Role of METTL3-Dependent N^6 -Methyladenosine mRNA Modification in the Promotion of Angiogenesis

Mu-Di Yao,^{1,2,3,6} Qin Jiang,^{2,3,6} Yan Ma,^{2,3,6} Chang Liu,^{1,2,3,6} Chuan-Yan Zhu,^{2,3,6} Ya-Nan Sun,¹ Kun Shan,¹ Hui-Min Ge,^{2,3} Qiu-Yang Zhang,^{2,3} Hui-Ying Zhang,^{2,3} Jin Yao,^{2,3} Xiu-Miao Li,^{2,3} and Biao Yan^{1,4,5}

¹Eye Institute and Department of Ophthalmology, Eye & ENT Hospital, Fudan University, Shanghai 200031, China; ²The Affiliated Eye Hospital, Nanjing Medical University, Nanjing, China; ³The Fourth School of Clinical Medicine, Nanjing Medical University, Nanjing, China; ⁴NHC Key Laboratory of Myopia (Fudan University), Key Laboratory of Myopia, Chinese Academy of Medical Sciences, Shanghai 200031, China; ⁵Shanghai Key Laboratory of Visual Impairment and Restoration, Shanghai 200031, China

Epigenetic alterations occur in many physiological and pathological processes. N^6 -methyladenosine (m^6A) modification is the most prevalent modification in eukaryotic mRNAs. However, the role of m^6A modification in pathological angiogenesis remains elusive. In this study, we showed that the level of m^6A modification was significantly upregulated in endothelial cells and mouse retinas following hypoxic stress, which was caused by increased METTL3 levels. METTL3 silencing or METTL3 overexpression altered endothelial cell viability, proliferation, migration, and tube formation in vitro. METTL3 knockout in vivo decreased avascular area and pathological neovascular tufts in an oxygen-induced retinopathy model and inhibited alkali burn-induced corneal neovascularization. Mechanistically, METTL3 exerted its angiogenic role by regulating Wnt signaling through the m^6A modification of target genes (e.g., LRP6 and dishevelled 1 [DVL1]). METTL3 enhanced the translation of LRP6 and DVL1 in an YTH m^6A RNA-binding protein 1 (YTHDF1)-dependent manner. Collectively, this study suggests that METTL3-mediated m^6A modification is an important hypoxic stress-response mechanism. The targeting of m^6A through its writer enzyme METTL3 is a promising strategy for the treatment of angiogenic diseases.

INTRODUCTION

Blood vessels supply oxygen and nutrients for eukaryotic cells to maintain oxidative phosphorylation for adenosine triphosphate (ATP) production.¹ Continuous oxygen supply is critical for the development and maintenance of vascularized tissues. Vascular dysfunction caused by vessel occlusion or rupture can lead to decreased oxygen supply and tissue hypoxia.² Hypoxia has become a critical driver of several human diseases, such as diabetic retinopathy, stroke, ischemic heart disease, and cancers.³ Hypoxia and subsequent hypoxia-inducible factors' (HIF's) pathway activation orchestrates the adaptive responses to hypoxic stress by controlling gene networks that govern angiogenesis and metabolism.⁴ Thus, the

targeting of hypoxia-induced angiogenesis is a promising strategy for the treatment of ischemic diseases.

The retinal vasculature offers an easily accessible site for noninvasive evaluation of the circulation condition and vascular remodeling.⁵ Pathological angiogenesis caused by retinal hypoxia is a pathological component of vision-threatening disorders, such as diabetic retinopathy, age-related macular degeneration (AMD), and retinopathy of prematurity (ROP).⁶ Ophthalmologists usually ablate the hypoxic retina through the inhibition of hypoxia-induced retinal angiogenesis.⁷ Neuronal cell apoptosis and vision loss in these diseases are caused by hemorrhage and vascular permeability, which are tightly associated with pathological angiogenesis. The front-line treatments of retinal angiogenesis include laser photocoagulation and anti-angiogenic reagents. Laser photocoagulation ablates ischemic retinal tissue through reducing angiogenic factor production. However, it compromises visual field and usually causes the scotomas.⁸ Vascular endothelial growth factor (VEGF) acts as an angiogenic and neurotrophic factor for normal neural and vascular development. Long-term anti-VEGF treatment can induce several neurological and developmental side effects.⁹ Thus, it is urgent to elucidate the mechanism underlying hypoxic regulation and hypoxia-induced angiogenesis.

Retinal angiogenesis is usually driven by hypoxia-induced gene dysregulation. The number of reported deregulated mRNAs in angiogenesis is growing fast.^{10–12} However, the mechanism underlying gene dysregulation is not fully understood. N^6 -methyladenosine (m^6A) mRNA modification is the most prevalent and reversible modification in eukaryotic mRNA.¹³ It acts as a critical regulator of gene expression via affecting mRNA stability, translation, subcellular localization, and

Received 16 December 2019; accepted 21 July 2020;
<https://doi.org/10.1016/j.ymthe.2020.07.022>.

⁶These authors contributed equally to this work.

Correspondence: Biao Yan, Eye Institute and Department of Ophthalmology, Eye & ENT Hospital, Fudan University, #83 Fen Yang Road, Shanghai 200031, China.
E-mail: biao.yan@fdeent.org

Figure 1. Hypoxic Stress Leads to Increased Levels of m⁶A Modification and Hypoxia-Responsive Genes

(A and B) HUVECs were cultured in normoxia (21% O₂) and hypoxia (1% O₂) for 24 h or 48 h. The levels of m⁶A RNA modification were detected by colorimetric quantification (A; n = 4, one-way ANOVA, Bonferroni test) or dot blot assays (B; n = 4, one-way ANOVA, Bonferroni test). (C and D) The levels of m⁶A RNA modification in the hypoxic P13 retinas and the corresponding controls were detected by colorimetric quantification (C; n = 8 retinas per group, Mann-Whitney *U* test, Bonferroni test) or dot blot assays (D; n = 8 retinas per group, Mann-Whitney *U* test, Bonferroni test). (E) qRT-PCR assays were conducted to detect the levels of HIF-1α, VEGFA, and EPO in the hypoxic retinas and the matched control retinas (n = 8 retinas per group, Mann-Whitney *U* test, Bonferroni test).

alternative splicing.¹⁴ Dynamic regulation of m⁶A modification has been implicated in several biological processes, such as metabolism, embryogenesis, and developmental processes.¹⁵ Aberrant m⁶A modification causes many human diseases, such as obesity, cancer, type 2 diabetes mellitus (T2DM), infertility, and developmental arrest.¹⁶ Although m⁶A mRNA methylation has been recognized as the conserved modification in eukaryotic mRNAs, its feature, regulatory mechanism, and function in pathological angiogenesis is still unknown.

