THE ROLE OF MICRORNA24 IN OCULAR FIBROSIS

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The topic for this thesis is the role of microRNA24 in ocular fibrosis, with an emphasis on this small biomolecule’s potential to minimize a fibrotic response in ocular pathologies. This thesis investigates the hypothesis that microRNA24 is capable of preventing endothelial to mesenchymal cell transition (EndoMT) as well as epithelial to mesenchymal transition (EMT) to thus minimize subretinal fibrotic scarring and associated damage in retinal pathologies such as age-related macular degeneration. Furthermore, this investigation seeks to further elucidate the mechanisms by which microRNA24 may act to minimize or prevent fibrosis. Chapter 1 discusses the rationale for this investigation, detailing the role and mechanisms of ocular fibrosis, particularly in retinal pathologies, and reviewing the established effects of microRNA24. Additionally, Chapter 1 addresses the therapeutic relevance of this research and focuses the research goals into explicit aims. Chapter 2 presents results from in vitro experiments which suggest microRNA24 has the potential to prevent EMT of retinal pigment epithelium cells, while Chapter 3 discusses experimental data which may indicate that microRNA24 can also prevent EndoMT of endothelial cells. Chapter 4 assesses the contributions of the thesis to the field, evaluates completion of research aims, and suggests further experiments. This thesis presents novel findings that suggest microRNA24 may have the potential to prevent both EMT and EndoMT, which could ameliorate ocular fibrosis and have significant therapeutic impact.
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CHAPTER 1

Ocular Fibrosis and Associated Pathologies

Fibrosis is a wound healing response to tissue injury in which excess extracellular matrix connective tissue is formed. This tissue injury can be mechanical or caused by disease. Pathological fibrosis, in which this excess fibrous tissue occurs as a pathological process and replaces normal parenchymal tissue, is known to occur in various organs including the lungs, heart, kidney, liver, and eye. Ocular fibrosis is associated with many different ocular diseases and can occur in several different eye tissues, harming patients’ vision. Many of the most prevalent causes of vision deterioration, such as cataracts, diabetic retinopathy, glaucoma, myopic choroidal neovascularization, and age-related macular degeneration (AMD), are associated with ocular fibrosis [1]. Fibroblasts also play important roles in other ocular conditions such as Graves’ Ophthalmopathy, pterygia, proliferative vitreoretinopathy (PVR) and the eye’s response to trauma resulting from injury or surgery e.g., corneal epithelial-stromal injury and glaucoma filtration surgery [1]. Though the molecular mechanisms of ocular fibrosis have not yet been fully elucidated, recent research in the field has revealed many of the potential cell sources, factors, and pathways involved.

AMD is the leading cause of blindness in the elderly and characterized by drusen deposits and retinal pigment epithelium (RPE) deterioration leading to photoreceptor loss and progressive degeneration of the macula (the area of the retina with a high density of photoreceptors and responsible for central vision). Early stages of AMD can progress to two late stages: dry/atrophic AMD and wet/neovascular AMD. Dry AMD is defined by atrophy of the macula while wet/neovascular AMD (nAMD) refers to the abnormal
growth of new blood vessels into the macula. The current treatment for nAMD is the intravitreal injection of vascular endothelial growth factor inhibitors (anti-VEGF therapy) to restrict this neovascularization by inhibiting angiogenic cytokines [2]. Unfortunately, subretinal fibrosis develops in about one third of the AMD patients who are treated with anti-VEGF therapy, preventing their condition from improving and causing further damage to the RPE and photoreceptors [3]. Macular fibrosis developed even more frequently in patients before anti-VEGF treatment was introduced, with the prevalence having ranged from 39%-100% [3]. In addition to AMD, CNV is also a frequent and serious complication of pathological myopia (PM) which is a leading cause of vision impairment worldwide. Myopic CNV (mCNV) is developed by 5%-11% of PM patients and the primary treatment method is anti-VEGF injection like it is for nAMD patients. A 2020 clinical study found the incidence of subretinal fibrosis after anti-VEGF treatment to be 40.7% in eyes with mCNV [4].

Figure 1. Stages of AMD (ARMD)[5]
Macular fibrosis is identified clinically 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” [3]. This can be visualized through fundoscopy and confirmed by optical coherence tomography (OCT), fluorescein angiography (FA), and color fundus tomography [4]. Subretinal fibrosis is classified into three stages based on its progression: minimal, prominent, and hyper-reflexive and imaging has shown the fibrotic lesions to be “vascularized fibrotic membranes” [3]. Clinical nAMD is divided into three categories based on the source of the abnormal blood vessel growth: polypoidal choroidal vasculopathy (PCV), retinal angiomatous proliferation (RAP), and choroidal neovascularization (CNV) which is classified as classic or occult based on the CNV growth pattern [3]. Significant photoreceptor cell loss is associated with regions over subretinal fibrotic tissues thicker than 200 µm [4].

![Subretinal fibrotic scarring in late-stage nAMD](image)

**Figure 2. Subretinal fibrotic scarring in late-stage nAMD [6]**

**Cellular Sources for Ocular Fibrosis**

The excess extracellular matrix (ECM) connective tissue that is formed during pathological fibrosis arises primarily from myofibroblasts (activated fibroblasts) which deposit ECM proteins and “exert synchronized tractional forces across the ECM, resulting in distortion of tissue architecture and subsequent scarring” [2]. Myofibroblast
transdifferentiation is a key process in fibrosis but the mechanism and sources of these myofibroblasts in ocular fibrosis have not yet been fully identified. Myofibroblasts have been identified in surgically removed CNV membranes from nAMD patients based on the presence of myofibroblast markers such as α-smooth muscle actin (α-SMA); other cell types such as endothelial cells, RPE cells, macrophages, and fibroblast-like cells have also been identified in these membranes [2]. These various cell types interact with growth factors and inflammatory cytokines causing considerable modifications to ECM structure [7]. Macular fibrosis in diseases such as AMD differs from fibrosis in other areas of the body in that it arises from the preexisting neovascular membrane, converting these diseased vessels into fibrous tissue which frequently has a vascular network [3]. There are multiple potential sources of myofibroblasts in subretinal fibrosis which must arise from differentiation of other cell types or recruitment from other places in the body since fibroblasts are not contained by the retina or blood vessels in nAMD [3].

**Epithelial-Mesenchymal Transition of RPE Cells**

RPE cells contribute to retinal fibrosis through Epithelial-Mesenchymal transition (EMT), a process which is well defined in its contribution to other types of fibrosis in the body and which “is the process by which polarized epithelial cells undergo several morphologic and molecular changes to give rise to motile, extracellular matrix-producing mesenchymal cells” [2]. These mesenchymal cells are migratory and invasive and can become myofibroblasts. EMT is classified into Type I, Type II, and Type III, with Type II being activated normally during tissue regeneration but which can be activated abnormally by dysregulated inflammatory responses during fibrosis [2]. When the cell-cell contacts of RPE cells are disrupted and EMT is initiated, the cells decrease
expression of epithelial markers such as E-cadherin and ZO-1 and increase production of mesenchymal markers such as N-cadherin, vimentin, and α-SMA [7]. Damaged RPE cells may become myofibroblasts through EMT which has been demonstrated in many *in vitro* experiments [3]. Studies have also shown that although senescent cells can produce matrix metalloproteinases (MMPs) which have a role in degrading ECM components and inhibiting fibrosis, chronic presences of these MMPs may actually induce EMT, indicating that by secreting MMPs, senescent RPE cells may contribute to fibrosis in diseases such as nAMD by inducing EMT [3].

