



## Review

# Myofibroblasts in macular fibrosis secondary to neovascular age-related macular degeneration - the potential sources and molecular cues for their recruitment and activation


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## ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly in developed countries. Neovascular AMD (nAMD) accounts for 90% of AMD-related vision loss. Although intravitreal injection of VEGF inhibitors can improve vision in nAMD, approximately 1/3 of patients do not benefit from the therapy due to macular fibrosis. The molecular mechanism underlying the transition of the neovascular lesion to a fibrovascular phenotype remains unknown. Here we discussed the clinical features and risk factors of macular fibrosis secondary to nAMD. Myofibroblasts are key cells in fibrosis development. However, fibroblasts do not exist in the macula. Potential sources of myofibroblast precursors, the molecular cues in the macular microenvironment that recruit them and the pathways that control their differentiation and activation in macular fibrosis were also discussed. Furthermore, we highlighted the challenges in macular fibrosis research and the urgent need for better animal models for mechanistic and therapeutic studies.

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## 1. Introduction

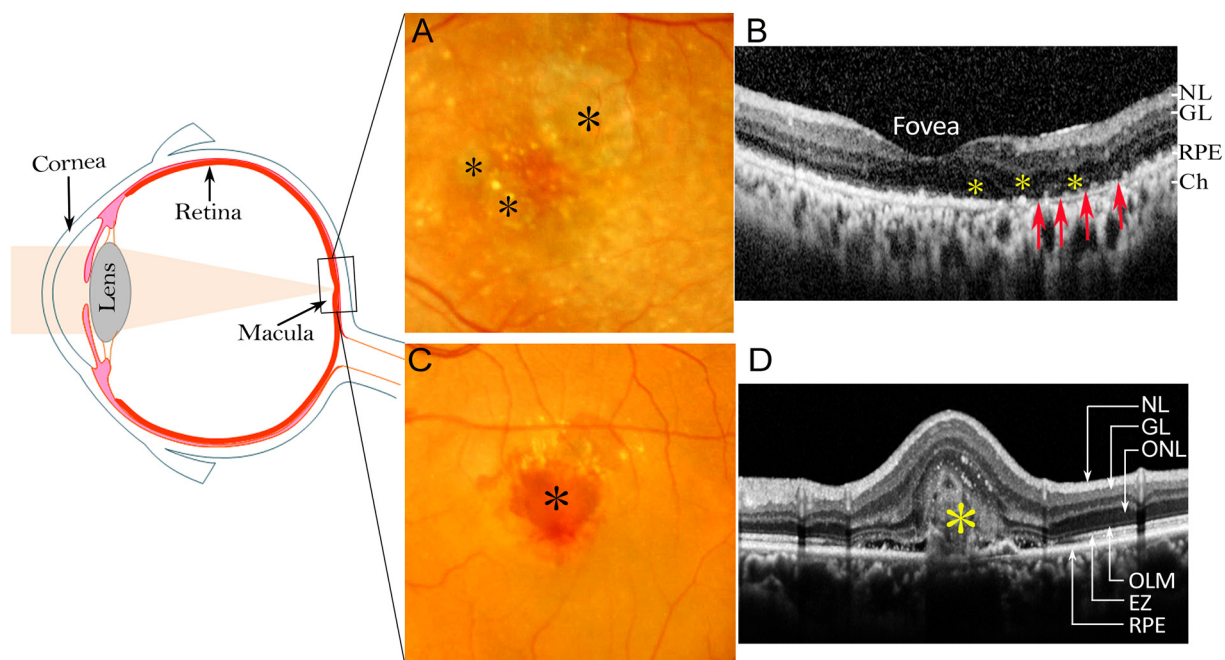
Age related macular degeneration (AMD) is the progressive degeneration of the central part of the neuronal retina, the macula, in people older than 55 years of age, and is the leading cause of blindness in the elderly in industrialized countries [1]. Ageing, urbanization, obesity and cigarette smoke are risk factors for AMD [1]. Due to the growing ageing population and increased life expectancy, it has been projected that 288 million people will be affected by AMD by 2040 [2,3]. The early stage of AMD is characterized by large drusen and disturbance of retinal pigment epithelial (RPE) cells. The disease can advance into two late stages, “dry” and “wet”. Dry AMD refers to the geographic atrophy of the macula (Fig. 1A, B); whereas wet AMD is the growth of abnormal blood vessels into the macula (Fig. 1C, D), also known as neovascular AMD (nAMD). nAMD is treated by intravitreal injection of vascular endothelial growth factor inhibitors (e.g., anti-VEGF). Although the treatment can prevent the decrease or even improve vision acuity, it is burdensome, costly and invasive. Importantly, approximately one-third of patients do not benefit from the therapy due to the development of macular fibrosis or atrophy. The pathogenesis of macular fibrosis in nAMD is poorly defined, and currently there is no therapy for it. This review discusses the clinical manifestations and the underlying mechanisms of macular fibrosis secondary to nAMD with a strong focus on the potential source of myofibroblast

precursors and the pathways involved in their recruitment and activation.

