BASIC SCIENCES

METTL3 Regulates Angiogenesis by Modulating let-7e-5p and miRNA-18a-5p Expression in Endothelial Cells

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OBJECTIVE: Postnatal angiogenesis is critical in vascular homeostasis and repair. m^6A (N6-methyladenosine) RNA methylation is emerging as a new layer for fine-tuning gene expression. Although the contribution of the m^6A-catalyzing enzyme, METTL3 (methyltransferase-like 3), in cancer biology has been described, its role in endothelial cell (EC) function, particularly during angiogenesis, remains unclear.

APPROACH AND RESULTS: To characterize the relevance of METTL3 in angiogenesis regulation, we performed gain- and loss-of-function studies in vitro. We demonstrated that depletion of METTL3 in ECs reduced the level of m^6A and impaired EC function, whereas adenovirus-mediated METTL3 overexpression increased angiogenesis. Mechanistically, we showed that METTL3 depletion in ECs decreased mature angiogenic microRNAs let-7e-5p and the miR-17-92 cluster, and increased the expression of their common target, Tsp1 (thrombospondin 1). Conversely, Ad.METTL3 increased the expression of let-7e-5p and miR-17-92 cluster and reduced protein levels of Tsp1 in ECs. Moreover, overexpression of let-7e-5p and miR-18a-5p restored the angiogenic potential of METTL3-depleted ECs. We corroborated our data in vivo employing 3 mouse models. When tested in an in vivo Matrigel plug assay, METTL3-depleted ECs had diminished ability to vascularize the plug, whereas overexpression of METTL3 promoted angiogenesis. Local Ad.METTL3 gene transfer increased postischemic neovascularization in mice with either unilateral limb ischemia or myocardial infarction.

CONCLUSIONS: METTL3 regulates m^6A RNA methylation in ECs. Endogenous METTL3 is essential for EC function and angiogenesis, potentially through influencing let-7e and miR-17-92 cluster processing. Thus, the therapeutic modulation of METTL3 should be considered as a new approach for controlling angiogenic responses in the clinical setting.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: endothelial cell | gene expression | homeostasis | microRNA | thrombospondin

Postnatal angiogenesis is the physiological process through which capillaries and arterioles are formed from preexisting vasculature. This process requires the proliferation, migration, and differentiation of endothelial cells (ECs) and is regulated by the fine interplay of proangiogenic and antiangiogenic mediators. Impairment of EC function contributes to several pathological conditions, including ischemic disease and tumor growth. MicroRNAs (miRNAs) are important regulators of EC function under physiological and pathological settings. MI RNAs are transcribed by PolII into primary miRNA (pri-miRNA) transcripts, which are sequentially processed by
the endonucleases Drosha-DGCR8 (DiGeorge syndrome critical region 8) and Dicer into precursor and mature miRNAs, respectively. Canonically, miRNAs repress gene expression at the post-transcriptional level in the RISC (RNA-induced silencing complex) complex by targeting mRNAs in their 3′ UTRs (untranslated regions), thus promoting mRNA degradation or translational repression.3,4 Recently, multiple studies have proposed RNA methylation, specifically on the RNA adenosine base at the nitrogen-6 position (m⁶A \[N6-methyladenosine\]), as a new layer of fine-tuned gene expression.5–7 m⁶A is the most abundant internal modification in eukaryotic mRNA and occurs within the conserved RRACH (R=A or G, H=A, C or U) motif that localizes near stop codons, in 3′ and 5′ UTRs and within long exons.8,9 m⁶A RNA methylation is achieved by the methyltransferase complex, which is composed of the writer proteins METTL3 and METTL14 (methyltransferase-like 3 \[catalytic domain\] and methyltransferase-like 14),10,11 and their cofactors, WTAP (Wilms tumor 1 \[WT1\]-associated protein),12 KIAA1429, RBM15 (RNA-binding protein 15), and ZC3H13 (zinc finger CCCH domain-containing protein 13). The m⁶A mark is dynamically reversed by the eraser demethylases, FTO (fat mass and obesity-associated protein)13 and ALKBH5 (ALKB homolog-5).14,15 The function of m⁶A is partly mediated by reader proteins, which have been identified as members of the YTH domain-containing protein and the HNRNP (heterogeneous nuclear ribonucleoproteins) protein families.16,17 Recent reports have shown that m⁶A RNA methylation plays a crucial role in miRNA maturation.18,19 In particular, METTL3 methylates primary miRNAs within the RRACH motif, which facilitates their processing by the RNA-binding protein DGCR8, leading to a global upregulation of mature miRNAs.18 The dysregulation of m⁶A RNA methylation has been associated with cardiovascular disease and cancer.20–22 Although the role of METTL3 in cancer cell biology has been widely described as a regulator of oncogenes and tumor suppressors,23,24 its role in ECs function remains unclear.