![Figure 3. Epithelial to mesenchymal transition](image)

*Figure 3. Epithelial to mesenchymal transition* [8]

**Endothelial to Mesenchymal Transition**

Endothelial to mesenchymal transition (EndoMT) is an analogous process to EMT in which endothelial cells stop expressing endothelial markers such as vascular endothelial-cadherin (VE-cadherin), platelet-endothelial cell adhesion molecule 1 (PECAM-1), and von Willebrand Factor (vWF) and begin expressing mesenchymal markers [9]. This process is known to contribute to fibrosis in other organs and preliminary studies suggest this process may also contribute to retinal fibrosis since endothelial cells in the neovascular membrane of nAMD are very active and SNAI1, an EndoMT transcription factor, has been established to be an important contributor to early ocular angiogenesis in AMD and CNV development [3, 9].
Macrophage to Myofibroblast Transition

Macrophages are prevalent in CNV tissue and have a crucial, complex role in inflammatory responses. Macrophages can produce the ECM proteins fibronectin and collagen 1, and both type M1 and M2 macrophages can contribute to fibrosis by secreting profibrotic factors such as transforming growth factor-β (TGFβ), the “master regulator” of fibrosis, and platelet-derived growth factor (PDGF) though these cells may later secrete factors that actually suppress fibrosis [3, 7]. Moreover, macrophages are known to directly transdifferentiate to myofibroblasts in other organs such as the kidney and experiments have shown that TGFβ treatment of in vitro cultured macrophages can generate expression of myofibroblast markers including α-SMA, fibronectin, and collagen I. This evidence indicates Macrophage to Myofibroblast Transition (MMT) may contribute to ocular fibrosis [3].

Other Cellular Sources of Myofibroblasts

In addition to the aforementioned potential myofibroblast precursors in retinal fibrosis, evidence from studies of CNVMs has suggested that bone-marrow derived cells
other than and inclusive of bone-marrow derived macrophages may be systemic sources for α-SMA-positive cells [7]. In diseases such as AMD and diabetic retinopathy, astrocytes and Müller glial cells generate ECM proteins; cells positive for glial fibrillary acidic protein (GFAP) have been found in subretinal fibrosis models and Müller glial cells have been shown to start expressing α-SMA and halting expression of GFAP, indicating they may be able to transdifferentiate into myofibroblasts in diseases such as nAMD [3]. Other potential sources of myofibroblasts in retinal fibrosis include circulating fibrocytes, which are blood-borne inactive mesenchymal cells and have been identified in CNVMS, and choroidal stromal cells, some of which have been shown to penetrate the CNV and differentiate to express collagen-1 [3].

**Mechanisms of Retinal Fibrosis**

Just as the sources of myofibroblasts in retinal fibrosis are not completely known, the mechanism by which these myofibroblast precursors are recruited is not fully elucidated. Based on research so far it appears that macrophages, RPE cells, and active endothelial cells have roles in recruiting myofibroblast precursors in nAMD [3]. CCR2+ macrophages can be recruited by the chemokine CCL2 which can be produced by RPE, macrophages, and microglia. In laser-induced CNV, cyclooxygenase-2 (COX-2) has also been shown to be involved in macrophage recruitment. Additionally, several different chemokines influence where fibrocytes are directed and likely play a role in directing fibrocytes to the retina, although which specific chemokines these are have not yet been identified. PDGF and thymic stromal lymphopoietin affect fibrocyte recruitment and could be important in retinal fibrosis [3].
There are several complex pathways known to be associated with the
differentiation and activation of myofibroblasts in ocular fibrosis though the underlying
molecular mechanisms are not yet fully understood. As previously mentioned, TGFβ is a
cytokine known as the master regulator of fibrosis and has been shown to influence both
EMT and MMT. Of the roughly 30 members of the TGFβ family, most of them utilize
the SMAD signaling pathway -- causing the recruitment and activation of SMAD family
transcription factors and leading to the expression of mesenchymal genes [3]. Past
research has demonstrated that induction of EMT in RPE cells by TGFβ involves the
SMAD2 and AKT signaling pathways, which are mitogen-activated protein kinases and
that activation of the retinoic acid receptor-γ (RARγ) pathway can diminish this response
[3]. Experiments have indicated that TGFβ can only induce RPE cells that have
undergone EMT to transdifferentiate into myofibroblasts and does not induce EMT in
differentiated RPE cells with “well-established cell contacts,” indicating that the tight
junctions between RPE cells must be disrupted for EMT to occur [7, 11]. Other signaling
pathways such as the wnt/β-catenin pathway and Notch signaling pathway can also
mediate EMT [7]. The signaling pathways that induce EMT do so by causing up-
regulation of downstream transcription factors including Snai1, Slug, zinc finger E-box
binding homeobox (ZEB)1, and ZEB2 (SIP1) [7]. Furthermore, the elevated intracellular
levels of reactive oxygen species (ROS) associated with oxidative stress and involved in
the pathogenesis of AMD may also contribute to retinal fibrosis because ROS convert
TGFβ from its inactive form to its active one which activates fibroblasts [3]. In addition
to TGFβ signaling, mechanical tension resulting from “increased stiffness of the
underlying ECM” is also required for full myofibroblast differentiation; the underlying
mechanism is unclear but recently myocardin-related transcription factor (MRTF) has been identified as playing a role in this process [11]. Another growth factor, PDGF, is also known to be involved in fibrosis. PDGF triggers mitosis in mesenchymal cells including myofibroblasts and regulates these cells’ proliferation and migration though PDGF’s mechanistic role in retinal fibrosis is still unclear [3]. Another factor which has long been known to influence ocular fibrosis is connective tissue growth factor (CTGF) which also can activate fibroblasts and trigger their proliferation [3]. Epidermal growth factor (EGF) binding to its receptor (EGFR) has been known to stimulate cell differentiation, proliferation, and survival. Activation of the EGFR signaling pathway has also been shown to be involved in “TGFβ fibrotic gene expression” and cigarette smoke-induced EMT in RPE cells though its mechanisms are not fully understood [3]. Fibroblast growth factor 2 (FGF2) has been shown to play a role in fibrotic development in the lungs, bone marrow, and retina [3]. These growth factors can promote fibrotic activities in myofibroblasts such as cell proliferation, migration, and ECM remodeling through multiple complex pathways with signaling network interplay including P38 Mitogen-activated protein kinase (MAPK), p44/p42 MAPK (ERK1/2), phosphatidylinositol-3 kinase (PI3K)/Akt (protein kinase B), and SMAD [7]. Additionally, sphingosine 1-phosphate (S1P) is a sphingolipid involved in EMT and the differentiation of fibroblasts to myofibroblasts which likely contributes to retinal fibrosis [3]. The heat-shock family protein αB-crystallin has been shown to induce EMT and boost TGFβ-induced EMT through the SMAD4 pathway and is implicated in retinal fibrosis [3]. Although the role of galectins in retinal fibrosis is not fully understood, upregulated galectin-1 has been associated with EMT induction in CNV-associated sub-retinal fibrosis and galectin-3 has
been shown to be “involved in TGFβ-mediated myofibroblast activation” and upregulated in fibrosis in other organs [3]. Deletion of Lgals1 (the gene encoding Galectin-1) has been shown to suppress CNV and attenuate subretinal fibrosis, expression of EMT markers including Snai1, and phosphorylation of SMAD2 [12]. Degradation of the proteoglycan decorin is reportedly associated with lung fibrosis, but it is still unclear whether decorin function is modified in conditions that lead to ocular fibrosis such as nAMD [3]. Dysregulation of the immune system’s complement system is associated with AMD and may be involved in retinal fibrosis; certain complement protein abnormalities have been identified as promoting EMT and MMT, particularly C3a and C5a, which may play a role in macular fibrosis [3]. Adiponectin is an inflammatory cytokine which is known to play both pro- and anti-fibrotic roles depending on the location in the body and has been suggested as a pro-fibrotic agent in retinal fibrosis based on its increased presence in PVR and PDR patients [13].