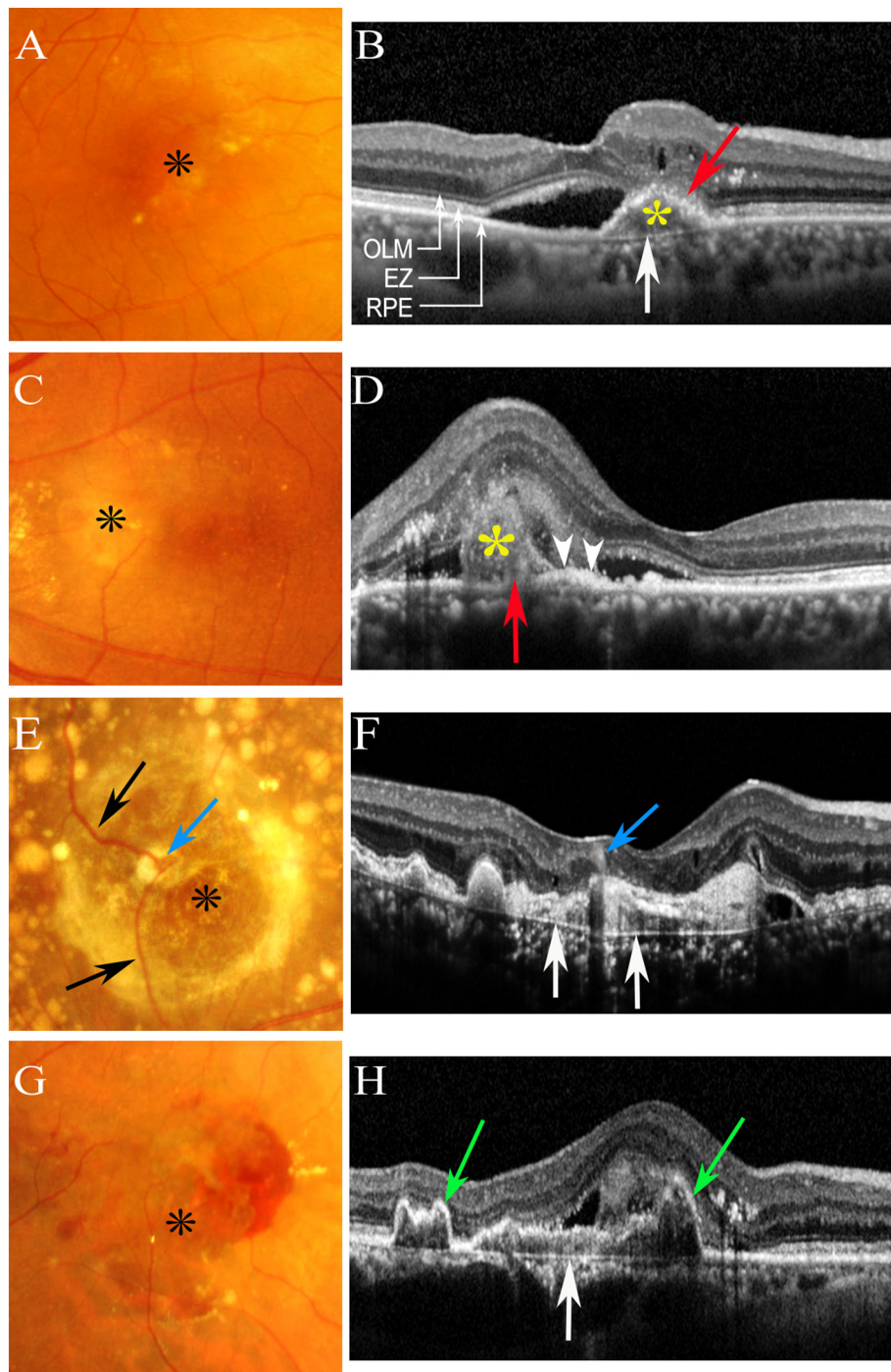
## 2. Clinical features of macular fibrosis in nAMD

### 2.1. Neovascular AMD

The growth of new blood vessels into the macula is the hallmark of nAMD. The new vessels cause leakage and hemorrhage beneath the RPE or photoreceptors resulting in severe visual loss. nAMD is classified into three subtypes according to the origin of diseased vessels, choroidal neovascularization (CNV) (Fig. 2A–D), retinal angiomatous proliferation (RAP) (Fig. 2E, F), and polypoidal choroidal vasculopathy (PCV) (Fig. 2G, H). CNV originates from choroidal blood vessels, which can be further classified into occult (CNV spreads beneath RPE cells, Fig. 2A, B) and classic (CNV breaks through RPE layer and grows in subretinal space, Fig. 2C, D) subtypes. RAP is the proliferation of retinal blood vessels that progress posteriorly into the subretinal space causing retinal-choroidal anastomosis (Fig. 2E, F) [4]. RAP accounts for 15% to 20% of all nAMD cases in Caucasian population [4]. PCV is the polypoidal dilations of an abnormal branching choroidal vascular network (BVN) (Fig. 2G, H), which can penetrate Bruch's membrane and spread into subretinal space of the macula [5]. PCV is more prevalent than RAP in Asian populations [5].