This study investigated the role of the m⁶A-catalyzing enzyme, METTL3, in regulating EC processes that are conducive to angiogenesis. We also examined if METTL3 affects the expression and functional activity of miRNAs involved in angiogenesis.

### MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

#### Cell Culture

Human umbilical vein ECs (HUVEC) and human cardiac microvascular ECs (HCMEC) were purchased from Promocell and cultured on 0.1 % gelatin (Sigma-Aldrich) in EGM2 (endothelial cell growth medium 2) medium (PromoCell). ECs were used between passages 2 to 6 for the experiments. Culture conditions were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

#### Transfection of Short Interfering RNA and Mimic miRNA

HUVEC were transfected with 30 nM of METTL3 short interfering RNA (siRNA; 15 nM of METTL3 siRNA \[s32142\] and 15 nM METTL3 siRNA \[s32143\]) or control siRNA (4390843; Ambion, Life Technologies), and hsa-miR-18a-5p mimic (C-300487-05), hsa-let-7e-5p mimic (C-300479-05) or miRNA mimic negative control (CN-001000-01-05; Dharmacon) using Oligofectamine (Life Technologies) for 48 hours, as previously described.25
Stable METTL3 Knockdown Generation
HUVEC (1.8×10⁵) were infected with commercially available lentiviral (LV) particles expressing shRNAs (short hairpin RNAs) against METTL3 (TRCN0000034715 or TRCN0000034717, Sigma-Aldrich; LV-METTL3-shRNA) or control shRNA (SHC002, Sigma-Aldrich; LV-CT-shRNA) and polybrene (4 µg/mL, Millipore). Cells were then selected in the presence of 1 µg/mL puromycin (Life Technologies) for 1 week.

Adenoviral Vector Generation
Wild-type human METTL3 clone was purchased from Addgene,10 and cloned into pDC-515IO vector to generate recombinant adenovirus (Microbix). HUVEC (1.8×10⁵) were seeded on a 6-well plate in 1 mL complete EGM2 medium and grown until 80% confluent. Cells were then infected overnight with adenoviruses carrying METTL3 (Ad.METTL3) or control adenoviruses (Ad.Null) at 50 MOI (multiplicity of infection) in EGM2 complete medium. The following day, the cells were washed with PBS, and the medium was replaced with normal complete or serum-free EGM2 medium for 36 hours.

BrdU Cell Proliferation Assay
HUVEC were seeded (1×10⁴) in a 96-well plate and transfected with METTL3 or control siRNA or infected with adenoviral vectors as mentioned above. Cell proliferation was determined using the BrdU (bromodeoxyuridine) Cell Proliferation ELISA Kit (Roche), as previously described.26

Caspase Activity Assay
HUVEC were seeded (1×10⁴) on a white-walled 96-well plate and transfected with METTL3 or control siRNA, as mentioned above. Apoptosis was quantified 48 hours post-transfection by measuring caspase-3 and caspase-7 activity using a luminescent cell death detection kit (Caspase-Glo Assay, Promega), following the instruction of the manufacturer.26

Scratch Wound Assay
HUVEC were transfected with METTL3 or control siRNA or infected with adenoviral vectors, as mentioned above. At 36 hours post-transfection, cells were serum-starved overnight in basal EBM2 supplemented with 0.1% bovine serum albumin. The following day, a p200 pipette tip was used to scratch the cell monolayer. Images were captured at 0, 6, and 12 hours using a Nikon Eclipse TE200 inverted microscope at ×4 magnification. Wound closure was analyzed using the migration plug-in for National Institutes of Health (NIH) ImageJ software.

Cord Formation Assay
HUVEC and HCMEC were transfected with METTL3 or control siRNA or infected with Ad.METTL3 or Ad.Null. After 48 hours post-transfection, HUVEC (7×10⁴) were seeded on a 24-well plate coated with 200 µL Growth Factor Reduced Matrigel (BD Biosciences). Images were captured at 6 hours using a Nikon Eclipse TE200 inverted microscope at ×4 magnification. Angiogenesis was assessed using the angiogenesis analyzer plugin for NIH ImageJ software.