**Subretinal Fibrotic Markers and ECM Components**

In subretinal fibrosis, the most prevalent components of the ECM are fibronectin (FN) and collagen I and IV with smaller amounts of collagen types III, V, and VI present [7]. Collagen IV is present in the basal membrane of healthy RPE, but collagen I, which supports the structure of the fibrotic tissue, is not normally present and is synthesized during retinal fibrosis [7]. Fibronectin secretion is induced by TGFβ and CTGF and offers a scaffold for RPE cells and macrophages to migrate to the fibrotic lesion [7]. Thrombospondin 1 (TSP1), tenascin (TNC), and osteonectin (SPARC) are matricellular proteins which have been identified in fibrotic lesions of nCNV and act as regulators of fibrosis by “binding to growth factor receptors or integrins on the cell surface and
influencing the down-stream signaling associated with cell proliferation, migration, and adhesion” [7]. TSP1 is known to activate TGFβ through integrin binding and both TSP1 and SPARC increase RPE migration [7]. Periostin (POSTN) acts like an autocrine agent, activating focal adhesion kinase (FAK) and AKT phosphorylation which promotes collagen synthesis, cell adhesion, and migration [7].

**MicroRNA24 and Fibrosis**

MicroRNAs are small endogenous noncoding RNA molecules which are involved in post-transcriptional gene regulation. They repress expression of target genes by binding complementary regions of specific target mRNA molecules and either specifying cleavage or blocking translation [14]. MicroRNA24 (miR-24) is one such molecule which is thought to act as a “tumor suppressor by regulating cell cycle progression, apoptosis, and DNA damage responses through several validated targets” [15]. A 2014 study from Dr. Shusheng Wang’s lab demonstrated that miR-24 regulates actin cytoskeleton pathways by targeting Pak4, Limk2, and Diahp1 proteins which are downstream of Rho signaling [16]. Overexpression of miR-24 in endothelial cells was found to repress endothelial cell migration, proliferation, and tube formation, as well as inhibit stress fiber and lamellipodia (cytoskeletal actin projections at the leading edge of motile cells) formation [16]. This investigation’s findings suggest that miR-24 can repress angiogenesis by regulation of actin cytoskeleton pathways, indicating that it could be a potential therapy for neovascularization as seen in diseases such as nAMD [16]. Furthermore, a 2020 investigation found that overexpression of miR-24 in human umbilical vein endothelial cells inhibited these cells’ migration, proliferation, and tube formation abilities by targeting endothelial nitric oxide synthase (eNOS) and Sp1, which
is a transcription factor for eNOS, although the regulatory effects of miR-24 on angiogenesis have not been fully elucidated [17].

MiR-24 has also been implicated in fibrosis in various physiological processes. In skeletal muscle fibrosis mediated by TGFβ, miR-24 was found to be downregulated and SMAD2 (involved in the TGFβ pathway) was identified as a target of miR-24. In vitro experiments demonstrated that overexpression of miR-24 downregulated fibrotic markers in cells treated with TGFβ to induce fibrosis and murine in vivo experiments indicated that overexpression of miR-24 downregulated fibrosis in injured skeletal muscle [18]. Additionally, a study on trabecular meshwork cells from the human eye identified FURIN as a novel target of miR-24 and determined that upregulating miR-24 may reduce the extent of fibrosis by reducing the amount of TGFβ activated by mechanical stress [15]. Preliminary results from our previous experiments indicate that overexpression of miR-24 in an in vitro EMT and fibrosis model downregulated both mesenchymal and fibroblast markers compared to controls (Wu, Y.; Byrnes, K.; Wang, S., unpublished results). In combination with the current understanding of the mechanisms and cell sources of retinal fibrosis, as outlined in this chapter, these findings inform the hypothesis that miR-24 may prevent EMT of RPE cells, EndoMT, and MMT to reduce pathological fibrosis in diseases such as AMD.

**Risk Factors and Epidemiology of Retinal Fibrosis**

Although it is still unclear exactly what determines whether certain patients develop subretinal fibrosis after anti-VEGF therapy, certain risk factors have been identified. Identified risk factors for subretinal fibrosis among nAMD patients include lower 25-hydroxyvitamin D plasma levels, the nAMD genotype CFH Y402H CC, the
presence of subretinal hyper-reflective material (SHRM) in RPE, classic CNV, poorer visual acuity at first presentation, a longer interval between diagnosis and anti-VEGF treatment, macular hemorrhage, and refractory intraretinal cysts. Furthermore, nAMD patients with RAP develop fibrosis more often than those with PCV. Researchers have used the SHRM thickness, location, and border definition to predict a patient’s likelihood of developing subretinal fibrosis [3]. In patients with mCNV treated with anti-VEGF therapy, “baseline CNV size and leakage were significantly progressive or more extensive in eyes with subretinal fibrosis compared with eyes without subretinal fibrosis” [4]. For mCNV patients who undergo anti-VEGF therapy, possible risk factors for fibrosis include a baseline best-corrected visual acuity (BCVA) score below 60 Early Treatment Diabetic Retinopathy Study (ETDRS) letters, a baseline macular integrity index (MI) score below 20, and patient age of less than 45 years [4]. mCNV location has also been shown to influence fibrotic scarring likelihood, with subfoveal mCNV being highly linked to subretinal fibrosis [4]. In proliferative diabetic retinopathy (PDR), studies have shown an increased ratio of pro-fibrotic connective tissue growth factor to VEGF levels in the vitreous after anti-VEGF treatment may be a cause of fibrosis [7]. According to histopathologic research on human AMD eyes, the diameter and thickness of subretinal fibrotic lesions is proportional to the progression of photoreceptor degeneration and “subretinal fibrosis may evolve along with regression of CNV in nAMD” [7]. Further research in large longitudinal human studies using detailed clinical phenotyping, imaging, and tissue banking have been proposed to deepen the field’s knowledge of risk factors for ocular fibrosis [19].
As discussed previously, nAMD often results in retinal fibrosis even after anti-VEGF therapy. AMD is responsible for 8.7% of all blindness worldwide and is the most prevalent cause of blindness in developed countries [21]. A 2014 meta-analysis which integrated 39 studies on the prevalence of AMD projected 288 million people to have AMD in 2040 and found the pooled prevalence to be 8.01% for early stage AMD, 0.37% for late stage AMD, and 8.69% for any stage of AMD [21]. Furthermore, Europeans were found to exhibit a higher prevalence of early and any stage AMD compared to Asians and a higher prevalence of early, late, and any stage AMD compared to Africans, while no significant differences were found between genders [21]. Another epidemiological study determined that AMD affected more than 1.75 million people in the United States in 2004 and projected this number to increase to nearly 3 million by 2020 [22]. Results from a study published in 2020 indicated that the incidence of subretinal fibrosis after up to 10 years of anti-VEGF therapy increased from 20.4% during the first year interval of treatment to 40.7% at year interval 9-10 [23]. Given that retinal fibrosis develops so prevalently in nAMD patients after anti-VEGF treatment and even more frequently without anti-VEGF treatment, as well in patients suffering from other ocular pathologies, the need for an effective treatment for retinal fibrosis is immense.
Treatments and Therapeutic Considerations for Retinal Fibrosis

