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miR-146a is upregulated during retinal pigment epithelium (RPE)/choroid aging in mice and represses *IL-6* and *VEGF-A* expression in RPE cells

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Abstract

Purpose—MicroRNA-146a (miR-146a) has been proposed as a marker for age-associated inflammation, or “inflammaging”, acting as a negative regulator of cellular senescence and pro-inflammatory signaling pathways. However, the regulation and function of miR-146 during ocular aging remains unclear. Here we propose that miR-146 is regulated during aging of the retina and choroid, and functions in retinal pigment epithelial (RPE) cells to regulate key genes involved in inflammation and angiogenesis.

Methods—The expression of miR-146a and miR-146b was examined in the neuroretina and RPE/choroid in mice aged from 2 months to 24 months. Then, the effect of synthetic miR-146a mimetic on *IL-6* and *VEGF-A* expression was analyzed in RPE cells treated with and without TNF- α .

Results—miR-146a and miR-146b was upregulated during aging of RPE/choroid but not neuroretina, supporting tissue-specific regulation of aging-related miRNAs in retinal tissues. Overexpression of miR-146a by miRNA mimics inhibited *VEGF-A* and TNF- α -induced *IL-6* expression.

Conclusions—Elevation of miR-146a and miR-146b in the aging RPE/choroid but not neuroretina suggests a role for miRNAs in inflammaging in the RPE/choroid. miR-146a overexpression inhibits the expression *IL-6* and *VEGF-A* in the RPE cells, supporting a negative feedback regulation mechanism by which inflammatory pathways may be dysregulated in RPE during aging.

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Introduction

Aging represents a significant risk factor for many diseases, including cardiovascular diseases, type 2 diabetes mellitus and degenerative diseases such as age-related macular degeneration (AMD). However, the pathophysiologic basis by which age contributes to these diseases is still unclear. Recently, dysregulation of inflammatory and immune pathways have become increasingly accepted as key regulators of age-related pathophysiology. Thus, the term “inflammaging” has been used to describe a state of chronic low-grade inflammation associated with age-related diseases (1).

AMD is a degenerative disease of the central retina and the leading cause of legal blindness in adults over 55 in the United States (2). Age is one of the top risk factors for AMD: AMD affects 14–24% of the U.S. population aged 65–74 years but 35–40% of people aged 74 years or more. AMD has both dry and wet forms (3). Wet AMD is characterized by choroidal neovascularization (CNV), a process involving abnormal growth of blood vessels from the choroid into the retina. In wet AMD, growth factors including vascular endothelial growth factor (VEGF) drive the growth of immature, leaky vessels, resulting in vision loss. By contrast, end-stage dry AMD is defined by geographic atrophy manifested by scattered or confluent areas of degeneration of retina pigment epithelial (RPE) cells. The pathophysiology of AMD is complex, with age, genetic, life style, and environmental factors each contributing to its pathogenesis. The molecular mechanism of retinal aging and its association with AMD pathogenesis remain unclear.

MicroRNAs (miRNAs or miRs) are small, non-coding RNAs that negatively regulate gene expression post-transcriptionally (4). Since their discovery in 1993, miRNAs have been shown to contribute to the pathogenesis of numerous diseases (5). The study of miRNAs in aging processes has emerged in recent years (6). One of the founding miRNAs, lin-4, has been shown to regulate lifespan in *C. elegans* (7). Numerous miRNAs have been shown to be significantly up- or down-regulated during aging; many of them have been identified as regulators of aging at cell, tissue or organism levels. One particular example is miR-34a, which is upregulated during aging and regulates cell senescence, life-span and aging in multiple species (8–14). The miRNA expression pattern during aging in mammals appears to be tissue-specific, which is in line with the tissue-specific aging signaling pathways (15). In the eye, age-dependent expression of miR-34a in the mouse retina and RPE cells has been observed, with steady increase from 4 months to 24 months, but a slight decrease between 24 and 32 months of age (16). Overall, the involvement of miRNAs in ocular aging is largely unknown.