**Fig. 1.** Clinical features of late stages of AMD. (A, B) “dry” AMD (Geography atrophy), sharply circumscribed areas of RPE atrophy (black asterisks) associated scattered pre-existing drusen were seen in color photography (A). Loss of RPE (red arrows) and morphologic alteration of the outer retina (yellow asterisks) on SD-OCT B-scan (B). (C, D) “wet” AMD (nAMD). (C) Subretinal exudation and hemorrhage (black asterisk) were shown on the fundus photograph. (D) Fibrovascular lesion penetrates the RPE on SD-OCT (yellow asterisk). NL: Nerve fiber layer; GL: ganglion layer; ONL: outer nuclear layer; OLM: outer limiting membrane; EZ: ellipsoid zone; RPE: retinal pigment epithelia; Ch: Choroid.



**Fig. 2.** Clinical features of different types of nAMD. (A, B) Occult CNV (type 1 CNV). Whitish hard exudation scattered around the swollen foveola (A). SD-OCT showed dome-shape elevation of intact RPE (B, red arrow) with intermediate hyper-reflective mass (yellow asterisk). Bruch's membrane was intact (white arrow). OLM: outer limiting membrane; EZ: ellipsoid zone; RPE: retinal pigment epithelium. (C, D) Classic CNV (type 2 CNV). Subretinal exudation (C, black asterisk) with fibrovascular lesion (D, yellow asterisk) penetrates the RPE (D, red arrow). The adjacent RPE contain subretinal hyper-reflective materials (SHRM, arrowheads). (E, F) Retinal angiomatous proliferation (RAP), proliferating vessels within and below the retina. Retinal vessels (E, black arrows) connecting to the disciform scar. SD-OCT shows retinal vessel (F, blue arrow) inserts into the sub-retinal hyper-reflective lesion (F, white arrows). (G, H) Polypoidal choroidal vasculopathy (PCV). Orange lesion (G, black asterisk) and subretinal hemorrhage were seen on color photograph. SD-OCT shows the thumb-like RPE detachment (H, green arrow) well correspond to polypoidal lesions, and the double layer sign (H, intermediate hyper-reflective membrane located between RPE and Bruch's membrane (white arrow)), which corresponds to the branched vascular network.

## 2.2. Macular fibrosis in nAMD

Macular fibrosis is the end stage of the natural history of nAMD [6,7]. It was originally described as disciform lesions by Pagenstecher in 1875, and was later found to be fibrotic scarring in histopathological examinations [8]. Macular fibrosis damages the RPE and photoreceptors resulting

in irreversible visual impairment even with anti-VEGF agent treatment [9,10]. The incidence of macular fibrosis in nAMD was reported to range from 39% [11] to 100% [12] 10–15 years ago; and was decreased to 20–37–61–4% after the introduction of anti-VEGF therapy [9,13,14].

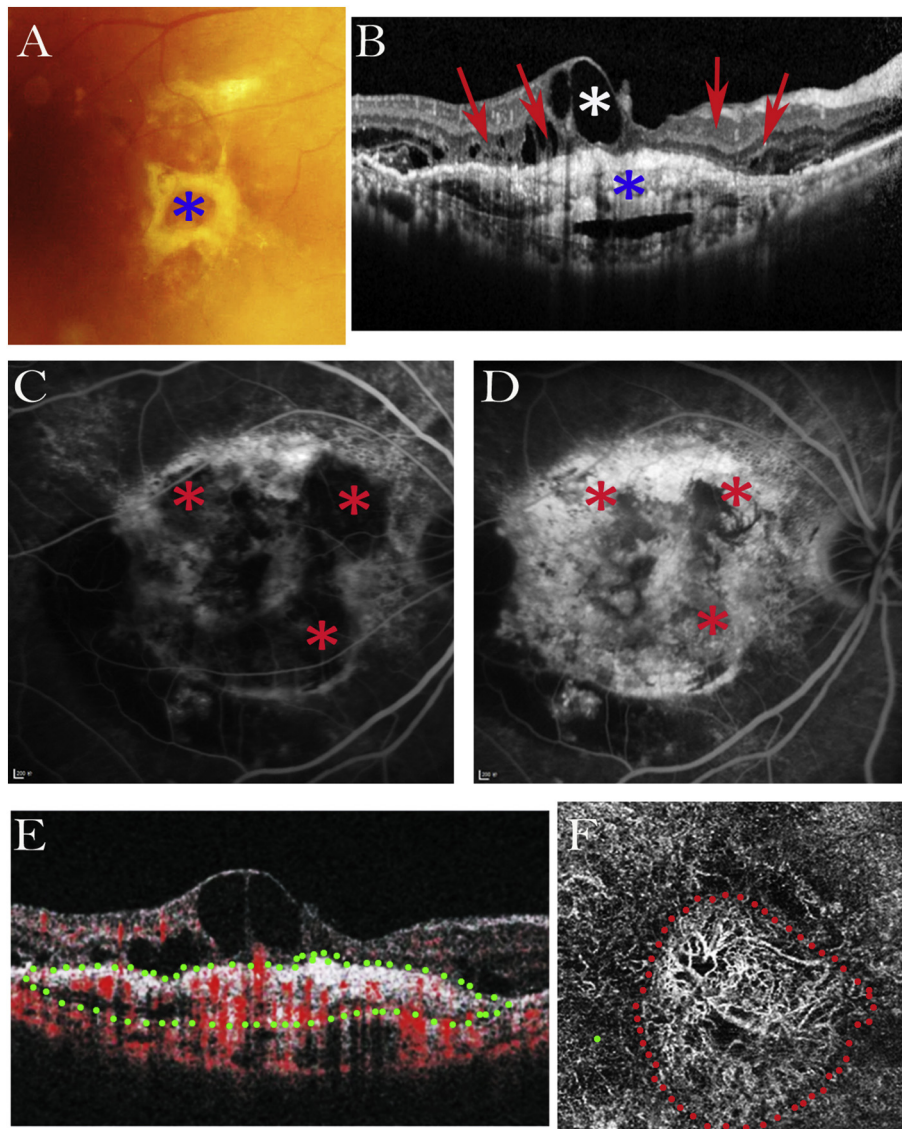
Clinically, macular fibrosis is defined as a well-demarcated, elevated mound of whitish or yellowish material within or under the retina that

