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MicroRNA-26a Regulates Pathological and Physiological Angiogenesis by Targeting BMP/SMAD1 Signaling

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Abstract

Rationale—The rapid induction and orchestration of new blood vessels are critical for tissue repair in response to injury, such as myocardial infarction, and for physiological angiogenic responses, such as embryonic development and exercise.

Objective—We aimed to identify and characterize microRNAs (miR) that regulate pathological and physiological angiogenesis.

Methods and Results—We show that miR-26a regulates pathological and physiological angiogenesis by targeting endothelial cell (EC) bone morphogenic protein/SMAD1 signaling in vitro and in vivo. MiR-26a expression is increased in a model of acute myocardial infarction in mice and in human subjects with acute coronary syndromes. Ectopic expression of miR-26a markedly induced EC cycle arrest and inhibited EC migration, sprouting angiogenesis, and network tube formation in matrigel, whereas blockade of miR-26a had the opposite effects. Mechanistic studies demonstrate that miR-26a inhibits the bone morphogenic protein/SMAD1 signaling pathway in ECs by binding to the SMAD1 3′-untranslated region, an effect that decreased expression of Id1 and increased $p21^{WAF/CIP}$ and p27. In zebrafish, miR-26a overexpression inhibited formation of the caudal vein plexus, a bone morphogenic protein-responsive process, an effect rescued by ectopic SMAD1 expression. In mice, miR-26a overexpression inhibited EC SMAD1 expression and exercise-induced angiogenesis. Furthermore, systemic intravenous administration of an miR-26a inhibitor, locked nucleic acid-anti–miR-26a, increased SMAD1 expression and rapidly induced robust angiogenesis within 2 days, an effect associated with reduced myocardial infarct size and improved heart function.

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Disclosures

Mark W. Feinberg, Basak Icli, and The Brigham and Women's Hospital have a patent pending related to the work that is described in the present study. The other authors report no conflicts.

Conclusions—These findings establish miR-26a as a regulator of bone morphogenic protein/SMAD1-mediated EC angiogenic responses, and that manipulating miR-26a expression could provide a new target for rapid angiogenic therapy in ischemic disease states.

Keywords

angiogenesis effect; endothelial cells; microRNAs; myocardial infarction

The appropriate progression of events in angiogenesis is controlled by a balance between pro- and antiangiogenic factors. In response to proangiogenic stimuli, vascular endothelial cells (ECs) need to be activated rapidly to migrate to distant sites and proliferate to form new primary capillaries from existing ones.[1] Failure to do so may delay tissue repair in an array of pathological or physiological conditions. Growth factors, such as vascular endothelial cell growth factor (VEGF), tumor necrosis factor-α (TNF-α), basic fibroblast growth factor, or placenta growth factor, are potent regulators of angiogenesis. Impaired EC angiogenic responses have been linked to exacerbation of a wide range of disease states, including poor cardiovascular function and outcomes,[1,2] diabetic wound healing,[3] and neurodegenerative disorders.[4]

Accumulating studies highlight an important role for bone morphogenic proteins (BMPs) and SMAD1 signaling in promoting angiogenesis.[5-8] BMPs and receptor-activated SMADs, SMAD1 in particular, induce the expression of Id1, a helix–loop–helix transcription factor, that lacks a basic DNA-binding domain, which stimulates migration and growth of ECs.[9-11] SMAD1 knockout mice fail to develop a mature vascular system and die at embryonic day 9.5.[12] Id1 primarily acts as a dominant-negative inhibitor of helix–loop–helix transcription factors by heterodimerization.[9,13,14] One example of this heterodimerization is with the cell cycle kinase inhibitor $p21^{WAF/CIP}$, an effect that inhibits cell cycle growth arrest and favors cell cycle progression.[15-17] Overexpression of Id1 mimicked BMP-induced effects in ECs, including cell growth, migration, and network tube formation in vitro.[18-20] Furthermore, overexpression of Id1 in mature ECs conferred proangiogenic properties both in vitro and in vivo in response to hindlimb ischemia.[21] However, the upstream molecular events governing SMAD1 expression in EC angiogenic responses remain poorly defined.

MicroRNAs (miRs) are small evolutionarily conserved, 20 to 22 nt, noncoding RNAs capable of repressing gene expression at the post-transcriptional level by base pairing at the 3′-untranslated regions (3’-UTRs) of mRNA targets and have been found to regulate a variety of physiological and cellular functions in heath and disease.[22,23] Several reports have identified miRs in regulating various aspects of the angiogenic response to diverse pathophysiological stimuli. For example, miR-126, miR-130a, miR-210, and the miR-23–miR-27–miR-24 cluster promote proangiogenic activity, whereas miR-221/miR-222, miR-92a, and miR-217 inhibit angiogenic activity in ECs.[24-30] Although the role of miRs in tumor-associated angiogenesis has garnered considerable attention, the identification and function of miRs regulating the angiogenic response in cardiovascular disease remain poorly understood.