Unfortunately, there is not currently a treatment that can eliminate retinal fibrosis. Macular fibrosis can be essentially prevented if the neovascular membrane is eliminated during early nAMD since the fibrosis arises from the existing neovascular membrane [3]. However, in most cases, anti-VEGF therapy cannot completely eliminate CNV lesions and about 30% of patients do not respond to anti-VEGF therapy for reasons which are still unclear [3]. Therefore, treatments that inhibit the progression of fibrosis in conjunction with anti-VEGF treatment should offer patients the best chance of preserving retinal function and sight [3]. A treatment that inhibits fibrosis progression could work by “inhibiting ECM production at the initiation/progression stages and promoting ECM degradation and uptake at late stages of fibrosis” [3]. Current research seeks to identify certain molecules with these capabilities, particularly focusing on what compounds could “prevent the recruitment of myofibroblast precursors or inhibit the activation or transdifferentiation of precursors.” Several promising therapies have been established, but of the growth factors discussed in this chapter, only the inhibition of CTGF and

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Figure 6. 6A: U.S. projections for AMD (2010-2030-2050); 6B: 2010 U.S. prevalent cases of AMD (in thousands) by race/ethnicity [24]
PDGF has been tested in human nAMD patients, with results so far being inconclusive for CTGF inhibition and indicating that PDGF inhibition may not further benefit patients undergoing anti-VEGF treatment in phase 3 clinical trials [3].

One potential therapeutic development is the neutralization of S1P, which as previously mentioned is a pro-fibrotic molecule involved in EMT and the differentiation of fibroblasts to myofibroblasts and which interacts with “profibrotic mediators such as PDGF, TGFβ, and CTGF,” as a potential target [7]. Studies using intravitreal monoclonal antibody treatment against S1P in a laser-induced CNV mouse model investigation resulted in less collagen deposition, indicating this has the potential to suppress fibrosis and be developed into a human treatment [7]. Because neutralizing S1P by this antibody treatment “addresses a neovascularization pathway separate from that activated by VEGF,” combining this treatment with anti-VEGF therapy could maximize these therapeutic benefits [25]. Human clinical trials are being conducted to evaluate the potential of S1P neutralization using monoclonal antibodies to inhibit subretinal fibrosis [2]. Another agent with therapeutic potential is WEB2086, a novel antagonist for the platelet-activating factor receptor (PAF-R) which is implicated in angiogenesis has been shown to inhibit CNV and subretinal fibrosis in a mouse model by inhibiting macrophage infiltration, VEGF production, and inflammatory molecule production [26]. Additionally, a preclinical study has indicated that combined inhibition of PDGF in addition to VEGF inhibition more effectively regresses CNV in a mouse model than solely anti-VEGF therapy [7]. Current research is also investigating the efficacy of regulating gene expression by microRNAs to inhibit both VEGF and PDGF [7]. Combining PDGF and VEGF inhibition as a treatment has the potential to not only more effectively reduce
CNV but also reduce subretinal fibrosis as a consequence of this improved CNV regression [7]. Current clinical trials are determining the therapeutic potential of these dual VEGF/PDGF inhibitors in nAMD: E10030 (Ophtotech), “an anti-PDGF pegylated aptamer” in combination with anti-VEGF treatment; Pazopanib, a VEGF receptor (VEGFR), PDGF receptor (PDGFR), and c-kit (a type of receptor tyrosine kinase) inhibitor; and Sorafenib, an inhibitor of Raf kinases, VEGFR, and PDGFR [7]. Doxycycline, a semi-synthetic tetracycline, has also been identified as a potential therapeutic agent which could “inhibit pro-fibrotic/angiogenic macrophages and the following angiogenesis, and then attenuate the EMT and subretinal fibrosis induced by the STAT6 pathway” and could be combined with anti-VEGF therapy for maximum efficacy [27]. Additionally, a 2019 study using a laser-induced CNV mouse model demonstrated the potential of a novel single-stranded RNA interference (RNAi) agent that targeted both human and mouse RNA involved in the production of the (pro)renin receptor in reducing CNV formation and attenuating subretinal fibrosis [28]. Additionally, studies have indicated SMAD7 gene therapy as promising for corneal stromal fibrosis, endothelial wound healing, injury-induced lens fibrosis, and reduction of EMT in RPE cells; bone morphogenetic proteins (which have been implicated with SMAD7 in inhibiting TGFβ signaling) and their downstream targets may also be effective in inhibiting TGFβ-induced corneal stromal injury, myofibroblast transdifferentiation of trabecular meshwork cells, and EMT of lens epithelial cells [2]. This thesis seeks to explore the potential of miR-24 as a novel therapeutic treatment for retinal fibrosis, as outlined in more detail in the following objectives. The results of this
research may have significant impact since ocular fibrosis often leads to permanent vision loss and accompanies many prevalent ocular diseases, as discussed in this chapter.

**Research Objectives**

**General Objective:** To investigate the role of microRNA24 in ocular fibrosis and preliminarily evaluate the biomolecule’s therapeutic potential to prevent subretinal fibrosis in pathologies such as age-related macular degeneration.

**Specific Objectives:**

- **Aim 1:** To evaluate the potential of miR-24 to prevent EMT of RPE cells
  - **Aim1A:** Determine the effects of upregulating miR-24 in cultured RPE cells under both normal and EMT-inducing conditions, evaluating mesenchymal and fibrotic marker expression, cell proliferation, and cell viability.
  - **Aim1B:** Determine the effects of neutralizing miR-24 in cultured RPE cells in both normal and EMT-inducing conditions, evaluating mesenchymal and fibrotic marker expression, cell proliferation, and cell viability.

