascular age-related macular degeneration: fundamentals of targeted combination therapy. Br J Ophthalmol 2011; 95:1631-7. [PMID: 21546514].

55. Fei P, Zaitoun I, Farnoodian M, Fisk DL, Wang S, Sorenson CM, Sheibani N. Expression of thrombospondin-1 modulates the angioinflammatory phenotype of choroidal endothelial cells. PLoS One 2014; 9:e116423-[PMID: 25548916].

56. Ildefonso CJ, Biswal MR, Ahmed CM, Lewin AS. The NLRP3 Inflammasome and its Role in Age-Related Macular Degeneration. Adv Exp Med Biol 2016; 854:59-65. [PMID: 26427394].

57. Gelfand BD, Wright CB, Kim Y, Yasuma T, Yasuma R, Li S, Fowler BJ, Bastos-Carvalho A, Kerur N, Uittenbogaard A, Han YS, Lou D, Kleinman ME, McDonald WH, Nunez G, Georgel P, Dunaief JL, Ambati J. Iron Toxicity in the Retina Requires Alu RNA and the NLRP3 Inflammasome. Cell Reports 2015; 11:1686-93. [PMID: 26074074].

58. Marneros AG. VEGF-A and the NLRP3 Inflammasome in Age-Related Macular Degeneration. Adv Exp Med Biol 2016; 854:79-85. [PMID: 26427397].

59. Saint-Geniez M, Kurihara T, Sekiyama E, Maldonado AE, D'Amore PA. An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris. Proc Natl Acad Sci USA 2009; 106:18751-6. [PMID: 19841260].

60. Saint-Geniez M, Maharaj AS, Walshe TE, Tucker BA, Sekiyama E, Kurihara T, Darland DC, Young MJ, D'Amore PA. Endogenous VEGF is required for visual function: evidence for a survival role on muller cells and photoreceptors. PLoS One 2008; 3:e3554-[PMID: 18978936].

61. Ford KM, Saint-Geniez M, Walshe T, Zahr A, D'Amore PA. Expression and Role of VEGF in the Adult Retinal Pigment Epithelium. Invest Ophthalmol Vis Sci 2011; 52:9478-87. [PMID: 22058334].

62. Maharaj ASR, Saint-Geniez M, Maldonado AE, D'Amore PA. Vascular Endothelial Growth Factor Localization in the Adult. Am J Pathol 2006; 168:639-48. [PMID: 16436677].

63. Saint-Geniez M, Maldonado AE, D'Amore PA. VEGF Expression and Receptor Activation in the Choroid during Development and in the Adult. Invest Ophthalmol Vis Sci 2006; 47:3135-42. [PMID: 16799060].

64. Henkind P, Gartner S. The relationship between retinal pigment epithelium and the choriocapillaris. Trans Ophthalmol Soc U K 1983; 103:444-7. [PMID: 6589861].

65. Korte GE, Reppucci V, Henkind P. RPE destruction causes choriocapillary atrophy. Invest Ophthalmol Vis Sci 1984; 25:1135-45. [PMID: 6480292].

66. Geltzer A, Turalba A, Vedula SS. Surgical implantation of steroids with antiangiogenic characteristics for treating neovascular age-related macular degeneration. Cochrane Database Syst Rev 2013; 1:CD005022-[PMID: 23440797].

67. Gehrs KM, Anderson DH, Johnson LV, Hageman GS. Age-related macular degeneration merging pathogenetic and therapeutic concepts. Ann Med 2006; 38:450-71. [PMID: 17101537].

68. Wykoff CC, Croft DE, Brown DM, Wang R, Payne JF, Clark L, Abdelfattah NS, Sadda SR. Prospective Trial of Treat-and-Extend versus Monthly Dosing for Neovascular Age-Related Macular Degeneration: TREX-AMD 1-Year Results. Ophthalmology 2015; 122:2514-22. [PMID: 26391465].

69. Scott AW, Bressler SB. Long-term follow-up of vascular endothelial growth factor inhibitor therapy for neovascular age-related macular degeneration. Curr Opin Ophthalmol 2013; 24:190-6. [PMID: 23492430].

70. Gragoudas ES, Adamis AP, Cunningham ET Jr, Feinsod M, Guyer DR. Group. tVISiONCT: Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 2004; 351:2805-16. [PMID: 15625332].