In this report, we show that miR-26a acts as a previously unrecognized pivotal regulator of pathological and physiological angiogenesis by targeting a SMAD1-Id1-$p21^{WAF/CIP1}$/p27

signaling axis to promote an antiangiogenic program in ECs. Furthermore, neutralization of miR-26a rapidly induced angiogenesis and reduced acute myocardial infarction (MI) size and improved heart function in mice. These findings may provide a novel approach for inducing rapid angiogenic therapy in ischemic cardiovascular disease.

Methods

Statistical Analysis

Data are presented as mean±SEM. All in vitro experiments are representative of 3 independent experiments. Data were subjected to Student *t* test or, where applicable, ANOVA with Bonferroni correction for multiple group comparisons using GraphPad Prism5, and $P<0.05$ was considered statistically significant. Human data were analyzed by the Mann–Whitney *U* test, and $P<0.05$ was considered statistically significant.

For more detailed experimental methods, refer to the Online Data Supplement.

Results

MiR-26a Is Regulated by Proangiogenic Stimuli and Inhibits Cell Growth in ECs

To identify how proangiogenic stimuli regulate EC function, microRNA (MiR) microarray profiling studies were undertaken using RNA from human umbilical vein ECs (HUVECs) exposed to vehicle alone or the proangiogenic stimulus TNF-α for 24 hours, and reduced expression of miR-26a was noted (data not shown). Using real-time polymerase chain reaction analysis, we verified that both miR-26a and its family member miR-26b were reduced by TNF-α by 70% and 21%, respectively (Online Figure IA and IB). Similarly, the prototypical proangiogenic growth factor VEGF reduced miR-26a and miR-26b by 56% and 71%, respectively (Figure 1A and Online Figure IC). The expression of miR-26a was ≈3-fold higher than miR-26b, suggesting that miR-26a is the dominant family member expressed in HUVECs (Figure 1B). The miR-26 family resides in the intronic region of carboxy-terminal domain RNA polymerase II polypeptide (CTDSP), a small phosphatase-like gene family including CTDSPL, CTDSP1, and CTDSP2.[31] Treatment of HUVECs with VEGF reduced CTDSP2 mRNA expression in a similar manner to miR-26a (Online Figure ID). The development of angiogenesis is important in response to ischemic injury. We examined whether miR-26a expression levels were regulated in human subjects with acute coronary syndromes (ACS) and in mice undergoing coronary ligation. As shown in Figure 1C, circulating miR-26a levels increased by 4.2-fold in ACS subjects with coronary angiograms bearing >70% stenotic lesions compared with non-ACS human subjects with coronary angiograms with lesions <20% stenosis. In mice, miR-26a expression increased in an analogous manner after 45 minutes of left anterior descending artery ligation (Figure 1D). Interestingly, 1 hour after 45 minutes of ischemiat-reperfusion–induced myocardial injury, miR-26a expression increased significantly in the ischemic region (apex) of the heart compared with sham controls. In contrast, by 24 hours, miR-26a expression in the ischemic region decreased compared with sham controls (Online Figure II). Collectively, these data suggest that miR-26a is dynamically regulated by proangiogenic stimuli in ECs, and its rapidly induced expression may correlate with acute injury states, such as MI, raising the

possibility that targeting this miR may facilitate the induction of angiogenesis. To assess the potential role of miR-26a in endothelial angiogenic functions, we examined the effect of miR-26a on EC growth by gain- and loss-of-function experiments. Overexpression of miR-26a mimics in HUVECs inhibited cell growth by 40% and cell proliferation by 20%, whereas miR-26a inhibitors (complementary antagonist) increased EC growth by 2.8-fold and cell proliferation by 10% (Figure 1E and Online Figure IIIA). Treatment of HUVECs with miR-26a mimics or miR-26a inhibitors did not induce apoptosis as shown by expression for caspase-3 and Annexin V (Online Figure IIIB). In accordance with the decreased EC growth properties in response to miR-26a, cell cycle analyses of cells transfected with miR-26a mimics demonstrated marked G1-phase cell cycle arrest (76% versus 61%, respectively) compared with nonspecific (NS) control mimics; conversely, miR-26a inhibition reduced G1/G0 phase (56% versus 65%, respectively; Figure 1F).

