IncRNA-MIAT Regulates Microvascular Dysfunction by Functioning as a Competing Endogenous RNA

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Rationale: Pathological angiogenesis is a critical component of diseases, such as ocular disorders, cancers, and atherosclerosis. It is usually caused by the abnormal activity of biological processes, such as cell proliferation, cell motility, immune, or inflammation response. Long noncoding RNAs (lncRNAs) have emerged as critical regulators of these biological processes. However, the role of lncRNA in diabetes mellitus–induced microvascular dysfunction is largely unknown.

Objective: To elucidate whether IncRNA-myocardial infarction–associated transcript (MIAT) is involved in diabetes mellitus–induced microvascular dysfunction.

Methods and Results: Using quantitative polymerase chain reaction, we demonstrated increased expression of lncRNA-MIAT in diabetic retinas and endothelial cells cultured in high glucose medium. Visual electrophysiology examination, TUNEL staining, retinal trypsin digestion, vascular permeability assay, and in vitro studies revealed that MIAT knockdown obviously ameliorated diabetes mellitus–induced retinal microvascular dysfunction in vivo, and inhibited endothelial cell proliferation, migration, and tube formation in vitro. Bioinformatics analysis, luciferase assay, RNA immunoprecipitation, and in vitro studies revealed that MIAT functioned as a competing endogenous RNA, and formed a feedback loop with vascular endothelial growth factor and miR-150-5p to regulate endothelial cell function.

Conclusions: This study highlights the involvement of lncRNA-MIAT in pathological angiogenesis and facilitates the development of lncRNA-directed diagnostics and therapeutics against neovascular diseases. (Circ Res. 2015;116:1143-1156. DOI: 10.1161/CIRCRESAHA.116.305510.)

Key Words: angiogenesis inhibitors ■ epigenomics ■ long noncoding RNA ■ vascular endothelial growth factor

Angiogenesis is an important physiological process that plays a role in growth, development, wound healing, and granulation tissue formation. Insufficient vessel growth and regression usually occurs in some ischemic diseases, such as myocardial infarction, stroke, and obesity-associated disorders, whereas excess vessel growth promotes tumorigenesis, inflammatory disorders, and ocular disorders. Blood vessels not only transport oxygen and nutrients to all parts of body but also nourish many human diseases. Thus, molecules with regulatory function in pathological angiogenesis are considered the potential targets for the treatment of neovascular diseases.

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Long noncoding RNAs (lncRNAs) represent a class of transcripts longer than 200 nucleotides with limited protein-coding potential. They regulate gene expression at transcription, epigenetic, or translation level, thereby altering cellular response to various stimuli. Aberrant lncRNA expressions have been implicated in many human disorders, especially those proliferative diseases, highlighting their importance in maintaining cellular homeostasis. Several biological processes are involved in pathological angiogenesis, including cell proliferation, cell motility, immune response, and inflammation. lncRNAs have emerged as key players in these biological processes. Inspired by these findings, we speculated that lncRNAs are potential regulators of pathological angiogenesis. Manipulation of lncRNAs may open up a novel avenue for molecular therapeutics of neovascular diseases.

Retinal vasculature can be viewed directly and noninvasively, providing an easily accessible window to explore the mechanism of pathological angiogenesis. Pathological angiogenesis...
is associated with many ocular disorders, such as diabetic retinopathy (DR), age-related macular degeneration, and retinopathy of prematurity.\textsuperscript{15,16} Severe ocular angiogenesis may ultimately cause a catastrophic loss of vision. The inhibitor of vascular endothelial growth factor (VEGF), such as ranibizumab (Lucentis) or bevacizumab (Avastin) has been clinically used for angiogenesis treatment. However, the requirement for repeated intraocular injection and the local or systemic adverse effects may pose great hurdles for clinical therapies,\textsuperscript{13,14} implying that our understanding of ocular angiogenesis is still incomplete.

Diabetes mellitus is associated with both microvascular and macrovascular complications.\textsuperscript{15,16} Structural or functional vessel abnormality represents an important cause of morbidity and mortality in diabetic patients.\textsuperscript{37} Here, we selected DR, a microvascular complication of diabetes mellitus, as the research direction. We characterized the expression pattern of IncRNA—myocardial infarction–associated transcript (MIAT) and investigated its role in the pathogenesis of microvascular dysfunction.

## Methods

### Ethics Statement and Clinical Sample Collection

All experiments were conducted in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals ophthalmic and vision research. The clinical study was approved by the ethics committees of Nanjing Medical University (Nanjing, China). The surgical specimens were handled according to the Declaration of Helsinki. All patients gave the informed consent before inclusion. The fibrovascular membranes were obtained from the patients undergoing pars plana vitrectomy for proliferative DR treatment or undergoing pars plana vitrectomy for the treatment of idiopathic macular holes.

### Induction of Diabetes Mellitus in Rats

After 24-hour fast, diabetes mellitus was induced using the male Sprague-Dawley rats with a single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma) in citrate buffer (10 mmol/L; pH 4.5). The nondiabetic controls received an equivalent amount of citrate buffer. Seven days after streptozotocin injection, animals with blood glucose levels >16.7 mmol/L were included in the diabetic group.

### Cell Culture

Human umbilical vein endothelial cells and human microvascular endothelial cells (HMVECs) were grown in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum. RF/6A, retinal pigment epithelium, retinal ganglion cell 5 (RGC-5), rat Müller cells (rMC-1), and human endothelial cells (EA.hy 926) were cultured in DMEM (Dulbecco’s modified Eagle’s medium): F12 (1:1) medium containing 10% fetal bovine serum. These cells were maintained at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}.

### Tube Formation Assay

The basement membrane matrix (BD Biosciences) was placed into the well of 24-well plate, and hardened at 37°C for 30 minutes; 2x10\textsuperscript{4} RF/6A cells were seeded on each well, and incubated with or without VEGF or tumor necrosis factor (TNF)-α at 37°C for 24 hours. Tube formation was observed using an Olympus IX-73 microscope.