Fibrin Gel Bead Assay
HCMEC were transfected with either Ad.METTL3 or Ad.Null, as mentioned above. Fibrin gel bead assay was performed, as previously described.26 Briefly, 1×10⁴ HUVEC were mixed with 2500 Cytodex-3 Beads (Amersham) in EGM2 for 4 hours at 37°C. Coated beads were transferred to T25 flask in 5 mL of EGM2 and left overnight. The following day, the coated beads were resuspended in fibrinogen solution (2.0 mg/mL fibrinogen, 0.15 Units/mL of aprotinin, Sigma-Aldrich) at a concentration of 200 beads/mL. Thrombin (0.625 Units/mL, Sigma-Aldrich) was added to each well of a 24-well plate before seeding 0.5 µL of the fibrinogen/bead suspension. Then, the plate was placed at 37°C for 20 minutes to generate a clot, and 1 mL of EGM2 was added to each well. HUVEC were allowed to undergo morphogenesis for 2 to 3 days. Angiogenic sprouting was quantified by measuring cumulative sprout length, branches, sprouts, and the number of detached cells using NIH ImageJ software.

Bioinformatic Analysis
From published data obtained in a metastatic breast cancer cell line (MDA-MB-231), we extracted a list of pri-miRNAs methylated by METTL3.18 The corresponding mature miRNAs were taken further for analyses of target genes with significance for angiogenesis. For miRNA target prediction, 3'-UTRs of the miRNAs were scanned to find miRNA binding sites using mirCode.27 To filter from all the predicted targets, only those candidates were considered that were conserved across vertebrates. Since mirCode predicts targets for miRNA families rather than individual members of the family, TargetScan was used to further find individual members corresponding to targets. Following this, Gene Ontology/KEGG pathways were identified for the conserved targets using DAVID.28 Candidates that corresponded to the term Angiogenesis with P<0.05 were incorporated into experimental design and probed throughout the project.

Western Blot Analysis
Samples were prepared as previously described.26 Briefly, cells were lysed in ice-cold RIPA buffer (Sigma-Aldrich) containing 1 mM orthovanadate, 1 mg/mL of protease inhibitor cocktail (Roche), and 0.25 mg/mL AEBSF (4-benzenesulfonyl fluoride hydrochloride; Roche). Western blots were performed using the following antibodies: monoclonal antibody anti-Tsp1 [A6.1] (SC-59887; 1:1000) and monoclonal antibody anti-β-Actin (SC47778; 1:3000) were purchased from Santa Cruz Biotechnologies. Rabbit recombinant anti-METTL3 antibody (ab195352; 1:1000) was purchased from Abcam. Mouse monoclonal HSP90 (heat shock protein; 610418; 1:3000) antibody was purchased from BD Bioscience. IRDye 800CW Donkey anti-Rabbit IgG (925-32212; 1:15 000) and IRDye 680RD Donkey anti-Mouse IgG (925-32212; 1:15 000) and IRDye 680RD Donkey anti-Rabbit IgG (925-68073; 1:15 000) were purchased from LI-COR. Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology). Densitometry analysis of the gels was carried out using NIH ImageJ software.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction
Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s protocol. For gene expression

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analysis, cDNA was synthesized using the PrimeScript RT-PCR kit (Takara), following the manufacturer’s protocol. Quantitative real-time polymerase chain reaction was performed in triplicate using TB Green Premix Ex Taq Kit (Takara) on QuantStudio 6 Flex Real-Time PCR System (Life Technologies). 18S rRNA (ribosomal RNA) was used for normalization.

For pri-miRNAs analysis, cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). Quantitative real-time polymerase chain reaction was performed using TaqMan Pri-miRNA Assays for pri-miR-17 (recognizes miR-17-miR-18a-miR-19a stem-loops), pri-miR-92 (recognizes miR-20a-miR-19b-miR-92a stem-loops), and pri-miR-let-7e.

Mature miRNA levels were analyzed using miRNA TaqMan Assays and TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). Both pri-miRNA and mature miRNA Quantitative real-time polymerase chain reaction was performed in triplicate using TaqMan Universal Master Mix (Life Technologies). 18S rRNA or small RNA U6 was used for normalization, respectively.

Primer references and sequences: pri-let-7e (hs03295917_s1), pri-miR-17 (hs03295901_s1), pri-miR-92 (hs03302603_s1), 18S (hs69999901_s1), Human Mettl3 (hs00219820_m1), mouse Mettl3 (Mm01316319_m1), and mouse Tsp1 (thrombospandin 1; Mm01335418_m1; Life Technologies).

Human 18S forward 5′ GTTTAAGGTGACTCAACACGGGA 3′ and reverse 5′ AGCTATCAATCTGTCAATCCTGTC 3′ and human Tsp1 forward 5′ TGTACTGAGAACAAAGAG 3′ and reverse 5′ CAGCTATCACAAGTGCATTC 3′.

m6A RNA Quantification
m6A RNA level was measured from 200 ng of total RNA per sample using an antibody-based m6A capture and colorimetric method (Epigentek) following the manufacturer’s protocol. Experiments were performed in triplicate.