MiR-26a Inhibits Proangiogenic Functions in ECs

To further characterize the role of miR-26a in HUVECs, we assessed vascular network formation assays in matrigel. Overexpression of miR-26a inhibited network tube formation in matrigel in vitro (Figure 2A, left) and angiogenesis in matrigel plugs in vivo (Figure 2B, left) by 60% and 40%, respectively, whereas miR-26a inhibition significantly increased tube formation in matrigel in vitro (Figure 2A, right) and angiogenesis in matrigel plugs in vivo (Figure 2B, right) by 30% and 70%, respectively. In addition, miR-26a overexpression decreased EC migration in response to proangiogenic stimuli TNF-α, BMP2, and VEGF by 41%, 31%, and 60%, respectively, compared with the NS control group, whereas miR-26a inhibition potently increased migration by 4.5-fold in response to VEGF and BMP2 and by 2.4-fold in response to TNF-α compared with the NS control group (Figure 2C). Furthermore, miR-26a overexpression in ex vivo aortic ring assays reduced sprouting by 36%, whereas miR-26a inhibition increased aortic ring sprouting by 2-fold (Figure 2D). Taken together, these data indicate that miR-26a inhibited EC angiogenic functions in vitro and in vivo.

MiR-26a Targets SMAD1 in ECs

To identify potential targets of miR-26a, we analyzed predicted targets according to the algorithms of TargetScan,[32] PITA,[33] and miRanda.[34] All the 3 predicted SMAD1 as a common target of miR-26a with the occurrence of 2 binding sites present in the 3′-UTR of the SMAD1 gene. We first verified that SMAD1 expression was significantly reduced (by 55%) in HUVECs overexpressing miR-26a, whereas miR-26a inhibition increased SMAD1 protein expression by 1.8-fold (Figure 3A). In addition, this regulation was specific to SMAD1 and not other SMAD family members, including SMAD2, SMAD4, and SMAD7 (Figure 3B). Overexpression of miR-26a in ECs inhibited the activity of a luciferase reporter construct containing the SMAD1 3′-UTR by 60%, whereas inhibition of miR-26a increased SMAD1 3′-UTR reporter activity by 29% (Figure 3C). Consistent with our prior observations, proangiogenic stimuli that decreased endogenous miR-26a in HUVECs, such as VEGF and TNF-α, increased SMAD1 3′-UTR reporter activity (Figure 3D). In addition, mutation of the SMAD1 sites blocked the increase of SMAD1 3′-UTR reporter activity by proangiogenic stimuli (Online Figure IVA). To further verify that miR-26a directly targets SMAD1 in ECs, we performed argonaute2 microribonucleoprotein immunoprecipitation

studies to assess whether SMAD1 mRNA is enriched in the RNA-induced silencing complex after miR-26a overexpression in HUVECs. A ≈5-fold enrichment of SMAD1 mRNA was observed after argonaute2 microribonucleoprotein immunoprecipitation in the presence of miR-26a as compared with the miRNA negative control. In contrast, argonaute2 microribonucleoprotein immunoprecipitation did not enrich the mRNA for *karyopherin alpha 4*, a gene that was not predicted to be an miR-26a target (Figure 3E). Moreover, lentiviral overexpression of SMAD1 lacking its 3′-UTR was able to rescue completely the inhibitory effect of miR-26a on EC growth (Figure 3F). Conversely, siRNA silencing of SMAD1 (Figure 3G) phenocopied the functional effects of miR-26a overexpression on EC network formation (Figure 3H) and growth (Figure 3I). Collectively, these data indicate that SMAD1 is a bona fide target of miR-26a in ECs and raise the possibility that miR-26a may be a molecular switch in which in response to proangiogenic stimuli reduced levels of miR-26a allow for increased SMAD1 expression, thereby facilitating EC growth and angiogenesis.

