MicroRNA-615-5p Regulates Angiogenesis and Tissue Repair by Targeting AKT/eNOS (Protein Kinase B/Endothelial Nitric Oxide Synthase) Signaling in Endothelial Cells

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Objective—In response to tissue injury, the appropriate progression of events in angiogenesis is controlled by a careful balance between pro and antiangiogenic factors. We aimed to identify and characterize microRNAs that regulate angiogenesis in response to tissue injury.

Approach and Results—We show that in response to tissue injury, microRNA-615-5p (miR-615-5p) is rapidly induced and serves as an antiangiogenic microRNA by targeting endothelial cell VEGF (vascular endothelial growth factor)-AKT (protein kinase B)/eNOS (endothelial nitric oxide synthase) signaling in vitro and in vivo. MiR-615-5p expression is increased in wounds of diabetic db/db mice, in plasma of human subjects with acute coronary syndromes, and in plasma and skin of human subjects with diabetes mellitus. Ectopic expression of miR-615-5p markedly inhibited endothelial cell proliferation, migration, network tube formation in Matrigel, and the release of nitric oxide, whereas miR-615-5p neutralization had the opposite effects. Mechanistic studies using transcriptomic profiling, bioinformatics, 3′ untranslated region reporter and microribonucleoprotein immunoprecipitation assays, and small interfering RNA dependency studies demonstrate that miR-615-5p inhibits the VEGF-AKT/eNOS signaling pathway in endothelial cells by targeting IGF2 (insulin-like growth factor 2) and RASSF2 (Ras-associating domain family member 2). Local delivery of miR-615-5p inhibitors, markedly increased angiogenesis, granulation tissue thickness, and wound closure rates in db/db mice, whereas miR-615-5p mimics impaired these effects. Systemic miR-615-5p neutralization improved skeletal muscle perfusion and angiogenesis after hindlimb ischemia in db/db mice. Finally, modulation of miR-615-5p expression dynamically regulated VEGF-induced AKT signaling and angiogenesis in human skin organoids as a model of tissue injury.

Conclusions—These findings establish miR-615-5p as an inhibitor of VEGF-AKT/eNOS–mediated endothelial cell angiogenic responses and that manipulating miR-615-5p expression could provide a new target for angiogenic therapy in response to tissue injury.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2019;39:1458-1474. DOI: 10.1161/ATVBAHA.119.312726.)

Key Words: angiogenesis • endothelial cells • microRNAs • tissue repair • AKT/eNOS signaling

The induction and orchestration of new blood vessels is critical for tissue repair in response to injury in ischemic cardiovascular diseases, such as myocardial infarction (MI), peripheral artery disease (PAD), and diabetic wound healing. In response to proangiogenic stimuli, activated vascular endothelial cells (ECs) migrate to distant sites to proliferate and generate new primary capillaries necessary for tissue repair. Impairment of these cellular and physiological processes can contribute to disease progression and have been linked to poor cardiovascular function and outcomes.

Over the years, the importance of angiogenesis has ignited numerous clinical investigations that have sought to stimulate angiogenesis to combat ischemic pathologies and tissue injury. These studies have primarily focused on the intramuscular and intraarterial introduction of a range of angiogenic growth factors, such as VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor), HGF (hepatocyte growth factor), hypoxia-inducible factor-1a, and Del-1 (developmental endothelial locus-1), to promote neovascularization and tissue perfusion in subjects with PAD or chronic stable...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
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<tr>
<td>3'-UTR</td>
<td>3'-untranslated region</td>
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<td>AGO2</td>
<td>argonaute2</td>
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<td>Ang</td>
<td>angiopoietin</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>Del-1</td>
<td>developmental endothelial locus-1</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>eNOS</td>
<td>endothelial NO synthase</td>
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<td>FGF</td>
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<td>HGF</td>
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<td>HUVECs</td>
<td>human umbilical vein ECs</td>
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<td>IGF2</td>
<td>insulin-like growth factor 2</td>
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<td>miRNA</td>
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<td>RASSF2</td>
<td>Ras-associating domain family member 2</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<td>VE-cadherin</td>
<td>vascular endothelial cadherin</td>
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<td>VEGF</td>
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Unfortunately, clinical trials using these growth factors either succeeded in improving patient outcomes nor led to effective therapeutic angiogenesis.\textsuperscript{14–16} The failure of these trials have been attributed to a plethora of reasons, including challenges in translating preclinical models to clinical disease, selection of patient cohorts, delivery routes, and therapeutic dosing.\textsuperscript{17}

However, accumulating studies have recently suggested that these failures in therapeutic angiogenesis may not be due to a deficiency of angiogenic growth factors but rather to an impairment in downstream signaling.\textsuperscript{18–21} In support, circulating proangiogenic growth factors (eg, VEGF-A) are increased in patients that present with myocardial ischemia with the highest levels observed in patients with the most severe myocardial ischemic burden.\textsuperscript{22,23} Similarly, VEGF-A, Tie2 (tyrosine kinase with Ig and EGF homology domains), and Ang (angiopoietin)-2 were also expressed higher in subjects with PAD compared with subjects without PAD. Similarly, patients with critical limb ischemia had the highest levels of these proangiogenic circulating factors compared with those with intermittent claudication or without PAD.\textsuperscript{24} These findings highlight that growth factor deficiency may not be a major contributor for the impaired neovascularization in response to ischemic disease states. Rather, impaired angiogenic signaling or angiogenic resistance may be a more dominant contributor through mechanisms analogous to the process by which impaired insulin signaling drives the development of insulin resistance in subjects with diabetes mellitus.