MiR-146 family contains miR-146a and miR-146b that have similar sequences in the mature miRNAs except for two bases toward the 3'-end. MiR-146a was the most upregulated miRNA during replicative senescence in human fibroblast cells, human umbilical vein endothelial cells (HUVEC) and human trabecular meshwork (HTM) cells (17–19). It was shown to be induced by bacterial lipopolysaccharide (LPS) and the inflammatory cytokines IL-1 β and TNF- α , and functions to negatively regulate IL-6 and IL-8 expression (19–23). Moreover, miR-146a was identified as marker of senescence-associated pro-inflammatory status in cells involved in vascular remodeling (24). Based on these studies, miR-146a has

been hypothesized as a key regulator of inflammaging (25). Consistently, miR-146a was upregulated in several canine models of early-onset retinal degeneration diseases (26). Given the regulation of miR-146 by cellular senescence and its documented function in inflammation, we hypothesized that miR-146 members are regulated by aging, and function to repress inflammation in the eye. Here we report that miR-146a and miR-146b show age-dependent upregulation in the RPE/choroid but not neuroretina in mice. Furthermore, overexpression of miR-146 by miRNA mimic blocks *VEGF-A* expression and the induction of cytokine *IL-6* by TNF- α in RPE cells. Our data implicate the miR-146 family in RPE/choroid aging, with potential implications for understanding mechanisms and therapeutic options for AMD.

Methods

Animals and tissue preparation

Animal studies were conducted in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center and Tulane University. C57BL/6J mice were obtained from the National Institute of Aging (NIA; Bethesda, MD). Mice were housed at 21 °C, under a 12 hr:12 hr light-dark cycle, with food and water supplied ad libitum. For retinal tissue collection, 3 male mice each from 2, 8, 12, 18 and 24 months old, were sacrificed by CO₂ overdose. Immediately after euthanization, enucleated eyes were cut along the limbus to separate the ciliary body and the lens from the neuroretina and RPE/choroid tissues. The neuroretina was then separated from the RPE/choroid. The tissues were then stored in RNAlater at -20°C before RNA isolation.

ARPE-19 cell culture, miRNA mimic transfection and TNF- α treatment

Human RPE cell line (ARPE-19, CLR-2302, ATCC) was cultured in DME/F-12 medium (HyClone) supplemented with 10% FBS (HyClone) as described (27). miRNA mimetic (or mimic) transfection was performed as described in (28). Briefly 4 μ l of 20 μ M miRNA mimic or control mimic, and 4 μ l of Lipofectamine RNAiMAX added to 50 μ l optiMEM, respectively. Lipofectamine was mixed with miRNA mimic at 5 minutes later, and incubated for another 25 minutes before adding to 6-well plates for transfection. After 72 hours, cells were treated with TNF- α (10ng/ml) for 24 hours before RNA isolation. miR-146a or control mimic were synthesized from Shanghai GenePharma Co. Sequences for control mimic are: [sense] 5'-p-fUfUfCfUfCfCGAAfCGfUGfUfCAfCsGfUsTsT-3' and [antisense] 5'-Chol-sAsfCGfUGAfCAfCGfUfUfCGGAGAAfTsT-3'. (f: 2'-deoxy-2'-fluro nucleotides, Chol: cholesterol, p: phosphate group, s: phosphorothioate linkages). Sequences for miR-146a mimic are: [sense] 5'-p-fUsGAGAAfCfUGAAfUfUfCfCAfUGGGsfUsfU-3' and [antisense] 5'-Chol-sfCsfcfCAfUGGAAfUfUfCAGfUfUfCfUfCAfUfUfU-3'.

RNA isolation and qRT-PCR

Total RNA was isolated from mouse tissues or cell lines using TRIzol reagent (Invitrogen) using a protocol modified from the manufacture manual. To enrich both mRNA and miRNA in the samples, 1 volume of isopropanol (instead of 0.5 volume listed in the manual) was added to the samples, and the samples were incubated at -80°C for 15 minutes before RNA

precipitation. mRNA and miRNA quantitative (q) RT-PCR were performed using qScript™ cDNA Synthesis and microRNA Quantification System (Quanta Biosciences). Primers used are: *IL-6*: 5′-CAC ACA GAC AGC CAC TCA CC-3′ and 5′-TTT TCT GCC AGT GCC TCT TT-3′; *VEGF-A*: 5′-AGT GTG TGC CCA AGG A-3′ and 5′-GGT GAG GTT TGA TCC GCA TA-3′; Cyclophilin A: 5′-CCAGTGCTCAGAGCACGAAA-3′ and 5′-CCCACCGTGTTCTTCGACAT-3′; Primers for miR-146a and miR-146b were ordered from Quanta Biosciences.