MiR-26a Regulates SMAD1 Signaling in ECs

We further explored the downstream signaling consequences of miR-26a–mediated repression of SMAD1 expression in ECs. Id1, a helix–loop–helix protein, has been identified as a downstream target of the BMP/SMAD1 signaling pathway and acts as a dominant negative to inhibit the cell cycle inhibitors $p21^{WAF/CIP1}$ or p27 in ECs.[16,17] We first verified that overexpression of miR-26a decreased Id1 expression and increased $p21^{WAF/CIP1}$ and p27 expression, whereas inhibition of miR-26a increased Id1 and decreased these cell cycle inhibitors in HUVECs (Figure 4A and 4B and Online Figure IVB and IVC). MiR-26a overexpression or inhibition had no effect on other miR-26a targets, cyclin D2 or E2 (Online Figure IVD), reported in hepatoma cells.[35] In response to BMP ligands, such as BMP9, ALK1-mediated phosphorylation of SMAD1/5/8 activates the Id1 promoter in microvascular ECs and promotes angiogenesis.[6] Accordingly, miR-26a overexpression effectively inhibited BMP9-mediated phosphorylation of SMAD1 (Figure 4B) and decreased Id1 reporter gene activity both in the absence and in the presence of BMP9 (Figure 4C), suggesting that miR-26a exerts its effects on EC growth inhibition and angiogenic functions predominantly through its regulation of the BMP/SMAD1/Id1 signaling pathway.

MiR-26a Regulates Caudal Vein Plexus Formation, a BMP-Responsive Process, in Zebrafish

In vertebrates, BMP signaling plays an important role in establishing the dorsoventral axis by promoting ventral fates. Genetic mutants of BMP signaling pathway exhibited a dorsalized axial pattern and pharmacological inhibitors of BMP signaling-inhibited sprouting angiogenesis along the caudal vein plexus (CVP) of the axial vein, a ventral structure in zebrafish.[36] Conversely, activation of BMP signaling (by overexpression of an activated BMP type II receptor) induced ectopic sprouts along the axial vein.[37] To examine the effect of miR-26a on BMP-dependent angiogenesis, we overexpressed miR-26a in zebrafish flk:eGFP embryos (Online Figure V) that severely impaired the development of the CVP of the axial vein, a BMP-responsive process,[37] by 48 hours after fertilization (Figure 5A). Notably, coexpression of SMAD1 RNA with miR-26a in flk:eGFP embryos

partially rescued the formation of the CVP by 69% (Figure 5B) and increased vascular branching by 68% (Figure 5C), suggesting that miR-26a regulates BMP-mediated angiogenesis in vivo.

MiR-26a Regulates Pathological Angiogenesis, Myocardial Infarct Size, and Left Ventricular Function

On the basis that miR-26a expression is enriched in the vascular endothelium of the heart and in noncardiomyocyte fractions (Online Figure VIA and VIB) and is induced in response to acute MI (Figure 1C and 1D), we explored the effect of inhibiting miR-26a on angiogenesis in acute MI consisting of 45 minutes of left anterior descending ischemia/reperfusion in the presence of systemically delivered locked nucleic acid (LNA)-anti–miR-26a (miR-26a inhibitors) or scrambled non-specific control anti-miRs (NS_i; Figure 6A). LNA-anti–miR-26a effectively decreased miR-26a expression both in the heart and in the circulation and increased SMAD1 protein expression in the heart (Online Figure VIC–VIE). Mice that received miR-26a anti-miRs exhibited not only significantly reduced myocardial infarct size compared with mice that received control LNA-anti-miRs (Figure 6B), but also remarkably generated rapid induction of myocardial angiogenesis by 48 hours as measured by CD31 and isolectin staining in the entire left ventricle (LV) by 2- and 1.7-fold, respectively (Figure 6C and 6D). In addition to inducing angiogenesis, inhibition of miR-26a significantly improved LV ejection fraction by 21% by 48 hours and by 32% by 8 days (Figure 6F and Online Tables I and II). Interestingly, LNA-anti-miR-miR-26a–injected mice displayed decreased myocardial apoptosis by Annexin V staining at 48 hours (Online Figure VIF), whereas no difference was observed in myocardial necrosis or in α-smooth muscle actin expression (Online Figure VIG and VIH). Expression of the inflammatory cytokines ICAM-1, VCAM-1, TNF-α, or E-selectin was not different in plasma of mice injected with NS_i or miR-26a inhibitors (Online Figure VII). Finally, there were no differences in the percentage of $lin^-Sca1^+KDR^+$ and $lin^-CD34^+KDR^+$ circulating progenitors in response to LNA-miR-26a or the NS control groups (data not shown). Thus, targeting miR-26a increased SMAD1 expression, induced myocardial angiogenesis and LV function, and reduced infarct size after acute MI.