Growth factors, such as VEGF, activate a broad range of canonical pathways, including AKT (protein kinase B), eNOS (endothelial nitric oxide synthase), p38, and MAPK (mitogen-activated protein kinase), to promote the proliferative, migratory, and organizational processes that are necessary for effective angiogenesis.\textsuperscript{25} Thus, reduced activation of key mediators of these pathways within the context of ischemic disease and tissue injury may significantly impede successful neovascularization. For example, despite no differences in VEGF expression, there is significantly reduced phosphorylation of AKT/eNOS in diabetic limbs compared with nondiabetic limbs.\textsuperscript{26} In addition, decreased AKT/eNOS phosphorylation in the vascular endothelium promoted vascular dysfunction and reduced angiogenesis in murine models of hindlimb ischemia and MI.\textsuperscript{27–30} Together, these studies suggest that defects in angiogenesis can be overcome by focusing on approaches to augment downstream effects of proangiogenic growth factor signaling pathways.

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs that suppress the expression of target genes at the post-transcriptional level and are involved in a variety of pathophysiological processes.\textsuperscript{31,32} The role of miRNAs in angiogenesis is well-established and has been implicated as important regulators of a range of pathophysiological processes, such as myocardial and limb ischemia and diabetic wound healing through their proangiogenic and antiangiogenic properties.\textsuperscript{18,19,33–38} The capacity of a few of these miRNAs, such as miR-126, miR-200b, miR-503, and miR-26a, to impede angiogenesis has been attributed to their ability to silence important downstream effectors of growth factor signaling in ECs.\textsuperscript{19,39–41} Thus, the identification of miRNAs and downstream growth factor targets that govern the angiogenic response may provide novel therapeutic approaches to improve tissue repair and ischemic cardiovascular pathologies.

In this study, we identified miR-615-5p as a novel regulator of pathological and physiological angiogenesis in ECs. MiR-615-5p expression is increased in response to tissue injury or a range of ischemic disease states and confers antiangiogenic properties by specifically targeting IGF2 (insulin-like growth factor 2) and RASSF2 (Ras-associating domain family member 2) to decrease AKT/eNOS signaling in the presence of proangiogenic stimuli. Consequently, miR-615-5p impairs EC functions critical for angiogenesis and wound healing. Conversely, neutralization of miR-615-5p can successfully restore angiogenesis and promote wound healing in diabetic mice and human skin organoid models by increasing activation of AKT signaling. Collectively, these findings establish a new approach to overcome impaired angiogenic signaling in response to tissue injury or ischemic cardiovascular disease.

Materials and Methods

All supporting data are available within the article and its online-only Data Supplement.

Tube-Like Network Formation on Matrigel (In Vitro)

Matrigel (BD Bioscience) basement membrane matrix was added to 96-well culture plates and incubated at 37°C until gelation occurred. Network tube formation was assessed 14 hours postplating and quantitated by counting the number of tubes formed per high power field as described.\textsuperscript{19,42,43}

Chemotaxis Assays

Migration assay was performed using ChemTX multwell system (Neuro probe Inc, MD). The number of cells migrating to the lower chamber was counted using a hemocytometer after 5 hours.\textsuperscript{44}
Microarray Transcriptomic Profiling and Bioinformatics

For DNA microarray gene chip analysis, human umbilical vein ECs (HUVECs) were transfected with 30 nmol/L miRNA-negative control or miR-615-5p mimics for 24 hours. Cells were collected into RNAeasy mini kit (Qiagen) and sent for Two-Color, 4x44 K format (Agilent Technologies), Human Whole Genome Oligo Microarray Service (ArrayStar, Inc.).

Differentially expressed genes that were identified as being at least 1.5-fold repressed (P<0.05) were subjected to gene set enrichment analyses. Gene Ontology (GOs) pathways were explored to determine whether a known biological network, process, or molecular function was suppressed by overexpression of miR-615-5p (Ingenuity Pathway Analysis, Qiagen). The top 10 GOs for molecular and cellular functions and the top 10 GOs for signaling networks were ranked on P value or score, respectively. The primers used for real-time quantitative polymerase chain reaction analyses are listed in Table I in the online-only Data Supplement.

Scratch Assay for EC Migration

Scratch wound assay was performed using Culture-Insert 2 Well 35 mm μ-Dishes (ibidi). HUVECs transfected with miR-615-5p mimic, miR-615-5p inhibitor, nonspecific miRNA controls, IGF2 small interfering RNA (siRNA), RASSF2 siRNA, or control siRNA were cultured for 60 hours in 12-well plates and plated at 21K cells per well into the μ-Dishes. Inserts were lifted at 72 hours after transfection, and cells were imaged using an Eclipse TE2000-U inverted microscope (Nikon) at x2 and x4 over time to assess for wound closure. Three technical replicates were performed per condition. Significance was determined by Student 2-tailed t test, P<0.05.