In this study, we showed that the level of global m⁶A RNA modification was significantly increased in endothelial cells and mouse retinas following hypoxic stress. METTL3 silencing decreased endothelial cell viability, proliferation, migration, and tube formation *in vitro*. Endothelial-specific METTL3 knockout suppressed pathological angiogenesis *in vivo*. Thus, the inhibition of METTL3-mediated m⁶A modification is an effective strategy for blocking pathological angiogenesis.

RESULTS

Hypoxic Stress Leads to Increased Levels of m⁶A Modification and Hypoxia-Responsive Genes

Angiogenesis relies on the proliferation and migration of endothelial cells for expansive growth of vascular network. Since hypoxia is a key driver of angiogenesis,¹¹ we thus exposed human umbilical vein

endothelial cells (HUVECs) to hypoxia and determined whether the levels of m⁶A RNA modification were altered. Colorimetric quantification and dot blot assays showed that the levels of m⁶A modification in total RNAs extracted from the hypoxic group were significantly higher than that in the control group (Ctrl; Figures 1A and 1B). Oxygen-induced retinopathy (OIR) is a well-established model to study ischemia-induced angiogenesis. OIR was induced in C57BL/6 mice by exposing the postnatal day 7 (P7) pups to 75% oxygen for 5 days and then returning them to room air at P12.¹⁷ The levels of m⁶A modification in total RNAs were significantly upregulated at the onset of retinal hypoxia on P13 (Figures 1C and 1D). Meanwhile, hypoxia-responsive genes, including HIF-1α, VEGFA, and erythropoietin (EPO), were significantly upregulated at P13 hypoxic retinas (Figure 1E). Collectively, these results provide the evidence that hypoxia is a critical inducer of m⁶A methylation *in vitro* and *in vivo*.

Hypoxic Stress-Induced METTL3 Upregulation Is Mediated in a HIF-Dependent Manner

To identify the regulators responsible for increased levels of m⁶A modification in endothelial cells, we detected the expression patterns of the known m⁶A writers (METTL3, METTL14, and WTAP) and erasers (FTO and ALKBH5) in endothelial cells. qRT-PCR and western blot assays revealed that METTL3 levels were significantly upregulated in endothelial cells' response to hypoxia both at mRNA and protein levels compared with their respective controls (Figures 2A and 2B). We also detected *Mettl3* levels in the hypoxic retinas and normoxic retinas. *Mettl3* levels were significantly upregulated in hypoxic retinas in comparison with the normoxic controls (Figures 2C and 2D).

To determine whether hypoxia-induced METTL3 expression was dependent on HIF-1α, HIF-2α, or both, HUVECs were transfected with HIF-1α small interfering RNA (siRNA; si1α), HIF-2α siRNA

Figure 2. Hypoxic Stress-Induced METTL3 Upregulation Is Mediated in a HIF-Dependent Manner

(A and B) HUVECs were cultured in normoxia (21% O₂) and hypoxia (1% O₂) for 24 h or 48 h. qRT-PCR assays (A) and western blots (B) were conducted to detect the levels of METTL3, METTL14, WTAP, FTO, and ALKBH5 (n = 4, one-way ANOVA, Bonferroni test). The representative immunoblots along with the quantification results were shown (B). *p < 0.05 versus the Ctrl group. (C and D) qRT-PCR assays (C) and western blots (D) were conducted to detect the levels of *Mettl3* in the hypoxic P13 retinas and the corresponding controls (n = 6 retinas per group, Mann-Whitney U test, Bonferroni test). The representative immunoblots along with the quantification results were shown (D). *p < 0.05 versus the normoxic group. (E and F) HUVECs were transfected with HIF-1α siRNA (si1α), HIF-2α siRNA (si2α), or both HIF-1α and HIF-2α siRNA (double knockdown [DKD]) as well as scrambled siRNA (Scr siRNA) as a negative control for 24 h. These cells were then cultured in normoxia (21% O₂) and hypoxia (1% O₂) for an additional 24 h. qRT-PCR assays (E) and western blots (F) were conducted to detect the levels of METTL3 (n = 4, one-way ANOVA, Bonferroni test). The representative immunoblots along with the quantification results were shown (F). *p < 0.05 versus the Scr group at 21% O₂; #p < 0.05 versus the Scr group at 1% O₂.

(si2α), or both HIF-1α and HIF-2α siRNA (double knockdown [DKD]) and scrambled siRNA (Scr) as a negative control. In contrast to the Scr group, hypoxia-induced METTL3 mRNA expression was abrogated in the si1α, si2α, or DKD group (Figure 2E). Immunoblot assays revealed that the levels of METTL3 protein were increased by hypoxia in the Scr group but not in the HIF-knockdown group (Figure 2F). Thus, hypoxia induces METTL3 expression in an HIF-dependent manner.

METTL3 Regulates m⁶A Level, and Its Silencing Inhibits Endothelial Angiogenic Function *In Vitro*

To determine whether METTL3 is a direct regulator of m⁶A modification in endothelial cells, we reduced METTL3 levels using METTL3 siRNA or upregulated METTL3 levels through the gain-

of-function analysis. Compared with the Ctrl group, METTL3 siRNA transfection significantly decreased the level of METTL3 expression. By contrast, METTL3 overexpression led to increased METTL3 expression in HUVECs, which was comparable to METTL3 expression in HUVECs followed by hypoxic stress (Figures 3A and S1). Moreover, METTL3 silencing induced a substantial decrease of m⁶A levels, whereas METTL3 overexpression induced a marked increase of m⁶A levels both under normoxic and hypoxic conditions (Figure S2A), suggesting that METTL3 is an important regulator of m⁶A methylation in endothelial cells. We observed similar phenotype in the primary endothelial cells isolated from hypoxic retinas, which had increased *Mettl3* levels and m⁶A abundance compared with that from the normoxic retinas (Figure S2B). Collectively, these results suggest that METTL3 levels are positively