71. Chakravarthy U, Harding SP, Rogers CA, Downes S, Lotery AJ, Dakin HA, Culliford L, Scott LJ, Nash RL, Taylor J, Muldrew A, Sahni J, Wordsworth S, Raftery J, Peto T, Reeves BC. A randomised controlled trial to assess the clinical effectiveness and cost-effectiveness of alternative treatments to Inhibit VEGF in Age-related choroidal Neovascularisation (IVAN). Health Technol Assess 2015; 19:1-298. [PMID: 26445075].

72. Bhisitkul RB, Mendes TS, Rofagha S, Enanoria W, Boyer DS, Sadda SR, Zhang K. Macular atrophy progression and 7-year vision outcomes in subjects from the ANCHOR, MARINA, and HORIZON studies: the SEVEN-UP study. Am J Ophthalmol 2015; 159:915-24. [PMID: 25640411].

73. Martin DF, Maguire MG, Ying GS, Grunwald JE, Fine SL, Jaffe GJ. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. N Engl J Med 2011; 364:1897-908. [PMID: 21526923].

74. Ying GS, Huang J, Maguire MG, Jaffe GJ, Grunwald JE, Toth C, Daniel E, Klein M, Pieramici D, Wells J, Martin DF. Baseline predictors for one-year visual outcomes with ranibizumab or bevacizumab for neovascular age-related macular degeneration. Ophthalmology 2013; 120:122-9. [PMID: 23047002].

75. Young M, Chui L, Fallah N, Or C, Merkur AB, Kirker AW, Albiani DA, Forooghian F. Exacerbation of choroidal and retinal pigment epithelial atrophy after anti-vascular endothelial growth factor treatment in neovascular age-related macular degeneration. Retina 2014; 34:1308-15. [PMID: 24451923].

76. Singh RS, Kim JE. Ocular hypertension following intravitreal anti-vascular endothelial growth factor agents. Drugs Aging 2012; 29:949-56. [PMID: 23179897].

77. Subfoveal neovascular lesions in age-related macular degeneration. Guidelines for evaluation and treatment in the macular photocoagulation study. Macular Photocoagulation Study Group. Arch Ophthalmol 1991; 109:1242-57. [PMID: 1718252].

78. Singerman LJ, Stockfish JH. Natural history of subfoveal pigment epithelial detachments associated with subfoveal or unidentifiable choroidal neovascularization complicating age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol 1989; 227:501-7. [PMID: 2483142].

79. Lukason M, DuFresne E, Rubin H, Pechan P, Li Q, Kim I, Kiss S, Flaxel C, Collins M, Miller J, Hauswirth W, Maclachlan T, Wadsworth S, Scaria A. Inhibition of choroidal neovascularization in a nonhuman primate model by intravitreal administration of an AAV2 vector expressing a novel anti-VEGF molecule. Mol Ther 2011; 19:260-5. [PMID: 20978476].

80. Coscas GJ, Lupidi M, Coscas F, Cagini C, Souied EH. Optical coherence tomography angiography versus traditional multimodal imaging in assessing the activity of exudative age-related macular generation: A New Diagnostic Challenge. Retina 2015; 35:2219-28. [PMID: 26398697].

81. Stevens TS, Bressler NM, Maguire MG, Bressler SB, Fine SL, Alexander J, Phillips DA, Margherio RR, Murphy PL, Schachat AP. Occult choroidal neovascularization in age-related macular degeneration. A natural history study. Arch Ophthalmol 1997; 115:345-50. [PMID: 9076206].

82. Hartnett ME, Weiter JJ, Staurenghi G, Elsner AE. Deep retinal vascular anomalous complexes in advanced age-related macular degeneration. Ophthalmology 1996; 103:2042-53. [PMID: 9003338].

83. Parravano M, Pilotto E, Musicco I, Varano M, Introini U, Staurenghi G, Menchini U, Virgili G. Reproducibility of fluorescein and indocyanine green angiographic assessment

for RAP diagnosis: a multicenter study. Eur J Ophthalmol 2012; 22:598-606. [PMID: 22139618].