In Vivo miR-615-5p Inhibition or Overexpression and Mouse Experiments

Animal protocols were approved by the Laboratory Animal Care at Harvard Medical School and Brigham and Women’s Hospital. For mouse dermal wound studies, male, 8 to 10 weeks old, db/db mice (Jackson Laboratories) were used for local intradermal injections of either scrambled control LNA (locked nucleic acid)–anti-miR or LNA–anti–miR-615-5p (Exiqon, Inc) at 0.63 mg/kg 48 and 24 hours before surgery. On day 0, dorsal full-thickness skin wounds (1 cm2) were cultured for 60 hours in 12-well plates and plated at 21K cells per well into the μ-Dishes. Inserts were lifted at 72 hours after transfection, and cells were imaged using an Eclipse TE2000-U inverted microscope (Nikon) at x2 and x4 over time to assess for wound closure. Sixty percent of the upper phase was collected. Samples that were red in color were excluded from the analyses, and only clear light yellow samples were homogenized for respective real-time quantitative polymerase chain reaction analyses. Anonymized plasma samples were generated from blood collected in EDTA-containing tubes at the time of the procedure and stored at −80°C. Diabetic patient plasma and skin samples collected as part of Lillehammer study (obtained from human subjects undergoing cardiac surgery) were generated from blood collected in EDTA-containing tubes or skin samples preserved in Allprotect tissue reagent (Qiagen). Plasma was isolated from whole blood, and skin samples were homogenized for respective real-time quantitative polymerase chain reaction analyses. Anonymized plasma samples were generated from blood collected in EDTA-containing tubes at the time of the procedure and skin samples were collected into Allprotect tissue reagent (Qiagen) and stored at −80°C. Plasma was isolated from whole blood at 1500g for 15 minutes at room temperature. Sixty percent of the upper phase was collected. Samples that were red in color were excluded from the analyses, and only clear light yellow plasma samples were utilized for RNA extraction. Total RNA was isolated from plasma by using total RNA purification kit from Norgen Biotek Corporation, and reverse transcription and real-time quantititative polymerase chain reaction was performed as described in the Section of Real-time qPCR in the online-only Data Supplement.

NO Assay

Nitrite content in cell culture supernatant was measured to indirectly reflect the content of NO using Griess reaction. Briefly, NO release from HUVECs was detected by enzymatic conversion of nitrate to nitrite by the enzyme nitrate reductase, followed by the Griess reaction to form a colored azo dye product per manufacturers protocols (Enzo, ADI-917-020). HUVECs transfected with miR-615-5p mimic, or nonspecific miRNA controls were cultured for 60 hours in 12-well plates and stimulated with 50 ng/mL VEGF for 1 or 3 hours as indicated. Supernatant from ECs was collected to detect total NO release.

Generation of Human Skin Organoids

Full-thickness circular (6 mm) human skin organoids were taken from surgical skin samples, and 3-mm full-thickness wounds were created as described previously. Briefly, human skin organoids were then embedded in collagen I matrix and maintained in DMEM (Life Technologies) supplemented with 10 mM HEPES, 50 μg/mL ascorbic acid, 100 μm adenine, 0.5 μm hydrocortisone, 0.1 nm cholaer toxin, 100 μM/L penicillin, and 10 μg/mL streptomycin (Sigma-Aldrich, St Louis, MO). The ex vivo organ culture were cultured at the air-liquid interface and maintained in the cell culture incubator at 37°C with 5% CO2 for every other day. The viability of cultured explants was validated by histological evaluation. Human skin organoids were transfected at the indicated time points using 30 nM miR-615-5p mimic.
miR-615-5p mimics or nonspecific controls or 100 nM miR-615-5p inhibitors or nonspecific controls. Neovascularization was measured at days 3 and 7 to determine miR-615-5p effects on wound healing.

Statistical Analysis
Data are presented as mean±SEM. All experiments are representative of 3 independent experiments unless indicated otherwise. Sample sizes for mouse and human organoid studies were chosen based on pilot studies or similar well-characterized studies in the literature. For 2 group comparisons, data were subjected to unpaired 2-sided Student t test if it passed normality and equal variance tests. If data for either normality or variance tests failed, then nonparametric Mann-Whitney U test was used. For ≥2 group comparisons, if data passed normality and variance testing, 1-way ANOVA with Bonferroni correction was used. If data did not pass either test, then nonparametric Kruskal Wallis test with Dunn post hoc test was used. P<0.05 was considered statistically significant.