MiR-26a Regulates Physiological Angiogenesis

To explore whether miR-26a also regulated angiogenesis under physiological conditions, we examined the effect of systemically delivered miR-26a mimics on exercise-induced angiogenesis in skeletal muscle (Figure 7A and Online Figure IXA). After systemic delivery of miR-26a or nonspecific miR mimic controls, miR-26a was overexpressed ≈4-fold higher in the quadriceps muscle (Figure IXB and IXC). After exercise for 8 days, overexpression of miR-26a decreased CD31 and Ki67 expression in the quadriceps of these mice compared with mice injected with scrambled miR mimic controls (Figure 7B–7E). Furthermore, miR-26a overexpression reduced SMAD1 expression that colocalized with CD31-positive cells and increased $p21^{WAF/CIP1}$ expression (Figure 7F and 7G). Collectively, these data indicate that increased miR-26a overexpression adversely affects physiological angiogenesis, such as in exercise.

Discussion

Impaired EC responses have been implicated in a variety of physiological conditions and pathological disease states.[1] We show here that miR-26a acts as a unique, rapid angiogenic switch in both physiological and pathological angiogenesis by suppressing endothelial SMAD1 expression, an effect leading to reduced Id1 and increased $p21^{WAF/CIP1}$ and p27 expression and EC cycle arrest. Importantly, these effects are distinct from other miRs that have been implicated in regulating angiogenic signals to date.[22,38] We identified that miR-26a expression is increased in response to acute MI in mice and in human subjects with ACS. MiR-26a overexpression impairs physiological angiogenic responses of CVP formation in zebrafish and exercise-induced angiogenesis in mice. In contrast, in vivo neutralization of miR-26a reduced myocardial infarct size by rapidly inducing robust angiogenesis by 2 days with improved LV function, suggesting a new therapeutic approach for diseases associated with pathological angiogenesis.

Several lines of evidence support a key role for the BMP/SMAD1/Id1 signaling pathway-regulating angiogenesis. Upstream activation of this pathway, for example, with administration of recombinant BMP2, stimulated angiogenesis in developing tumors.[6] SMAD1 knockout mice exhibit an immature vasculature and suffer early embryonic lethality.[9,12] Id1, a known SMAD1 target gene, has been implicated in tumor-associated angiogenesis. Id1 knockout mice exhibited reduced angiogenesis and increased $p21^{WAF/CIP1}$ expression in several cell types.[15] In addition, genetic ablation of $p21^{WAF/CIP1}$ in Id1 knockout mice restored a functional EC population and rescued the defective angiogenesis and tumor growth.[15] Furthermore, activation of BMP signaling in zebrafish increased sprouting along the CVP, whereas inhibition of BMP signaling reduced CVP formation.[37] Thus, our findings that miR-26a regulates the downstream SMAD1 signaling pathway in ECs, zebrafish, and mice is consistent with observed effects in response to perturbation of this signaling pathway in complementary pathological and physiological angiogenic paradigms. Increased miR-26a expression, as observed after myocardial injury in mice (Figure 1C), may dampen endogenous angiogenic responses required for early, effective tissue repair. Importantly, increased miR-26a expression is not only restricted to murine myocardial injury, but also detected in human subjects with ACS, thereby potentially providing a new target for ischemic cardiovascular disease.

Consistent with our findings, miR-26a has been shown to target SMAD1 in osteoblasts and myoblasts.[39,40] Interestingly, inhibition of miR-26a in osteoblasts increased bone marker genes and promoted osteoblast differentiation.[39] In contrast, inhibition of miR-26a decreased skeletal muscle myoblast differentiation.[40] Intriguingly, miR-26a inhibition in vascular smooth muscle cells in vitro[41] had the opposite effects on cell proliferation and migration that we observed herein on ECs (miR-26a inhibition reduced SMC proliferation and migration in vitro),[41] whereas our findings demonstrate that miR-26a increases EC proliferation, migration, and angiogenesis in vitro and in vivo (Figures 1-3, 5-6). Although it is unclear whether SMAD1 or its downstream signaling targets that we identified in this report may be playing an analogous role in these studies, an emerging paradigm from other studies indicates that cell type–specific MiR-mediated effects may be dependent on the relative expression of the proteins that are regulated by the MiR.[42,43] In support of this

concept, miR-26a expression in liver cancer cells induces cell cycle arrest through direct targeting of cyclins D2 and E2.[35] Although their cell cycle growth arrest findings are consistent with our study, the mechanism in hepatoma cells seems to be quite different than the results reported here. We showed that miR-26a causes cell cycle and growth arrest in ECs through direct targeting of the SMAD1-Id1-p21$^{WAF/CIP1}$-p27 pathway (Figures 3 and 4). Furthermore, there was no effect of miR-26a on cyclin D2 or E2 expression in HUVECs (Online Figure III). MiR-26a has also been implicated in cell apoptosis by predicting to target a BAK1 pathway[44]; however, we did not detect any effect of miR-26a overexpression or inhibition on EC apoptosis as quantified by caspase-3 and Annexin V expression (Online Figure IIIB). In addition, functional differences may exist between primary cells and transformed/tumor cell lines.[35] Thus, the signaling pathways and targets regulated by miR-26a are likely to be quite different depending on the cellular context.