- **Aim 2:** To evaluate the potential of miR-24 to prevent EndoMT
  - **Aim2A:** Determine the effects of upregulating miR-24 in cultured endothelial cells under both normal and EndoMT-inducing conditions on mesenchymal and fibrotic marker expression.
  - **Aim2B:** Determine the effects of neutralizing miR-24 in cultured endothelial cells in both normal and EndoMT-inducing conditions on mesenchymal and fibrotic marker expression.
CHAPTER 2

The Role of miR-24 in preventing EMT of RPE Cells

Methods

To investigate the capacity of miR-24 to prevent EMT of RPE cells, experiments were conducted on RPE cells in vitro. MiR-24 was upregulated in cultured ARPE-19 cells (an immortalized human RPE cell line) by transfection with precursor miR-24 (with Lipofectamine™ RNAiMAX Transfection Reagent [ThermoFisher, Invitrogen™, Catalog Number: 13778075] and Gibco™ Opti-MEM™ Reduced Serum Medium) or treatment with miR-24 containing adenovirus (ad-miR24) that was previously designed in the Wang Lab. ARPE-19 cells were cultured with HyClone™ DME/F-12 1:1 medium. Human precursor miR-24 (pre-miR-24) was obtained from ThermoFisher Scientific (miRBase ID: hsa-miR-24-1-5p; miRBase Accession Number: MIMA- T000079; Catalog Number: AM17100). As a negative control in the pre-miR-24 experiments, ARPE-19 cells were transfected with Pre-miR Negative Control #1 (pre-control, catalog number: AM17110) from ThermoFisher Scientific. Adenovirus not containing miR-24 (ad-empty) was used as a negative control in the ad-miR-24 experiments.

Figure 7. Stem-loop structure of precursor microRNA-24 (pre-miR-24) [29]

To investigate the effects of loss of function of miR-24 in RPE cells, anti-microRNA-24 microRNA inhibitor (anti-miR-24) was transfected into ARPE-19 cells.
Loss of strand specific function was evaluated using anti-miR-24-3’ or anti-miR-24-5’ to target the 3’ or 5’ stand/side, respectively. These were used in combination to knockdown both strands of miR-24. Anti-miR-24-3’ (species: human; miRBase ID: hsa-miR-24-3p; miRBase Accession Number: MIMAT0000080; Catalog Number: AM17000) and anti-miR-24-5’ (species: human; miRBase ID: hsa-miR-24-2-5p; miRBase Accession Number: MIMAT0004497; Catalog Number: AM17000) were obtained from Thermo-Fisher Scientific. To model EMT and fibrosis-inducing conditions in vitro, ARPE-19 cells were treated with human TGF-β2 (Sigma-Aldrich, Catalog Number: H8666-5UG) at a concentration of 10 ng/mL.

Results regarding protein expression were collected from these in vitro experiments by immunofluorescence staining and Western Blot, and results regarding cell viability were visualized by propidium iodide staining. Selected Western Blot data was quantified utilizing the gel analysis tools from Fiji ImageJ software and graphed using GraphPad Prism 8 software. Immunofluorescence staining images were also
quantified using Fiji ImageJ, by calculating the Integrated Density Value (IntDen, which is the sum of the brightness intensity of all pixels in the region of interest) for the complete area of each image taken with the 10x magnification objective lens, and then normalizing the data by calculating Relative Fluorescence, the ratio of samples’ IntDen values over the average of the respective experimental control group IntDen values. This data was also graphed using GraphPad Prism 8 software. Applicable statistical analyses were performed using GraphPad Prism 8 software.

**Results and Discussion**

The ability of ad-miR-24 and pre-miR-24 to overexpress miR-24 in RPE cells was validated by qPCR, as shown in figures 9 and 10. Additionally, the knockdown of miR-24-3’ and/or miR-24-3’ by transfection with anti-miR-24-5’, anti-miR-24-5’, or both, was confirmed by qPCR (Figure 11).

![Graph](image)

**Figure 9. Overexpression of miR-24 by Ad-miR-24 treatment**

9A: Fold-change expression of miR-24-3’ in ARPE-19 cells treated with Ad-empty or Ad-miR-24, normalized to U6. 9B: Fold-change expression of miR-24-5’ in ARPE-19 cells treated with Ad-empty or Ad-miR-24, normalized to U6. qPCR by Yinga Wu.
Figure 10. Overexpression of miR-24 by pre-miR-24 transfection
Fold-change expression of miR-24-3' in ARPE-19 cells treated with pre-control or pre-miR-24, normalized to U6. qPCR by Yinga Wu.

Figure 11. Knockdown of miR-24 by anti-miR-24 transfection
Fold-change expression normalized to U6 of miR-24-3' (11A) or miR-24-5' (11B) in ARPE-19 cells treated with negative control (NC), anti-miR-24-3', anti-miR-24-5', or both anti-miR-24-3' and -5'. qPCR by Yinga Wu.
Figure 12. miR-24 downregulates fibrotic and mesenchymal markers
APRE-19 cells transfected with Pre-miR24 or Pre-Ctrl (50 nM) for 3 days, then treated with TGF-β2 (10 ng/ml) for 6 days, show lowered expression of Fibronectin, a fibrotic marker (12A), and α-SMA, a mesenchymal marker (12B).

Figure 13. miR-24 downregulates fibrotic markers
13A: Schematic of experimental design. 13B: APRE-19 cells treated with Ad-miR24 or Ad-empty on Day 1, then treated with TGF-β2 (10 ng/mL) for 6 days beginning on Day 4, show lowered expression of Collagen III Fibronectin. Figure 13B results imaged by Yinga Wu.
Immunostaining results from ARPE-19 cells in which miR-24 was upregulated by pre-miR-24 transfection (see Figure 12) or by Ad-miR-24 (see Figure 13) and which were treated with TGF-β2 to induce EMT and fibrosis showed decreased expression of mesenchymal marker α-SMA and fibrotic markers Fibronectin and Collagen III. These results support the hypothesis that miR-24 upregulation prevents EMT of RPE cells. Furthermore, upregulation of miR-24 in ARPE-19 cells by Ad-miR-24 treatment according to the same experimental design led to decreased expression of SMAD3 and LIMK2, as shown in the Western Blots of Figure 14. As previously mentioned, SMAD2 is a known target of miR-24 in other tissues (skeletal muscle) but was not found to be downregulated by miR-24 overexpression in the RPE cell experiments. SMAD3 is involved in the canonical TGF-β signaling pathway which promotes EMT and fibrosis. The finding of lowered SMAD3 expression after miR-24 upregulation in these experiments is notable, and our lab has identified SMAD3 as a novel target of miR-24 in RPE cells by luciferase assay in 293T cells after predicting it as a target using software. After treatment of ARPE-19 cells with Ad-miR24 (with or without TGF-β2 treatment), the observed lowered expression of LIMK2 (Figure 14), which is involved in regulation of actin cytoskeleton dynamics and previously identified as a miR-24 target in endothelial cells by the Wang Lab, also supports the hypothesis that miR-24 can prevent EMT and fibrosis [16].
**Figure 14.** miR-24 overexpression downregulates SMAD3 and LIMK2 in ARPE-19 cells

14A: Schematic of experimental design with TGF-β2 treatment. 14B: Western Blot of SMAD3, LIMK2, SMAD2, and α-Tubulin (loading control) for these samples, by Yinga Wu

Propidium iodine stain, which is not permeable to live cells but stains dead cells red, was used to investigate the effects of miR-24 upregulation on cell viability. At high concentrations of pre-miR-24, cell death of human dermal fibroblast cells (HDEF) was observed (Figure 15A), while cell death of ARPE-19 cells was not observed from the concentrations of pre-miR-24 used (Figure 15B). This evidence is promising, suggesting miR-24 upregulation may lead to fibroblast cell death but not RPE cell death, which could be therapeutically beneficial. Similar results were observed when miR-24 was upregulated in ARPE-19 cells by treatment with ad-miR-24 at various concentrations, with very few dead cells observed in the negative control, ad-empty treatment, and ad-miR-24 conditions (Figure 15C). Hydrogen peroxide was used as a positive control as it is known to cause cell death, and yielded the expected results of cells stained red, indicating death.
Figure 15. miR-24 upregulation does not cause cell death in ARPE-19 cells

15A: PI staining of HDEF cells after transfection of pre-miR-24 at varying concentrations. 15B: PI staining of ARPE-19 cells after transfection of pre-miR-24 at varying concentrations. 15C: PI staining of ARPE-19 cells after treatment with ad-miR-24 or ad-empty for 4 days.
Figure 16. Effects of miR-24 knockdown on SMAD3 expression in ARPE-19 cells

16A: Western Blot for SMAD3 after anti-miR-24 transfection of ARPE-19 Cells (Day 1) with or without TGF-β treatment starting on Day 4 in ARPE-19 cells. Samples were collected on Day 8. 16B: Quantified SMAD3 expression.