Statistics

The *in vitro* experiment was repeated at least three times. Student's *t*-tests and TWO-WAY ANOVA (followed by Tukey's post hoc test) were used to determine statistical significance between groups. P values of less than 0.05 were considered to be statistically significant.

Results

Differential upregulation of miR-146a and miR-146b expression during Choroid/RPE aging in mice

To examine the expression of miR-146a and miR-146b during retinal aging, neuroretinal tissues and choroid/RPE tissues were isolated from male mice aged at 2, 8, 12, 18 and 24 months. Quantitative (q) RT-PCR was performed, and miR-146a/b expression was normalized to U6 and compared to their expression to 2-month samples. miR-146a expression was mildly increased in 8 month choroid/RPE tissues, but significantly increased at 18 months (~8 folds) and 24 months (~22 folds) (Fig. 1A). miR-146b expression was unchanged at 8 months, but started to increase gradually from 12 to 24 months (~2 folds at 18 months and ~5 fold increase at 24 months). On the contrary, the expression of neither miR-146a nor miR-146b was significantly changed in the neuroretina in mice from 2 months to 24 months (Fig. 1B). These results suggest differential regulation of miR-146a and miR-146b expression during the aging process of neuroretina and choroid/RPE in mice.

Inhibition of *IL-6* and *VEGF-A* gene expression in RPE cells by miR-146a mimic

MiR-146a has been shown to be induced by inflammatory cytokines IL-1 β and TNF- α , and function to target and negatively regulate IL-6 and IL-8 expression (19–22). In a recent study, miR-146a has been shown to downregulate VEGF expression in cancer cells (29). To examine the function of miR-146 in RPE cells, miR-146a or control mimic was transfected into ARPE-19 cells; cells without transfection were also used as an additional control. The cells were treated with TNF- α , and the expression of *IL-6* and *VEGF-A* was examined by qRT-PCR, with cyclophilin A used as normalization control. As shown in Fig. 2A, compared to the controls, miR-146a mimic resulted in drastic over-expression of miR-146a but not miR-146b at either control condition or with TNF- α treatment, confirming the specificity and efficiency of miR-146a mimic. Consistent with a previous report, TNF- α preferentially induced the expression of miR-146a (~10 fold induction) but not miR-146b (23). Of note, control mimic blunted the induction of miR-146a by TNF- α , suggesting the control miRNA mimic or the transfection reagents may have some non-specific effect on miR-146a expression at the concentration used.

After setting up the system, the expression of *IL-6* and *VEGF-A* was examined by qRT-PCR. Without TNF- α treatment, neither control mimic nor miR-146a mimic significantly affected *IL-6* expression compared to the non-transfection control (Fig. 2B). However, TNF- α induced *IL-6* expression completely blunted upon miR-146a mimic transfection.

When *VEGF-A* expression was analyzed, control mimics repressed *VEGF-A* expression to ~60% compared to the non-transfection control at the basal condition, again suggesting non-specific effects. Meanwhile, miR-146a mimic reduced expression of *VEGF-A* expression to less than 11% compared to the both controls. In addition, TNF- α also significantly increased the expression of *VEGF-A* expression, and this upregulation was reversed by miR-146a mimic transfection.

Taken together, these data demonstrate that miR-146a is induced by the inflammatory cytokine TNF- α , and miR-146a mimic represses the expression of inflammatory mediators *IL-6* and *VEGF-A* in ARPE-19 cells.