### Apoptosis Detection Using TUNEL Staining

Enucleated eyes were fixed in 4% paraformaldehyde for 3 hours. After the dehydration in a graded ethanol series, these samples were embedded in paraffin. Retinal sections were cut in the sagittal plane. The sections were stained using fluorescein-conjugated TUNEL (terminal transferase-mediated dUTP nick end-labeling) in situ cell death detection kit (Roche). For each retina, the number of TUNEL-positive cells was counted in 30 sections, and spaced every 200 μm centered on the optic nerve head.

### Retinal Trypsin Digestion

Trypsin digestion was performed to analyze retinal vasculature change.\textsuperscript{15} Briefly, enucleated eyes were fixed in 10% neutral-buffered formalin for 24 hours, and then incubated with 3% trypsin until the medium became cloudy. The retinas were gently shaken to free vessel network, washed, and mounted on the glass slides for dry. They were finally stained with PAS/hematoxylin. Endothelial cell nuclei are large and ellipsoid, whereas pericytes nuclei are smaller and darker located at the outer side of vessel wall.

### Data Analysis

All data were expressed as mean±SEM. One-way ANOVA combined with Bonferroni post-test was applied to determine the significance between different groups. \textit{p}<0.05 was considered as statistically significant.

### Results

#### In Vivo and In Vitro Expression Pattern of IncRNA-MIAT on High Glucose Stress

MIAT, also known as retinal noncoding RNA 2 or Gomafu, was first reported to be expressed in mitotic progenitors and postmitotic retinal precursor cells. It is highly conserved in placental mammals, and seems to be conserved back to amphibians.\textsuperscript{19,20} The information of gene location and genome comparison was shown in Online Figure I. RNA-fluorescent in situ hybridization suggested that MIAT was expressed in the retinal pigment epithelium layer, outer nuclear layer, inner nuclear layer, and ganglion cell layer in human and rat retinas. MIAT expression was significantly higher in the diabetic retinas than in the nondiabetic retinas (Figure 1A). MIAT was constitutively expressed in several retinal cells, including retinal pigment epithelium, RGC-5, RF/6A, and Müller cell. Notably, MIAT transcript was mainly localized in the nuclei (Figure 1B).

We next addressed whether MIAT expression is altered under diabetic condition in vivo, quantitative reverse transcription polymerase chain reactions indicated that retinal MIAT levels in diabetic rats were significantly higher than that in the nondiabetic rats (Figure 1C). A similar event occurred in the retinas of db/db mice, a type 2 diabetic model, compared with their nondiabetic controls (Figure 1D). Moreover, MIAT levels in the fibrovascular membranes of diabetic patients were significantly higher than that in the idiopathic epiretinal membranes of nondiabetic patients (Figure 1E; Online Table I).

We further investigated whether MIAT expression is altered on high glucose stress in vitro. We cultured RF/6A cells, a retinal endothelial cell line, in the high glucose medium to...
mimic diabetic condition. High glucose could lead to an obvious induction of MIAT expression in a time-dependant manner (Online Figure II). Similar results were observed in high glucose–treated retinal pigment epithelium, RGC-5, and rMC-1 cells (a cell line derived from rat Müller cells; Online Figure II). To determine whether MIAT induction by high glucose
Collectively, these results suggest that MIAT knockdown has the downregulation of phosphorylated Akt (Figure 2E), whereas MIAT knockdown partially reversed previously decreased the number of apoptotic cells (Figure 2C). Immunofluorescence analysis of activated caspase-3 was conducted to assess retinal cell apoptosis. A representative image was shown. Scale bar, 50 μm. *Significant difference compared with the wild-type nondiabetic group. #Significant difference between the marked experimental groups. D. Immunofluorescence analysis of cleaved caspase-3 showed that compared with high glucose–treated group, MIAT knockdown could further increase the number of dead or dying cells induced by high glucose (Online Figure III). In addition, caspase-3 was a major executioner of apoptosis, and cleaved caspase-3 level could quantify the apoptosis degree of retinal layers.21 Immunofluorescence analysis and western blots revealed that hyperglycemia increased the level of cleaved caspase-3, whereas MIAT knockdown partially decreased caspase-3 upregulation (Figure 2D and 2E). Akt is a serine/threonine kinase regulated by the membrane levels of phosphatidylinositol 3-phosphate. Activated Akt has an obvious antiapoptotic property.22 Hyperglycemia decreased phosphorylated Akt, whereas MIAT knockdown partially reversed the downregulation of phosphorylated Akt (Figure 2E). Collectively, these results suggest that MIAT knockdown has a beneficial effect on visual function under diabetic condition.

**MIAT Knockdown Alleviates Retinal Vessel Impairment In Vivo**

DR is usually characterized by abnormal change in retinal microvasculature, causing retinal nonperfusion, increased vasopermeability, and pathological intraocular proliferation of retinal vessels.23 Retinal trypsin digestion assay indicated that diabetes mellitus resulted in severe pericytes loss and aggravaed capillary degeneration. MIAT knockdown could partially reduce this detrimental effect (Figure 3A). Evans blue leakage assay indicated that MIAT knockdown could alleviate diabetes mellitus–induced retinal vascular leakage (Figure 3B).

Increased proinflammatory proteins, such as intercellular adhesion molecule-1, TNF-α, or upregulated VEGF plays a critical role in the pathogenesis of diabetes mellitus–induced microvascular dysfunction.24 We found that diabetic rats showed elevated retinal expression of VEGF, TNF-α, and intercellular adhesion molecule-1, whereas MIAT knockdown could partially reduce the upregulation of VEGF, TNF-α, and intercellular adhesion molecule-1 induced by diabetes mellitus (Figure 3C), suggesting that MIAT knockdown alleviates retinal inflammation under diabetic condition.