Animal Studies
Experiments involving live animals were performed at 3 different institutions, in accordance with The Guide For The Care And Use Of Laboratory Animals published by the US National Institutes of Health.29

In vivo Matrigel plug assay and limb ischemia protocols were performed with the prior approval of the UK Home Office and the University of Nottingham or University of Bristol ethics committees, respectively. Myocardial infarction (MI) protocol was performed following approval from the Animal Ethics Committee at the University of Otago, New Zealand.

In Vivo Matrigel Plug Assay
Two protocols were performed at the University of Nottingham under the project license (PPL) P435A9CF8.

Matrigel plug assay with METTL3 depleted ECs: HUVEC were infected with LV-METTL3-shRNA or LV-CT-shRNA particles, as mentioned above. Cells (3×10^5) were resuspended in 100 μL of medium without FBS and mixed with growth factor reduced matrigel premixed with VEGF (vascular endothelial growth factor)-A165 and bFGF (basic fibroblast growth factor). Samples were injected subcutaneously into the flank region of 10-week-old immunodeficient male mice (Crl:CD1-Foxn1nu, Charles River, United Kingdom; N=8 mice per group).

Matrigel plug assay with METTL3 overexpressing ECs: HUVEC were infected overnight with adenoviruses carrying METTL3 (Ad.METTL3) or control adenoviruses (Ad.Null) at 50 MOI in EGM2 complete medium. Cells were prepared and inoculated as described above in immunodeficient male and female mice in equal proportion (N= 6 mice per group).

For perfusion studies, mice were injected with 150 μL of biotinylated isoecltin-B4 (Molecular Probes) in the tail vein and allowed it to circulate for 15 minutes before sacrifice. After 14 days for the METTL3 overexpression experiment or 21 days for the METTL3 depletion experiment, mice were euthanized, and matrigel plugs were embedded in OCT prior to staining.

Limb Ischemia Model
The protocol was developed at the University of Bristol under PPL/30/2811 and PI/I77AD3F36. The animal procedure was performed on 10 to 12 week-old male CD1 mice. The use of only one sex group concerning this particular model is compliant with the guidelines on the consideration of sex differences in design and reporting recently published by the Arteriosclerosis, Thrombosis, and Vascular Biology Council.30

Unilateral limb ischemia was surgically induced in anesthetized CD1 mice by occlusion of the left femoral artery.31 Immediately after, either Ad.METTL3 or Ad.Null (10^9 plaque-forming units) was injected directly into the ischemic adductor muscle, at 3 equidistant points along the projection of the femoral artery. To confirm successful ischemia induction, the superficial blood flow of the ischemic and contralateral feet was analyzed immediately after surgery using a high-resolution laser color Doppler imaging system (Moor LDI2, Moor Instruments, Devon, United Kingdom).

At 3 days after limb ischemia surgery, mice were sacrificed to harvest the muscular ECs (N=4 samples per group, 3 mice pooled per sample). To this aim, ischemic and contralateral adductor muscles were collected from animals under terminal anesthesia. The muscles were rinsed in PBS and digested with collagenase II (600 U/mL, Worthington) and DNase I (1 mg/mL, Sigma-Aldrich) using gentle MACS dissociator (Miltenyi Biotech) following the manufacturer’s protocol. Microvascular cells were sorted using an immunomagnetic CD146 antibody (clone ME-9F1, Miltenyi Biotech) as previously reported.32 Additional subgroups of mice were euthanized at 21 days postischemia to assess the impact of METTL3 overexpression on postischemic angiogenesis. To this aim, mice were terminally anesthetized (Avertin), and their limbs were perfusion-fixed in situ. Adductor and gastrocnemius muscles were harvested and processed for immunohistochemical analysis.

MI Model
The protocol was performed at the University of Otago under AEC10/14. The animal procedure was performed on 10-week-old male C57BL/6J mice. Due to the reported effects of estrogen on the heart and cardiac miRNAs expression,33-35 only adult male mice were used to avoid potential effects of sex differences.32,33 MI was induced by permanent ligation of the left anterior descending coronary artery in mice as previously described.36 In brief, following anesthesia (2,2,2 tribromo ethanol, 0.3gm/kg, IP) and artificial ventilation, the chest cavity was opened, and after careful dissection of the pericardium, the left anterior descending coronary artery was located and permanently ligated using a 7-0 silk suture. Immediately after ligation, mice were randomized to receive either Ad.METTL3 or Ad.Null (10^9 plaque-forming units/mouse, N=10
each group). After confirming the absence of bleeding, the chest cavity was closed in layers. Animals were allowed to recover for at least 4 hours before being returned to the housing unit. Mice were monitored twice a day for the first 5 days postsurgery and thereafter once every day. All mice received analgesic and antibiotic prior to the surgery and then for 3 days postoperation. Cardiac function was assessed using echocardiography measurement at baseline (before MI) and at 14 days post-MI. At the end of the treatment period and following echocardiography, the heart was stopped under anesthesia in diastole using KCl, and ventricular tissue was collected following 4% paraformaldehyde perfusion-fixation using a perfusion pump set at 2 mL/min constant flow.\textsuperscript{36}