Discussion

We provide evidence that the expression of miR-146a and miR-146b shows age-dependent regulation in the RPE/choroid but not the neuroretina in mice. Moreover, overexpression of miR-146a by miRNA mimic inhibits TNF- α -induced *IL-6* expression and the expression of *VEGF-A*. Together, these data support a hypothesis for tissue-specific inflammaging mechanisms in RPE/choroid. In addition, our finding that miR-146a inhibits *VEGF-A* and TNF- α -induced *IL-6* expression may have implications in understanding the mechanism and therapeutics of AMD.

miR-146a as marker for RPE/choroid aging and inflammaging

How miRNAs are involved in ocular aging remains unclear. miR-34a upregulation in the blood and the brain have been identified as a senescence marker for brain tissue in mice (30). In the neuroretina and the RPE/choroid, miR-34a expression was recently shown to peak at 24 months of age in mice, but decreased gradually from 24–32 months of age, suggesting regulation of miRNAs during aging (16). Since miR-34a expression is not strictly age-dependent during neuroretina or RPE/choroid aging, we set to search for additional miRNAs involved in retinal aging. We found that miR-146a and miR-146b expression level increases steadily from 2 months to 48 months in the mouse RPE/choroid. MiR-146a has been shown to be highly up-regulated in senescent human fibroblast cells, HUVEC and HTM cells (17–19), and circulating miR-146a has been observed in aged individuals and in patients age-related diseases (17). Collectively, our work is consistent with prior studies, implicating miR-146 as a candidate marker for RPE/choroid aging.

Mechanistically, MiR-146a is induced by the Toll-like receptor (TLR) ligand LPS and the inflammatory cytokines IL-1 β and TNF- α , and functions to negatively regulate *IL-6* and *IL-8* expression (19–23). Two key adapter molecules downstream of TLRs, TRAF6 and IRAK1, have been confirmed as direct targets of miR-146a (31). These data suggest a negative regulatory loop where miR-146a gene is upregulated by NF- κ B and downregulates IRAK1 and TRAF6 to reduce NF- κ B activity. Based on these studies, miR-146a was

proposed as to be one of the key miRNAs involved in inflammaging (32). Differentially increased expression of inflammatory genes and proteins has been observed in models of neuronretina/RPE/choroid aging (33, 34). The normal aging process of the neuroretina is known to be less obvious than that of the choroid based on the fact that fewer age-related disease is associated with the retina, which correlates with the upregulation of miR-146a in the choroid/RPE but not neuroretina. Our findings that miR-146a is upregulated by inflammatory cytokine TNF- α and by choroid/RPE aging suggest a heightened inflammation in the RPE/choroid during aging, further supporting the inflammaging theory in the RPE/choroid. Future studies should address the regulation and function of miR-146 in AMD patients and in animal models of ocular disease.

Taken together, our data support the hypothesis that miR-146a/b is a marker for both aging and inflammaging in the RPE/choroid. Limitations of the study include the small sample size, limited time points and the lack of *in vivo* miR-146a functional studies. Future studies are needed to dissect the regulation of miR-146a/b in choroid and RPE separately, and rigorously test the inflammaging theory in RPE/Choroid.

Function and implications of miR-146a in RPE cells

miR-146a has been shown to repress multiple genes associated with inflammation, including *IRAK1*, *TRAF6*, *IL-6* and *IL-8* (19–23). It has been recently shown to inhibit cancer metastasis by downregulating *VEGF* expression in cancer cells (29). Our results that miR-146a mimic represses *VEGF-A* expression and TNF- α induced *IL-6* expression in ARPE-19 cells is consistent with the previous results using other cell types. Upregulation of miR-146a/b in senescent fibroblasts and HTM cells has been shown to downregulate the expression of multiple genes involved in the inflammatory response. Our data suggest that miR-146a serves as a negative feedback mechanism to prevent excessive inflammatory gene expression in the RPE cells (Fig. 3). Future study is required to determine whether miR-146a can regulate inflammatory pathways in aging RPE cells *in vivo*.

VEGF plays an important role in the pathological angiogenesis and vascular permeability in wet AMD, and anti-VEGF agents are the current mainstay in treating wet AMD (35–37). Therefore, our findings that miR-146a is upregulated during RPE/choroid aging *in vivo* and miR-146a mimic negatively regulates the expression of inflammatory and angiogenic genes may have implication in the therapeutics for AMD. Our published data and others have shown important function of miRNAs in the vascular system (28, 38–43). It would be thus interesting to test the function of miR-146a in both inflammation and angiogenesis in models of ocular and vascular disease.