**MIAT Knockdown Regulates Endothelial Cell Function In Vitro**

Endothelial cells are recognized as the primary cellular targets during diabetes mellitus–induced vascular damage.23,25 We thus selected an endothelial cell line to study the mechanistic aspects and functional significance of MIAT alteration in vitro. MIAT small interfering RNA transfection caused a marked reduction in the level of MIAT but not other lncRNAs. Compared with high glucose–treated group, MIAT knockdown could further decrease the number of viable cells and viability of RF/6A cell as detected by Trypan blue staining and MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide) assay (Online Figure III). To determine whether MIAT regulates the development of high glucose–induced RF/6A cell apoptosis, Hoechst 33342, and JC-1 staining was used to detect apoptosis change. We observed that MIAT knockdown plus high glucose treatment resulted in higher apoptotic percentage than high glucose alone, as shown by increased apoptotic nuclei (condensed or fragmented) and decreased mitochondrial depolarization (Figure 4A and 4B). MIAT knockdown could further accelerate the shift of fluorescence emission from red to green (Figure 4B). Ki67 staining revealed that compared with high glucose–treated group, MIAT knockdown significantly decreased the number of proliferating cells (Figure 4C). Calcein-AM (calcein-acetoxymethyl ester) and propidium iodide double staining indicated that MIAT knockdown could further increase the number of dead or dying cells induced by high glucose (Online Figure III). In addition, we designed 2 additional MIAT small interfering RNAs (small interfering RNA 2 and small interfering RNA 3) to assure the
specificity of MIAT knockdown. They only silenced the expression of MIAT but not other lncRNAs. On high glucose stress, MIAT knockdown further decreased the number of viable cells, reduced cell viability, accelerated apoptosis development, and inhibited cell proliferation (Online Figures IV and V).

High glucose stimulates free radical production and reactive oxygen species formation. Excess glucose stress could cause serious oxidative stress during diabetic complications.26 High glucose significantly increased endogenous ROS production in a time- and concentration-dependant manner (Online
Figure 4. Myocardial infarction-associated transcript (MIAT) knockdown affects endothelial cell function in vitro. A, RF/6A cells were transfected with MIAT small interfering RNA (siRNA), scramble (Scr) siRNA, or left untreated, and then these cells were exposed with or without high glucose (HG, 30 mmol/L) for 48 hours. Apoptotic cells were analyzed by Hoechst staining and quantitated. Scale bar, 20 μm. B, RF/6A cells were incubated with JC-1 probe at 37°C for 30 minutes, centrifuged, washed, transferred to a 96-well plate, and assayed using a fluorescence plate reader and observed using a fluorescence microscope. Scale bar, 50 μm. C, A representative image was shown for Ki67 staining along with the quantification of Ki67 positive cells. Scale bar, 10 μm. D, Cell migration was assessed using wound-healing assay. Images were taken 0, 24, and 48 hours after tumor necrosis factor (TNF-α) treatment (10 ng/mL). The horizontal line indicates the wound edge. Migration was estimated by measuring cell numbers within the wounded region. The data were shown as the relative change compared the control group without TNF-α treatment. Scale bar, 100 μm. E, RF/6A cells were seeded on the matrigel matrix, and stimulated with TNF-α (10 ng/mL). The tube-like structures were observed 24 hours after TNF-α treatment. The average number of tube formation for each field was statistically analyzed (n=50). Scale bar, 100 μm. *Significant difference compared with the control group. #Significant difference between the marked groups. WT indicates wild-type.

Figure VI). We then treated RF/6A cells with H2O2 to mimic oxidative stress. Compared with H2O2-treated group, MIAT knockdown could further decrease the number of viable cells, reduce cell viability, accelerate apoptosis development, and inhibit cell proliferation (Online Figure VI). Hypoxia is recognized as another important cause of pathological angiogenesis. RF/6A cells were treated with CoCl2 to mimic hypoxia stress. Compared with CoCl2-treated group, we observed that MIAT knockdown could further decrease viable cells, reduce cell viability, accelerate cell apoptosis, and inhibit cell proliferation (Online Figure VII).

Increased expression of pathogenic factors, such as TNF-α and VEGF, contributes to endothelial cell migration and tube formation, thereby affecting retinal microvascular stability. We found that TNF-α or VEGF treatment resulted in an increase in the number of migrated endothelial cells. MIAT knockdown obviously decreased the number of migrated cells (Figure 4D; Online Figure VIII). Moreover, we observed that MIAT knockdown significantly decreased TNF-α or VEGF-induced tube formation (Figure 4E; Online Figure VIII).

Taken together, MIAT is emerged as a critical regulator of endothelial cell function in vitro.

lncRNA-MIAT Functions as miR-150-5p Sponge in Endothelial Cell

lncRNAs could act as miRNA sponges, and regulate miRNAs available for binding their target mRNAs. Bioinformatics prediction using starBase suggested that MIAT sequence contained 4 putative miRNA binding sites, including miR-29a-3p, miR-29b-3p, miR-29c-3p, and miR-150-5p (Figure 5A). MIAT cDNA was cloned into the downstream of luciferase gene (RLuc-MIAT-WT) and transfected into RF/6A cells with different miRNA mimics. The activity of RLuc-MIAT-WT was significantly reduced by miR-150-5p mimic, but not by other miRNA mimics (Figure 5A). To avoid unspecific binding, we also mutated the miR-150-5p binding site of MIAT to generate RLuc-MIAT-Mut. miR-150-5p mimic transfection significantly reduced RLuc-MIAT-WT activity, but had no effect on RLuc-MIAT-Mut activity (Figure 5A). These data suggest that miR-150-5p directly targets MIAT in endothelial cell.

Having established MIAT targeting by miR-150-5p in vitro, we tested whether miR-150-5p targets MIAT in vivo. miR-150-5p mimic or antagonist was injected into the vitreous cavity of 1 eye of nondiabetic rats, and the other eye was received the same dose of scrambled control. miR-150-5p mimic injection resulted in an obvious reduction in MIAT levels, whereas miR-150-5p antagonist injection obviously upregulated MIAT levels in nondiabetic rats (Online Figure IX). In the diabetic rats, hyperglycemia significantly upregulated MIAT levels. miR-150-5p knockdown could further increase MIAT levels. By contrast, miR-150-5p mimic injection partially prevented MIAT upregulation induced by diabetes mellitus (Online Figure IX). These results indicate that miR-150-5p could regulate MIAT expression in vivo.