**Immunohistochemistry**

Explanted matrigel plugs were sectioned at a thickness of 15 μm and stained using Rabbit anti-human CD31 (Ab32457, Abcam; 1:200) and Goat anti-rabbit Alexa Fluor 594 (Ab150080, Abcam; 1:200) to detect human capillary–like structures. Perfused vessels stained with biotinylated isolectin-B4 (I21411, Molecular Probes; 1:100) and Streptavidin Alexa Fluor 488 conjugate (S11223, Molecular Probes; 1:200). The number of human CD31\textsuperscript{+} structures per mm\textsuperscript{2} and perfused vessels were quantified using NIH ImageJ software in 12 randomly selected fields at ×10 magnification. The extent of vessel perfusion was expressed as the percentage of perfused vessels among total human capillary–like structures.

For analysis of posts ischemic limb vascularization, adductor muscles were sectioned at a thickness of 7 μm and stained with biotinylated isoleucin-B4 (I21411, Molecular Probes; 1:100) and streptavidin Alexa Fluor 488 conjugate (S11223, Molecular Probes; 1:200) to detect ECs. The number of capillaries per mm\textsuperscript{2} was counted in 10 randomly selected high-power fields (×40 magnification) captured using a fluorescent microscope (Zeiss Axio Observer).

For analysis of post MI vascularization, the left ventricular tissue was sectioned at a thickness of 7 μm and stained with biotinylated isoleucin-B4 (B-1205-5, Vector Laboratories; 1:100) and α-smooth muscle actin (C6198, Sigma-Aldrich; 1:400) to identify capillaries and arterioles, respectively. The number of capillaries per mm\textsuperscript{2} was counted in 6 random images analyzed from each sample (×400 magnification), captured using a confocal microscope (Nikon).

**Statistics**

Statistical analyses were performed with GraphPad Prism 9 software (GraphPad). Normal distribution of each group was confirmed by the Shapiro-Wilk test. Statistical significance between 2 groups was analyzed by unpaired 2-tailed Student t test. For the comparison of multiple groups, 1-way ANOVA (Tukey post hoc test) or 2-way ANOVA (Šidák post hoc test) tests were used. Data are expressed as mean±SEM. P values ≤0.05 were considered statistically significant.

**RESULTS**

**METTL3 Regulates Angiogenic Cell Functions In Vitro**

To investigate if METTL3 regulates EC function, we performed different gain- and loss-of-function studies. First, we knocked down METTL3 in HUVEC using 2 different siRNA against METTL3, and we confirmed a decrease in the expression of METTL3 at mRNA and protein levels (Figure IA and IB in the Data Supplement). As expected, METTL3 depletion reduced the level of the m\textsuperscript{6}A RNA in HUVEC (Figure IC in the Data Supplement). We observed a significant reduction in cell number (Figure ID in the Data Supplement), associated with a decrease in cell proliferation and a significant increase in cell apoptosis evaluated by bromodeoxyuridine incorporation (Figure 1A) and caspase-3 activity assay (Figure 1E in the Data Supplement) respectively. In addition, METTL3 depleted HUVEC showed impairment in both migration and capacity to develop an in vitro endothelial network by performing a cord formation assay (Figure 1B and 1C). Similar data were observed in angiogenesis in vitro when we depleted the levels of METTL3 in HCMEC (Figure IIA in the Data Supplement). By using Ad.METTL3, we next overexpressed METTL3 and validated METTL3 expression in HUVEC at mRNA and protein level (Figure IF and IIG in the Data Supplement). Ad.METTL3 increased the level of m\textsuperscript{6}A RNA (Figure IH in the Data Supplement) and promoted in vitro angiogenesis as observed in a cord formation assay in both HUVEC (Figure 1F) and HCMEC (Figure IIIB in the Data Supplement) compared with Ad.Null. The angiogenic potential of METTL3 overexpression in vitro was further confirmed by performing a fibrin gel bead assay in HCMEC (Figure IIIC in the Data Supplement). However, METTL3 overexpression did not increase proliferation (Figure 1D and Figure II in the Data Supplement) or migration in HUVEC (Figure IE).