Figure 3. METTL3 Regulates m⁶A Level, and Its Silencing Inhibits Endothelial Angiogenic Function *In Vitro*

(A) HUVECs were transfected with scrambled (Scr) siRNA, METTL3 (M3) siRNA 1, METTL3 (M3) siRNA 2, pcDNA 3.1 vector, pcDNA 3.1-METTL3 (M3 OE), or left untreated (Ctrl) for 48 h. qRT-PCR assays were conducted to detect the levels of METTL3 ($n = 4$, $*p < 0.05$ versus the Ctrl group, 1-way ANOVA, Bonferroni test). (B) MTT assays were conducted to detect cell viability ($n = 4$, $*p < 0.05$ versus the Ctrl group, one-way ANOVA, Bonferroni test). (C) Ki67 staining and quantification analysis were conducted to detect cell proliferation ($n = 4$, $*p < 0.05$ versus the Ctrl group, 1-way ANOVA, Bonferroni test). Scale bar, 20 μm . (D) Transwell assay and quantification analysis were conducted to detect cell migration ($n = 4$, $*p < 0.05$ versus the Ctrl group, 1-way ANOVA, Bonferroni test). Scale bar, 50 μm . (E) HUVECs were seeded on the Matrigel matrix, and the tube-like structures were observed 6 h after cell seeding. Average tube length for each field was statistically analyzed ($n = 4$, $*p < 0.05$ versus the Ctrl group, one-way ANOVA, Bonferroni test). Scale bar, 100 μm .

correlated with the levels of m⁶A RNA modification in endothelial cells.

We then determined the functional significance of METTL3 in endothelial angiogenic effects *in vitro*. MTT (3-[4,5-dimethylthiazol-2-yl]-

2,5-diphenyl tetrazolium bromide) assays showed that METTL3 silencing led to a marked reduction of HUVEC viability (Figure 3B). Ki67 staining assays showed that METTL3 silencing decreased HUVEC proliferation (Figure 3C). Transwell and Matrigel tube formation assays showed that METTL3 silencing attenuated the migration

Figure 4. *Mettl3* Knockout Suppresses Pathological Angiogenesis In Vivo

(A–C) 7-day-old (P7) endothelial-specific *Mettl3* knockout (ecKO) or the control group (*Mettl3*^{+/+} Cdh5-Cre) mouse pups with their nursing mothers were subjected to hyperoxia (75% O₂) for 5 days and then returned to room air at P12. The retinas were harvested on P12 and P17 and then stained with isolectin B4 (IB4) to show retinal vasculature. (A and B) Red dashed lines highlight avascular areas; green staining indicates retinal vessels. (C) Red staining indicates pathologic angiogenic area. Scale bars, 500 μ m. Avascular area (at P12, A, and P17, B) and pathologic angiogenic area (C; at P17) were statistically analyzed ($n = 5$, Mann-Whitney U test, Bonferroni test). (D and E) Corneal neovascularization was observed by a slit-lamp biomicroscope (D). IB4 immunofluorescence staining was conducted at 2, 5, and 8 days after alkali burn injury. Green staining indicated corneal neovascularization (E). Statistical analysis was conducted to compare the difference of neovascular length and neovascular area between *Mettl3*-ecKO mice and the control group (*Mettl3*^{+/+} Cdh5-Cre). The representative images for the slit-lamp biomicroscope and IB4 staining were shown at 8 days after alkali burn injury. Scale bar, 200 μ m ($n = 5$, Mann-Whitney U test, Bonferroni test).

show gross or histological abnormalities. Endothelial-specific *Mettl3* knockout mice (*Mettl3*-ecKO) were generated by intercrossing *Mettl3*^{fllox/fllox} mice with cadherin-5 (Cdh5)-Cre mice under the control of tamoxifen activation after birth. *Mettl3*-ecKO mice did not show histological abnormalities or defects in vascular development. The levels of *Mettl3* in the primary endothelial cells isolated from *Mettl3*-ecKO mice were significantly lower than that in *Mettl3*^{+/+} Cdh5-Cre mice (Figure S4).

We then determined the role of *Mettl3* in retinal neovascularization *in vivo*. *Mettl3*-ecKO and the control (*Mettl3*^{+/+} Cdh5-Cre) mice were exposed to hyperoxygen from P7 to P12 and then returned to room air. Retinal avascular areas in the *Mettl3*-ecKO mice were not significantly different from the control mice at P12 (Figure 4A).

At P17, pathological neovascularization achieved the maximum amount. The *Mettl3*-ecKO mice had smaller avascular areas and pathological neovascular tufts (Figures 4B and 4C). Thus, *Mettl3*-ecKO suppressed pathological retinal angiogenesis *in vivo*.

We also used another angiogenesis model, the corneal neovascularization model, to investigate the role of endothelium-resident *Mettl3* in angiogenesis *in vivo*. The visibility, accessibility, and avascularity of the cornea are highly advantageous and provide an ideal model for investigating the angiogenic response.¹⁸ In all mice subjected to

and tube-formation ability of HUVECs (Figures 3D and 3E). We also conducted the gain-of-function analysis of METTL3 and determined whether METTL3 was sufficiently alone to drive endothelial angiogenic phenotypes. METTL3 overexpression increased the viability, proliferation, migration, and tube formation of HUVECs (Figure S3).