is not dehomoglobinized blood or hard exudation on fundus examination [13–15] (Fig. 3A). Fluorescein angiography (FA) usually demonstrates early hypofluorescent plaque masking the underneath (Fig. 3C) followed by non-leakage hyperfluorescein in the late stage (Fig. 3D). On spectral-domain optical coherence tomography (SD-OCT), fibrosis appears as a compact, subretinal hyper-reflective lesion, with aberrant RPE and ellipsoid zone (Fig. 3B, E) [15]. Subretinal fibrosis was classified into three stages using OCT and color fundus tomography [15]. Stage I: Minimal subretinal fibrosis - with a distinct pale patch lesion in fundus photograph and a thin continuous, highly reflective band on OCT between a grossly intact outer neurosensory retina and Bruch's membrane, with or without subretinal fluid. Stage II: Prominent subretinal fibrosis - with a thick, continuous, highly reflective subretinal mass and diffuse thickening of the neurosensory fovea. Stage III: Hyper-reflective subretinal fibrosis - with atrophy of the overlying neurosensory retina [15]. On optical coherence tomography angiography (OCTA), abnormal vascular network and collateral architectural changes in the outer retina and the choriocapillaris layer

are often evident, suggesting that the lesions are vascularized fibrotic membranes (Fig. 3E, F) [14].

### 2.3. Risk factors of retinal fibrosis in nAMD

AMD is related to several environmental and genetic risk factors. Age, smoking and diet are most consistently associated environmental risk factors of AMD [16]. Genetic studies have shown that the complement factor H (CFH) on chromosome 1 and the age-related maculopathy susceptibility 2/HtraA serine peptidase (ARMS2/HTRA1) genes on chromosome 10 have AMD susceptibility loci [17,18]. However, the environmental and genetic factors related to macular fibrosis in AMD have not been identified. Singh et al. reported that lower plasma levels of 25-hydroxyvitamin D is associated with subretinal fibrosis in nAMD [19]. The CFH Y402H CC genotype in nAMD patients is known to be related to poor response to anti-VEGF therapy, and patients with fibrosis are often poor responders [20]. This suggests that the CFH Y402H CC genotype may be a genetic risk factor



**Fig. 3.** Clinical features of macular fibrosis. (A) Well-circumscribed white fibrous tissue was seen (blue asterisk). (B) SD-OCT shows well-defined hyper-reflective sub-retinal tissue (blue asterisk) with overlying aberrant outer retinal components (red arrows) and cystic lesions (white asterisk). (C and D) Fluorescein angiography shows hypo-fluorescent at the early stage (C, 1 min 27 s) and hyper-fluorescent at late stage (D, 11 min 49 s) (red asterisks). (E) SD-OCT with blood flow overlay shows hyper-reflective subretinal fibrosis (green dotted line) with blood flow signals inside (red color dots). The overlying neurosensory retina was thinning and atrophic. (F) en Face OCT-A shows blood vessels within the fibrous tissue (within the red dotted circle).

for macular fibrosis, although direct evidence supporting the claim is lacking.

Among different types of nAMD, patients with RAP are more likely to develop macular fibrosis [21] than those with PCV [22]. Furthermore, classic CNV, poorer visual acuity at first presentation, a longer interval between diagnosis and treatment [13], refractory intraretinal cysts [23] and macular hemorrhage [24] are known risk factors of retinal fibrosis. SD-OCT investigations have shown that the presence of subretinal hyper-reflective material (SHRM, Fig. 2D) in RPE is related to macular fibrosis and poor vision prognosis [25]. In particular, the central location, a well-defined border and the thickness of SHRM can predict the likelihood of subretinal fibrosis [26]. The clinical observations suggest that damage to the neuronal retina and RPE put the macula at risk of developing fibrosis in nAMD.

### 3. Pathophysiology of macular fibrosis

Fibrosis is a pathological state of excess deposition of extracellular matrix (ECM) proteins in tissue repair. Wound healing occurs when injury to the tissue causes the recruitment and activation of inflammatory cells and fibroblasts. Activated fibroblasts (myofibroblasts) proliferate and deposit ECM proteins such as collagens, fibronectin, laminin and glycosaminoglycans. ECM spreads to cover and regenerate the damaged tissue. When repeated injuries occur or during chronic inflammation, this process becomes uncontrolled resulting in excessive fibrotic scarring. Macular fibrosis in nAMD has unique pathological features. First, it originates from a pre-existing neovascular membrane. Therefore, it is a conversion of the diseased vessels into fibrous tissues. Second, the fibrous tissue often has a vascular network (Fig. 3) i.e., a fibrovascular membrane [14].