84. Kuehlewein L, Dansingani KK, de Carlo TE, Bonini Filho MA, Iafe NA, Lenis TL, Freund KB, Waheed NK, Duker JS, Sadda SR, Sarraf D. Optical Coherence Tomography Angiography of Type 3 Neovascularization Secondary to Age-Related Macular Degeneration. Retina 2015; 35:2229-35. [PMID: 26502007].

85. Ueda-Arakawa N, Ooto S, Nakata I, Yamashiro K, Tsujikawa A, Oishi A, Yoshimura N. Prevalence and genomic association of reticular pseudodrusen in age-related macular degeneration. Am J Ophthalmol 2013; 155:260-9. [PMID: 23111182].

86. Arnold JJ, Sarks SH, Killingsworth MC, Sarks JP. Reticular pseudodrusen. A risk factor in age-related maculopathy. Retina 1995; 15:183-91. [PMID: 7569344].

87. Cho HJ, Yoo SG, Kim HS, Kim JH, Kim CG, Lee TG, Kim JW. Risk factors for geographic atrophy after intravitreal ranibizumab injections for retinal angiomatous proliferation. Am J Ophthalmol 2015; 159:285-92. [PMID: 25447115].

88. Hirschi KK, Rohovsky SA, D'Amore PA. Cell-cell interactions in vessel assembly: a model for the fundamentals of vascular remodelling. Review. 5 refs. Transpl Immunol 1997; 5:177-8. [PMID: 9402682].

89. Geisen P, McColm JR, Hartnett ME. Choroidal endothelial cells transmigrate across the retinal pigment epithelium but do not proliferate in response to soluble vascular endothelial growth factor. Exp Eye Res 2006; 82:608-19. [PMID: 16259980].

90. Wang H, Geisen P, Wittchen ES, King B, Burridge K, D'Amore PA, Hartnett ME. The Role of RPE Cell-Associated VEGF189 in Choroidal Endothelial Cell Transmigration across the RPE. Invest Ophthalmol Vis Sci 2011; 52:570-8. [PMID: 20811045].

91. Peterson LJ, Wittchen ES, Geisen P, Burridge K, Hartnett ME. Heterotypic RPE-choroidal endothelial cell contact increases choroidal endothelial cell transmigration via PI 3-kinase and Rac1. Exp Eye Res 2007; 84:737-44. [PMID: 17292356].

92. Garrett TA, Van Buul JD, Burridge K. VEGF-induced Rac1 activation in endothelial cells is regulated by the guanine nucleotide exchange factor Vav2. Exp Cell Res 2007; 313:3285-97. [PMID: 17686471].

93. Wang H, Wittchen ES, Jiang Y, Ambati B, Grossniklaus HE, Hartnett ME. Upregulation of CCR3 by Age-Related Stresses Promotes Choroidal Endothelial Cell Migration via VEGF-Dependent and -Independent Signaling. Invest Ophthalmol Vis Sci 2011; 52:8271-7. [PMID: 21917937].

94. Giehl K, Graness A, Goppelt-Struebe M. The small GTPase Rac-1 is a regulator of mesangial cell morphology and thrombospondin-1 expression. Am J Physiol Renal Physiol 2008; 294:F407-13. [PMID: 18045834].

95. Chen Y, Zhong M, Liang L, Gu F, Peng H. Interleukin-17 induces angiogenesis in human choroidal endothelial cells in vitro. Invest Ophthalmol Vis Sci 2014; 55:6968-75. [PMID: 25228547].

96. Bid HK, Roberts RD, Manchanda PK, Houghton PJ. RAC1: an emerging therapeutic option for targeting cancer angiogenesis and metastasis. Mol Cancer Ther 2013; 12:1925-34. [PMID: 24072884].

97. Ushio-Fukai M. VEGF Signaling Through NADPH Oxidase-Derived ROS. Antioxid Redox Signal 2007; 9:731-9. [PMID: 17511588].

98. Ushio-Fukai M, Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling. Role of NAD(P)H oxidase. Mol Cell Biochem 2004; V264:85-97. [PMID: 15544038].

99. . Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS Report No. 8. Arch Ophthalmol 2001; 119:1417-36. [PMID: 11594942].

100. Lutein + zeaxanthin and omega-3 fatty acids for age-related macular degeneration: the Age-Related Eye Disease Study 2 (AREDS2) randomized clinical trial. JAMA 2013; 309:2005-15. [PMID: 23644932].