**Endogenous METTL3 May Be Required for the Maturation of let-7e and the miR-17-92 Cluster in ECs**

In cancer cells, METTL3 has been shown to play a crucial role in the processing of primary miRNAs that contain RRACH motifs,\textsuperscript{18} which are included in pri-let-7e and the polycistronic pri-miR-17-92. Importantly, the mature forms of these miRNAs are important regulators of angiogenesis.\textsuperscript{26,37–41} To confirm the relevance of endogenous METTL3 in the processing of these miRNAs in ECs, we used 2 independent LV-METTL3-shRNAs (sequence no. 1 or no. 2) or LV-CT-shRNA in HUVEC. Successful depletion of METTL3 was confirmed at both mRNA and protein levels (Figure IIIA and IIIB in the Data Supplement). Both METTL3-shRNAs reduced the level of m\textsuperscript{6}A RNA, which was accompanied by a decrease in cell number and increased apoptosis (Figure IIIC through IIIE in the Data Supplement). Importantly, METTL3-depleted ECs showed an increase in the levels of both pri-let-7e and pri-miR-17-92 (Figure 2A and 2B), whereas the levels of mature let-7e-5p and the individual components of the miR-17-92 cluster were reduced in ECs (Figure 2C and 2D).
Taken together, these data suggest that METTL3 depletion leads to a decrease in pri-miRNA processing and a corresponding drop in mature miRNA levels, including endothelial miRNAs that play a crucial role in angiogenesis.

**METTL3 Modulates the Expression of Tsp1 and Regulates Angiogenesis in ECs**

To further investigate the potential impact of METTL3 on the functional capacity of let-7e-5p and miR-17-92 cluster, we explored if the modulation of METTL3 levels could affect the expression of targets of these miRNAs. To do so, we used miRcode and TargetScan to identify common targets for let-7e-5p and miR-18a-5p. Let-7 family members and miR-18a-5p within the miR-17-92 cluster have been previously shown to regulate Tsp1 in ECs. We next confirmed that the overexpression of mRNAs of let-7e-5p and miR-18a-5p reduced Tsp1 protein levels in HUVEC (Figure 2E). In agreement with these data, we observed an increase in Tsp1 mRNA and protein expression in METTL3 depleted HUVEC and HCMEC by using METTL3 shRNAs (Figure 2F and 2G, Figure IIIH in the Data Supplement). Conversely, the overexpression of METTL3 increased the expression of both let-7e-5p and the miR-17-92 cluster and reduced the level of Tsp1 in HUVEC (Figure 3). The overexpression of let-7e-5p and miR-18a-5p did not change the level of m6A RNA in METTL3 depleted ECs (Figure 4A). However, the mimic of miR-18a-5p partially rescued the reduction of proliferation (Figure 4B), while the mimic of let-7e-5p abrogated the induction of apoptosis (Figure 4C) and promoted migration (Figure 4D). Finally, the overexpression of both miRNAs restored the angiogenic capacity in METTL3 depleted ECs (Figure 4E).

**METTL3 Regulates Angiogenesis in 3 In Vivo Murine Models**

To further assess the physiological significance of endogenous METTL3 in vivo, we performed a Matrigel plug assay, with HUVEC transduced with either 2 independent LV-METTL3-shRNAs or LV-CT-shRNA. Plugs embedding METTL3 depleted ECs exhibited a markedly decreased number of total CD31+ capillary-like structures (Figure 5A) and the subfraction of prfused vessels, which were identified after staining by the intravenously infused isolecin-B4, compared with control plugs (Figure 5B). Conversely, plugs from METTL3 overexpressing ECs showed an increase in the number of total CD31+ capillary-like structures (Figure 5C) and perfused vessels compared with Ad.
Null plugs (Figure 5D). As shown in Figure IV in the Data Supplement, the proangiogenic response to the implantation of METTL3 OE ECs was equivalent in mice of both sexes.

To determine the impact of endothelial METTL3 in pathological angiogenesis, we utilized 2 mouse models of ischemia, limb ischemia, and MI. This also allowed us to study the therapeutic potential of Ad.METTL3 on post-ischemic reparative angiogenic responses.

In the limb ischemia model, successful local gene transfer after Ad.METTL3 injection into the ischemic muscle was first verified by quantitative polymerase chain reaction analysis of human METTL3 in ECs isolated from ischemic adductor muscle. A 10-fold increase in METTL3 mRNA level was observed in ECs isolated from ischemic limb injected with Ad.METTL3 compared with those injected with Ad.Null (Figure 6A). Ad.METTL3 increased the level of m6A RNA but did not modify the endogenous level of METTL3 in the ischemic adductor muscle (Figure VI in the Data Supplement). Ischemic limb muscles treated with Ad.METTL3 showed an increase in capillary density (Figure 6B) and a reduction in the expression of Tsp1 at an mRNA level compared with Ad.Null muscle (Figure VC in the Data Supplement).