Ago2 is a core component of RNA-induced silencing complex (RISC) that binds miRNA complexes to target mRNA transcripts. We further investigated whether MIAT expression is under the control of miRNAs by knocking down Ago2 in vitro. A marked increase in MIAT level was detected in Ago2 knockdown cells, whereas, as expected, miR-150-5p stability was impaired by Ago2 knockdown (Figure 5B). miRNAs are known to play their roles, if not exclusively, in the cytoplasm. However, MIAT was found to be a nuclear-localized lncRNA, raising the possibility that miR-150-5p targets MIAT in the nucleus. RNA-fluorescent in situ hybridization experiment revealed that miR-150-5p was expressed in the cytoplasm and nucleus of RF/6A cells (Figure 5C), which was consistent with previous studies. Western blots further revealed that Ago2 was expressed in the nucleus and cytoplasm fraction of RF/6A cells (Figure 5D). Thus, it is not surprise that miR-150-5p potentially regulates MIAT expression in the nucleus.

We next investigated whether miR-150-5p regulates MIAT in the nucleus in an Ago2-dependent manner. RF/6A cells were transfected with miR-150-5p antagonist or the scramble control. miR-150-5p knockdown could upregulate MIAT level in the nucleus fraction and total cell fraction (Figure 5E). To determine whether MIAT associates with Ago2 in the nuclear fraction further, RNA immunoprecipitation were performed in the nuclear and cytoplasmic fraction of RF/6A cells using Ago2 antibody. MIAT or miR-150-5p was found to be preferentially enriched in Ago2-containing miRNPs relative to IgG immunoprecipitates in the nuclear fraction but not cytoplasmic fraction (Figure 5F). These results suggest that MIAT is targeted by miR-150-5p in the nucleus in an Ago2-dependent manner.

MIAT Regulates the Expression of miR-150-5p Target Gene, VEGF

Among the putative targets of miR-150-5p, we focused on VEGF (with 3 miR-150-5p sites), a key angiogenic factor involved in pathological angiogenesis (Figure 6A). The 3′ untranslated region of VEGF was fused to the luciferase coding region (RLuc-VEGF-WT) and transfected into RF/6A cells with miR-150-5p mimic in parallel to the negative control. Luciferase assay showed that VEGF was a target of miR-150-5p. The use of mutant derivatives (-Mut) in the miRNA recognition site confirmed the specificity of repressing activity (Figure 6B).
Figure 5. Long noncoding RNA-myocardial infarction–associated transcript (MIAT) is a target gene of miR-150-5p. A, StarBase prediction indicated that MIAT sequence contained 4 putative microRNA (miRNA) sites, including miR-29a-3p, miR-29c-3p, miR-29b-3p, and miR-150-5p. MIAT cDNA containing the putative miRNA recognition sites was cloned into the downstream of luciferase vector. RF/6A cells were cotransfected with RLuc-MIAT-wild-type (WT) with different miRNA mimics. Luciferase activity was detected using the dual luciferase assay (Promega). RLuc-MIAT-WT or RLuc-MIAT-Mut was cotransfected with miR-150-5p mimic into RF/6A cells in parallel with the vector. Luciferase activity was detected 48 hours after transfection. B, RF/6A cells were transfected with Ago2 siRNA, scramble siRNA, or left untreated. miR-150-5p or MIAT levels were detected using quantitative reverse transcription polymerase chain reactions (qRT-PCRs). C, RNA–fluorescent in situ hybridization (FISH) for miR-150-5p in RF/6A cells. Nuclei, blue; miR-150-5p, red. U6 was detected as the positive control, whereas NS indicated no probe addition. Scale bar, 10 μm. D, Western blots were conducted to detect Ago2 expression distribution. Proliferating cell nuclear antigen (PCNA) was detected as the nuclear marker, and Tubulin as the cytoplasmic marker. E, Nuclear and total cellular fractions were isolated from RF/6A cells transfected with the scramble control or miR-150-5p antagomir, and immunoprecipitated using Ago2 or IgG antibody. miR-150-5p and MIAT levels in the nuclear and total fractions used as inputs for RNA immunoprecipitation were detected by qRT-PCRs (n=4). F, Nuclear and cytoplasmic fractions were isolated from RF/6A cells, and immunoprecipitated using Ago2 or IgG antibody. MIAT and miR-150-5p levels were detected using qRT-PCRs (n=4). CMV indicates cytomegalovirus; DAPI, 4′,6-diamidino-2-phenylindole; and WT, wild-type.
RLuc-VEGF-WT was subsequently transfected together with miR-150-5p mimic or MIAT. Luciferase assays indicated that miR-150-5p mimic significantly reduced the activity of RLuc-VEGF-WT, whereas MIAT overexpression partially abolished miR-150-5p–mediated repression on RLuc-VEGF-WT activity, suggesting the existence of MIAT–VEGF

**Figure 6.** Myocardial infarction–associated transcript (MIAT) regulate the expression of miR-150-5p target gene, vascular endothelial growth factor (VEGF). A, VEGF was predicted as a target gene of miR-150-5p using miRTarBase. The positions of miR-150-5p binding sites on VEGF (Transcript ID: NM_0010253) were shown. B, VEGF (RLuc-VEGF-WT) and mutant (RLuc-VEGF-Mut) were cloned into the downstream of luciferase vector. Luciferase activity was detected using the dual luciferase assay. C, RLuc-VEGF-WT and miR-150-5p was cotransfected into RF/6A cells with MIAT plasmid or the vector to verify the competing endogenous RNA activity of MIAT. Histogram indicated the data of luciferase activity measured 48 hours after transfection. D and E, RF/6A cells were transfected with different combinations of MIAT and miR-150-5p mimic. Quantitative reverse transcription polymerase chain reactions were conducted to detect VEGF expression. (+) corresponds to 100 ng MIAT construct or 20 ng of miR-150-5p mimic. (+++) corresponds to 200 ng MIAT construct or 50 ng of miR-150-5p mimic. Data were shown as mean±SEM, and expressed as the relative change compared with the control group (n=4). The control group was shown in black color. *Significant difference compared with the corresponding control group. #Significant difference between the marked groups. UTR indicates untranslated region.
cross-talk through the competition for miR-150-5p binding (Figure 6C).

