RNCR3: A regulator of diabetes mellitus-related retinal microvascular dysfunction

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ABSTRACT
Retinal microvascular abnormality is an important pathological feature of diabetic retinopathy. Herein, we report the role of IncRNA-RNCR3 in diabetes mellitus-induced retinal microvascular abnormalities. We show that RNCR3 is significantly up-regulated upon high glucose stress in vivo and in vitro. RNCR3 knockdown alleviates retinal vascular dysfunction in vivo, as shown by decreased acellular capillaries, decreased vascular leakage, and reduced inflammatory response. RNCR3 knockdown decreases retinal endothelial cell proliferation, and reduces cell migration and tube formation in vitro. RNCR3 regulates endothelial cell function through RNCR3/KLF2/miR-185-5p regulatory network. RNCR3 inhibition may be a treatment option for the prevention of diabetes mellitus-induced retinal microvascular abnormalities.

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1. Introduction
Diabetic retinopathy (DR) is one of the most common complications of diabetes mellitus and a leading cause of blindness in industrialized nations [1]. DR can be divided into several stages: mild, moderate, severe and proliferative DR [2,3]. The non-proliferative stage is characterized by retinal vascular permeability, retinal vein dilation, retinal microaneurysms, capillary nonperfusion, and retinal hemorrhages [4]. In the proliferative stage, ischemia causes the formation of new blood vessels. Over-proliferation of capillary endothelial cells leads to retinal neovascularization, and abnormal formation of new vessels in the retina and the vitreous [5].

Long non-coding RNAs (lncRNAs) are defined as non-coding transcripts longer than 200 nt. They affect gene expression and signaling pathways at various levels [6,7]. Increasing studies have revealed that lncRNAs play important roles in vascular system. The endothelial-expressed IncRNAs, such as MALAT1 and Tie-1-AS, can regulate vessel growth and function. The smooth-muscle-expressed IncRNAs can control the contractile phenotype of smooth muscle cells [8–11]. Inspired by these studies, we speculated that IncRNAs were potential regulators of diabetes mellitus-induced microvascular dysfunction.

Retinal non-coding RNA3 (RNCR3), also known as LINC00599, is a long intergenic non-coding RNA. RNCR3 was first reported to be dynamically expressed during mouse retinal development [12]. RNCR3 knockdown alleviates retinal vascular dysfunction in vivo and in vitro. RNCR3 knockdown decreases retinal endothelial cell proliferation, and reduces cell migration and tube formation in vitro. RNCR3 regulates endothelial cell function through RNCR3/KLF2/miR-185-5p regulatory network. RNCR3 inhibition may be a treatment option for the prevention of diabetes mellitus-induced retinal microvascular abnormalities.

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received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health.

2.2. Cell culture and gene silencing

RF/6A cells were cultured in DMEM-F12 medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/ml streptomycin, and 2 mM l-glutamine. The medium was changed every 2 days. RNCR3 knockdown was achieved by siRNA transfection with lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

2.3. MTT assay

RF/6A cells (5 × 10^5) were plated in each well of a 96-well plate and allowed to adhere and spread for 24 h. After the required treatment, MTT solution (10 μl of 10 mg/ml) was added to each well, and the cultures were incubated for an additional 3 h. After the medium removal, 100 mM DMSO solution was added to dissolve formazan crystals. The absorbance at 540 nm was determined in each well using a microplate reader (Molecular Devices).

2.4. Transwell assay

The migration of RF/6A cell was detected using 12-well Transwell units. The undersides of insert membranes were coated with fibronectin (10 μg/ml) overnight at 4 °C and blocked with 1% BSA for 1 h at 37 °C. RF/6A cells (3 × 10^4) suspended in 500 μl DMEM was added to the upper compartment of the Transwell unit. Cells were allowed to migrate for 6 h at 37 °C. Non-migrated cells were scraped off using a cotton swab. Migrated cells on the bottom surface were counted after staining with crystal violet.

2.5. Tube formation assay

After the required treatment, RF/6A cells (1 × 10^5 cells/well) were seeded on the growth factor-reduced Matrigel (BD Biosciences) a 24-well plate. After 24 -h culture, the tube-like network was observed using a fluorescent IX70 microscope (Olympus, Tokyo) and photographed using a DP-70 digital camera (Olympus).