Finally, we used the mouse MI model to investigate the functional relevance of Ad.METTL3 on myocardial therapeutic angiogenesis and cardiac function. Successful gene transfer was verified by western blotting analysis of human METTL3 in the murine infarcted heart (Figure VI in the Data Supplement). Ventricular tissue treated with Ad.METTL3 showed an increase of capillary and arteriole density identified by positive isolectin-B4 and smooth muscle actin structures respectively, compared with infarcted hearts injected with Ad.Null (Figure 6C). Baseline indexes of left ventricular ejection fraction function did not differ between groups. Importantly, METTL3 overexpression dramatically improved contractile function, as indicated by a higher % of left ventricular ejection fraction (Figure 6D).

**DISCUSSION**

In this study, we have provided in vitro and in vivo evidence demonstrating the importance of METTL3 in angiogenesis. Mechanistically, we have shown that METTL3 maintains the level of m6A RNA and enables
the processing of endothelial let-7e-5p and the miR-17-92 cluster, which have been reported to be involved in angiogenesis,9,25,37–41 thereby improving EC angiogenic capacity.

The first step in miRNA biogenesis is the processing of pri-miRNAs by the endonucleases Drosha-DGCR8 into mature miRNAs.3,4 Alarcón et al18 provided initial evidence that m6A RNA facilitates the binding between pri-miRNA and DGCR8, thus promoting miRNA processing. The authors also described several RRACH motifs in the pri-miRNA-let-7e and pri-miR-17-92. Additionally, the METTL3 depletion in human cancer cells, MDA-MB-231, resulted in the reduction of mature let-7e concomitantly accumulating the unprocessed pri-let-7e form.18 Similarly, we found an increase in the expression of pri-miR-let-7e and pri-miR-17-92, and a reduction of their mature forms in METTL3 depleted ECs. Importantly, this switch in miRNA expression was associated with a loss of their functional capacity, as demonstrated by the inability to repress their common target, Tsp1. Supporting these findings, the overexpression of let-7e-5p and miR-18a-5p (member of the miR-17-92 cluster), restored the proangiogenic capacity of METTL3 depleted ECs. Conversely, the overexpression of METTL3 increased the levels of both let-7e-5p and individual miRNAs of the miR-17-92 cluster while reducing the expression of Tsp1. These data suggest that the processing of angiogenic miRNAs is the potential underlying mechanism by which METTL3 regulates angiogenesis in ECs.

Recent studies have provided evidence for the role of m6A RNA methylation in regulating various biological processes, whereas m6A dysregulation has been associated with several diseases.20–22 In agreement with our data, the depletion of METTL3 was previously shown to decrease tumor proliferation, whereas METTL3 overexpression promoted cancer cell growth, survival, and invasion.23,24

**Figure 3.** METTL3 (methyltransferase-like 3) overexpression increases the levels of let-7e and miR-17-92 and decreases Tsp1 (thrombospondin 1) expression.

Human umbilical vein endothelial cells (HUVEC) were infected with 50 MOI (multiplicity of infection) of Ad.METTL3 or Ad.Null for 12 h. Quantitative real-time polymerase chain reaction analysis of (A) let-7e-5p, (B) miR-17-92, and (C) Tsp1 at 48 h after infection (N=4). 18s was used as housekeeping gene. D, Western blot analysis of METTL3 and Tsp1 at 48 h after infection. Representative blots are shown (N=4). HSP90 (heat shock protein 90) was used as loading control. Data are expressed as mean±SEM. Results in A, C–D were assessed by unpaired Student t test and B by 1-way ANOVA (Tukey post hoc test). *P<0.05, **P<0.01, ***P<0.001 vs Ad.Null infected cells.
Figure 4. The overexpression of let-7e and miR-18a restores the angiogenic capacity of METTL3 (methyltransferase-like 3) depleted endothelial cells (ECs) in vitro.