In addition to miR-26a, other miRs have been implicated in molecular mechanisms controlling EC-driven angiogenesis.[22,38] For example, inhibition of miR-92a, a part of the miR-17 to miR-92 cluster, also reduced infarct size and promoted neovascularization in response to MI. However, the targets identified were quite different than targets of miR-26a and included integrin-α5 and eNOS.[25] The role of MiR-92a in physiological angiogenesis remains unknown. MiR-132 was found enriched in ECs of the tumor vasculature and regulated tumor- and retinal-angiogenesis by targeting p120RasGAP.[45] Another MiR, miR-126, targets sprouty-related EVH-1 domain-containing 1 and regulated developmental angiogenesis in zebrafish and neovascularization after ischemic myocardial injury in mice.[46] Finally, members of the miR-23–miR-27–miR-24 cluster target several known angiogenic factors, including semaphorin 6A, Sprouty2, GATA2, and p21-activated kinase PAK4, to promote myocardial and retinal neovascularization.[28,47-49] Thus, we have identified an unexpected role for miR-26a in its unique ability to inhibit rapidly angiogenesis via targeting the BMP/SMAD1/Id1 signaling, an effect that is distinct from other miRs that have been implicated in regulating angiogenic signals.

Accumulating studies demonstrate that strategies to enhance myocardial angiogenesis after myocardial injury are often associated with improved LV function.[50-53] Angiogenesis is a critical component in the early reparative process of granulation tissue after acute MI and can participate in limiting infarct size and reducing myocardial apoptosis.[50-53] Consistent with this premise, injection of mice with LNA-anti–miR-26a markedly increased myocardial angiogenesis by 2-fold, an effect associated with improved LV function and reduced myocardial apoptosis by 48 hours (Figure 6 and Online Table I). Although LV function improved even further by 8 days, there was only a nonsignificant trend of increased angiogenesis by 8 days (≈20%; Online Figure VIII and Tables I and II). Interestingly, miR-26a expression was significantly induced in the ischemic apex 1 hour after acute MI, but there were no differences in the LV mid and base regions at this time point compared with sham controls, whereas by 24 hours, miR-26a expression was reduced in the apex and mid regions but not in the base (Online Figure IIA–IIC). As such, there may be endogenous pressure to reduce miR-26a expression in the ischemic myocardium to promote angiogenesis and myocardial repair. Collectively, these findings raise the possibility that earlier therapeutic intervention to enhance angiogenesis may confer a more favorable enduring effect for ongoing myocardial remodeling.

In summary, our findings establish miR-26a as an EC-enriched miR that has a key role in regulating both physiological and pathological angiogenesis by targeting a SMAD1-Id1-$p21^{WAF/CIP1}$/p27 signaling axis to promote an antiangiogenic program in ECs. MiR-26a expression is increased in response to acute MI in mice and in human subjects with ACS. Inhibition of miR-26a in mice induced rapid angiogenesis and attenuated myocardial infarct size. These effects were associated with improved LV function. As impaired EC responses have been implicated in a variety of disease states, therapeutic neutralization of miR-26a could be applied for rapid angiogenic induction in other ischemic disease states, including chronic ischemic heart disease, peripheral artery disease, and diabetic wound healing, whereas application of miR-26a mimetics could offer new opportunities in treating angiogenesis associated with cancer, age-associated macular degeneration, rheumatoid arthritis, and psoriasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbrevations and Acronyms

ACS	acute coronary syndromes
BMP	bone morphogenic protein
CTDSP	carboxy-terminal domain RNA polymerase II polypeptide
CVP	caudal vein plexus
EC	endothelial cell
HUVECs	human umbilical vein ECs
MI	myocardial infarction
MiR	microRNA
NS	nonspecific
TNF-α	tumor necrosis factor-α
3′-UTR	3′-untranslated region
VEGF	vascular endothelial cell growth factor

Novelty and Significance

What Is Known?

- Angiogenesis, the generation of new blood vessels from pre-existing ones, is an important event in growth, development, and tissue repair.
- Impaired angiogenesis is implicated in a number of disease states, such as ischemic cardiovascular diseases (ie, acute myocardial infarction), diabetic wound healing, and neurodegenerative disorders.
- Although several microRNAs (miR) have been shown to regulate tumor angiogenesis, their role in the angiogenic response relevant to ischemic cardiovascular disease remains inadequately understood.