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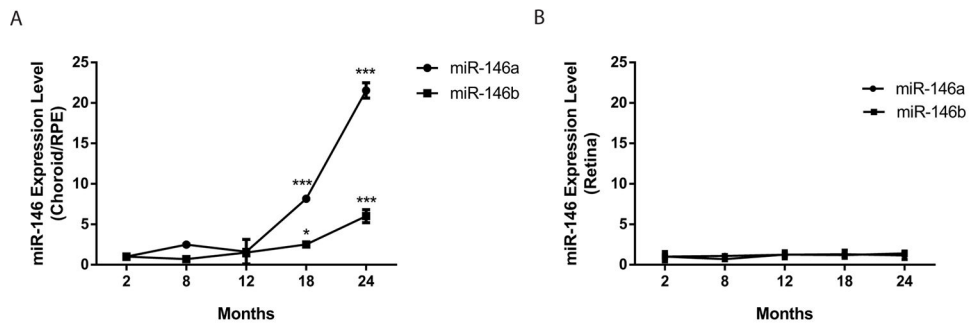


Figure 1. Upregulation of miR-146a and miR-146b during aging of RPE/choroid and neuroretina. Three samples were used for each age group. qRT-PCR results showed relative miR-146a and miR-146b expression in the RPE/choroid (A) and neuroretina (B). *, $p < 0.05$ ***, $p < 0.001$.

