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Expression, regulation and function of miR-126 in the mouse choroid vasculature

Fangkun Zhao[a,b], Chastain Anderson[b], Sara Karnes[b], Qinbo Zhou[b], Jing Ma[b], Zheng-Gen Jin[c], Partha S. Bhattacharjee[d], Shusheng Wang[b,e,*]

[a] Department of Ophthalmology, The Fourth Affiliated Hospital of China Medical University, Eye Hospital of China Medical University, Key Lens Research Laboratory of Liaoning Province, China
[b] Department of Cell and Molecular Biology, Tulane University, New Orleans, LA 70118, USA
[c] Aab Cardiovascular Research Institute, University of Rochester Medical Center, 601 Elmwood Avenue, Box CVRI, Rochester, NY 14620, USA
[d] Department of Biology, Xavier University, New Orleans, LA 70125, USA
[e] Department of Ophthalmology, Tulane University, New Orleans, LA 70112, USA

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ABSTRACT

MicroRNA miR-126 has been shown to be required for proper angiogenesis in several models. However, its expression, regulation and function in the mouse choroid remain unclear. Our previous data has shown that miR-126 expression is enriched in the endothelial cells (ECs) of the mouse choroid. Here we report that a 5.5 kb *Egfl7/miR-126* promoter drives the expression of miR-126 in the choroid ECs during choroidal vascular development. The expression of miR-126 in the ECs is regulated by flow stress likely through Krüppel-like transcriptional factors. *miR-126*$^{-/-}$ mice show mildly delayed choroidal vascular development, but adult knockout mice develop periphery choroidal vascular lesions. This study suggests that miR-126 is largely dispensable for mouse choroidal development but required for maintaining choroidal vasculature integrity.

1. Introduction

The choroid is a highly vascularized tissue positioned between the retinal pigment epithelium (RPE)/Bruch's membrane and the sclera in the eye (Lutty et al., 2010). It contains three layers, including the inner capillary layer, Sattler's layer of intermediate arteries in the middle, and the outer Haller's layer with large veins. Choroidal circulation is responsible for the supply and removal of metabolites to and from the outer retina. Not surprisingly, a defective choroidal vasculature is associated with ocular diseases, including age-related macular degeneration (AMD) (Grunwald et al., 1998; McLeod et al., 2009). Choroidal neovascularization (CNV) is the major cause of vision loss in patients with wet AMD. The development of CNV begins with new vessel formation and sprouting from the choroid, through Bruch's membrane and into the RPE and subretinal space. Choroidal capillary degeneration was also observed in both wet AMD and late-stage dry AMD subjects (McLeod et al., 2009). Human choroidal vascular development originates from periocular mesenchyme through haemovasculogenesis (Lutty et al., 2010; Saint-Geniez and D'Amore, 2004). Subsequently, angiogenesis accounts for the new vessel formation from the existing vessels. The mouse eye has been an excellent model system to study vascular development and disease. Vascular endothelial growth factor (VEGF) has been shown expressed in the RPE cells throughout mouse choroidal vascular development and in the adults. VEGF receptor VEGFR2 is expressed in the choroicapillaries underlying the RPE (Saint-Geniez et al., 2006). RPE-derived VEGF is critical for choroidal vascular development and maintenance (Le et al., 2010; Saint-Geniez et al., 2009). Besides, by studying the phenotype of transgenic mice expressing dominant-negative FGF receptor 1 (FGFR-1) in the RPE, RPE-derived FGF signaling is also shown to be important for choroid vascular development (Rousseau et al., 2000). Despite the research progress, the genetic, cellular and molecular mechanisms underlying mouse choroidal vascular development and maintenance remain unclear.

microRNAs (miRNA or miRs) are ~22-nucleotide noncoding RNAs that function by repressing the expression of multiple target genes through inducing mRNA cleavage or repressing protein translation (Bartel, 2004). Recent studies have shown that a list of miRNAs are enriched in the vasculature, and play important regulatory functions in angiogenesis and vascular diseases (Bonauer et al., 2010; Qin and Zhang, 2011; Wang and Olson, 2009). The term “angiomiR” has been used to name miRNAs that regulate angiogenesis either cell autonomously or non-cell autonomously (Wang and Olson, 2009; Wurdinger

* Corresponding author. Tulane University, 2000 Percival Stern Hall, 6400 Freret Street, New Orleans, LA 70118, USA.
E-mail address: swang1@tulane.edu (S. Wang).

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et al., 2008). miR-126 is one of the best characterized angiomiRs. It has been shown to be required for proper angiogenesis and maintaining vascular integrity (Fish et al., 2008; Kuhnert et al., 2008; van Solingen et al., 2009; Wang et al., 2008; Zhou et al., 2016). miR-126 expression has been shown to be enriched in vascular endothelium in the choroid (Zhou et al., 2016). However, the regulation of miR-126 in vascular EC and its function in choroidal vascular development and maintenance are still unclear. Here we provide evidence that miR-126 expression is regulated by flow in ECs, and miR-126 promoter activity is enriched in the choroidal ECs during choroidal vascular development. miR-126 is largely dispensable for mouse choroidal vascular development but required for the maintenance of choroidal vascular integrity in the adult.

2. Materials and methods

2.1. Animals

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 Tulane University. *miR-126*$^{-/-}$ mice and their WT littermate controls were generated from *miR-126*$^{+/-}$ (backcrossed to C57BL6J background for 10 generations) breeding as described (Wang et al., 2008). *Egfl7/miR-126* promoter-LacZ transgenic mice were generated as described (Zhou et al., 2016).