If MIAT effectively functions as a decoy, one would expect that the relative concentration of MIAT and miRNAs affects target gene expression. We gradually increased miR-150-5p amount in the presence or absence of MIAT. MIAT overexpression significantly upregulated VEGF level, and was gradually reduced when miR-150-5p level was increased (Figure 6D). We also gradually increased MIAT amount in the presence or absence of miR-150-5p. miR-150-5p overexpression markedly decreased VEGF level, whereas the decrease was gradually restored when MIAT level was increased (Figure 6E). These results suggest that there is interplay among IncRNA-MIAT, miR-150-5p, and VEGF.

**MIAT-miR-150-5p Regulatory Loop is Critical for Endothelial Cell Function**

To determine the role of miR-150-5p in endothelial cell, RF/6A cells were transfected with miR-150-5p mimic to upregulate its level. miR-150-5p overexpression significantly reduced the viability and proliferation of RF/6A cells. MIAT overexpression could partially abrogate miR-150-5p repression effect (Figure 7A and 7B). On high glucose stress, miR-150-5p also played an inhibitory role in cell viability (Figure 7C). miR-150-5p overexpression could accelerate RF/6A cell apoptosis, whereas MIAT overexpression partially prevented apoptosis development (Figure 7D). We also found that miR-150-5p-regulated endothelial cell function on oxidative stress (Online Figure X). In addition, we found that miR-150-5p overexpression significantly reduced the number of migrated cells and tube formation by TNF-α or VEGF treatment (Figure 7E and 7F; Online Figure XI).

We also determined whether the MIAT–VEGF cross-talk is involved in endothelial cell function. MIAT knockdown obviously inhibited the viability and proliferation of RF/6A cells. Exogenous VEGF addition could partially abrogate the inhibitions. In response to high glucose stress, exogenous VEGF addition could partially prevent MIAT knockdown-induced RF/6A cell damage (Online Figure XII). Collectively, these results suggest that MIAT–VEGF cross-talk regulate endothelial cell function.

**Discussion**

Although most IncRNAs seem to be expressed at lower levels than protein-coding genes, they recently gain extensive attention from molecular biologists and clinicians because of their versatile roles in biological processes and human disorders.33 Here, we provide direct evidence that IncRNA-MIAT is involved in regulating endothelial cell function and pathological angiogenesis. Several IncRNAs have been implicated as the regulators of cardiovascular development. IncRNA-Braveheart regulates cardiovascular development through the activation of a core cardiovascular gene network.33 IncRNA-Fendrr regulates chromatin modification, and thereby affects developmental signaling in cardiovascular system.33 IncRNA-MALAT1 is enriched in endothelial cells, and regulates some endothelial functions, such as migration and vascular sprouting.3 In addition, some lncRNAs are associated with angiotensin-II action and vascular diseases, including coronary heart disease and atherosclerosis.36 We extended IncRNA study into a novel research field, microvascular regulation. IncRNA-MIAT is shown as a regulator of endothelial cell function, and is worthy for further investigation because of its potential for neovascular disease treatment.

DR, a microvascular complication of diabetes mellitus, is the major cause of catastrophic vision loss. Visual deterioration is usually accompanied by inflammation, neovascularization, vascular hyperpermeability, and vascular cell dysfunction.12,23 Hyperglycemia could significantly upregulate IncRNA-MIAT levels in endothelial cells and diabetic retinas. MIAT knockdown could alleviate diabetic-induced retinal neovascularization, vascular leakage, and inflammation in vivo. During normal retinal vascular development, retinal endothelial cells proliferate and migrate through the extracellular matrix on the stimulus of cytokines and inflammatory factors, causing the formation of new blood vessels. In certain disease states, an unceasing and excessive proliferation and migration of endothelial cells occur in retinal vascular system.5 Any factor altering endothelial cell proliferation and migration could cause retinal microvascular dysfunction. MIAT knockdown significantly reduces the proliferation and mobility of endothelial cells in vitro. Thus, it is not surprise that MIAT is involved in the pathogenesis of microvascular dysfunction.

On the basis of in vivo and in vitro evidence, we speculated that MIAT upregulation was a potential stress response on hyperglycaemia stimulus. Early retinal microvascular dysfunctions mainly include blood flow disruption, basement membrane thickening, pericyte loss, and acellular capillary genesis,37 which are accompanied by upregulated MIAT levels. MIAT knockdown could partially prevent the development of these pathological processes. With disease progression, capillary dropout lead to a hypoxic inner retina, alterations in growth factors, increased inflammatory response, and preretinal microvessel generation. During these processes, increased MIAT is tightly associated with abnormal endothelial cell proliferation and migration, causing pathological angiogenesis.38 Its knockdown could alleviate diabetic-induced retinal neovascularization, vascular leakage, and inflammation.

Hyperglycemia contributes directly or indirectly to capillary injury. Potential mechanisms mainly include (1) an increase in the flux of glucose to sorbitol via the polyol pathway, (2) an increase in glucosamine-6-phosphate via the hexosamine pathway, (3) protein kinase C activation, and (4) nonenzymatic
The emergence of lncRNAs as regulators of gene expression has been confirmed in many clinical and preclinical studies.32 VEGF is significantly upregulated in many ocular neovascular diseases, such as DR, retinopathy of prematurity, neovascular glaucoma, and retinal vein occlusion.43 lncRNA-MIAT functions as a ceRNA to regulate VEGF levels by sponging miR-150-5p in retinal endothelial cells. MIAT overexpression may become a sink for miR-150-5p, thereby affecting the depression of VEGF. The ceRNA regulatory network, MIAT/miR-150-5p/VEGF, would provide a novel insight into ocular angiogenesis.