The implications of neutralizing miR-24 in ARPE-19 cells were also investigated, and Figure 16 shows the effect of miR-24 knockdown by anti-miR-24 transfection on SMAD3 expression. The results depicted in the blot (Figure 16A) were quantified and graphed (Figure 16B), but this experiment has not yet been repeated successfully to strengthen these observed results and generate statistics. However, the preliminary data from Figure 16 may indicate that knockdown of miR-24 in ARPE-19 cells by this method
does not cause a significant change in SMAD3 expression in ARPE-19 cells. Though several other experiments were performed investigating the biological effects of miR-24 loss of function in ARPE-19 cells, these experiments were unsuccessful and the effects of miR-24 knockdown on other miR-24 targets and EMT-associated gene expression is yet to be determined.
CHAPTER 3

Potential of miR-24 to Prevent EndoMT of Endothelial Cells

Methods

To investigate the capacity of miR-24 to prevent EndoMT, which can contribute to retinal fibrosis, experiments were conducted on endothelial cells in vitro. EndoMT has previously been demonstrated in vitro on human umbilical vein endothelial cells (HUVECs), which are a primary human cell line derived from the endothelium of umbilical cord veins. IL-1β and TGF-β2 have been shown to act synergistically to induce pathological EndoMT in HUVECs in an NFκB-dependent manner, while TNF-α is known to enhance TGFβ-induced EndoMT by activating TGFβ signals from increased TGFβ type I receptor (ALK5) expression [31, 32]. Previous experiments in the Wang Lab have shown treatment of HUVECs with these three factors in combination (TGF-β2: 5 ng/mL, IL1β: 0.1 ng/mL, TNF- α: 5 ng/mL) is sufficient to induce EndoMT. In consideration of difficulties encountered with culturing HUVECs for these experiments, immortalized human aortic endothelial cells (TeloHAECs) were also used for experiments in this investigation. The capacity of TGF-β2, IL1β, and TNF- α, both separately and in combination, to induce EndoMT in this cell line as in HUVECs was evaluated by immunostaining before this model of EndoMT was used in miR-24 experiments. As was performed for ARPE-19 cells, miR-24 was upregulated in cultured HUVECs and TeloHAECs by transfection with precursor miR-24 (with Lipofectamine™ RNAiMAX Transfection Reagent and Gibco™ Opti-MEM™ Reduced Serum Medium) or treatment with ad-miR24. Both HUVECs and TeloHAECs were cultured with Lonza Clonetics® EGM™-2 Medium [containing EBM-2 Basal Medium (Catalog Number:
CC-3156) and EGM™-2 SingleQuots™ Supplements (Catalog Number: CC-4176)]. A line of immortalized endothelial cells derived from mouse pancreatic islet endothelium, MS1 cells, were also cultured and used for experiments to investigate the effects of miR-24 in mouse endothelial cells. MS1 cells were cultured with Cytiva HyClone™ Dulbecco's Modified Eagles High Glucose Medium. Pre-control and ad-empty were used as negative controls, as in the RPE cell experiments. In the same manner as for the RPE cell experiments, anti-miR-24-3’, anti-miR-24-5’, or both were used to investigate the effects of neutralizing miR-24 in endothelial cells. Results were collected by immunofluorescence staining and Western Blot and subsequently analyzed as described in Chapter 2.

Results and Discussion

Figure 17. Effects of miR-24 upregulation on Fibronectin expression in TeloHAECs P13 TeloHAECs were treated with 2 uL Ad-empty or Ad-miR-24 (Day 1) and samples were collected on Day 9. Half of the samples were treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4 (right side).
Immunostaining experiments investigating the effects of miR-24 upregulation by Ad-miR-24 revealed notable differences in Fibronectin expression in TeloHAECs (Figures 17 and 18). Under modeled EndoMT and fibrosis inducing conditions (by treatment with TGF-β2, IL1β, and TNF-α), TeloHAEC cells treated with Ad-miR-24 expressed notably less Fibronectin compared to cells treated with Ad-empty, as can be visually appreciated in Figures 17 and 18. This reduction in observed fibronectin in these samples may partially be attributed to fewer cells, in accordance with miR-24’s previously discovered repressive influence on endothelial cell proliferation and modest promotion of cell death [16]. After repeating the experiment from Figure 17 similar results were yielded and thus compiled in Figure 18.

**Figure 18. Effects of miR-24 upregulation on Fibronectin expression in TeloHAECs (repeated experiment)**

P12 TeloHAECs were treated with 2 μL Ad-empty or Ad-miR-24 (Day 1) and samples were collected on Day 9. Half of the samples were treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4 (right side).
Figure 19. Ad-miR-24 treatment reduces Fibronectin expression in TeloHAECs treated with EndoMT-inducing factors

Fibronectin expression quantified from relative fluorescent intensity after immunostaining of TeloHAECs treated with Ad-empty or Ad-miR-24 (Day 1) and collected on Day 9. Half of the samples were treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4.

Quantification of these Fibronectin immunostaining results (duplicate experiments, with 4 images in each of the 4 treatment groups quantified) also shows that overexpression of miR-24 by ad-miR-24 significantly reduces Fibronectin expression in TeloHAECs treated with EndoMT-inducing factors. Although triplicate results are desired to reach a stronger conclusion, two-way ANOVA statistical analysis of this data (see full statistics in Appendix) identified a very statistically significant interaction between the two variables of Ad-empty/Ad-miR-24 treatment and no treatment/EndoMT factor treatment (P value = 0.0058), accounting for 30.48% of the observed variance, and also a very statistically significant influence of Ad-empty/Ad-miR-24 treatment (P value = 0.0055), accounting for 30.99% of the observed variance in the results. Although further experiments are required, this data supports the hypothesis that miR-24 may be capable of preventing EndoMT as well as EMT to thus minimize subretinal fibrotic scarring.
Figure 20. Effects of miR-24 upregulation on Collagen III expression in TeloHAECs
P13 TeloHAECs were treated with 2 µL Ad-empty or Ad-miR-24 (Day 1) and samples were collected on Day 9. Half of the samples were treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4 (right side).