Results
To identify miRNAs that regulate angiogenesis in response to tissue injury, miRNA microarray profiling studies were undertaken using plasma from human subjects with ACS with coronary angiograms showing >70% stenotic lesions compared with non-ACS human subjects with coronary angiograms with lesions <20% stenosis, and increased expression of miR-615-5p was noted with ACS (data not shown). On further investigation using real-time polymerase chain reaction analysis, we found that miR-615-5p was reduced significantly by both proangiogenic stimuli VEGF or bFGF (basic fibroblast growth factor) over the course of 24 hours (Figure 1A), whereas antiangiogenic stimuli TSP1 (thrombospondin) and TSP2 significantly increased miR-615-5p expression.
Furthermore, costimulation of ECs with VEGF and TSP1 resulted in the loss of VEGF-mediated suppression of miR-615-5p expression (Figure 1B in the online-only Data Supplement). VEGF represses target genes via an HDAC (histone deacetylase) class IIa dependent mechanism. Pretreatment of ECs with an inhibitor of HDAC class IIa blocked the VEGF repression of miR-615-5p suggesting that VEGF-mediated repression of miR-615-5p was mediated in part by HDAC class IIa (Figure IC in the online-only Data Supplement). We also verified by real-time polymerase chain reaction that miR-615-5p expression levels were increased in a larger cohort of human subjects with ACS. As shown in Figure 1B, circulating levels of miR-615-5p increased by 2.2-fold in ACS subjects with coronary angiograms bearing >70% stenotic lesions compared with non-ACS human subjects with coronary angiograms with lesions <20% stenosis. Similarly, miR-615-5p was significantly increased in the skin and plasma of patients with diabetes mellitus by 2.2- and 2-fold, respectively (Figure 1C and 1D). We also examined miR-615-5p expression in response to tissue injury from dermal wounds generated by punch biopsy db/db mice. Under basal conditions, miR-615-5p was expressed 4.6 fold higher in the skin of db/db mice compared with wild-type mice (day 0). MiR-615-5p expression remained elevated in wounds of db/db mice by 2.4-fold on day 7 and 1.95-fold on day 9 post-wounding compared with wild-type controls (Figure 1E).

To assess the potential role of miR-615-5p in endothelial angiogenic functions, we examined the effect of miR-615-5p on EC growth by gain- and loss-of-function experiments. Overexpression of miR-615-5p mimics (miR-615-5pm) in HUVECs inhibited cell growth by 32% under basal conditions and by 62% and 53% in response to VEGF and bFGF stimulation, respectively, whereas miR-615-5p inhibitors...
(miR-615-5p, complementary antagonist) increased EC growth by 3.2-fold under basal conditions and 2-fold and 1.85-fold in response to VEGF and bFGF stimulation, respectively (Figure 1F). To further characterize the role of miR-615-5p in HUVECs, we assessed vascular network formation assays in Matrigel. Overexpression of miR-615 inhibited network tube formation in Matrigel in vitro (Figure 2A, top) by 40%, whereas miR-615-5p inhibition significantly increased tube formation.

Figure 3. Bioinformatics and miR-615-5p gene profiling predict AKT (protein kinase B) as a targeted signaling pathway. A, Gene ontology (GO) analysis of 337 genes repressed by miR-615-5p overexpression in endothelial cells (ECs) identified from transcriptomic profiling. B and C, AKT signaling pathway is predicted to be the top regulated signaling network regulated by miR-615-5p.
in Matrigel in vitro (Figure 2A, bottom) by 28%. In addition, miR-615-5p overexpression decreased EC migration by ≈17%, compared with the nonspecific control, whereas miR-615-5p inhibition increased migration by 20% compared with the nonspecific control under basal conditions (Figure 2B). Interestingly, overexpression of miR-615-5p in the presence of VEGF (50 ng/mL) or bFGF (50 ng/mL) stimulation further inhibited the EC migration by 68% and 60%, respectively, whereas inhibition of miR-615-5p increased EC migration by 18% and 24%, respectively (Figure 2B). Finally, consistent with its effects on EC growth and migration, HUVECs overexpressing miR-615-5p decreased wound closure in scratch assays by 32% under basal conditions and by 81% in the presence of VEGF, whereas inhibition of miR-615-5p increased...

Figure 4. MiR-615-5p regulates the expression of downstream AKT/eNOS (protein kinase B/endothelial nitric oxide synthase) signaling in endothelial cells (ECs), human umbilical vein ECs transfected with (A) miR-negative mimic control (NSm) or miR-615-5p mimics (miR-615-5pm) or (B) miR inhibitor negative control (NSi) or miR-615-5p inhibitor (miR-615-5pi) were subjected to Western analysis using antibodies to p-AKT (phosphorylated protein kinase B), p-eNOS (phosphorylated endothelial nitric oxide synthase), AKT, eNOS, p-p38, p38, p-ERK1/2 (extracellular signal regulated), ERK1/2, and β-actin (n=3–5 experiments). C, NO release was measured by Griess assay. All data represent mean±SEM. *P<0.05 compared with controls.
To identify potential target signaling pathways of miR-615-5p, we performed a microarray gene chip profiling approach from HUVECs overexpressing miR-615-5p followed by GO analyses. In accordance with our in vitro findings from ECs, cellular growth and proliferation were predicted to be one of the top biological pathways to be regulated by miR-615-5p (Figure 3A). In addition, the AKT signaling pathway is predicted to be the top regulated signaling pathway by miR-615-5p in ECs (Figure 3B and 3C). Based on the GO analyses, we first verified that AKT phosphorylation was significantly reduced by ≈2-fold in response to 15, 30, and 60 minutes of VEGF stimulation in HUVECs overexpressing miR-615-5p (Figure 4A), whereas miR-615-5p inhibition increased AKT phosphorylation by 40% under baseline conditions and by ≈2-fold in response to 15, 30, and 60 minutes of VEGF stimulation (Figure 4B). Because activation of eNOS in ECs is dependent on AKT phosphorylation, we assessed for phosphorylation of eNOS at Ser 1177. Similarly, overexpression of miR-615-5p decreased eNOS phosphorylation by ≈2-fold (Figure 4A), whereas miR-615-5p inhibition increased eNOS phosphorylation by 48% under basal conditions and by ≈2-fold in response to VEGF stimulation (Figure 4B). This regulation was specific to AKT/eNOS signaling and not other signaling pathways including p38 and ERK1/2 (extracellular signal regulated; Figure 4 and Figure II in the online-only Data Supplement). Furthermore, miR-615-5p overexpression reduced NO release from HUVECs by 60% under baseline conditions and by 62% and 50% in response to 1 or 3 hours, respectively, of VEGF treatment (Figure 4C).