The origin of myofibroblasts and the underlying mechanisms of ECM deposition in macular fibrosis remain poorly defined. It has been suggested that the macular scar may originate from retinal glial cells (e.g., Müller cells, microglia and astroglia) [27]. Histological studies in surgically removed CNV membranes (CNVMs) from nAMD patients have reported multiple cell types, including endothelial cells, RPE, macrophages and fibroblast-like cells [28]. Importantly,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) positive cells, a characteristic marker of myofibroblasts have also been detected in CNVMs [29]. The results suggest that multiple cells may contribute to macular fibrosis.

### 4. Molecular mechanism of macular fibrosis in nAMD

The function of the myofibroblast is to synthesise and maintain ECM proteins. Since the retina and new blood vessels in nAMD do not contain fibroblasts, myofibroblasts in macular fibrosis must have been recruited or differentiated from other cells.

#### 4.1. The potential sources of myofibroblast precursors in macular fibrosis

##### 4.1.1. Glial cells

Müller glia and astrocytes provide framework support to retinal neurons and vasculatures. Under disease conditions, such as diabetic retinopathy and AMD, reactive Müller glia and astrocytes produce a variety of ECM and participate in retinal repair and remodeling [27]. Glial fibrillary acidic protein (GFAP) positive cells have been detected in an experimental model of subretinal fibrosis [30]. Under in vitro culture conditions, Müller cells lose the expression of glutamine synthetase and GFAP but gain the expression of  $\alpha$ -SMA suggesting that they have the potential to transdifferentiate into myofibroblasts [31]. Whether Müller cells can transdifferentiate into myofibroblasts in nAMD warrants further investigation.

##### 4.1.2. RPE cells

RPE cells are known to contribute to retinal fibrosis, including macular fibrosis [32,33] through Epithelial-Mesenchymal Transition (EMT).

The EMT is well-known as a contributing process in kidney, liver and lung fibrosis [34–36]. Clinical studies have observed higher incidence of fibrosis in classic CNV (compared with occult CNV) [13], in which the neovascular membrane penetrates the Bruch's membrane-RPE complex into the subretinal space. Damaged RPE cells may become myofibroblasts through EMT and contribute to macular fibrosis. Various in vitro studies have shown that RPE cells can undergo EMT under experimental conditions [32,37]. Senescent cells can secrete a number of matrix metalloproteinases (MMPs) that are important for limiting fibrosis [38]. However, chronic presence of MMPs by senescent cells can damage tissue structure and function by inducing EMT [38]. Senescent RPE cells in nAMD may induce EMT by secreting MMPs.

##### 4.1.3. Endothelial cells

Endothelial to mesenchymal transition (EndoMT) is known to contribute to organ fibrosis [39]. Endothelial cells of the neovascular membrane in nAMD are highly active, and they may transdifferentiate into myofibroblasts through EndoMT contributing to macular fibrosis. An EndoMT transcription factor, SNAI1 has been shown to critically contribute to the development of CNV [40]. The overall incidence of macular fibrosis has been reduced since the approval of intravitreal injection of VEGF inhibitors as a standard of care for nAMD. The results suggest that endothelial cells may contribute to macular fibrosis in nAMD.

##### 4.1.4. Macrophages

Macrophages play an important role in tissue repair and wound healing [41]. A previous study has shown that 20% of the cells in experimental CNV were macrophages, and the majority (70%) of macrophages originated from the bone marrow [42]. During the early stages of inflammation, pro-inflammatory M1-macrophages produce cytokines to eliminate pathogens. After the inflammatory response subsides, M2-macrophages facilitate tissue repair by stimulating cellular proliferation and differentiation. Both M1 and M2 macrophages can participate in tissue fibrosis by secreting transforming growth factor- $\beta$  (TGF $\beta$ ) [41]. Later, macrophages may switch to a pro-resolving phenotype that suppresses the tissue-damaging inflammation and dissolves excessive ECM proteins [41]. When the pathogenic stimuli persist for a prolonged period, such as in AMD, the inflammatory and repair process may be dysregulated or maladapted leading to destructive fibrosis.

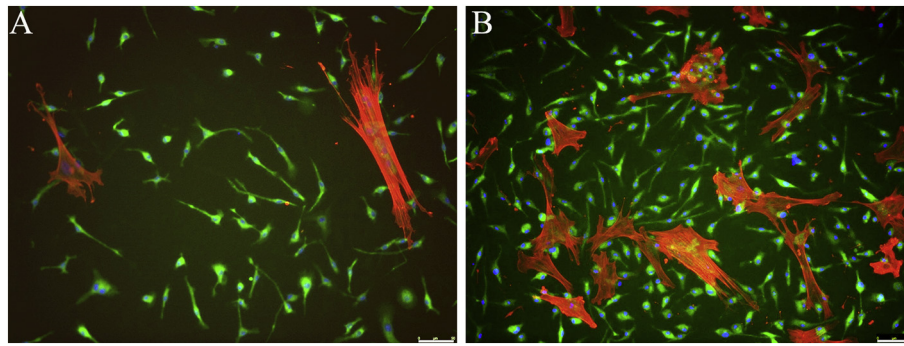
Macrophages can directly transdifferentiate to myofibroblasts, and this phenomenon is known to contribute to renal fibrosis [43]. Using lineage tracing studies, it was reported that the majority of macrophages undergoing MMT were CD206<sup>+</sup> M2 macrophages [44]. Macrophages are capable of producing ECM proteins. Under in vitro culture conditions, TGF $\beta$  can induce macrophage expression of  $\alpha$ -SMA (Fig. 4), collagen 1 and fibronectin, suggesting that they can undergo Macrophage to Myofibroblast Transition (MMT).