The emergence of lncRNAs as regulators of gene expression has undoubtedly altered our understanding of the mechanisms of pathological angiogenesis. We found that lncRNA-MIAT was shown as a regulator of microvascular dysfunction. Its knockdown significantly alleviated microvascular dysfunction in vivo, and altered specific signaling pathways involved in cell proliferation, migration, and survival of endothelial cells. It is worth mentioning that lncRNA-MIAT functions as a ceRNA, which may sequester miR-150-5p, thereby relieving its repressive effect on VEGF expression. The identification of ceRNA regulatory network will allow us to understand the mechanisms of vascular endothelial dysfunction further, and ultimately facilitate the development of lncRNA-directed diagnostics and therapeutics.

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Disclosures
None.

References
MIAT knockdown inhibits endothelial cell proliferation, migration, and tube formation in vitro.

What New Information Does This Article Contribute?

- MIAT knockdown ameliorates diabetes mellitus–induced microvascular dysfunction in vivo.

What Is Known?

- Long noncoding RNAs regulate several biological processes, such as cell proliferation, cell motility, immune, and inflammation response.
- Myocardial infarction–associated transcript (MIAT) is highly conserved throughout evolution and expressed in mitotic progenitors and postmitotic retinal precursor cells.

Novelty and Significance

- MIAT functions as a competing endogenous RNA, and forms a feedback loop with vascular endothelial growth factor and miR-150-5p to regulate endothelial cell function.

The eukaryotic genome harbors a large number of long noncoding RNAs. These RNAs have been described to play important role in many cellular processes and human disorders. However, the role of long noncoding RNA in diabetes mellitus–induced microvascular dysfunction has not been studied. Here, we report that long noncoding RNA-MIAT is significantly upregulated in diabetic retinas and high glucose–treated endothelial cells. Genetic ablation of MIAT levels ameliorates diabetes mellitus–induced retinal microvascular impairment. Therefore, the reduction of MIAT expression could have therapeutic benefit against microvascular complications of diabetes mellitus.
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Supplemental Material

Supplemental Methods

Western blot

To detect protein expression of interest, the retinas were homogenized in 0.1% SDS buffer containing 125 mM NaCl, 10 mM EDTA, 25 mM HEPES, 10 mM Na₃VO₄, 0.5% deoxycholic acid, 0.1% SDS, 1% Triton X-100 with Complete™ protease inhibitor cocktail (Roche). The lysate was centrifuged at 12,000 rpm for 15 min. The supernatant was then collected and the protein concentration was determined using protein assay kit (Bio-Rad). The extracted protein was separated on SDS-PAGE gel, and transferred onto PVDF membrane (Millipore). The membrane was blocked with 5% bovine serum albumin (BSA) for 1 h to reduce non-specific binding. Then, the blot was incubated with the primary antibody for 12 h at 4°C. After washing, the blot was incubated with HRP-conjugated secondary antibody (Santa Cruz) for 1 h at room temperature. Finally, the signal was detected using the enhanced chemiluminescence (ECL) kit (Amersham) and exposed to X-film. The used antibodies included Akt (1:1,000, Cell Signaling), Caspase 3 (1:1,000, Santa Cruz), VEGF (1:1,000, Santa Cruz), TNF-α (1:1,000, Santa Cruz), and ICAM-1(1:1,000, Santa Cruz).

RNA fluorescence in situ hybridization

To detect the distribution of MIAT or miR-150-5p expression, cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature, and then permeabilized with 1% Triton X-100 on ice for 15 min. These cells were washed in
phosphate-buffered saline (PBS) 3×10 min and rinsed once in 2×SSC prior to hybridization. Hybridization was carried out at 37°C for 8-12 h using Cy3-labeled cDNA probe. Slides were counterstained Tubulin antibody to show cell boundary. Finally, these cells were stained with DAPI to show the nuclear.

Eyes were enucleated, and then fixed with 4% PFA at 4°C for 12 h. They were transferred to 30% sucrose solution for 12 h, embedded in Tissue-Tek OCT compound (Miles), and cut into 10 μm cryosections. Retinal sections were immersed in pre-hybridization buffer containing 50% formamide, 5×Denhardt’s solution, and 5×SSC (1×SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) for 3 h at room temperature. The sections were then hybridized using U6, sense or antisense Cy3-labeled MIAT probe at 60°C for 18-24 h. Slides were washed, and then incubated in RNase A (20 mg/ml) at 37°C for 30 min. Slides were mounted and observed using an Olympus IX-73 microscope.

**Measurement of blood-retinal barrier breakdown**

Blood-retinal barrier breakdown was determined by detecting Evans blue leakage from retinal blood vessels. Briefly, under deep anesthesia, the right jugular vein and right iliac artery were cannulated with 0.28- and 0.58-mm internal diameter polyethylene tube (Becton Dickinson), respectively, and filled with heparinized saline. Evans blue (45 mg/kg) was injected through the jugular vein. Subsequently, at 15 min intervals, 0.1 ml blood was drawn from the iliac artery for 2 h to obtain the time-averaged plasma Evans blue concentration. After the dye circulated for 2 h, the chest cavity was opened. The rats were perfused for 2 min via the left ventricle with
citrate-buffered paraformaldehyde (0.05 M, pH 3.5, Sigma). After the perfusion, both eyes were enucleated and bisected at the equator. The retinas were carefully dissected away under an operating microscope and thoroughly dried for 6 h. Evans blue was extracted by incubating each retina in 120 μl formamide (Sigma) for 18 h at 70°C. The supernatant was filtered through Ultrafree-MC tubes at 3000 rpm for 2.5 h, and 50 μl of the filtrate was used for triplicate spectrophotometric measurement. The background-subtracted absorbance was determined at 620 nm, and the absorbance maximum for Evans blue in formamide. The concentration of Evans blue was calculated from a standard curve and normalized to the dry weight of retina.

**Detection of intracellular ROS**

Intracellular ROS was detected by loading cells with an oxidation-sensitive fluorescent probe dye, 2,7-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes). Retinal cells were grown in 6-well plates in phenol red-free DMEM, and then incubated with normal glucose (5 mM) or high glucose (30 mM) for the indicated times. These cells were washed in PBS buffer. The probe (10 mM) in serum-free medium was added at 37°C for 30 min. DCF fluorescence in the supernatant was detected using an excitation wavelength for 485 nm and an emission wave length of 525 nm.