To identify a direct target of miR-615-5p and narrow down the potential targets from the microarray gene chip profiling data, we took a rigorous, systematic approach using a combination of bioinformatics and prediction algorithms (eg, miRWalk, Micro T4, miRNAmap, RNAhybrid, and Targetscan) and validation by expression on the mRNA and protein levels (Figure 5A). From 337 genes repressed by 2-fold, 24 genes contained at least 1 potential binding site in the 3′-untranslated region (3′-UTR) and 11 genes showed significantly decreased mRNA expression in HUVECs overexpressing miR-615-5p (Figure 5A and data not shown). Of these, overexpression of miR-615-5p in HUVECs decreased the mRNA (Figure 5B) and protein expression (Figure 5C) and 3′-UTR activity (Figure 5D) of only 2 genes—IGF2 and RASSF2. In contrast, inhibition of miR-615-5p increased IGF2 and RASSF2 expression (Figure 5B and 5C). Overexpression of miR-615-5p in ECs inhibited the activity of luciferase reporter constructs containing the IGF2 or RASSF2 3′-UTR by 72% and 30%, respectively; in contrast, inhibition of miR-615-5p increased IGF2 and RASSF2 3′-UTR reporter activity by 2- and ≈3.9-fold, respectively (Figure 5D). In addition, deletion of the miR-615-5p binding sites on the IGF2 or RASSF2 3′UTRs blocked the miR-615-5p-mediated inhibition (Figure 5E and Figure III in the online-only Data Supplement). To further verify that miR-615-5p directly targets IGF2 and RASSF2 in ECs, we performed AGO2 (Argonaute2) miRNP-IP studies to assess whether IGF2 and RASSF2 mRNA is enriched in the RNA-induced silencing complex after miR-615-5p overexpression in HUVECs. A ≈7-fold enrichment of IGF2 and a 1.7-fold enrichment of RASSF2 mRNA were observed after AGO2 miRNP-IP in the presence of miR-615-5p, as compared to the miRNA-negative control. In contrast, AGO2 miRNP-IP did not enrich the mRNA for SMAD1, a gene that was not predicted to be a miR-615-5p target (Figure 5F). In addition, overexpression of miR-615-5p decreased the protein expression of IGF2 in mouse skeletal muscle microvascular ECs (Figure 1A and in the online-only Data Supplement). siRNA silencing of RASSF2 (Figure 6A) and IGF2 (Figure 6D) phenocopied the effects of miR-615-5p overexpression on AKT phosphorylation (Figure 6B and 6E), eNOS phosphorylation (Figure IVF and IVG in the online-only Data Supplement), EC proliferation by BrdU (5-bromo-2′-deoxyuridine) assay (Figure IVB and IVC in the online-only Data Supplement), and wound closure in scratch assays (Figure IVD and IVE in the online-only Data Supplement). Furthermore, siRNA knockdown of both RASSF2 and IGF2 had a cooperative inhibitory effect on both AKT and eNOS phosphorylation (Figure IVF through IVH in the online-only Data Supplement) and exacerbated endothelial proliferation in EC scratch assays compared with RASSF2 or IGF2 knockdown alone (Figure 6G). To explore whether the miR-615-5p-mediated inhibitory effects on EC proliferation were dependent on RASSF2 and IGF2, we performed siRNA knockdown studies using EC scratch assays and quantified EC wound closure. Functionally in the absence of RASSF2 (Figure 6C) or IGF2 (Figure 6F), miR-615-5p overexpression had markedly impaired ability to inhibit EC wound closure showing that the miR-615-5p-mediated effects are dependent in part on RASSF2 and IGF2. In addition, siRNA-mediated knockdown of AKT or eNOS blocked the inhibitory effect of miR-615-5p on EC proliferation (Figure VA in the online-only Data Supplement) and migration (Figure VB in the online-only Data Supplement). Collectively, these data indicate that IGF2 and RASSF2 are bona fide targets of miR-615-5p in ECs and raise the possibility that miR-615-5p may be a molecular switch in which increased levels of miR-615-5p reduce IGF2 and RASSF2 expression, thereby suppressing EC growth and angiogenesis.