##### 4.1.5. Circulating fibrocytes

Circulating fibrocytes are a distinct population of blood-borne inactive mesenchymal cells that can enter tissue and become fibroblasts. They express the hematopoietic marker CD45, the progenitor marker CD34, and collagen. During tissue injury and inflammation, the levels of circulating fibrocytes are increased [45] and they may participate in wound healing and fibrotic tissue repair. Fibrocytes have been detected in CNVMs [28] and they may be an important source of myofibroblasts in macular fibrosis.

##### 4.1.6. Choroidal stromal cells

Choroidal stroma contains melanocytes, macrophages, fibroblasts, lymphocytes and mast cells, as well as collagen and elastic fibres. Various choroidal stromal cells have been detected in the experimental model CNV and fibrosis [28]. A recent study in a mouse model of CNV/fibrosis has shown that choroidal pericytes can infiltrate the CNV and differentiate into collagen 1-expressing cells [46]. The choroidal stromal cells may be a potential source of myofibroblast precursors.



**Fig. 4.** Macrophage-to-myofibroblast transition. A proportion of murine bone marrow derived macrophages treated with recombinant TGF- $\beta$  for (96 h). The cells were stained for F4/80 (green) and  $\alpha$ SMA (red) and imaged by confocal microscopy. (A) Two macrophages express  $\alpha$ SMA in control macrophages. (B) Many more cells express  $\alpha$ SMA in TGF $\beta$  treated macrophages.

#### 4.2. Molecular pathways involved in the recruitment of myofibroblast precursors

Clinical studies have shown nAMD eyes with persistent retinal and RPE disruption are more likely to develop fibrosis. This highlights the importance of retinal microenvironment in disease pathogenesis. A low-grade chronic inflammation is known to play an important role in AMD [47]. During inflammation, retinal cells (e.g., RPE and microglia) and infiltrating immune cells produce various cytokines and chemokines that may recruit myofibroblast precursors to the macula. For example, RPE, microglia and macrophages can all produce CCL2, a chemokine that plays an important role in the recruitment of CCR2<sup>+</sup> macrophages [48] and fibrocytes [49]. CCR2<sup>+</sup> macrophages critically contribute to the development of retinal neovascular membrane in laser-induced CNV [50] and chronic autoimmune uveoretinitis [51]. The cyclooxygenase-2 (COX-2) is also implicated in the recruitment of macrophages in the laser-induced CNV and selective inhibition of COX-2 suppresses CNV and subretinal fibrosis [52].

Fibrocytes express the chemokine receptors CCR2, CCR7 and CXCR4 that control their trafficking to sites of injury [53]. Different chemokine/chemokine receptor pathways are involved in fibrocyte trafficking under different pathological conditions. For example, in asthma, the CXCR4/CXCL12 axis critically controls fibrocyte infiltration during acute inflammation, whereas, the CCR7/CCL19 axis plays an important role in fibrocyte trafficking in chronic obstructive asthma [54]. In addition, the platelet-derived growth factor (PDGF) [55] and thymic stromal lymphopoietin [56] are also known to play a role in fibrocyte trafficking. The cells responsible for the recruitment of myofibroblast precursors in nAMD may include infiltrating macrophages, active endothelial cells and RPE. However, the underlying molecular pathways remain elusive.

#### 4.3. Molecular pathways involved in the differentiation and activation of myofibroblasts

##### 4.3.1. Transforming growth factor- $\beta$ (TGF $\beta$ )

TGF $\beta$  is the ‘master regulator’ of fibrosis. The TGF $\beta$  family consists of around 30 members, and many of them signal via the SMAD signaling pathway. TGF $\beta$  has been implicated in the EMT [32,34] and MMT [44]. Previous studies have shown that the TGF $\beta$ -induced EMT in RPE cells involves the mitogen-activated protein kinases (ERK, p38 and JNK), SMAD2 and AKT pathways [57], which can be attenuated by activating the retinoic acid receptor- $\gamma$  (RAR $\gamma$ ) pathway [57]. EMT may also be induced by non-SMAD signaling pathways such as the wnt/ $\beta$ -catenin pathway [58]. Reactive Oxygen Species (ROS) is known to activate fibroblasts and increase collagen deposition in fibrosis through converting the latent form of TGF $\beta$  into its active form [59]. Oxidative stress is a major contributor of AMD, and ROS production by RPE or inflammatory cells may participate in macular fibrosis through activating TGF $\beta$ .