What New Information Does This Article Contribute?

- MiR-26a is an important regulator of pathological and physiological angiogenesis.
- MiR-26a targets a SMAD1-Id1-p21$^{WAF/CIP1}$/p27 signaling axis to promote an antiangiogenic program in endothelial cells.
- Targeted inhibition of miR-26a rapidly increases angiogenesis, decreases infarct size, and improves cardiac function after myocardial infarction in a mouse model.

In this report, we identified and characterized miR-26a as a previously unknown regulator of physiological and pathological angiogenesis. We found that expression of miR-26a is increased in acute myocardial infarction in mice. In addition, circulating level of miR-26a was increased in human subjects with acute coronary syndromes. MiR-26a regulates EC growth, proliferation, and migration and early angiogenesis in animal models of acute myocardial infarction, and during exercise, or development. These findings suggest that neutralization of miR-26a expression may provide a novel approach for inducing rapid angiogenic therapy in ischemic cardiovascular disease.

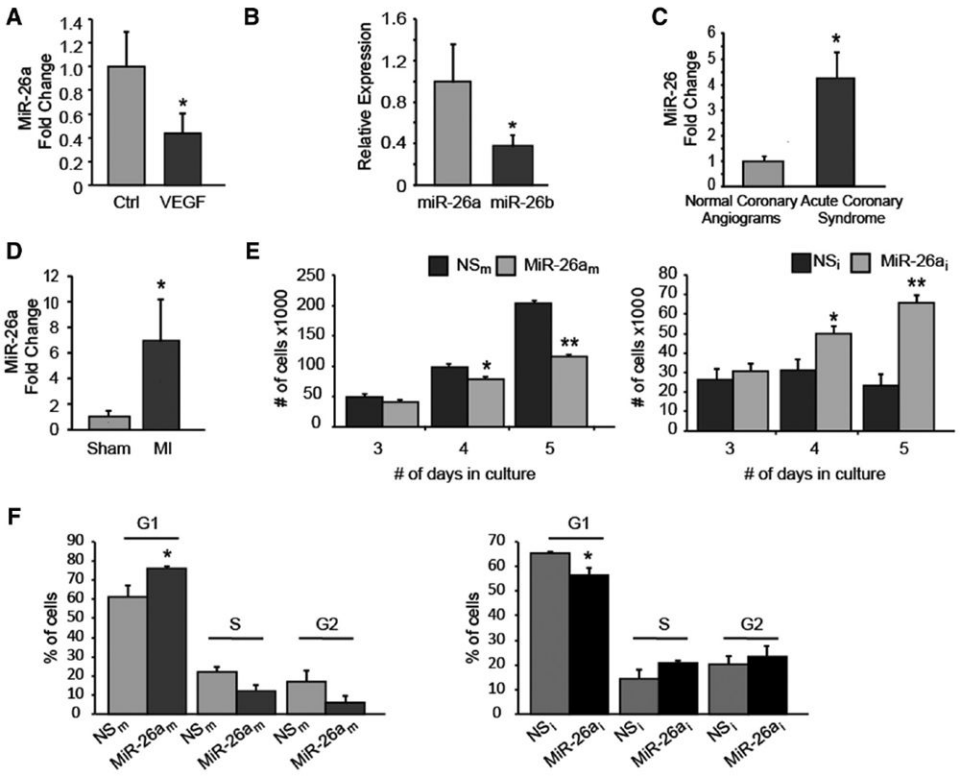

Figure 1. MicroRNA (miR)-26a is regulated by proangiogenic stimuli and inhibits cell growth

A, Real-time quantitative polymerase chain reaction (qPCR) analysis of miR-26a expression in response to vascular endothelial cell growth factor (VEGF) in human umbilical vein endothelial cells (HUVECs). **P*<0.005 compared with control (Ctrl). **B**, Real-time qPCR analysis of miR-26a and miR-26b in HUVECs. **P*<0.001. **C**, Circulating miR-26a levels are increased in plasma from human subjects with acute coronary syndrome (n=14) compared with subjects with normal coronary angiograms (n=16). **P*<0.05 compared with normal coronary angiogram. **D**, Circulating miR-26a levels are increased in plasma of mice after 45 minutes of ischemia/reperfusion of the left anterior descending artery (n=4 per group). **P*<0.05 compared with sham. HUVECs transfected with miR negative control (NS_m), miR-26a mimics ($miR\text{-}26a_m$), miR inhibitor negative control (NS_i), or miR-26a inhibitor ($miR\text{-}26a_i$) were subjected to cell growth assays (**E**; n=6 per group). **P*<0.05 compared with NS_m or NS_i; ***P*<0.001 compared with NS_m or NS_i; or cell cycle profiling (**F**) by propidium iodide staining and fluorescence activated cell sorting (data representative of n=3 experiments). **P*<0.001 compared with NS_m (G0/G1) or NS_i (G0/G1).