Diabetic wound healing represents a complex disease state associated with significant morbidity and mortality.18 Accumulating studies reveal that impaired angiogenesis is a hallmark in the pathogenesis of such wounds.35-38 On the basis that miR-615-5p inhibition promoted features of EC angiogenesis in vitro (Figures 1F and 2), we explored the effect of inhibiting miR-615-5p on angiogenesis in a diabetic db/db model of dermal wound healing generated by punch biopsy of the skin on the dorsal surface of the mice (Figure 7A). Compared with scrambled nonspecific control anti-miRs (miR inhibitor negative control [NSi]), local delivery of LNA–anti–miR-615-5p (MiR-615-5p) did not only significantly improved wound closure (Figure 7B) but also increased granulation tissue thickness by 2.6-fold (Figure 7C) and robust induction of angiogenesis as measured by CD31 by 1.9-fold (Figure 7D). In contrast, overexpression of miR-615-5p not only significantly delayed wound closure by 68% (Figure 7E) and
decreased granulation tissue thickness (Figure 7F) but also reduced angiogenesis in wounds by 35% compared with mice that received local intradermal delivery of control LNA–anti-miRs (Figure 7G). In addition, whereas miR-615-5p neutralization had no effect on the expression of epithelial markers keratin 10 or 14 (Figure VIA and VIB in the online-only Data Supplement) or accumulation of M1 or M2 macrophages in wounds (Figure VIC in the online-only Data Supplement), there was a significantly increase in VE-cadherin (vascular endothelial cadherin) and α-SMA.

Figure 5. IGF2 (insulin-like growth factor 2) and RASSF2 (Ras-associating domain family member 2) are bona fide targets of miR-615-5p in ECs. A. Discovery and validation of MiR-615-5p target genes. Human umbilical vein endothelial cells (HUVECs) transfected with miR-negative mimic control (NSm), and miR-615-5p mimics (miR-615-5pₘ) were subjected to microarray gene profiling. Potential gene targets were further narrowed down by sequential use of bioinformatics and prediction algorithms, real-time quantitative polymerase chain reaction (RT-qPCR), Western blot analyses, 3′-untranslated region (3′-UTR) reporter studies, and microribonucleoprotein immunoprecipitation (miRNP-IP) analysis. B and C, HUVECs transfected with NSₘ or miR-615-5pₘ were subjected RT-qPCR for IGF2 and RASSF2 expression (B) or Western blot analyses using antibodies to IGF2, RASSF2, and GAPDH (n=3 experiments; C). D, Luciferase activity of IGF2 3′-UTR and RASSF2 3′-UTR normalized to total protein was quantified in HUVECs transfected with NSₘ or miR-615-5pₘ (n=3 experiments). E, Luciferase activity of IGF2 or RASSF2 3′-UTRs bearing a deletion of the miR-615 binding site (miR-615 deletion [DEL]) normalized to total protein was quantified in HUVECs transfected with NSₘ or miR-615 m (n=3 experiments). F, miRNP-IP analysis of enrichment of IGF2 and RASSF2 mRNA in HUVECs transfected with NSₘ or miR-615-5pₘ. *P<0.01. RT-qPCR was performed to detect IGF2, RASSF2 or SMAD1. Results are representative of n=3 replicates per group and 2 independent experiments. *P<0.01. All data represent means±SEM.
(α-smooth muscle actin) expression (Figure VIIA and VIIB in the online-only Data Supplement). Finally, using a mouse model of femoral artery ligation in diabetic db/db mice, miR-615-5p neutralization significantly improved blood flow recovery and skeletal muscle neovascularization after 15 days compared with controls (Figure VIII in the online-only Data Supplement). Thus, targeting miR-615-5p induced angiogenesis and wound healing under diabetic conditions.

To evaluate whether neutralization of miR-615-5p may regulate angiogenesis from human tissues, we developed a modified human skin organoid assay in which a 6 mm circular full-thickness punch biopsy is generated. In the middle of each biopsy, a 3 mm full-thickness wound is created using a 3 mm punch biopsy which can be maintained at the air-liquid interface in culture for several days (Figure 8A). To
explore whether miR-615-5p also regulated angiogenesis in human skin organoids, we transduced human skin organoids with anti–miR-615-5p (MiR-615-5pi) or scrambled nonspecific control anti-miRs (miR inhibitor negative control [NSi]; n=11–12 per group), mice underwent dorsal skin wounding. B–D, Wound analyses included (B) wound closure areas, granulation tissue thickness (GTT; C), and (D) confocal immunofluorescence staining for CD31. E–G, After 2 local injections in mice of miR-615-5p mimics (MiR-615-5pm) or scrambled nonspecific control miRs (miR-negative mimic control [NSm]; n=10 per group), mice underwent dorsal skin wounding. E–G, Wound analyses included (E) wound closure areas, granulation tissue thickness (GTT; F), and (G) confocal immunofluorescence staining for CD31. Scale bars, 5 mm (B and E), 500 μm (C and F) and 100 μm (D and G). All data represent means±SEM. *P<0.05, **P<0.001.