##### 4.3.2. Platelet-derived growth factor (PDGF)

PDGF is a potent mitogen for mesenchymal cells such as myofibroblasts [60]. It also critically controls their migration and proliferation [60] and plays an important role in tissue repair, remodeling and organ fibrosis [60]. PDGF is critically involved in the pathogenesis of nAMD. A phase-2 clinical trial in nAMD using a dual antagonism of PDGF (Fovista) and VEGF (Ranibizumab) has observed significant benefits, including reduced macular fibrosis, compared with Ranibizumab monotherapy [61]. However, a follow-up phase-3 study (NCT01940900) did not confirm the improvement in visual acuity. Interestingly, a recent in vitro study has shown that a combined VEGF/PDGF inhibition induced a pro-fibrotic phenotype in human pericytes [62]. Further mechanistic studies may help to understand the role PDGF in retinal fibrosis.

##### 4.3.3. Connective tissue growth factor (CTGF)

CTGF is an extracellular matrix-associated heparin-binding protein produced by many tissue cells including vascular endothelial cells [63]. CTGF is critically involved in angiogenesis, tissue repair, and fibrosis through stimulating activation and proliferation of fibroblasts [64]. The role of CTGF in choroidal neovascularization [65] and retinal fibrosis [66] has been recognized for over a decade. A phase 1/2 trial with RXI-109, a self-delivering CTGF-RNAi for retinal fibrosis in nAMD has shown that the compound is well-tolerated when injected intravitreally (NCT02599064) although the therapeutic potential remains to be evaluated.

##### 4.3.4. Epidermal growth factor (EGF)

EGF is a small polypeptide of 53 amino acid residues produced by many cells and is one of the best characterized epithelial cell mitogens [67]. EGF binds to its receptor EGFR resulting in cellular proliferation, differentiation, and survival [67]. Recent studies have shown that EGFR signaling is also critically involved in TGF $\beta$ -induced fibrotic gene expression [68]. Significantly higher levels of EGF have been detected in the aqueous humour of nAMD patients compared with cataract patients [69]. A recent study has shown that cigarette smoke-induced EMT in RPE cells is mediated through activation of the EGFR signaling pathway [70]. The role of EGF-EGFR signaling pathway in macular fibrosis warrants further investigation.

##### 4.3.5. Fibroblast growth factor (FGF)

The FGFs are a family of cell signaling proteins consisting of at least 22 members and 4 receptors (FGFR1–4) [71]. The FGF-FGFR signaling responses are involved in diverse biological processes, including proliferation, migration, differentiation and apoptosis [71]. It has been reported that FGF2 is involved in the development of fibrosis in the bone marrow [72], lung [73], and retina [74]. Pharmacological inhibition

of FGF2 in combination with VEGF inhibition is more effective in reducing CNV than inhibition of VEGF alone [74].

#### 4.3.6. Sphingosine 1-phosphate (S1P)

S1P is implicated in the process of differentiation of fibroblasts to myofibroblasts and the EMT [75]. A previous study has shown that S1P is expressed at the lesion site in a mouse model of laser-induced CNV/subretinal fibrosis and intravitreal injection of anti-S1P monoclonal antibodies reduces experimental CNV and retinal fibrosis [76]. S1P may play a role in macular fibrosis in nAMD.

#### 4.3.7. Alpha B-crystallin

$\alpha$ B-crystallin is a major protein of the eye lens belonging to the small heat-shock protein family.  $\alpha$ B-crystallin is also found in various non-ocular tissues and acts as a molecular chaperone by preventing aggregation of proteins, inhibiting apoptosis and inflammation, and maintaining cytoskeletal architecture [77].  $\alpha$ B-crystallin is reported to be involved in tissue fibrosis including the retina [33].  $\alpha$ B-crystallin can induce EMT and enhance TGF $\beta$ -induced EMT in RPE cells through the SMAD4 pathway [33].

#### 4.3.8. Galectin

Galectins are S-type soluble lectins that bind specifically to  $\beta$ -galactoside sugars with intra- and extracellular functions. Galectins are known to play critical roles in cell-cell adhesion, cell-matrix interactions, macrophage activation, angiogenesis, metastasis, and apoptosis. Previous studies have shown that galectin-3 is involved in TGF $\beta$ -mediated myofibroblast activation [78] and increased plasma levels of galectin-3 are associated with myocardial and vascular fibrosis [79] and systemic sclerosis [80]. Although macular fibrosis in nAMD is a vascularized fibrotic lesion, we did not detect any difference in the plasma levels of galectin-3 between nAMD patients with and without macular fibrosis and age-matched healthy controls (Yang et al., unpublished observation). Galectin-1 was found to be upregulated in RPE in the laser-CNV, and was reported to induce CNV-associated sub-retinal fibrosis through EMT [81]. The role of galectins in macular fibrosis in nAMD patients remains to be elucidated.

#### 4.3.9. Decorin

Decorin is a proteoglycan that binds to collagen fibrils in the ECM. It can also interact with various growth factors and their receptors, including TGF $\beta$  and participate in angiogenesis and fibrosis [82]. A recent study has shown that increased decorin degradation is related to fibrotic lung disorders [83]. Interestingly, we did not observe any difference in the serum levels of decorin between nAMD patients and age-matched controls. There was also no difference in serum levels of decorin between patients with macular fibrosis and those without (Yang et al., unpublished observation). Whether the function of decorin is altered in nAMD patients warrant further investigation.