2.6. Immunofluorescence staining

RF/6A cell proliferation was detected by Ki67 immunofluorescence staining. Briefly, RF/6A cells were fixed in 4% formaldehyde for 10 min, and then blocked with 5% BSA for 0.5 h. They were incubated overnight with Ki67 antibody (Abcam, dilution: 1:200) for 24 h at 4 °C. After washing, they were incubated with Cy3-conjugated secondary antibody (Life Technologies). The slides were mounted using the anti-fade medium containing DAPI.

2.7. Detection retinal vascular leak

Retinal vascular leak was estimated by calculating Evans blue (EB) leakage from retinal blood vessels. Briefly, the right jugular vein and iliac artery were cannulated, and filled with heparinized saline. EB was injected from the jugular vein (75 mg/kg), and the chest cavity was opened after 1 h dye circulation. About 0.1 ml blood was drawn from the iliac artery to detect plasma EB amount. Retinal EB amount was calculated from the standard curve and normalized to dry retina weight [16].

2.8. Retinal trypsin digestion

The eyes were fixed in 10% neutral buffered formalin for 3 h, and then digested using 3% trypsin until the medium became cloudy. Retinas were gently shaken to free vessel from the adherent retinal

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**Fig. 1.** LncRNA-RNCR3 is significantly up-regulated upon high glucose stress. (A, B) RF/6A cells were exposed to high glucose (HG, 30 mM) for the indicated time periods. qRT-PCR were conducted to detect RNCR3 expression levels (n = 4, *P < 0.05). GAPDH was detected as the internal control. RNCR3 expression levels were shown as the relative change compared with the untreated group (0 h). (B) qRT-PCR were conducted to detect RNCR3 expression levels in mouse retinas of 2 months, 4 months, and 6 months after diabetes mellitus induction (n = 5 animals per group, *P < 0.05). GAPDH was detected as the internal control. RNCR3 expression levels were shown as the relative change compared with the un-diabetic group (Ctrl). (C) qRT-PCRs were conducted to detect RNCR3 levels in the fibrovascular membranes of diabetic patients and the idiopathic epiretinal membranes of non-diabetic patients (n = 10, *P < 0.05). GAPDH was detected as the internal control. All data were from three independent experiments.
tissue, washed, and mounted on glass slides for dry. The retinas were stained with PAS/hematoxylin to observe retinal vasculature [17].

2.9. Statistical analysis

All data were shown as mean ± SEM from at least 3 independent experiments. All statistical analyses were performed using SPSS version 17.0 software. Statistical comparison between two groups was evaluated by Student t-test. P < 0.05 was considered statistically significant.

3. Results

3.1. LncRNA-RNCR3 is significantly up-regulated upon high glucose stress

We first determined whether RNCR3 expression levels were altered upon high glucose stress in vitro. RF/6A cells were exposed to the culture medium containing high glucose (HG, 30 mM). We found that high glucose significantly up-regulated RNCR3 expression levels in a time-dependant manner (Fig. 1A). We then determined whether RNCR3 expression levels were altered under diabetic condition in vivo. Retinal RNCR3 expression levels in diabetic mice were obviously higher than that in the non-diabetic mice (Fig. 1B). We also found that RNCR3 expression levels in the fibrovascular membranes of diabetic patients were significantly higher than that in the idiopathic epiretinal membranes of the non-diabetic patients (Fig. 1C).

3.2. RNCR3 knockdown alleviates retinal vascular dysfunction in vivo

Retinal microvascular dysfunction is an important event in the pathogenesis of DR [18]. RNCR3 shRNA injection could significantly

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Fig. 2. RNCR3 knockdown alleviates retinal vascular dysfunction in vivo. (A) C57BL/6 diabetic mice (4-month old, male) received an intravitreal injection of scrambled shRNA (Scr), RNCR3 shRNA, or left untreated (Ctrl). To maximize virus delivery, these mice were injected twice during the first week, then once weekly thereafter, for a total of six month. qRT-PCRs were performed to detect RNCR3 expression levels (*P < 0.05 versus Ctrl group; n = 5 animals per group). (B and C) C57BL/6 diabetic mice (4-month old, male) were received an intravitreous injection of Scr shRNA or RNCR3 shRNA. Six months after diabetes onset, retinal trypsin digestion was conducted to detect the number of acellular capillaries. Red arrows indicated the acellular capillaries. Acellular capillaries were quantified in 20 random fields per retina. Scale bar, 20 μm (*P < 0.05, n = 5, B). The animals were infused with Evans blue dye for 2 h. The fluorescent signaling of flat-mounted retina was observed using a fluorescence microscope. The quantification of Evans blue leakage was also conducted. Scar bar, 50 μm (*P < 0.05, n = 5, C). All data were from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
down-regulate RNCR3 expression (Fig. 2A). Acellular capillaries were quantified in trypsin-digested retinas of RNCR3 shRNA-injected diabetic C57BL/6 mice and age-matched scrambled shRNA-injected diabetic mice. Retinal acellular capillaries were significantly decreased in RNCR3 shRNA-injected group, compared with scrambled shRNA-injected group (Fig. 2B). We then employed Evans blue method to detect retinal vascular leakage. RNCR3 knockdown could significantly alleviate diabetes mellitus-induced retinal microvascular leakage (Fig. 2C). Taken together, these results suggest that RNCR3 knockdown alleviates retinal vascular dysfunction in vivo.