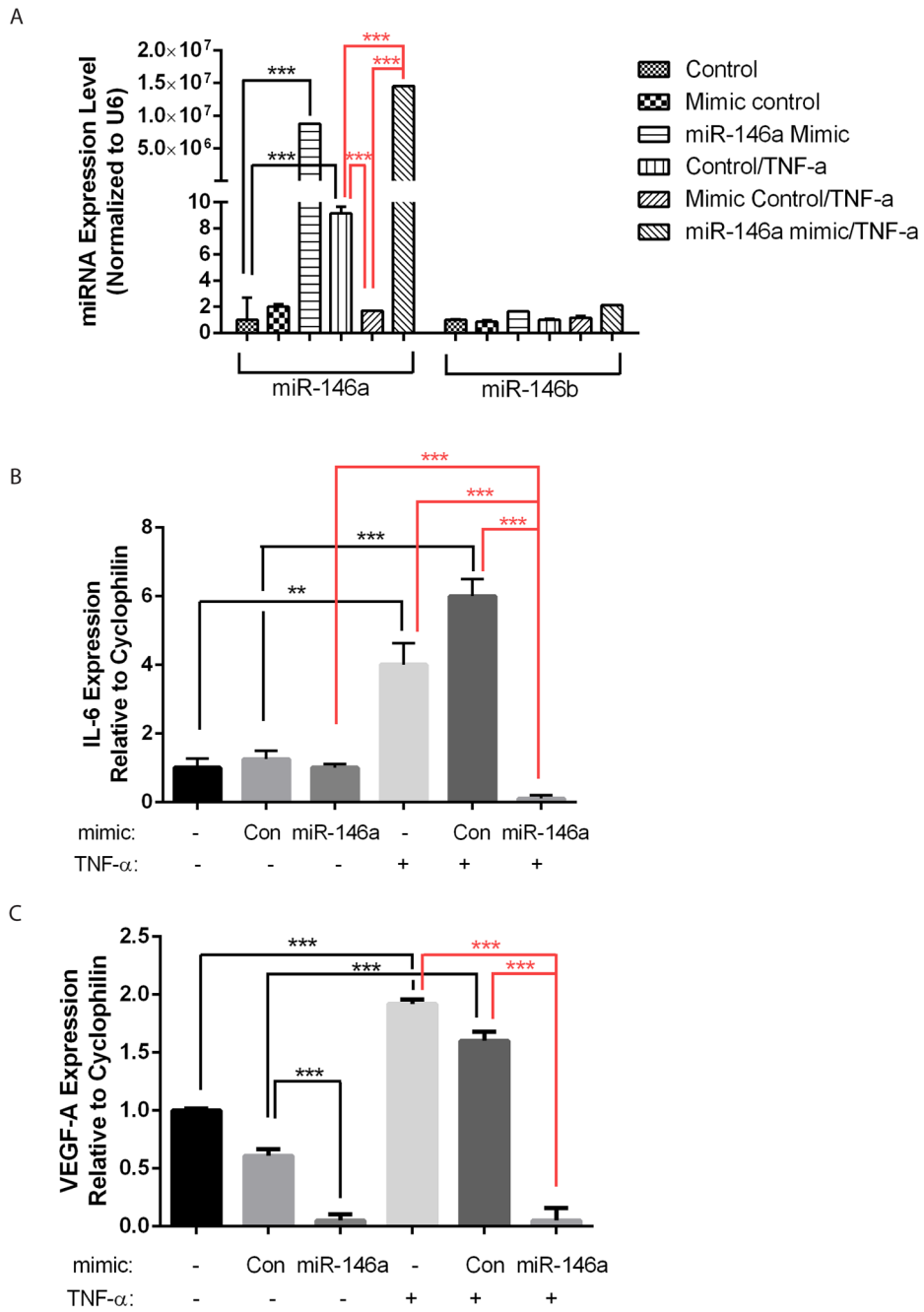


Figure 2. Expression of miR-146a, miR-146b, *IL-6* and *VEGF-A* in ARPE-19 cells after miRNA mimic transfection and TNF- α treatment. qRT-PCR results showed miR-146a and -b expression (A), *IL-6* expression (B) and *VEGF-A* expression (C) in ARPE-19 cells with/without miR-146a mimic transfection and TNF- α treatment. **, $p < 0.01$; ***, $p < 0.001$.

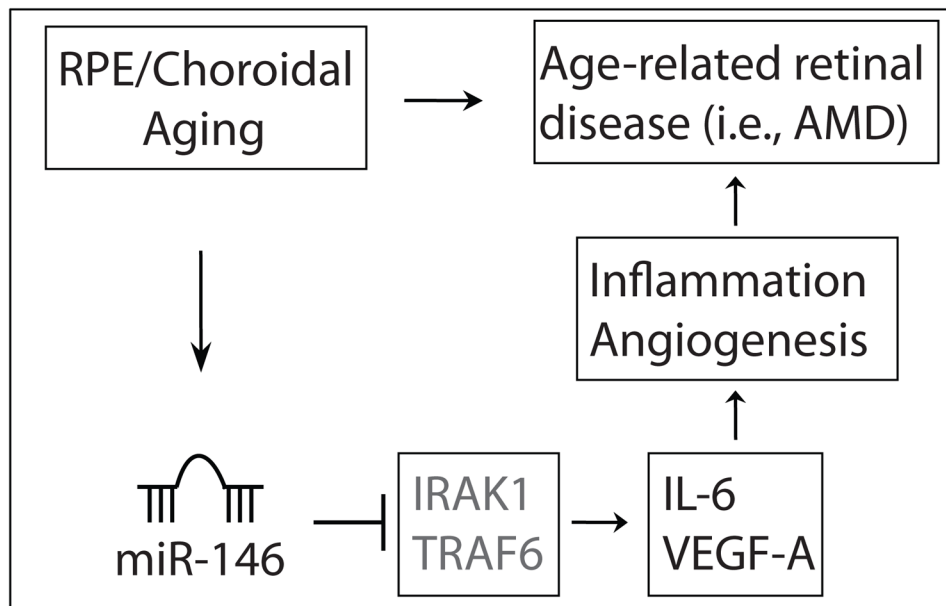


Figure 3.

Working model. In this model, RPE/choroid aging induces the expression of miR-146a, which in turn represses the *IL-6* and *VEGF-A* expression in RPE cells. Therefore miR-146a functions to alleviate the inflammatory and angiogenic response in the aging RPE/choroid. Regulation of *IL-6* and *VEGF-A* is likely mediated by its target genes IRAK1, TRAF6, which function in the NF κ B pathway.