Figure 7. Local delivery of LNA (locked nucleic acid)—anti–miR-615-5p promotes wound healing in db/db mice. A, After 2 local injections in mice of LNA—anti–miR-615-5p (MiR-615-5p) or scrambled nonspecific control LNA—anti–miRs (miR inhibitor negative control [NSi]; n=11–12 per group), mice underwent dorsal skin wounding. B–D, Wound analyses included (B) wound closure areas, granulation tissue thickness (GTT; C), and (D) confocal immunofluorescence staining for CD31. E–G, After 2 local injections in mice of miR-615-5p mimics (MiR-615-5pm) or scrambled nonspecific control miRs (miR-negative mimic control [NSm]; n=10 per group), mice underwent dorsal skin wounding. E–G, Wound analyses included (E) wound closure areas, granulation tissue thickness (GTT; F), and (G) confocal immunofluorescence staining for CD31. Scale bars, 5 mm (B and E), 500 μm (C and F) and 100 μm (D and G). All data represent means±SEM. *P<0.05, **P<0.001.
respectively (Figure 8E). Finally, we investigated the downstream effects of miR-615-5p knockdown or overexpression on RASSF2 or IGF2 in human skin organoids. In accordance with the in vitro findings in ECs, overexpression of miR-615-5p in human skin organoids significantly decreased RASSF2 mRNA (Figure IXA, left in the online-only Data Supplement) and protein expression (Figure IXB, left in the online-only Data Supplement), whereas the miR-615 inhibitor
had the opposite effects (Figure IXA and IXB, right) in the online-only Data Supplement. Similarly, overexpression of miR-615-5p in human skin organoids significantly decreased IGF2 mRNA (Figure IXC, left in the online-only Data Supplement) and protein expression (Figure IXC, left in the online-only Data Supplement), whereas the miR-615-5p inhibitor had the opposite effects (Figure IXC and IXD right in the online-only Data Supplement). Collectively, these data indicate that increased miR-615-5p expression adversely affects angiogenesis in response to tissue injury, whereas its neutralization can potently promote angiogenesis in human tissue.

**Discussion**

Impaired neovascularization after tissue injury is a major hallmark in a range of conditions, such as ischemic cardiovascular disease and diabetic wound healing, and can lead to maladaptive or delayed tissue recovery. The inability of ECs to perform their physiological functions (known as EC dysfunction), including EC proliferation, migration, lumen formation, and formation of new basement membranes is a major obstacle in therapeutic angiogenesis.5,59–62 Over the past few decades, experimental treatment strategies have focused on addressing EC dysfunction and angiogenesis by delivering recombinant growth factors, such as VEGF, and a diverse set of molecules that can modestly or indirectly target angiogenic signaling pathways, such as free radical scavengers63–65 receptors such as angiotensin receptor66 or Ang11,67 and many others68,69—all have shown limited efficacy with transitory effects. Indeed, pathways, such as free radical scavengers, growth factors, such as VEGF, and a diverse set of molecules regulating the VEGF-AKT/eNOS signaling pathway through the selective regulation of VEGF-induced AKT/eNOS pathway and not the p38 or pERK (phosphorylated extracellular signal regulated kinase) pathways (Figure 8 and Figure I in the online-only Data Supplement). Systemic inhibition of miR-615-5p overexpression had the opposite effects in both diabetic wounds in mice and in human skin organoids. Our findings can potentially be utilized to develop new therapeutic approaches to address impaired neoangiogenesis observed in response to tissue injury, such as diabetic wound healing or ischemic cardiovascular disease states.

The PI3K (phosphoinositide 3-kinase)/AKT signaling pathway is known to be involved in key EC functions, such as cell growth, migration, vascular tone, survival, and angiogenesis. Of the 2 isoforms of AKT, AKT1 phosphorylates eNOS and promotes NO release.70 Furthermore, genetic deletion of AKT1 under ischemic conditions resulted in impairment in ischemia-induced arteriogenesis and VEGF-induced postnatal angiogenesis.71–73 Endothelium-secreted NO not only plays an important role in the maintenance of vascular tone through activating smooth muscle relaxation but is also known to drive EC angiogenic responses.74,75 For example, vascular endothelium with decreased AKT/eNOS phosphorylation resulted in vascular dysfunction and reduced angiogenesis in mouse models of hindlimb ischemia and MI.74–76 Our findings showing that miR-615-5p regulates EC growth and angiogenesis through the selective regulation of VEGF-induced AKT/eNOS pathway and not the p38 or pERK (phosphorylated extracellular signal regulated kinase) pathways (Figures 3 and 4 and Figure I in the online-only Data Supplement) is consistent with the above paradigms. Moreover, siRNA-mediated knockdown of AKT or eNOS significantly attenuated the inhibitory effect of miR-615 on EC proliferation (Figure IVA in the online-only Data Supplement) and migration (Figure IVB in the online-only Data Supplement), thereby highlighting miR-615-5p dependency on the AKT/eNOS signaling pathway.

We used an unbiased transcriptomic profiling approach in combination with bioinformatics to identify and validate miR-615-5p target genes. After filtering 337 genes repressed by at least 2-fold by miR-615-5p in transcriptomic profiling, only 2 target genes, IGF2 and RASSF2, were verified by expression, 3′-UTR reporter assays, mIRNP-IP studies, and siRNA dependency (Figure 5). Interestingly, IGF2 was identified as a target of miR-615-5p in pancreatic ductal adenocarcinoma where overexpression of miR-615-5p decreased cell proliferation, migration, and cell invasion.77 Our findings...
demonstrate that miR-615-5p overexpression significantly decreased EC growth, migration, network tube formation in Matrigel, and NO release (Figures 1 through 2) are consistent with the observed effects in this study. Moreover, our findings implicate that miR-615-5p is dependent in part on IGF2 for exerting downstream EC functional effects (Figure 6F). In addition, IGF2 deficiency is associated with reduced VEGF-induced activation of AKT in an analogous manner as miR-615-5p overexpression (Figure 6E), an effect consistent with this gene serving as a miR-615-5p target (Figure VI in the online-only Data Supplement).