3.3. RNCR3 regulates retinal endothelial cell function in vitro

We then determined the functional significance of RNCR3 up-regulation upon high glucose stress. RNCR3 expression levels were significantly down-regulated upon RNCR3 siRNA transfection in RF/6A cells (Fig. 3A). MTT assay showed that RNCR3 knockdown

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

![Graph E](image5.png)

**Fig. 3.** RNCR3 regulates retinal endothelial cell function in vitro. (A) RF/6A cells were transfected with scrambled (Scr) siRNA, RNCR3 siRNA, or left untreated (Ctrl) for 48 h qRT-PCRs were performed to detect RNCR3 expression levels (*P* < 0.05 versus Ctrl group; n = 4). (B) RF/6A cells were transfected with scrambled (Scr) siRNA, RNCR3 siRNA, or left untreated (Ctrl) for 48 h. Cell viability was determined using MTT method (*P* < 0.05 versus Ctrl group; n = 4). (C and D) RF/6A cells were transfected with scrambled (Scr) siRNA or RNCR3 siRNA for 48 h Ki67 immunofluorescent staining and quantitative analysis was conducted to detect cell proliferation. Blue: nuclei, Red: Ki67. Scale bar, 20 μm (*P* < 0.05 versus Scr group; n = 4). Transwell assays were conducted to detect the migration of RF/6A cells. Scale bar, 50 μm (*P* < 0.05 versus Scr group; n = 4). (E) RF/6A cells were seeded onto the matrigel matrix, and tube-like structures were observed by a light microscopy 24 h after seeding. The average length of tube formation for each field was analyzed (*P* < 0.05 versus Scr group; n = 4). Scale bar, 200 μm. All data were from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
significantly decreased RF/6A cell viability (Fig. 3B). Ki67 immunofluorescence staining showed that RNCR3 knockdown significantly reduced the proliferation of RF/6A cells (Fig. 3C). We also revealed that RNCR3 knockdown could significantly inhibit cell migration and tube formation of RF/6A cells (Fig. 3D and E). Collectively, these results suggest that RNCR3 plays an important role in regulating retinal endothelial cell function in vitro.

3.4. RNCR3 regulates retinal endothelial cell function through RNCR3/KLF2/miR-185-5p regulatory network

We previously show that RNCR3 acts as a competing endogenous RNAs (ceRNAs), and forms a feedback loop with Kruppel-like factor 2 and miR-185-5p to regulate HUVEC and VSMC function [14]. We thus determined whether miRNA-185-5p regulates RNCR3 level in RF/6A cells. miR-185-5p mimic injection significantly decreased the expression levels of RNCR3 and KLF2 in RF/6A cells, suggesting that miR-185-5p regulates RNCR3 and KLF2 expression (Fig. 4A). We also determined the role of miR-185-5p in RF/6A cell function. We revealed that miR-185-5p mimic transfection significantly decreased RF/6A cell viability (Fig. 4B), inhibited the proliferation of RF/6A cells (Fig. 4C). We further determined whether RNCR3-KLF2 crosstalk is involved in the regulation of RF/6A cell function (Fig. 4D). MTT assay revealed that RNCR3 knockdown significantly decreased RF/6A cell viability, whereas KLF2 overexpression could reverse the decrease of cell viability (Fig. 4E). Ki67 immunofluorescence staining showed that RNCR3 knockdown significantly reduced the proliferation of RF/6A cells (Fig. 4C).