**MTT assay**

Cell viability was detected using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium-bromide assay (MTT). Briefly, RF/6A cells were plated at a density of 1×10⁴ cells per well in 96-well plates. After the specific treatments, they
were incubated with MTT (0.5 mg/ml) at 37°C for 3 h. Finally, 100 mM DMSO solution was added to dissolve the formazan crystals after the medium removal. The absorbance was detected at 570 nm wavelength using a microplate reader (Molecular Devices).

**Measurement of mitochondrial membrane potential (Δψm)**

The treated cells (2×10^6 cells/ml) were incubated with the fluorescent dye, JC-1 (Molecular Probes), at 37 °C for 2 h to detect the mitochondrial membrane potential. The fluorescence intensity was detected using a microplate reader (Molecular Devices) (excitation at 485 nm, emission spectra between 530-620 nm). In the control cells, an intact Δψm allows JC-1 having a delocalized positive charge, which shows red fluorescence, whereas Δψm collapse allows JC-1 dye to remain in the cytoplasm showing green fluorescence in apoptotic cells. Mitochondrial depolarization was expressed as a decrease in the red-to-green fluorescence intensity ratio.

**Quantitative RT-PCRs (qRT-PCRs)**

Total RNAs from retinas or cells were extracted using TRIzol reagent (Invitrogen). The quality and purity of RNA was controlled spectrophotometrically and estimated by electrophoresis. qRT-PCRs were performed using PikoReal Real-Time PCR System (Thermo Scientific). The amounts of target genes were compared among the groups of interest.

**Hoechst staining**

Following the specific treatment, cells grown on coverslips were fixed in 4% PFA for 15 min at room temperature, and then permeabilized with Triton-X 100 for an
additional 10 min. After PBS wash, these cells were stained using Hoechst 33342 (100 μg/ml) for 10 min. The excess staining was removed by a series of PBS washes. The stained nuclei were observed using an Olympus IX-73 microscope.

**Luciferase assay**

The 3’-untranslated regions (UTR) of VEGF was cloned into the downstream of the firefly luciferase gene to generate RLuc-VEGF-WT vector. The 3’-UTR without predicted miR-150-5p binding site was constructed to generate RLuc-VEGF-Mut vector. For the luciferase reporter assays, cells were plated in 24-well culture plates, and then transfected with either wild-type or mutant construct with and without miRNA mimic or negative control mimic. Firefly and *Renilla* luciferase activities were measured using a Dual Luciferase Reporter Assay System (Promega) and each transfected well was detected in triplicate.

**Calcein-AM and propidium iodide (PI) double staining**

Calcein-AM and PI double staining was used to discriminate live and dead cells. After the specific treatment, RF/6A cells were fixed with 4% PFA for 15 min, and then these cells were stain with Calcein-AM (10 μmol/l) for 5 min. After washing with PBS for three times, these cells were stained with PI (10 μmol/l) for 10 min. The living cells were observed using 490 nm excitation filter, while the dead cells were observed using 545 nm excitation filter.
Online Figures

Online Figure I: Genomic information of IncRNA-MIAT

The information of gene location and genome comparison in different species for IncRNA-MIAT

Online Figure II: Expression pattern of IncRNA-MIAT in different cell lines upon high glucose stress

(A-G) RF/6A, RPE, RGC-5, rMC-1, EA.hy 926 cells, HUVECs, and HMVECs were cultured in the medium containing 5 mM D-glucose (Untreated), 30 mM D-glucose (high glucose), or 30 mM D-mannitol (osmotic control). qRT-PCRs were conducted to compare MIAT levels. MIAT levels were expressed as the fold change compared with untreated group.

Online Figure III: Effect of MIAT knockdown on RF/6A cell function

(A) RF/6A cells were transfected with scramble siRNA (Scr), MIAT siRNA, or left untreated for 48 h. qRT-PCRs were conducted to detect the expression of MIAT, XIST, MALAT1, RP11-197N18.2, LINC00657, LOC100505736, CERKL, and LOC102724801. (B-D) RF/6A cells were transfected with MIAT siRNA, scramble siRNA (Scr), or left untreated, and then these cells were exposed with or without high glucose (30 μM). Viable cells were assessed by cell counting after trypan blue exclusion (B). (C) Cell viability was detected using MTT method. (D) Cell death was analyzed using Calcein-AM and PI double staining. Green: live cells, Red: dead or dying cell. Scale bar: 50 μm.

Online Figure IV: MIAT siRNA2 transfection affected RF/6A cell function upon
high glucose stress

(A) RF/6A cells were transfected with scramble siRNA (Scr), MIAT siRNA2 (GGUGUUAAGACUUGGUUUCTT), or left untreated for 48 h. qRT-PCRs were conducted to detect the expression of MIAT, XIST, MALAT1, RP11-197N18.2, LINC00657, LOC100505736, CERKL, and LOC102724801. (B-E) Cell viability was detected using MTT method (B). Apoptotic cells were analyzed using Hoechst staining and quantitated (C). The treated cells were incubated with JC-1 probe at 37 °C for 30 min in complete cell culture medium, centrifuged, washed, transferred to a 96-well plate, and assayed using a fluorescence plate reader (D). The quantification of Ki67 positive cells showed that MIAT knockdown affected RF/6A cell proliferation upon high glucose stress (E).