101. Monaghan-Benson E, Hartmann J, Vendrov AE, Budd S, Byfield G, Parker A, Ahmad F, Huang W, Runge M, Burridge K, Madamanchi N, Hartnett ME. The Role of Vascular Endothelial Growth Factor-Induced Activation of NADPH Oxidase in Choroidal Endothelial Cells and Choroidal Neovascularization. Am J Pathol 2010; 177:2091-102. [PMID: 20802176].

102. Xu H, Chen M, Forrester JV. Para-inflammation in the aging retina. Prog Retin Eye Res 2009; 28:348-68. [PMID: 19560552].

103. Rodrigues MM, Wiggert B, T'so MM, Chader GJ. Retinitis pigmentosa: Immunohistochemical and biochemical studies of the retina. Can J Ophthalmol 1986; 21:[PMID: 3085907].

104. Wang H, Fotheringham L, Wittchen ES, Hartnett ME. Rap1 GTPase Inhibits Tumor Necrosis Factor-alpha-Induced Choroidal Endothelial Migration via NADPH Oxidase- and NF-kappaB-Dependent Activation of Rac1. Am J Pathol 2015; 185:3316-25. [PMID: 26476350].

105. Wittchen ES, Aghajanian A, Burridge K. Isoform-specific differences between Rap1A and Rap1B GTPases in the formation of endothelial cell junctions. Small GTPases 2011; 2:65-76. [PMID: 21776404].

106. Wilson CW, Ye W. Regulation of vascular endothelial junction stability and remodeling through Rap1-Rasip1 signaling. Cell Adhes Migr 2014; 8:76-83. [PMID: 24622510].

107. Wittchen ES, Nishimura E, McCloskey M, Wang H, Quilliam LA, Chrzanowska-Wodnicka M, Hartnett ME. Rap1 GTPase activation and barrier enhancement in rpe inhibits choroidal neovascularization in vivo. PLoS One 2013; 8:e73070-[PMID: 24039860].

108. Chrzanowska-Wodnicka M, Kraus AE, Gale D, White GC 2nd, Vansluys J. Defective angiogenesis, endothelial migration, proliferation, and MAPK signaling in Rap1b-deficient mice. Blood 2008; 111:2647-56. [PMID: 17993608].

109. Wang H, Jiang Y, Shi D, Quilliam LA, Chrzanowska-Wodnicka M, Wittchen ES, Li DY, Hartnett ME. Activation of Rap1 inhibits NADPH oxidase-dependent ROS generation in retinal pigment epithelium and reduces choroidal neovascularization. FASEB J 2014; 28:265-74. [PMID: 24043260].

110. Zhou T, Hu Y, Chen Y, Zhou KK, Zhang B, Gao G, Ma JX. The pathogenic role of the canonical Wnt pathway in age-related macular degeneration. Invest Ophthalmol Vis Sci 2010; 51:4371-9. [PMID: 19875668].

111. Seddon JM, Willett WC, Speizer FE, Hankinson SE. A prospective study of cigarette smoking and age-related macular degeneration in women. JAMA 1996; 276:1141-6. [PMID: 8827966].

112. Fliesler SJ. Cholesterol homeostasis in the retina: seeing is believing. J Lipid Res 2015; 56:1-4. [PMID: 25421059].

113. Amaral J, Lee JW, Chou J, Campos MM, Rodriguez IR. 7-Ketocholesterol induces inflammation and angiogenesis in vivo: a novel rat model. PLoS One 2013; 8:e56099-[PMID: 23409131].

114. Blaauwgeers HGT, Holtkamp GM, Rutten H, Witmer AN, Koolwijk P, Partanen TA, Alitalo K, Kroon ME, Kijlstra A, van Hinsbergh VWM, Schlingemann RO. Polarized vascular endothelial growth factor secretion by human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris: evidence for a trophic paracrine relation. Am J Pathol 1999; 155:421-8. [PMID: 10433935].

115. Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, Keshet E, Neufeld G. VEGF145, a Secreted Vascular Endothelial Growth Factor Isoform That Binds to Extracellular Matrix. J Biol Chem 1997; 272:7151-8. [PMID: 9054410].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 27 February 2016. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.