Mettl3 Knockout Suppresses Pathological Angiogenesis In Vivo

We then investigated the role of endothelium-resident *Mettl3* in angiogenesis *in vivo*. The mating between heterozygous *Mettl3*^{fllox/wt} mice yielded homozygous floxed *Mettl3* mice (*Mettl3*^{fllox/fllox}) at the expected Mendelian ratio. *Mettl3*^{fllox/fllox} mice were viable, fertile, and did not

Figure 5. High-Throughput m⁶A Sequencing Identified the Wnt Signaling Pathway as the Target of METTL3

(A) Metagene plots showed the abundance of the m⁶A peak along the normalized transcript composed of three rescaled nonoverlapping segments: 5' UTR, CDS, and 3' UTR in the control and METTL3-overexpression groups. (B) Pie charts showed the distribution of m⁶A peaks throughout mRNAs. (C) Volcano plots showed the mRNAs that were m⁶A hypermethylated (orange dots) and hypomethylated (green dots) in the METTL3-overexpression group over the control group (n = 3/group). The red threshold lines were set at 1.5-fold (p < 0.05) between the METTL3-overexpression and control group. (D) Gene ontology (GO) analysis was conducted to predict the molecular function of upregulated m⁶A modified mRNA transcripts upon METTL3 overexpression. (E) KEGG pathway analysis was conducted to predict the signaling pathways associated with METTL3 overexpression. (F) Integrative Genomics Viewer (IGV) plots showed the MeRIP reads of the selected members of Wnt signaling, such as DVL1, LRP6, and CARM1.

alkali burn injury, corneal neovascularization started on day 2 and reached its maximum on day 8. Moreover, the area of corneal neovascularization in *Mettl3*-ecKO mice was smaller than that in the control mice at all detected time points (Figures 4D and 4E).

High-Throughput m⁶A Sequencing Identified the Wnt Signaling Pathway as the Target of METTL3

To identify the target of METTL3, RNA samples were isolated from HUVECs, including the METTL3-overexpression group and the

Ctrl group. The RNA samples were subjected to m⁶A-modified RNA immunoprecipitation (MeRIP) followed by sequencing. MeRIP reads were normalized to the corresponding nonimmunoprecipitation reads to identify differential m⁶A modifications independent of varying transcription. Transcriptome-wide methylation represented by the abundance of m⁶A peak distribution revealed the globe hypermethylation in the METTL3-overexpression group compared with the Ctrl group (Figure 5A). m⁶A peaks were abundant in the coding sequences (CDSs) (Figure 5B). MeRIP, followed by sequencing,

revealed that 574 genes were differentially m⁶A methylated between the METTL3-overexpression and Ctrl group, including 429 m⁶A hypermethylated genes and 145 m⁶A hypomethylated genes (Figure 5C; Table S1). With the consideration of the role of METTL3 in the m⁶A writer complex, only m⁶A peaks with increased abundance upon METTL3 overexpression were considered as authentic m⁶A peaks. Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were conducted to predict the potential function of upregulated m⁶A methylated transcripts induced by METTL3 overexpression. GO analysis showed that regulation of transcription from the RNA polymerase II promoter was the most important molecular function of the upregulated m⁶A methylated transcripts induced by METTL3 overexpression (Figure 5D). Pathway analysis showed that 8 signaling pathways were associated with the upregulated m⁶A methylated transcripts induced by METTL3 overexpression. Notably, the Wnt signaling pathway was ranked as the top 1 signaling pathway that was potentially affected by METTL3 overexpression (Figure 5E). MeRIP sequencing (MeRIP-seq) maps of individual transcripts showed the hypermethylated sites in the METTL3-overexpression group in comparison with the Ctrl group for some members of the Wnt signaling pathway, such as dishevelled 1 (DVL1), LRP6, and CARM1 (Figure 5F).

METTL3 Regulates Wnt Signaling Activation by Targeting LRP6 and DVL1

Based on the above-mentioned evidence, we knew that Wnt signaling was the major pathway affected by METTL3 intervention. In particular, the m⁶A abundance of LRP6 and DVL1 was markedly decreased upon METTL3 knockdown, as shown by gene-specific m⁶A quantitative polymerase chain reactions (qPCRs) (Figure 6A), suggesting LRP6 and DVL1 are the potential targets of METTL3. We then examined whether METTL3 regulated the expression of LRP6 and DVL1 in HUVECs. METTL3 knockdown decreased the levels of LRP6 and DVL1 (Figure 6B). Conversely, METTL3 overexpression induced a significant increase in LRP6 and DVL1 levels in HUVECs (Figure 6B). Moreover, METTL3 knockdown decreased the expression of the members of Wnt signaling, which have been well curated, including matrix metalloproteinase-7 (MMP-7), hepatocyte growth factor (HGF), and VEGF (Figure 6C).

To determine whether METTL3 overexpression-induced Wnt signaling was responsible for increased endothelial angiogenic effects, we performed the rescue experiments using Wnt pathway inhibitors, such as ICG-001 and ETC-159. We showed that METTL3 overexpression-induced endothelial angiogenic effects could be interrupted by the inhibitors of Wnt signaling, ICG-001 and ETC-159 (Figures 6D–6F).

METTL3 Enhances the Translation of LRP6 and DVL1 in a YTH m⁶A RNA-Binding Protein 1 (YTHDF1)-Dependent Manner

We next investigated the mechanism how m⁶A modification regulated the expression of LRP6 and DVL1 in HUVECs. It is known that m⁶A is selectively recognized by the specific m⁶A-binding proteins to exert its biological functions. YTHDF1, YTHDF2, YTHDC2,

and YTHDC1 have been reported to regulate the translation and stability of m⁶A methylated transcripts.¹³ Overexpression of YTHDF1 but not YTHDF2, YTHDC2, or YTHDC1 led to increased levels of LRP6 and DVL1 in HUVECs (Figures 7A and S5). As expected, RIP-qPCR analysis revealed that LRP6 and DVL1 were the target genes of YTHDF1 (Figure 7B). Overexpression of YTHDF1 recovered the decreased levels of LRP6 and DVL1 in METTL3-knockdown HUVECs (Figure 7C). Overexpression of YTHDF1 could partially rescue the reduction of endothelial angiogenic effects induced by METTL3 knockdown (Figures 7D–7F). Taken together, the above-mentioned results show that METTL3 regulates LRP6 and DVL1 levels by modulating the translation in a YTHDF1-dependent pathway.

With the consideration that initiation is typically the rate-limiting step of translation, we tested whether YTHDF1 might interact with the translation initiation factors. We conducted the coimmunoprecipitation (coIP) experiments with the FLAG affinity-purified complexes and tested for the association of YTHDF1 with translation initiation proteins. The result showed that CBP80 and eukaryotic translation initiation factor 4E (eIF4E) were coimmunopurified with FLAG-YTHDF1 in an RNA-independent manner (Figure 7G). However, none of these factors was found associated with FLAG-FTO (Figure 7G).