Human umbilical vein endothelial cells (HUVEC) were cotransfected with 30 nM of control (CT) short interfering RNA (siRNA) and control mimic (CM) sequences (CT siRNA/CM), METTL3 siRNA in the presence of CT siRNA and CM (METTL3 siRNA/CT siRNA/CM), or METTL3 siRNA in the presence of mimic miR-18a-5p or let-7e-5p for 48 h. A, Quantification of m6A (N6-methyladenosine) level in total RNA (N=3). B, BrdU (bromodeoxyuridine) assay (N=3). C, Caspase-3/7 activity was measured as relative light units (RLU; N=3). D, Scratch assay. Representative microscopy images are shown (×4 magnification, scale bar 50 µm; N=3). Migration was quantified as the rate of gap closure after 12 h. E, Cord formation assay. Representative microscopy images are shown (×4 magnification, scale bar 50 µm; N=3). Angiogenesis was quantified as number of junctions, meshes, branches, and total length after 6 h. Data are expressed as mean±SEM. Results in A–E were assessed by 1-way ANOVA (Tukey post hoc test). *P<0.05, **P<0.01, ***P<0.001, n.s [nonsignificant] vs cells transfected with CT siRNA/CM or †P<0.05 vs cells transfected with METTL3 siRNA/CT siRNA/CM.
The contribution of m⁶A RNA methylation to cardiac homeostasis, hypertrophy, and fibrosis has been recently reported, even if with contradictory results.⁴³⁻⁴⁵ The role of METTL3 in the cardiac endothelium and myocardial angiogenesis remains unexplored. Importantly, our study is the first to demonstrate that post-MI local delivery of Ad.METTL3 supports reparative neovascularization in the peri-infarct area and improved cardiac function at 2 weeks post-MI. Mathiyalagan et al.⁴⁵ reported an increased level of m⁶A RNA in human and mouse failing hearts, which they attributed to a decrease in the demethylase FTO. In possible contrast with the proposed proangiogenic role of METTL3, the authors describe an increase in CD31⁺ cardiac ECs 4 weeks post-MI and FTO gene transfer in mice. However, angiogenesis was not the focus on this study, and these data were obtained from a small sample size requiring further validation. In the same study, the authors demonstrated that FTO gene transfer improved MI-induced cardiac dysfunction.⁴⁵

The relevance of METTL3 in the broader endothelial setting has also been partially investigated. For instance, Lv et al.⁴⁶ demonstrated that the loss of endothelial METTL3 impairs definitive hematopoiesis via Notch signaling during mouse embryogenesis. Moreover,
Yao et al. recently reported that endothelial depletion of METTL3 decreased pathological angiogenesis in an oxygen-induced retinopathy model.

Apart from miRNA processing, m^6^A can control the biogenesis and function of long noncoding RNAs and circular RNAs, as well as any aspect of mRNA post-transcriptional regulation, including splicing, export, stability, and translation. Therefore, METTL3 levels in ECs could also influence potential noncoding RNAs and mRNA transcripts involved in angiogenesis. In summary, our findings have provided the first evidence that METTL3 is an important regulator of angiogenesis by modulating the expression of let-7e and the miR-17-92 cluster.

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**Figure 6.** METTL3 (methyltransferase-like 3) overexpression improves angiogenesis under ischemic conditions and increases cardiac function in vivo.

**A** and **B**, Limb ischemia was surgically induced in male CD1 mice by occlusion of the left femoral artery. Immediately after ligation, the mice were randomized to receive either Ad.METTL3 or Ad.Null injection (10^8 plaque-forming units [pfu]/mouse, N=8 mice in each group) in the ischemic adductor muscle. **A**, Endothelial cells from Ad.METTL3 or Ad.Null-treated ischemic adductor muscles were isolated 3 d after surgery to validate human METTL3 gene delivery by Quantitative real-time polymerase chain reaction (N=4 samples per group, 3 mice pooled per sample). 18s was used as housekeeping gene. **B**, Grafts from Ad.METTL3 or Ad.Null ischemic adductor muscles were isolated 3 wk after surgery. Representative microscopy images showing vascular density in the study groups (×40 magnification, scale bar 50 µm). Angiogenesis was quantified as isolectin-B4 (IsoB4) positive structures. Cell nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole). **C** and **D**, Myocardial infarction (MI) was induced in male C67BL/6J mice by left anterior descending coronary artery ligation. Immediately after ligation, the mice were randomized to receive either Ad.METTL3 or Ad.Null (10^8 pfu/mouse, N=10 mice in each group) intracardiac injection. Ad. METTL3 or Ad.Null-treated hearts were isolated 2 wk post-MI. **C**, Representative confocal microscopy images showing vascular density in the study groups (×400 magnification, scale bar 50 µm). Capillaries and arterioles were quantified as isolectin-B4 and α-smooth muscle actin positive structures (white arrowheads) respectively. **D**, Graph showing basal and final echocardiography indexes. For Ad.Null: N=12 basal, N=8 final; Ad.METTL3: N=12 basal, N=10 final. Data are expressed as mean±SEM. Results in **A**–**C** were assessed by unpaired Student t test, and (**D**) by 2-way ANOVA (Šídák post hoc test). LVEF indicates left ventricular ejection fraction. *P<0.05, **P<0.01, ***P<0.01, ****P<0.0001 vs Ad.Null ischemic adductor muscle or Ad.Null infarcted heart.
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