Figure 21. Effects of miR-24 upregulation on Collagen III expression in TeloHAECs (repeated experiment)
P12 TeloHAECs were treated with 2 µL Ad-empty or Ad-miR-24 (Day 1) and samples were collected on Day 9. Half of the samples were treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4 (right side).
Equivalent immunofluorescence experiments were performed to investigate the effects of miR-24 overexpression on Collagen III expression in TeloHAECs as for Fibronectin. As shown in Figures 20 and 21, similar results were also observed, with the TeloHAECs treated with Ad-miR-24 expressing noticeably less Collagen III than those treated with Ad-empty under modeled Endo-MT inducing conditions. As was done for the Fibronectin immunofluorescence data, these results were quantified and that this observed effect is statistically significant (Figure 22). Again, two-way ANOVA statistical analysis of this data (see full statistics in Appendix) identified a very statistically significant interaction between the two variables of Ad-empty/Ad-miR-24 treatment and no treatment/EndoMT factor treatment (P value = 0.0077), accounting for 36.71% of the observed variance, and also a statistically significant influence of Ad-empty/Ad-miR-24 treatment (P value = 0.0361), accounting for 19.99% of the observed variance in the results.

![Collagen III Expression](image)

**Figure 22.** *Ad-miR-24 treatment reduces Collagen III expression in TeloHAECs treated with EndoMT-inducing factors*

Collagen III expression quantified from relative fluorescent intensity after immunostaining of TeloHAECs treated with 2 uL Ad-empty or Ad-miR-24 (Day 1) and collected on Day 9. Half of the samples were treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4.
Figure 23. miR-24 upregulation downregulates SMAD3 expression in HUVECs
23A: Western blot: HUVECs were treated with 3 uL Ad-empty or Ad-miR24 (Day 1) and then treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4. Samples were collected on Day 9. 23B: Quantification of 23A results.

Western blots were also used to investigate the effects of miR-24 upregulation on expression of proteins related to EndoMT and fibrosis. Figure 23 depicts a novel result: miR-24 overexpression by ad-miR-24 reduces SMAD3 expression in HUVECs, both in treated and untreated conditions. Although this is a single result and has not yet been successfully repeated to strengthen the results and generate statistics, it is promising and suggests SMAD3 may also be a target of miR-24 in endothelial cells.
Figure 24. miR-24 upregulation downregulates SMAD3 in TeloHAECs

24A: Western blot: TeloHAECs were treated with 4 uL Ad-empty or Ad-miR24 (Day 1) and then treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4. Samples were collected on Day 10. 24B: Quantified SMAD3 expression.

Western blots of TeloHAEC samples also showed downregulation of SMAD3 in samples treated with Ad-miR-24 (Figure 24), though these results also need to be confirmed by repetition. Similar to the results from ARPE-19 experiments, the current results do not indicate that Ad-miR-24 downregulates SMAD2 in TeloHAECs. In TeloHAECs, samples treated with Ad-miR-24 also exhibited reduced expression of the mesenchymal marker α-SMA (in both treated and untreated conditions) and the profibrotic marker CTGF (in EndoMT-inducing factor treated conditions) as shown in Figure 25.
Figure 25. miR-24 upregulation downregulates EndoMT and fibrosis-related proteins in TeloHAECs

25A: Western blot: TeloHAECs were treated with 4 μL Ad-empty or Ad-miR24 (Day 1) and then treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4. Samples were collected on Day 10. 25B: Quantified CTGF expression. 25C: Quantified α-SMA expression.

Although triplicate results are needed for stronger conclusions, similar experiments were repeated for TeloHAECs in which both SMAD3 and CTGF were again observed to be downregulated in samples treated with Ad-miR-24 (Figure 26), which further suggests that miR-24 may target SMAD3 in endothelial cells and could potentially reduce progression of EndoMT and fibrosis.
Figure 26. miR-24 upregulation downregulates SMAD3 and CTGF in TeloHAECs
26A: Western blot: TeloHAECs were treated with 4 uL Ad-empty or Ad-miR24 (Day 1) and then treated with TGF-β2, IL1β, and TNF-α for 6 days beginning on Day 4. Samples not treated with these factors were collected on Day 7 and samples treated with these factors were collected on Day 10. 26B: Quantified SMAD3 expression. 26C: Quantified CTGF expression.

The effects of miR-24 upregulation on SMAD3 expression in MS1 cells was also investigated. Because the software analysis used by the Wang Lab to predict SMAD3 as a target of miR-24 used human sequences, it is unclear whether SMAD3 is likely to be a target of miR-24 in mouse cells, but of interest since the lab is using a mouse model to investigate the effects of miR-24 on subretinal fibrosis in vivo. Western blot results from a preliminary experiment do not show a significant difference in SMAD3 expression caused by treatment with Ad-miR-24 (Figure 27), but further experimentation and a
treatment group with EndoMT-inducing factors is imperative before drawing any conclusions.

Figure 27. Effects of miR-24 upregulation on SMAD3 expression in MS1 cells
27A: Western blot: MS1 cells were treated with 4 uL Ad-empty or Ad-miR24 (Day 1) and then samples were collected on Day 5. 27B: Quantified SMAD3 expression; T-test performed to generate P value (not significant).
CHAPTER 4
Conclusions and Future Directions

The general objective of this thesis research is to investigate the role of miR-24 in ocular fibrosis and preliminarily evaluate the biomolecule’s therapeutic potential to prevent subretinal fibrosis in pathologies such as age-related macular degeneration. Although numerous experiments did not yield end results for a variety of reasons, including cells dying, using an ineffective anti-miR-5’ (before replacing it), interference from fluorescence channels during microscopy, and samples having too low a concentration of protein to use for Western Blot, just to name a few of the hurdles encountered, the evidence gathered predominantly suggests that miR-24 may have therapeutic potential to prevent subretinal fibrosis.

With regard to Aim 1, multiple results presented in Chapter 2 indicate miR-24 has the potential to prevent EMT of RPE cells. The results suggest miR-24 can act in this way by targeting SMAD3, leading to downstream effects such as the observed downregulation of Collagen III, Fibronectin, and α-SMA, and prevention of EMT and fibrosis. Additionally, the results of the cell viability experiments in ARPE-19 cells, which suggest miR-24 upregulation can lead to cell death of fibroblasts but not RPE cells are therapeutically promising in two ways. Not only does this suggest a potential therapy involving miR-24 overexpression might not harm RPE cells, but also that miR-24 expression could act to ameliorate fibrosis not only by preventing its progression, but also by killing fibroblasts themselves. To further address Aim 1A (determine the effects of upregulating miR-24 in cultured RPE cells under both normal and EMT-inducing conditions, evaluating mesenchymal and fibrotic marker expression, cell proliferation,
and cell viability), experiments must be repeated to generate triplicate results, the effects of miR-24 on cell proliferation needs to be evaluated, and the effects of miR-24 overexpression on additional mesenchymal and fibrotic markers such as N-cadherin and Vimentin should be investigated. Aim 1B (Determine the effects of neutralizing miR-24 in cultured RPE cells in both normal and EMT-inducing conditions, evaluating mesenchymal and fibrotic marker expression, cell proliferation, and cell viability) is still largely unaddressed due to difficulties encountered with the anti-miR experiments. The biological consequences of miR-24 neutralization in ARPE-19 cells are still unclear and should be investigated by repeated experiments assessing any changes in expression of miR-24 known and potential targets, as well as mesenchymal and fibrotic markers. Secondarily, the influence of miR-24 neutralization on cell proliferation and cell viability in ARPE-19 cells should be explored.