Rapamycin is a specific inhibitor of cap-dependent protein translation. It can inhibit eIF4E-binding protein 1 (4E-BP1) phosphorylation and induce increased interaction between 4E-BP1 and eIF4E. We treated the control and YTHDF1-overexpressed HUVECs with or without rapamycin. Rapamycin treatment markedly inhibited the increase of LRP6 and DVL1 levels in YTHDF1-overexpressed cells, indicating that YTHDF1 mediates the translation of LRP6 and DVL1 in a cap-dependent manner (Figure 7H).

DISCUSSION

m⁶A modification is the most prevalent modification that occurs in eukaryotic mRNAs. It plays important roles in many biological processes, such as ultraviolet-induced DNA damage, maternal mRNA clearance, neuronal functions, T cell homeostasis, and progenitor cell specification.^{19–22} In this study, we report a novel function of m⁶A modification in pathological angiogenesis. Hypoxic stress induces increased levels of m⁶A methylation in endothelial cells and mouse retinas. Enhanced m⁶A RNA methylation contributes to the progression of pathological angiogenesis, whereas decreased m⁶A methylation alleviates vascular dysfunction, highlighting the importance of m⁶A methylation in vascular homeostasis.

Hypoxia is usually recognized as the critical driver of angiogenesis. Under physiological condition, the vasculature grows toward hypoxic areas in an organized fashion.¹² However, the immature neovascular nearly has no therapeutic effect on ischemic injury. Restoration of a proper angiogenic response would be beneficial for reoxygenizing the ischemic condition and resolving disease pathogenesis.^{23,24} The regulation of angiogenesis by hypoxia is an important component of homeostatic mechanism that links vascular oxygen supply to

Figure 6. METTL3 Regulates Wnt Signaling Activation by Targeting LRP6 and DVL1

(A) Reduction of m^6A modification in the specific regions of LRP6 and DVL1 transcripts upon METTL3 knockdown as determined by the gene-specific m^6A -qPCR assays in HUVECs ($n = 4$, Student's t test). (B) Western blots were conducted to detect the levels of LRP6 and DVL1 in HUVECs after METTL3 intervention. Representative immunoblots were shown ($n = 4$). (C) qRT-PCRs were conducted to detect the levels of MMP-7, HGF, and VEGF ($n = 4$, Student's t test). (D–F) HUVECs were treated as shown. Ki67 staining and quantification analysis were conducted to detect cell proliferation. Scale bar, 20 μm (D; $n = 4$, one-way ANOVA, Bonferroni test). Transwell assay and quantification analysis were conducted to detect cell migration. Scale bar, 50 μm (E; $n = 4$, one-way ANOVA, Bonferroni test). HUVECs were seeded on the Matrigel matrix. The tube-like structures were observed 6 h after cell seeding. Average tube length for each field was statistically analyzed. Scale bar, 100 μm (F; $n = 4$, one-way ANOVA, Bonferroni test). *Significant difference compared with the control group. #Significant difference between the marked groups.

metabolic demand, which is regulated at different gene levels. m^6A methylation is the most prevalent post-transcriptional modification on mRNAs. Compared with the transcriptional regulation of mRNAs, the post-transcriptional regulation has several advantages, such as prompt stimuli response, fine-tuning protein amount, and localized regulation control.^{25,26} We show that pathological angiogenesis is regulated by m^6A methylation. Hypoxic stress induces increased levels of m^6A RNA methylation in the hypoxia-treated endothelial cells and the hypoxic retinas. m^6A modification is essential for the normal function of endothelial cells and vascular physiology. Its disruption would lead to vascular pathology.

m^6A homeostasis is achieved through the coordinated regulation of the m^6A methylase complex and demethylases.^{27,28} We show that hypoxic stress induces an increased METTL3 level but has no effects on the levels of other m^6A writers (METTL14 and WTAP) and erasers (FTO and ALKBH5) in endothelial cells. The role of METTL3 in tissue homeostasis and embryogenesis has been verified. Zhang et al.²² have reported that METTL3-mediated m^6A modification determines cell fate during the endothelial-to-hematopoietic transition (EHT) to specify the earliest hematopoietic stem/progenitor cells (HSPCs) during embryogenesis. Dorn et al.²⁹ have reported that METTL3-mediated methylation of mRNA on N^6 -adenosines is

Figure 7. METTL3 Enhances the Translation of LRP6 and DVL1 in a YTHDF1-Dependent Manner

(A) Western blot analysis of LRP6 or DVL1 level in HUVECs transfected with FLAG-YTHDF1, vector, or left untreated (Ctrl). GAPDH was detected as the loading control ($n = 3$). Representative immunoblots were shown. (B) RIP analysis of the interaction of LRP6 and DVL1 in HUVECs transfected with FLAG-YTHDF1 plasmid. Enrichment of LRP6 and DVL1 was determined by qPCRs and normalized to the input ($n = 3$, Student's t test). (C) Western blot analysis of LRP6 or DVL1 level in METTL3-knockout HUVECs transfected with control and plasmid ($n = 3$). (D–F) HUVECs were treated as shown. Ki67 staining and quantification analysis were conducted to detect cell proliferation. Scale bar, 20 μm (D; $n = 4$, one-way ANOVA, Bonferroni test). Transwell assay and quantification analysis were conducted to detect cell migration. Scale bar, 50 μm (E; $n = 4$, one-way ANOVA, Bonferroni test). The tube-like structures were observed 6 h after cell seeding on the Matrigel matrix. Average tube length for each field was statistically analyzed. Scale bar, 100 μm (F; $n = 4$, one-way ANOVA, Bonferroni test). *Significant difference compared with the control group. #Significant difference between the marked groups. (G) The binding between YTHDF1 with CBP80 and eIF4E in HUVECs was checked by immunoprecipitation ($n = 3$). (H) HUVECs were transfected with or without YTHDF1 and then treated with or without rapamycin (Rap; 20 nM). Western blots were conducted to detect the levels of LRP6 or DVL1 ($n = 3$).

enhanced in response to hypertrophic stimuli. METTL3 is involved in the regulation of cardiac homeostasis and hypertrophy.²⁹ We show that METTL3 silencing reduces aberrant proliferation, mobility, and tube formation of endothelial cells. METTL3 silencing plays an anti-angiogenic role in the models of OIR and corneal neovascularization. With the consideration of the complexity and intersection of multiple signaling mechanisms in angiogenesis, the targeting of m⁶A modification through the regulation of the METTL3 level is a promising strategy for the treatment of angiogenic diseases.

