The Role of microRNA24 in Ocular Fibrosis

Abstract

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 investigation aims to evaluate the hypothesis that microRNA24 is capable of preventing endothelial to mesenchymal cell transitions and macrophage to myofibroblast transitions as well as epithelial to mesenchymal transitions 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. A possible additional aim of this research is to evaluate the efficacy of microRNA24 in minimizing fibrosis in ocular pathologies localized to other regions of the eye, such as the cornea.

Background and Introduction

Fibrosis is a wound healing response to tissue injury in which excess extracellular matrix (ECM) connective tissue is formed. 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 tissues of the eye, harming the patient's vision. Many of the most prevalent causes of vision deterioration and blindness, such as cataracts, diabetic retinopathy, glaucoma, myopic choroidal neovascularization, and age-related macular degeneration (AMD), are associated with ocular fibrosis (O'brien & Clark, 2015). 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 (O'brien & Clark, 2015). 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 in this fibrosis.

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. 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) to restrict this neovascularization by inhibiting angiogenic cytokines (Shu & Lovicu, 2017). 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 (Little et al., 2018). 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. In a 2020 clinical study, the incidence of subretinal fibrosis after anti-VEGF treatment was found to be 40.7% in eyes with mCNV (Xiao et al., 2020). This investigation will focus primarily on subretinal fibrosis, since this type of ocular fibrosis is associated with some of the most prevalent ocular pathologies and can lead to severe vision loss.

The ECM connective tissue that is formed during pathological fibrosis arises primarily from myofibroblasts (activated fibroblasts) which deposit ECM proteins and also “exert synchronized tractional forces across the ECM, resulting in distortion of tissue architecture and subsequent scarring” (Shu & Lovicu, 2017). 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 as well as other cell types, including endothelial cells, RPE cells, macrophages, and fibroblast-like cells (Shu & Lovicu, 2017). These various cell types interact with growth factors and inflammatory cytokines causing considerable modifications to the ECM structure (Ishikawa et al., 2015). Subretinal fibrosis 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, and this fibrous tissue frequently has a vascular network (Little et al., 2018).

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 (Little et al., 2018). 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" (Shu & Lovicu, 2017). These mesenchymal cells are invasive and can become myofibroblasts. 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 (Ishikawa et al., 2015). Damaged RPE cells may become myofibroblasts through EMT which has been demonstrated in many in vitro experiments (Little et al., 2018). Studies have also shown that although senescent cells can produce matrix metalloproteinases (MMPs) which have a role in inhibiting fibrosis, chronic presences of these MMPs may actually induce EMT, indicating that by secreting MMPs, senescent RPE cells may contribute to ocular fibrosis by inducing EMT (Little et al., 2018).

Additionally, it is known that the process of endothelial to mesenchymal transition (EndoMT) contributes 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 as an important contributor to CNV development (Little et al., 2018).

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 (Little et al., 2018; Ishikawa et al., 2015). 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 (Little et al., 2018).

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 (Bartel, 2004). 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" (Luna et al., 2011). 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. 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 (Zhou et al., 2014). 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 (Zhou et al., 2014). 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 (Luo et. Al, 2020).

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 (Sun et al., 2018). 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 (Luna et al., 2011). 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).

Proposed Methods

To address the aim of determining whether miR-24 is capable of preventing endothelial to mesenchymal cell transitions, *in vitro* experiments will be performed on HUVEC cells. Treatment with TGFβ will be used to induce fibrosis. MiR-24 will be upregulated in these cells by two different methods: transfection with pre-miR-24 and treatment with a miR-24 recombinant adenovirus. The effect of miR-24 on HUVEC viability and proliferation will be evaluated using propidium iodide staining and BrdU assays, respectively. MiR-24's potential influence on endothelial to mesenchymal cell transitions and differentiation to fibroblasts will be evaluated by immunostaining and Western Blot for both mesenchymal and fibroblast markers. MiR-24 will also be knocked down by treatment with anti-miR-24-3p and anti-miR-24-5p (strand specific function can be investigated by only using one or the other) to further evaluate the role of miR-24 in fibrosis. To address the aim of determining whether miR-24 is capable of preventing MMT, similar *in vitro* experiments will be performed on macrophages. TGFβ-mediated fibrosis and miR-24 overexpression will be induced as described for the endothelial cells and

results will be collected using similar assays as for the endothelial cells. Ongoing experiments will also be continued using ARPE-19 cells to further investigate our preliminary results that indicate miR-24 may repress EMT and RPE-derived fibroblast differentiation. Upon thorough fulfillment of these aims, *in vivo* experiments will be continued to further substantiate our claims utilizing laser-induced choroidal neovascularization to induce fibrotic injury in mice and miR-24 delivery by lentivirus injection. Immunohistochemical analysis and Western blot will be performed with these samples.

Conclusion

This investigation seeks to elucidate the role of miR-24 in ocular fibrosis and evaluate whether miR-24 can inhibit endothelial to mesenchymal transition, MMT, and EMT. The results of this research may have significant impact since ocular fibrosis often leads to permanent vision loss and accompanies many prevalent ocular diseases. If the proposed experiments indeed demonstrate that miR-24 plays a protective role in ocular fibrosis, translational research investigating miR-24's efficacy as a gene therapy for subretinal fibrosis could further evaluate miR-24's potential as a clinical intervention.

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