#### 4.3.10. Complement proteins

The complement system is an important arm of the innate immune system, and dysregulated complement activation is known to be involved in AMD [84,85]. Uncontrolled complement activation may contribute to retinal pathology directly by damaging retinal cells using the membrane attack complex (MAC, C5b-9) or indirectly by modulating inflammation with complement fragments e.g., C3a, C3b, and C5a [84]. Previously, we reported that the plasma levels of C3a and C5a were significantly increased in nAMD patients, particularly those with macular fibrosis [86].

C5 polymorphisms have been reported in liver fibrosis [87]. Deletion of C5 or blocking C5a receptor suppresses fibrosis in a mouse model of late stage pancreatitis [88]. Furthermore, C3a can induce EMT in proximal tubular epithelial cells [89] and C3aR1 deletion inhibits interstitial fibrosis in mice [89]. We have found that C3a can induce MMT (Little

et al. ARVO 2018 Abstract). C3a and C5a may participate in macular fibrosis in nAMD.

## 5. Therapeutic considerations

Macular fibrosis in nAMD develops from existing neovascular membrane. If the neovascular membrane is eliminated at the very early stage of nAMD, fibrosis is unlikely to occur. Unfortunately, the majority of CNV lesions cannot be completely eliminated with existing anti-VEGF therapy. Furthermore, ~30% of nAMD patients do not respond to anti-VEGF therapy, and the reasons behind this remain unclear. Future studies may seek to understand this sub-set of patients, with aims to provide a preventative treatment strategy for those susceptible to macular fibrosis.

When the neovascular lesion cannot be eliminated promptly, approaches should be taken to prevent fibrosis in conjunction with VEGF inhibition. This may include inhibiting ECM production at the initiation/progression stages and promoting ECM degradation and uptake at late stages of fibrosis (e.g., the development of anti-fibrotic macrophages).

Myofibroblasts are key for the development of macular fibrosis. Strategies to prevent the recruitment of myofibroblast precursors or inhibit the activation or transdifferentiation of the precursors should benefit nAMD patients. The challenge is to identify key molecules responsible for the recruitment or the activation/transdifferentiation of myofibroblast precursors. Among various growth factors discussed here, only PDGF and CTGF have been tested in nAMD patients. Whilst the therapeutic effect of CTGF inhibition remains to be evaluated, the inhibition of PDGF failed to show any additional benefit in patients with VEGF inhibition in phase 3 trials. This highlights the complexity of the disease mechanism and the need for further understanding of the pathogenesis of the disease.

## 6. Outstanding questions and challenges

Recent clinical studies have uncovered the morphological features and the dynamic changes of the fibrotic lesion during different stages of nAMD and following anti-VEGF therapy. However, the underlying mechanism remains elusive. The pathogenesis of macular fibrosis in nAMD is different from a normal wound-healing fibrotic scar as it arises from a pre-existing neovascular membrane. The neovascular membranes that originate from the retina (i.e., RAP) or invade the retina from the choroid (i.e., classic CNV) or those with refractory intraretinal cysts are more likely to convert into fibrous lesions, highlighting the importance of retinal microenvironment in disease process. Fibroblasts do not exist in the macula, nor in the abnormal new blood vessels. Future researches should aim to understand the sources of myofibroblast precursors, the molecular cues that attract them to macular neovascular membranes and the pathways underlying their differentiation and activation.

To address these important outstanding questions, we need to overcome a number challenges. For example, laboratory examination of clinically well-documented fibrotic samples will be extremely helpful to understand the cellular and molecular base of clinical presentations of macular fibrosis. However, as surgery is no longer a treatment option, fibrotic macula can only be obtained through donations, and matching the clinical phenotype of the donated eye is often not possible.

Animal models are powerful tools to dissect disease pathways. Currently, rodents are most commonly used to study retinal fibrosis. As rodents have no macula, the models cannot truthfully replicate the complexity of macular fibrosis in patients. Nevertheless, they are valuable in dissecting specific pathways or testing drug candidates. For example, the subretinal Matrigel implantation induced CNV and fibrosis in rats has been used to test the therapeutic effect of VEGF traps [90]. The model may also be useful in testing anti-fibrosis drugs. Although it can persist for 35 days [91], the subretinal fibrosis following the

laser-induced CNV, contains no blood vessels – a key characteristic of human macular fibrosis in nAMD (Fig. 3) [29]. The model, therefore, has limited value in dissecting the molecular mechanism of macular fibrosis. The injection of peritoneal macrophages into the subretinal space immediately after the laser-induced CNV has resulted in a larger size of subretinal fibrosis [30]. The model has not been widely used due to the technical challenges (e.g., macrophages need to be injected precisely into the laser spot). Better animal models are urgently needed to understand the pathogenesis of macular fibrosis.

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## Search strategy and selection criteria

Search strategy and selection criteria Data for this Review were identified by searches of MEDLINE, Current Contents, PubMed, and references from relevant articles using the search terms “neovascular AMD”, “macular fibrosis”, “optical coherence tomography”, “optical coherence tomography angiography”, and “myofibroblast”. Abstracts and reports from meetings were included only when they related directly to previously published work. Articles published in English between 1875 and 2018 were included.

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