Pathway analysis shows that Wnt signaling is mainly affected by m⁶A methylation in endothelial cells. Wnt signaling regulates several biological phenomena throughout development and adult life. Aberrant Wnt signaling contributes to a wide range of pathologies, including angiogenesis.³⁰ Previous study has revealed that Wnt signaling contributes to retinal angiogenesis via increased β -catenin nuclear signaling. Wnt signaling can mediate pathological vascular growth in proliferative retinopathy through claudin5-mediated signaling. Loss of LRP5 and Wnt signaling protein Dvl2 leads to decreased levels of pathological neovascularization in oxygen-induced proliferative retinopathy.^{31,32} Intervention of Wnt signaling can affect the development of angiogenesis. Angiogenesis accelerates the transport of nutrients and oxygen to hypoxic regions, which is important for combating against ischemic stress. Thus, intervention of Wnt signaling via regulating m⁶A methylation is critical for vascular homeostasis.

We reveal that m⁶A methylation affects two members of Wnt signaling: LRP6 and DVL1. The binding of Wnt to the LRP receptor complex stabilizes β -catenin in the cytoplasm, promoting its translocation to the nucleus.³³ Elevated LRP6 levels, which correlate with VEGF levels, have been detected in the vitreous of proliferative diabetic retinopathy.³⁴ DVL1 is a central mediator of Wnt signal transduction. It encodes intracellular scaffolding proteins, acting directly downstream of the transmembrane Wnt receptors.³⁵ Previous studies have revealed that null mutations and dysregulation of Frizzled-4, LRP5, Lef-1, and Norrin affect Wnt signaling activation and induce vascular dysfunction, such as intraretinal capillary absence, vascular patterning defect, arteriovenous anastomosis, and intraocular hemorrhages.^{31,36} METTL3 knockdown decreases the levels of LRP6 and DVL1 and reduces the expression of MMP-7, HGF, and VEGF, the target genes of Wnt signaling. The roles of MMP-7, HGF, and VEGF in angiogenesis have also been recognized.³⁷ Thus, it is not surprising that METTL3-mediated Wnt signaling is involved in pathological angiogenesis.

In summary, we utilized the endothelial cells and *Mettl3*-ecKO mice to investigate the role of METTL3-mediated m⁶A modification in pathological angiogenesis. METTL3 silencing reduces endothelial angiogenic effects *in vitro* and suppresses pathological angiogenesis *in vivo*. Mechanistically, METTL3 exerts its proangiogenic role through the aberrant activation of Wnt signaling. METTL3 can methylate LRP6 and DVL1 mRNA and induce abnormal translation of LRP6 and DVL1. This study provides strong evidence for deciphering the function of METTL3-mediated m⁶A modification in vascular biology.

MATERIALS AND METHODS

Animals

All animal experiments were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee of Eye and Ears, Nose, and Throat Hospital and Nanjing Medical University. *Mettl3*^{flox/flox} mice were gifts from Prof. Ming-Han Tong (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). *Cdh5*-CreERT2 mice were gifts from Prof. Yu-Zhen Zhang (Tongji University, Shanghai, China). *Mettl3*^{flox/flox} mice were crossed with the transgenic *Cdh5*-CreERT2 mice to generate the *Mettl3*-ecKO mice. In all experiments, the littermates from the same breeding pairs were used as controls. All mice were bred under the specific pathogen-free condition with free access to diet and water or their nursing mothers in a 12-h day/night cycle (lights on at 0800 and off at 2000).

Cell Culture

HUVECs were maintained in endothelial basal medium (EBM-2; Lonza; cc-3156), supplemented with endothelial cell growth medium (Lonza; cc-3162) at 37°C in a mixture of 95% air and 5% CO₂. HUVECs were passaged approximately 2 times per week, and the culture medium was exchanged every 2 days. HUVECs at passages 5–8 were used for all experiments.

OIR Model

Cdh5-Cre *Mettl3*^{flox/flox} mice received an intragastric injection of 50 μ L tamoxifen (1 mg/mL) at P1–P3 and P5 for Cre activation and *Mettl3* knockout. After *Mettl3* knockout, the mouse pups and their nursing mothers were exposed to 75% oxygen (hyperoxia) from P7 to P12 in an incubator chamber. Then, the pups were returned to normal oxygen conditions (normoxia). The age-matched and gender-matched mice were kept continuously in room air as the Ctrl group. This model was served as a proxy to ocular neovascular diseases characterized by pathological angiogenesis.

Alkali Burn-Induced Corneal Angiogenesis Model

For the induction of *Mettl3* knockout, *Cdh5*-Cre *Mettl3*^{flox/flox} mice (2 months old) received an intraperitoneal injection of 75 mg/kg tamoxifen for 5 consecutive days between 6 and 8 weeks of age. After *Mettl3* knockout, the mice were anesthetized with isoflurane (4% vol/vol), followed by the topical application of 0.5% proparacaine hydrochloride on the corneal surface. The central region of cornea was injured by soaking NaOH (0.1 M) for 30 s. Then, the corneas were washed with phosphate buffered saline (PBS) to remove the residual NaOH. Finally, ofloxacin ophthalmic ointment was instilled immediately after the operation to prevent the potential infection.

Quantification of m⁶A RNA Level

The levels of m⁶A RNA modification were determined by the EpiQuik m⁶A RNA Methylation Quantification Kit (Colorimetric; EpiGentek; P-9008-48), according to the manufacturer's instruction. Briefly, total

RNAs (100 ng) were bound to each well, followed by the addition of the capture antibody solution and detection antibody solution, according to the manufacturer's protocol. The levels of m⁶A modification were quantified by detecting the absorbance at 450 nm. All samples were detected in triplicate, and the standard curves were used for the quantification of m⁶A RNA levels.