Our study also identified a previously unknown target of miR-615-5p, RASSF2. Interestingly, RASSF2 is known to function as a tumor suppressor inhibiting growth of lung cancer cells via its association with farnesylated K-RAS (Kirsten rat sarcoma viral oncogene homolog).\(^{79,80}\) In addition, in 293T embryonic kidney cells, expression of RASSF2 resulted in growth inhibition enhanced by activated K-Ras.\(^{81}\) Our findings in ECs are distinct from the reported effects of RASSF2 in tumor cells and in 293T embryonic kidney cells. We and others have shown that these miRNA functional differences can be attributed to cell-type-specific miRNA-mediated effects that are often dependent on the relative expression of the proteins targeted by the miRNA.\(^{19}\) In addition, differences in basal miRNA expression may be different in primary and transformed cells lines, resulting in distinct effects on downstream signaling events.\(^{82-84}\) Similar to IGF2, our findings demonstrate that miR-615-5p is dependent in part upon RASSF2 for exerting downstream EC functional effects (Figure 6C). RASSF2 deficiency is associated with reduced VEGF-induced activation of AKT in an analogous manner as miR-615-5p overexpression (Figure 6B), an effect consistent with this gene serving as a miR-615-5p target. In addition, combined RASSF2 and IGF2 siRNA knockdown further reduced p-AKT (phosphorylated protein kinase B) and p-eNOS (phosphorylated endothelial nitric oxide synthase) expression in ECs (Figure IVG in the online-only Data Supplement) and exacerbated endothelial proliferation in EC scratch assays compared with RASSF2 or IGF2 knockdown alone (Figure 6G), implicating potential cooperativity of these targets in mediating miR-615-5p downstream effects.

In addition to miR-615-5p, a handful of other miRNAs have been implicated in regulating the VEGF-AKT/eNOS signaling pathway. However, most studies have not identified direct miRNA target genes that may underlie these observations. For example, miR-126 is an endothelial-enriched miRNA that was found in human cardiac microvascular ECs to protect against hypoxia/reoxygenation-induced injury by activating the AKT/eNOS signaling pathway, albeit no miR-126 direct targets were identified that may account for these observations.\(^{85}\) Using retinal ECs, miR-152 was found to repress VEGF by altering prorenin receptor expression, although effects on AKT/eNOS signaling were not assessed.\(^{86}\) Finally, miR-223 overexpression attenuated VEGF-induced EC proliferation and inhibited VEGF-induced phosphorylation of VEGFR2 and AKT activation by targeting \(\beta 1\) integrin. It will be of interest to determine whether miR-615-5p in combination with these or other miRNAs that activate VEGF-AKT/eNOS signaling can more effectively overcome angiogenic resistance as a basis for therapeutic intervention.

There are several limitations that we wish to acknowledge in this study. It is unclear whether miR-615-5p may serve as a biomarker of ischemic injury in other clinical contexts, or may be associated with specific baseline demographics, including sex. Future studies will be required to prospectively assess the clinical utility of this miRNA as a biomarker or therapeutic. For the human skin organoid studies, it is possible that effects of miR-615-5p inhibitors may be more pronounced in specific patient subgroups or in skin derived from specific locations; however, these were anonymized samples and future-focused studies will be needed to clarify this point further. For the human skin organoid studies, Finally, although we demonstrated that miR-615-5p mediates EC angiogenic functional responses dependent on the 2 targets IGF2 and RASSF2, other targets may certainly be involved in response to divergent pathophysiological stimuli as we have observed for other miRNAs.\(^{45,87,88}\)

In summary, we identified miR-615-5p as a novel negative regulator of angiogenesis by regulating the VEGF-AKT/eNOS pathway and direct targeting of IGF2 and RASSF2 genes. Inhibition of miR-615-5p improved wound healing and angiogenesis under diabetic conditions in addition to markedly increasing angiogenesis in human skin organoids. Because miR-615-5p is increased under conditions associated with impaired microvascular disease, therapies focusing on neutralization of miR-615-5p may be beneficial for promoting neovascularization and tissue repair.

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Disclosures

None.

References


**Highlights**

- MiR-615-5p expression is rapidly increased in response to diverse stimuli, including in wounds of diabetic db/db mice, in plasma of human subjects with acute coronary syndromes, and in plasma and skin of human subjects with diabetes mellitus.
- MiR-615-5p serves as an antiangiogenic microRNA by targeting RASSF2 (Ras-associating domain family member 2) and IGF2 (insulin-like growth factor 2) and subsequently endothelial cell VEGF (vascular endothelial growth factor)-AKT (protein kinase B)/eNOS (endothelial nitric oxide synthase) signaling.
- Local miR-615-5p neutralization markedly increased angiogenesis, granulation tissue thickness, and wound closure rates in diabetic db/db mice, whereas local delivery of miR-615-5p mimics impaired these effects.
- Systemic miR-615-5p neutralization improved skeletal muscle perfusion and angiogenesis after hindlimb ischemia in db/db mice.
- Gain- and loss-of-function studies of miR-615-5p in human skin organoids demonstrated dynamic regulation of VEGF-induced AKT signaling and angiogenesis.