The results compiled from this thesis research partially address Aim 2 (to evaluate the potential of miR-24 to prevent EndoMT). The results presented in Chapter 3 primarily pertain to Aim 2A (Determine the effects of upregulating miR-24 in cultured endothelial cells under both normal and Endo-MT-inducing conditions on mesenchymal and fibrotic marker expression). Although more extensive data is necessary to draw conclusions, the results compiled thus far support the hypothesis that miR-24 has the potential to prevent EndoMT. The finding that Ad-miR-24 treatment is associated with decreased SMAD3 expression in both HUVECs and TeloHAECs is novel and encouraging. This result, in conjunction with the statistically significant influence of miR-24 overexpression on expression of Fibronectin and Collagen III found by immunostaining, and suppression of CTGF and α-SMA expression identified by Western Blot, supports the hypothesis that
miR-24 has the potential to prevent EndoMT. Further research required includes replication of the presented results and investigation into the effect of miR-24 overexpression on other fibrotic and mesenchymal markers. It would also be interesting to repeat the immunofluorescence staining experiments for Fibronectin and Collagen III and analyze the samples at higher magnification using a confocal microscope to assess secretion of these proteins. Although several experiments were performed on HUVECs and TeloHAECs to investigate the effects of miR-24 neutralization, these experiments were not successful and Aim 2B (determine the effects of neutralizing miR-24 in cultured endothelial cells in both normal and EMT-inducing conditions on mesenchymal and fibrotic marker expression) remains unaddressed. Additional experimentation should be performed to successfully meet this aim.

In summary, the results that have been gathered suggest miR-24 may prevent EMT of RPE cells and EndoMT of endothelial cells to potentially minimize ocular fibrosis by reducing the contributions of RPE and endothelial cells to the fibrotic process. However, further research is compulsory to more thoroughly satisfy the aims and determine if further research is warranted to evaluate miR-24’s therapeutic potential in ocular pathologies such as AMD.
REFERENCES


[20] OCTMD, OCT of AMD, OCTMD, OCTMD.


APPENDIX

Table Analyzed: Fibronectin

Two-way ANOVA

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ANOVA table

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Difference between outcome means:

Mean of (No Treatment) 1.375
Mean of Treatment with Factors 1.227
Difference between means -0.123
95% CI of difference -0.215 to 0.158

Difference between ad-empty:

Mean of ad-empty 1.375
Mean of ad-eR-24 0.85
Difference between means 0.53
95% CI of difference 0.155 to 0.863

Interaction CI

Mean diff. A1 - B1 0.73
Mean diff. A2 - B2 2.913

Interaction

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Figure 28. Two-way Anova statistical analysis for Fibronectin immunostaining data
Table Analyzed: Collagen III

Two-way ANOVA

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<td>12</td>
<td>0.05272</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Difference between column means

| Mean of No Treatment | 1.648 |
| Mean of Treatment with Factors | 1.619 |
| Difference between means | 0.0295 |
| SE of difference | 0.1148 |
| 95% CI of difference | -0.2211 to 0.2702 |

Difference between row means

| Mean of ad-empty | 1.169 |
| Mean of ad-miR-24 | 0.8981 |
| Difference between means | 0.2709 |
| SE of difference | 0.1148 |
| 95% CI of difference | 0.0279 to 0.5211 |

Interaction CI

| Mean diff, A1 - A2 | -0.3391 |
| Mean diff, A1 - B1 | 0.3962 |
| (A1 - A2) - (A1 - B1) | -0.7353 |
| 95% CI of difference | -1.223 to -0.2240 |
| (B1 - A1) - (B2 - A2) | 0.7343 |
| 95% CI of difference | 0.2348 to 1.235 |

2-way ANOVA: Multiple Comparisons

Compare cell means regardless of rows and columns

Number of families: 1
Number of comparisons per family: 6

Tukey's multiple comparisons test

<table>
<thead>
<tr>
<th>Test details</th>
<th>Mean Diff</th>
<th>95.00% CI of - Same?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ad-empty No Treatment vs. ad-empty Treatment with Factors</td>
<td>-0.3391</td>
<td>-0.4201 to -0.258</td>
<td>ns</td>
<td>0.2134</td>
</tr>
<tr>
<td>ad-empty No Treatment vs. ad-miR-24 No Treatment</td>
<td>-0.0921</td>
<td>-0.1792 to -0.005</td>
<td>ns</td>
<td>0.9324</td>
</tr>
<tr>
<td>ad-empty No Treatment vs. ad-miR-24 Treatment with Factors</td>
<td>0.0382</td>
<td>0.0182 to 0.0582</td>
<td></td>
<td>0.9680</td>
</tr>
<tr>
<td>ad-empty Treatment with Factors vs. ad-miR-24 No Treatment</td>
<td>0.2419</td>
<td>0.2401 to 0.2437</td>
<td>ns</td>
<td>0.4727</td>
</tr>
<tr>
<td>ad-empty Treatment with Factors vs. ad-miR-24 Treatment with Factors</td>
<td>0.6381</td>
<td>0.6159 to 0.6603</td>
<td>**</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Test details

<table>
<thead>
<tr>
<th>Test details</th>
<th>Mean 1</th>
<th>Mean 2</th>
<th>Mean Diff</th>
<th>SE of diff</th>
<th>N1</th>
<th>N2</th>
<th>q</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ad-empty No Treatment vs. ad-empty Treatment with Factors</td>
<td>1</td>
<td>1.228</td>
<td>-0.2281</td>
<td>0.1624</td>
<td>4</td>
<td>4</td>
<td>2.945</td>
<td>12</td>
</tr>
<tr>
<td>ad-empty No Treatment vs. ad-miR-24 No Treatment</td>
<td>1</td>
<td>1.086</td>
<td>-0.09621</td>
<td>0.1624</td>
<td>4</td>
<td>4</td>
<td>6.318</td>
<td>12</td>
</tr>
<tr>
<td>ad-empty No Treatment vs. ad-miR-24 Treatment with Factors</td>
<td>1</td>
<td>0.7</td>
<td>0.3</td>
<td>0.1624</td>
<td>4</td>
<td>4</td>
<td>2.613</td>
<td>12</td>
</tr>
<tr>
<td>ad-empty Treatment with Factors vs. ad-miR-24 No Treatment</td>
<td>1.338</td>
<td>1.086</td>
<td>0.2419</td>
<td>0.1624</td>
<td>4</td>
<td>4</td>
<td>2.107</td>
<td>12</td>
</tr>
<tr>
<td>ad-empty Treatment with Factors vs. ad-miR-24 Treatment with Factors</td>
<td>1.338</td>
<td>0.7</td>
<td>0.6381</td>
<td>0.1624</td>
<td>4</td>
<td>4</td>
<td>5.558</td>
<td>12</td>
</tr>
<tr>
<td>ad-miR-24 No Treatment vs. ad-miR-24 Treatment with Factors</td>
<td>1.496</td>
<td>0.7</td>
<td>0.3962</td>
<td>0.1624</td>
<td>4</td>
<td>4</td>
<td>3.451</td>
<td>12</td>
</tr>
</tbody>
</table>

Figure 29. Two-way Anova statistical analysis for Collagen III immunostaining data