M⁶A Dot Blot Assay

Total RNAs were isolated using the TRIzol reagent (Invitrogen; 15596018), according to the manufacturer's instructions. RNA concentration was determined by the NanoDrop ND-1000 Spectrophotometer (Agilent). m⁶A dot blot assay was conducted as shown below. Briefly, the RNA samples were loaded onto the Amersham Hybond-N⁺ membrane (GE Healthcare; 45-000-763) and UV crosslinked. The membrane was blocked with 5% nonfat milk for 1 h, incubated with m⁶A antibody (1:1,000 dilution; Synaptic Systems; 202003) overnight at 4°C, and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology; sc-2030) for 1 h at room temperature. The membrane was finally visualized using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). The relative signal density of each dot was quantified by ImageJ software.

MeRIP-Seq and MeRIP-qPCR

Total RNAs were extracted from METTL3-overexpression HUVECs and their corresponding controls. They were sonicated into 100–150 nt fragments, and the fragmented RNA was incubated with m⁶A antibody (Synaptic Systems; 202003) for immunoprecipitation (IP). m⁶A RNA enrichment was determined by qRT-PCR or high-throughput sequencing. Briefly, 4 µg fragmented mRNA was incubated with 2 µg m⁶A antibody or rabbit normal immunoglobulin G (IgG; negative control) in 1 × IP buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40 [NP-40]) for 2 h at 4°C. Then, the m⁶A-IP mixture was incubated with Dynabeads protein A (Life Technologies; 10002D) at 4°C for 2 h. The m⁶A-IP mixture was washed with 1 × IP buffer, 3 times, and 1 × wash buffer, 2 times (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1% NP-40). The bound RNA was eluted by the elution buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.05% SDS, 40 U proteinase K) at 50°C for 30 min and purified. The immunoprecipitated, purified RNA fragments from MeRIP or input RNAs were used for library construction using the KAPA Stranded mRNA-Seq Kit (Illumina; KK8421) and sequenced with Illumina HiSeq 4000. For the MeRIP-qPCR, the immunopurified RNAs were purified. The first-strand cDNA synthesis was conducted using the PrimeScript RT Reagent Kit (Takara Bio; RR037A). mRNA enrichment in the m⁶A-immunopurified sample was expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in bound samples and expressed as fold change between groups.

M⁶A-Seq Data Analysis

The quality of sequencing data was evaluated using FastQC v.0.11.5 software. Raw data filtration was conducted using Trimmomatic v.0.32 software. The sequencing reads were aligned to human genome GRCh37/hg19 using HISAT2 (v.2.1.0) software. Differential peak

analysis of m⁶A MeRIP-seq data was conducted using the exomePeak and MACS2 algorithm to compare the ratio of the absolute number of MeRIP reads with the nonimmunoprecipitation reads at a given peak between 2 conditions. The Database for Annotation, Visualization and Integrated Discovery (DAVID) database was used for GO analysis and pathway enrichment analysis of the genes with differentially methylated MeRIP peaks.

Statistical Analysis

All results were presented in the form of mean ± SEM unless otherwise mentioned. The normality tests were conducted to evaluate the normal distribution of data. For normally distributed data with equal variance, the difference was analyzed by the two-tailed Student's *t* test (2-group comparisons) and analysis of variance (ANOVA) followed by post hoc Bonferroni's test (multi-group comparisons) as appropriate. For non-normally distributed data or data with unequal variances, the difference was analyzed by the nonparametric Mann-Whitney *U* test (2-group comparisons) or Kruskal-Wallis test followed by post hoc Bonferroni's test (multi-group comparisons). The results were considered significantly different if the *p* value was <0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.ymthe.2020.07.022>.

AUTHOR CONTRIBUTIONS

B.Y. conceived and supervised this study. B.Y. and Q.J. were responsible for all aspects of study design. M.-D.Y., Y.M., C.L., C.-Y.Z., Y.-N.S., K.S., H.-M.G., Q.-Y. Z., and H.-Y.Z. conducted the experiments and interpreted all results. B.Y. wrote the manuscript. J.Y. an X.-M.L. provided advice. All authors critically reviewed the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

This work was generously supported by grants from the National Natural Science Foundation of China (grant nos. 81770945, 81970809, 81470594, 81570859, and 81800858); Medical Science and Technology Development Project Fund of Nanjing (grant no. ZKX1705); Innovation Team Project Fund of Jiangsu Province (grant no. CXTDB2017010); and Science and Technology Development Plan Project Fund of Nanjing (grant no. 201716007).

REFERENCES

- Li, X., Sun, X., and Carmeliet, P. (2019). Hallmarks of endothelial cell metabolism in health and disease. *Cell Metab.* 30, 414–433.
- Li, X., Kumar, A., and Carmeliet, P. (2019). Metabolic pathways fueling the endothelial cell drive. *Annu. Rev. Physiol.* 81, 483–503.
- Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873–887.
- Carmeliet, P., and Jain, R.K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473, 298–307.
- Flammer, J., Konieczka, K., Bruno, R.M., Virdis, A., Flammer, A.J., and Taddei, S. (2013). The eye and the heart. *Eur. Heart J.* 34, 1270–1278.