Online Figure V: MIAT siRNA3 transfection affects RF/6A cell function upon high glucose stress

(A) RF/6A cells were transfected with scramble siRNA (Scr), MIAT siRNA3 (GCUUUAGAUCAGAGUAUUCTT), or left untreated for 48 h. qRT-PCRs were conducted to detect the expression of MIAT, XIST, MALAT1, RP11-197N18.2, LINC00657, LOC100505736, CERKL, and LOC102724801. (B-E) Cell viability was detected using MTT method (B). Apoptotic cells were analyzed using Hoechst staining and quantitated (C). The treated cells were incubated with JC-1 probe at 37 °C for 30 min in complete cell culture medium, centrifuged, washed, transferred to a 96-well plate, and assayed using a fluorescence plate reader (D). The quantification of Ki67 positive cells showed that MIAT knockdown affected RF/6A cell proliferation.
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**Online Figure VI**: MIAT regulates endothelial cell function upon oxidative stress *in vitro*

(A) RF/6A cells were incubated in the medium containing high glucose (30 mM) for 12 h, 24 h, and 48 h, or in the medium containing different glucose concentration (5 mM, 20 mM, 25 mM, and 30 mM) for 48 h. Intracellular ROS production was detected using DCFDA dye. The control group was shown in black color. The data was expressed as the relative change compared with the control group. “*” indicated a significant difference compared with the control group. (B) RF/6A cells were transfected with scramble siRNA (Scr), MIAT siRNA, or left untreated, and exposed to H$_2$O$_2$ (50 μm) for 48 h. Viable RF/6A cells were assessed by cell counting after trypan blue exclusion. (C) Cell viability was detected using MTT method. (D) Apoptotic cells were analyzed using Hoechst staining and quantitated. Scale bar: 20 μm. The results were shown as means ± S.E.M., and represented four individual experiments in which more than 500 cells were counted. (E and F) The treated cells were incubated with JC-1 probe at 37 °C for 30 min in complete cell culture medium, centrifuged, washed, transferred to a 96-well plate, and assayed using a fluorescence plate reader (E) and observed using a fluorescence microscope (F). Scale bar: 50 μm. (G) The quantification of Ki67 positive cells showed that MIAT knockdown affected RF/6A cell proliferation upon oxidative stress. “*” indicated a significant difference compared with the untreated group. “#” indicated a significant difference between high glucose-treated group and high glucose plus MIAT knockdown group.
Online Figure VII: MIAT regulates endothelial cell function upon hypoxia stress

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(A) RF/6A cells were transfected with scramble siRNA (Scr), MIAT siRNA, or left untreated, and exposed to CoCl₂ (200 μm) for 48 h. Viable RF/6A cells were assessed by cell counting after trypan blue exclusion. (B) Cell viability was detected using MTT method. (C) Apoptotic cells were detected using Hoechst staining and quantitated. The results were shown as means ± S.E.M., and represented four individual experiments in which more than 500 cells were counted. (D) The treated cells were incubated with JC-1 probe at 37 °C for 30 min in complete cell culture medium, centrifuged, washed, transferred to a 96-well plate, and assayed using a fluorescence plate reader. (E) The quantification of Ki67 positive cells showed that MIAT knockdown affected RF/6A cell proliferation upon hypoxia stress. “*” indicated a significant difference compared with the untreated group. “#” indicated a significant difference between high glucose-treated group and high glucose plus MIAT knockdown group.

Online Figure VIII: MIAT knockdown affects VEGF-induced cell migration and tube formation in vitro

(A) RF/6A cells were transfected with MIAT siRNA, scramble siRNA (Scr), or left untreated, and then stimulated with VEGF (10 ng/ml). Cell migration was assessed using wound-healing assay. Images of wounded monolayer were taken at times 0, 24, and 48 h after VEGF treatment. The horizontal line indicated the wound edge. A representative image was shown. Cell migration was estimated by measuring cell
numbers within the wounded region. The data was shown as the relative change compared with the control group without VEGF treatment. (B) RF/6A cells were transfected with MIAT siRNA, scramble siRNA (Scr), or left untreated. These cells were seeded on the matrigel matrix, and stimulated with VEGF (10 ng/ml). The tube-like structures was observed 24 h after VEGF treatment. A representative image was shown. The average number of tube formation for each field was statistically analyzed (n = 50). Scale bar: 100 μm. “*” indicated a significant difference compared with the corresponding control group. “#” indicated a significant difference between the marked groups.

**Online Figure IX : Effect of miR-150-5p intervention on lncRNA-MIAT expression**

(A and B) miR-150-5p mimic or antagonim was injected into the vitreous cavity of one eye of the non-diabetic (A) or diabetic rats (B), and the other eye was received the same dose of scramble miRNA control. MIAT expression was detected using qRT-PCRs. “*” indicated a significant difference compared with the non-diabetic control group. “#"""" indicated a significant difference compared with the diabetic control group.

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RF/6A cells were transfected with miR-150-5p mimic, scramble mimic, or left untreated, and exposed to H₂O₂ (50 μm) for 48 h. Cell viability was detected using MTT assay (A). Apoptotic cells were analyzed by Hoechst staining (B). The change in
mitochondrial potential was detected using JC-1 staining (C).

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RF/6A cells were transfected with miR-150-5p mimic, scramble mimic, or left untreated, and then treated with VEGF (10 ng/ml) for the indicated time. The tube-like structures was observed 24 h after VEGF treatment. A representative image was shown. The average number of tube formation for each field was statistically analyzed (n = 50) (A). Cell migration was assessed using a wound-healing assay (B). “*” indicated a significant difference compared with the corresponding control group. “#” indicated a significant difference between the marked groups. Scale bar: 100 μm.

**Online Figure XII**: MIAT-VEGF crosstalk is involved in the regulation of endothelial cell function

(A, B) RF/6A cells were transfected with MIAT siRNA or scramble siRNA, and then treated with or without VEGF (10 ng/ml) for 48 h. Cell viability was detected using MTT assay (A). Cell proliferation was determined using Ki67 staining, and then quantified (B). (C, D, E) RF/6A cells were transfected as shown, and exposed to high glucose for 48 h. Cell viability was detected using MTT assay (C). Apoptotic cells were analyzed by Hoechst staining (D). The change in mitochondrial potential was detected using JC-1 staining (E). “*” indicated a significant difference compared with the corresponding control group. “#” indicated a significant difference between the marked groups.

**Online Tables**
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Online Tables I. Clinical characteristics of the patients for fibrovascular membrane collection

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Data was shown as mean±S.E.M.. ** indicates a significant difference between the non-diabetic group and experimental group.