2.2. Fluorescein angiography

Fluorescein angiography was performed using a Micron III Fundus Camera connected to the Image system (Phoenix Research Labs, CA) as described (Zhou et al., 2017). 3 months WT and *miR-126*$^{-/-}$ mice were anesthetized with an intraperitoneal injection of Ketamine (100 mg/kg) plus Xylazine (5 mg/kg). Eyes were dilated with one drop of each of 2.5% Phenylephrine HCL and 1% Cyclopentolate HCL. Topical analgesic was applied (1 drop of proparacaine 0.5%). Fundus pictures were taken before or after fluorescein injection. Fluorescein dye (0.1 mL/kg of 10% fluorescein sodium) was injected intraperitoneally, and multiple pictures were taken at 30–300 s after injection of the dye. The same mice were reexamined by fluorescent angiography at 6, 12 and 18 months old.

2.3. Hematoxylin & Eosin (H&E), LacZ antibody staining, and immunostaining

Hematoxylin & Eosin staining was performed as described (Wang et al., 2008). For flat mount staining of the choroidal vasculature, pigment bleaching in RPE cells and choroidal melanocytes was performed as described (Kim and Assawachananont, 2016). Briefly, pigmented eyes were fixed in 4% paraformaldehyde PBS for 30 min to 2 h. After washing with PBS three times, the cornea and lens were removed before 3% hydrogen peroxide bleaching at 55 °C for 30 min to 2 h depending on the stage, or at 4 °C for several days. Immunofluorescence staining was performed using standard procedures. Primary antibodies used include: Beta-galactosidase (LacZ) from chicken (1:300, Abcam), Endomucin from rat (1:200, Santa Cruz Biotechnology), PECAM-1 from rat (1:50, BD Pharmingen), ICAM-2 from rat (1: 200, BD Pharmingen). These three EC markers, Endomucin, PECAM-1 and ICAM-2, were used interchangeably, but some antibodies worked better than others due to different sample processing. Secondary antibodies include: goat anti-chicken (or rat) AlexaFluor 488 (1:500, Invitrogen) and goat anti-rat AlexaFluor 594 (1:500, Invitrogen).

2.4. Cell culture, flow treatment and RT-PCR

HUVEC (ATCC) cells were grown in EC growth medium EGM-2 (Lonza). Steady laminar flow experiments was performed as described (Kwon et al., 2014). Briefly, confluent HUVECs were cultured in the absence of serum for 24 h and exposed to fluid shear stress (12 dynes/cm^2) for 4 and 24 h before processing for miRNA gene expression analysis. Total RNA was isolated using TRIzol reagent (Invitrogen). miRNA real-time RT-PCR was performed using miRCURY LNA Universal microRNA RT-PCR system (Exiqon). miR-126-3p, miR-92 primers from Exiqon, with U6 as control, were used as primers for PCR.

2.5. Plasmid construction and reporter assay

The 0.9 kb region enhancer of mouse *Egfl7/miR-126* was amplified by PCR, and cloned into pGL3 vector upstream of an engineered ANF basal promoter. Of note, the 0.9kb region is also the region-2 shown in our previous paper (Wang et al., 2008). Primer sequences include: 5′-ACAGAGGTCTGGGCATGTTC-3′ and 5′- TGCACAGAGGCTCCTCCCG GGT-3'. COS-7 cells in 24-well plates were transfected with 50 ng of reporter plasmid in the presence or absence of ETS1 and/or KLF2 expression plasmids (Meadows et al., 2009; Wang et al., 2008). Reporter assays were performed as described (Zhou et al., 2016).

2.6. Statistics

Number of animals was indicated in the text when necessary. Student's t-Tests were used to determine statistical significance between groups. P-values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Egfl7/miR-126 promoter activity during mouse choroidal vascular development

miR-126 is an EC-enriched miRNA located in the intron of *Egfl7* gene. A 5.5kb promoter has been shown to drive EC-enriched Egfl7/miR-126 expression (Wang et al., 2008). Our published data has shown that miR-126 is expressed in the adult mouse choroidal vasculature (Zhou et al., 2016). To define a role for miR-126 in choroidal vascular development, *Egfl7/miR-126* promoter activity was visualized by LacZ antibody staining and EC marker co-staining in a stable transgenic mouse line that expresses LacZ reporter under the control of the 5.4 *Egfl7/miR-126* promoter. The development of the mouse choroidal vascular system has not been systematically defined in literature. In humans, the ocular vascular system emerges from the mesoderm surrounding the newly formed optic cup (Saint-Geniez and D'Amore, 2004). In the E9.5 mouse embryo, isolated Endomucin-positive EC cells were observed at the presumptive choroidal region that surrounds the optic vesicle laterally (arrows in Fig. 1A). A few of the Endomucin-positive EC cells also showed *Egfl7/miR-126* promoter activity as shown by LacZ co-staining (Fig. 1B–C). At E10.5, when the prospective retina and retinal pigment epithelium (RPE) start to form, co-localization of Endomucin and LacZ staining was observed in the cells behind the RPE that surround the optic cup. At E13.5, Endomucin and LacZ co-staining persists in the choroid plexus behind the retina/RPE layer. Of note, double-positive staining was also seen in the hyaloid vasculature at this stage. Based on these, we conclude that *Egfl7/miR-126* promoter activity is enriched in the choroidal ECs during early choroidal vascular development.

3.2. Regulation of miR-126 by flow in ECs

The choroid blood flow has multiple functions, including vascular remodeling and supplying nutrients and oxygen for the outer retina (Nickla and Wallman, 2010). Impairment of the choroid blood flow may cause Age-related Macular Degeneration (AMD) (Grunwald et al., 1998). miR-126 has been shown to integrate mechanosensory stimulus and growth factor signaling to guide angiogenesis in zebrafish (Nicoli