6. Lee, J., Kim, K.E., Choi, D.K., Jang, J.Y., Jung, J.J., Kiyonari, H., Shioi, G., Chang, W., Suda, T., Mochizuki, N., et al. (2013). Angiopoietin-1 guides directional angiogenesis through integrin $\alpha v\beta 5$ signaling for recovery of ischemic retinopathy. *Sci. Transl. Med.* 5, 203ra127.
7. Moravski, C.J., Kelly, D.J., Cooper, M.E., Gilbert, R.E., Bertram, J.F., Shahinfar, S., Skinner, S.L., and Wilkinson-Berka, J.L. (2000). Retinal neovascularization is prevented by blockade of the renin-angiotensin system. *Hypertension* 36, 1099–1104.
8. Ishida, S., Yamashiro, K., Usui, T., Kaji, Y., Ogura, Y., Hida, T., Honda, Y., Oguchi, Y., and Adamis, A.P. (2003). Leukocytes mediate retinal vascular remodeling during development and vaso-obliteration in disease. *Nat. Med.* 9, 781–788.
9. Ferrara, N., and Adamis, A.P. (2016). Ten years of anti-vascular endothelial growth factor therapy. *Nat. Rev. Drug Discov.* 15, 385–403.
10. Hou, X., Kumar, A., Lee, C., Wang, B., Arjunan, P., Dong, L., Maminishkis, A., Tang, Z., Li, Y., Zhang, F., et al. (2010). PDGF-CC blockade inhibits pathological angiogenesis by acting on multiple cellular and molecular targets. *Proc. Natl. Acad. Sci. USA* 107, 12216–12221.
11. Pugh, C.W., and Ratcliffe, P.J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat. Med.* 9, 677–684.
12. Krock, B.L., Skuli, N., and Simon, M.C. (2011). Hypoxia-induced angiogenesis: good and evil. *Genes Cancer* 2, 1117–1133.
13. Patil, D.P., Pickering, B.F., and Jaffrey, S.R. (2018). Reading m⁶A in the transcriptome: m⁶A-binding proteins. *Trends Cell Biol.* 28, 113–127.
14. Liu, J., Harada, B.T., and He, C. (2019). Regulation of gene expression by N⁶-methyladenosine in cancer. *Trends Cell Biol.* 29, 487–499.
15. Meyer, K.D., and Jaffrey, S.R. (2014). The dynamic epitranscriptome: N⁶-methyladenosine and gene expression control. *Nat. Rev. Mol. Cell Biol.* 15, 313–326.
16. Klungland, A., and Dahl, J.A. (2014). Dynamic RNA modifications in disease. *Curr. Opin. Genet. Dev.* 26, 47–52.
17. Selvam, S., Kumar, T., and Fruttiger, M. (2018). Retinal vasculature development in health and disease. *Prog. Retin. Eye Res.* 63, 1–19.
18. Liu, C.H., Wang, Z., Sun, Y., and Chen, J. (2017). Animal models of ocular angiogenesis: from development to pathologies. *FASEB J.* 31, 4665–4681.
19. Lin, X., Chai, G., Wu, Y., Li, J., Chen, F., Liu, J., Luo, G., Tauler, J., Du, J., Lin, S., et al. (2019). RNA m⁶A methylation regulates the epithelial mesenchymal transition of cancer cells and translation of Snail. *Nat. Commun.* 10, 2065.
20. Lence, T., Akhtar, J., Bayer, M., Schmid, K., Spindler, L., Ho, C.H., Kreim, N., Andrade-Navarro, M.A., Poeck, B., Helm, M., and Roignant, J.Y. (2016). m⁶A modulates neuronal functions and sex determination in *Drosophila*. *Nature* 540, 242–247.
21. Xiang, Y., Laurent, B., Hsu, C.H., Nachtergaele, S., Lu, Z., Sheng, W., Xu, C., Chen, H., Ouyang, J., Wang, S., et al. (2017). RNA m⁶A methylation regulates the ultraviolet-induced DNA damage response. *Nature* 543, 573–576.
22. Zhang, C., Chen, Y., Sun, B., Wang, L., Yang, Y., Ma, D., Lv, J., Heng, J., Ding, Y., Xue, Y., et al. (2017). m⁶A modulates haematopoietic stem and progenitor cell specification. *Nature* 549, 273–276.
23. Howangyin, K.Y., and Silvestre, J.S. (2014). Diabetes mellitus and ischemic diseases: molecular mechanisms of vascular repair dysfunction. *Arterioscler. Thromb. Vasc. Biol.* 34, 1126–1135.
24. Zhang, T., Suo, C., Zheng, C., and Zhang, H. (2019). Hypoxia and metabolism in metastasis. *Adv. Exp. Med. Biol.* 1136, 87–95.
25. Wang, X., Zhao, B.S., Roundtree, I.A., Lu, Z., Han, D., Ma, H., Weng, X., Chen, K., Shi, H., and He, C. (2015). N(6)-methyladenosine Modulates Messenger RNA Translation Efficiency. *Cell* 161, 1388–1399.
26. Moore, M.J. (2005). From birth to death: the complex lives of eukaryotic mRNAs. *Science* 309, 1514–1518.
27. Wu, B., Li, L., Huang, Y., Ma, J., and Min, J. (2017). Readers, writers and erasers of N⁶-methylated adenosine modification. *Curr. Opin. Struct. Biol.* 47, 67–76.
28. Meyer, K.D., and Jaffrey, S.R. (2017). Rethinking m⁶A readers, writers, and erasers. *Annu. Rev. Cell Dev. Biol.* 33, 319–342.
29. Dorn, L.E., Lasman, L., Chen, J., Xu, X., Hund, T.J., Medvedovic, M., Hanna, J.H., van Berlo, J.H., and Accornero, F. (2019). The N6-Methyladenosine mRNA Methylase METTL3 Controls Cardiac Homeostasis and Hypertrophy. *Circulation* 139, 533–545.
30. Clevers, H., and Nusse, R. (2012). Wnt/ β -catenin signaling and disease. *Cell* 149, 1192–1205.
31. Chen, J., Stahl, A., Krah, N.M., Seaward, M.R., Dennison, R.J., Sapieha, P., Hua, J., Hatton, C.J., Juan, A.M., Aderman, C.M., et al. (2011). Wnt signaling mediates pathological vascular growth in proliferative retinopathy. *Circulation* 124, 1871–1881.
32. Nusse, R., and Clevers, H. (2017). Wnt/ β -catenin signaling, disease, and emerging therapeutic modalities. *Cell* 169, 985–999.
33. González-Sancho, J.M., Brennan, K.R., Castelo-Soccio, L.A., and Brown, A.M. (2004). Wnt proteins induce dishevelled phosphorylation via an LRP5/6-independent mechanism, irrespective of their ability to stabilize β -catenin. *Mol. Cell. Biol.* 24, 4757–4768.
34. Gao, X., Ma, K., Lu, N., Xu, Y., Hong, T., and Peng, X. (2015). Elevated LRP6 levels correlate with vascular endothelial growth factor in the vitreous of proliferative diabetic retinopathy. *Mol. Vis.* 21, 665–672.
35. Masckauchán, T.N., and Kitajewski, J. (2006). Wnt/Frizzled signaling in the vasculature: new angiogenic factors in sight. *Physiology (Bethesda)* 21, 181–188.
36. Zerlin, M., Julius, M.A., and Kitajewski, J. (2008). Wnt/Frizzled signaling in angiogenesis. *Angiogenesis* 11, 63–69.
37. Das, A., and McGuire, P.G. (2003). Retinal and choroidal angiogenesis: pathophysiology and strategies for inhibition. *Prog. Retin. Eye Res.* 22, 721–748.