![Graph A](A) RF/6A cells were transfected with miR-185-5p mimic, scrambled (Scr) mimic, or left untreated (Ctrl) for 48 h. RNCR3 and KLF2 expression levels were detected using qRT-PCRs, and shown as the relative change compared with Ctrl group (*P < 0.05 versus Ctrl group; n = 4). (B and C) RF/6A cells were transfected with miR-185-5p mimic, scrambled mimic, or left untreated (Ctrl) for 48 h. MTT assay was performed to determine cell viability (*P < 0.05 versus Ctrl group; n = 4, B). Ki67 staining was conducted to detect cell proliferation (*P < 0.05 versus Ctrl group; n = 4, C). (D) A diagram showed that potential interaction among RNCR3, miR-185-5p, and KLF2. (E and F) RF/6A cells were transfected with RNCR3 siRNA or scrambled siRNA with or without KLF2 overexpression for 48 h. Cell viability were detected using MTT assay (*P < 0.05 versus Ctrl group; n = 4, E). Ki67 staining was conducted to detect cell proliferation (*P < 0.05 versus Ctrl group; n = 4, F). "#" indicated significant difference between the marked groups. All data were from three independent experiments.
staining revealed that RNCR3 knockdown obviously inhibited RF/6A cell proliferation, whereas KLF2 overexpression could increase RF/6A cell proliferation (Fig. 4F). Taken together, these results suggest that RNCR3/KLF2/miR-185-5p regulatory network is involved in RF/6A cell function regulation.

4. Discussion

Diabetic retinopathy is an ischemic retinal vasculopathy that affects individuals who suffer from diabetes mellitus. Microvascular dysfunction is associated with retinal ischemia and neovascularization in diabetic retinopathy [19,20]. Recently, the role of lncRNAs in vascular biology has been gradually recognized. LncRNA can regulate vascular smooth muscle cell and endothelial cell function, which participate in the pathogenesis of vascular disorders [8,10,21]. In this study, we show that RNCR3 expression levels are significantly up-regulated upon high glucose stress. RNCR3 knockdown alleviates retinal vascular dysfunction in vivo, and regulates retinal endothelial cell function in vitro. This study provides novel insights into the mechanism of diabetes mellitus-induced microvascular dysfunction.

Diabetic retinopathy is associated with microvascular dysfunction characterized by an initial microvascular degeneration followed by a compensatory but pathological hypervascularization [19]. Retinal angiogenesis, vascular hyperpermeability, and endothelial cell dysfunction would lead to visual deterioration [22,23]. We show that high glucose leads to a significant increase in RNCR3 expression levels. RNCR3 knockdown significantly decreases diabetes-induced retinal angiogenesis, and reduces vascular leakage. RNCR3 knockdown also decreases the proliferation, migration, and tube formation of endothelial cells. Thus, it is not surprising that RNCR3 is involved in diabetes mellitus-related microvascular dysfunction.

RNCR3 was first identified as a lncRNA dynamically expressed during mouse retinal development. It plays an important role in neurons and oligodendrocyte differentiation, implicating a crucial role of RNCR3 in nervous system [13]. Nervous and vascular systems are usually anatomically tied to each other. They often share the common regulators for function maintenance [24,25]. RNCR3 has been reported to be involved in atherosclerosis-related vascular dysfunction [14]. We herein show that RNCR3 is involved in diabetes mellitus-induced retinal microvascular dysfunction. Collectively, these results suggest that RNCR3 is a critical regulator of vascular dysfunction and neurodegeneration.

LncRNAs play their biological roles through various molecular mechanisms, such as functioning as scaffolds for chromatin remodeling, complexes and biological functions of miRNAs [26,27]. In our previous study, we show that RNCR3 acts as a ceRNA, and forms a feedback loop with KLF2 and miR-185-5p to regulate HUVEC and VSMC function during atherosclerosis-related vascular dysfunction. We speculated that RNCR3/miR-185-5p/KLF2 was also involved in diabetes-related vascular dysfunction [14]. Upon high glucose stress, RNCR3 is significantly up-regulated, which alleviates miR-185-5p repression effect, thereby up-regulating the level of miR-185-5p target gene, KLF2. This regulatory loop maintains a relative balance in retinal endothelial cell function to resist high glucose stress.

Taken together, this study reveals a crucial role of RNCR3 in diabetes mellitus-induced microvascular dysfunction. RNCR3 is significantly up-regulated in diabetic retinas and high glucose-treated endothelial cells. RNCR3 knockdown decreases diabetes mellitus-induced microvascular dysfunction in vivo and decreases endothelial cell proliferation, migration, and tube formation in vitro. Thus, RNCR3 is a promising therapeutic target for treating diabetes mellitus-related microvascular complications.

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