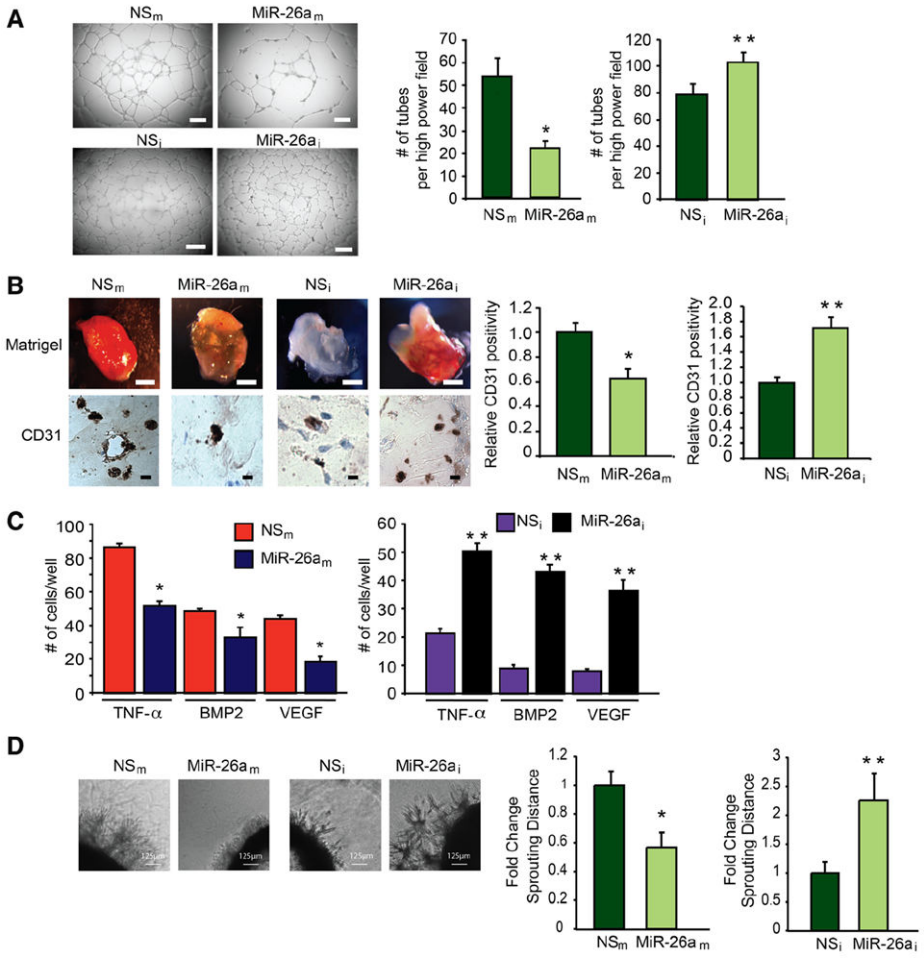

Figure 2. MicroRNA (miR)-26a inhibits proangiogenic functions in endothelial cells (ECs) in vitro and in vivo

A and **B**, Human umbilical vein ECs (HUVECs) transfected with miR negative control (NS_m), miR-26a mimics (miR-26a_m), miR inhibitor negative control (NS_i), or miR-26a inhibitor (miR-26a_i) were subjected to tube-like network formation in matrigel (**A**; n=6 per group) or admixed in matrigel plugs (**B**) placed subcutaneously in nude mice (n=5 per group). CD31 staining was examined in matrigel plugs 1 week later. **P*<0.05 compared with NS_m; ***P*<0.05 compared with NS_i. Scale bars, 500 μm (**upper**), 25 μm (**lower**). EC migration (**C**) or aortic ring sprouting (**D**) was examined in response to transfection with NS_m, miR-26a_m, NS_i, or miR-26a_i. Transwell Boyden chambers were used for EC migration (**C**) with the indicated growth factors (n=6 per group). **P*<0.001 compared with NS_m; ***P*<0.001 compared with NS_i. **D**, Sprouting distance was measured from n=4 to 6 aortic rings per group. **P*<0.05 compared with NS_m; ***P*<0.05 compared with NS_i. Scale bar, 125 μm.

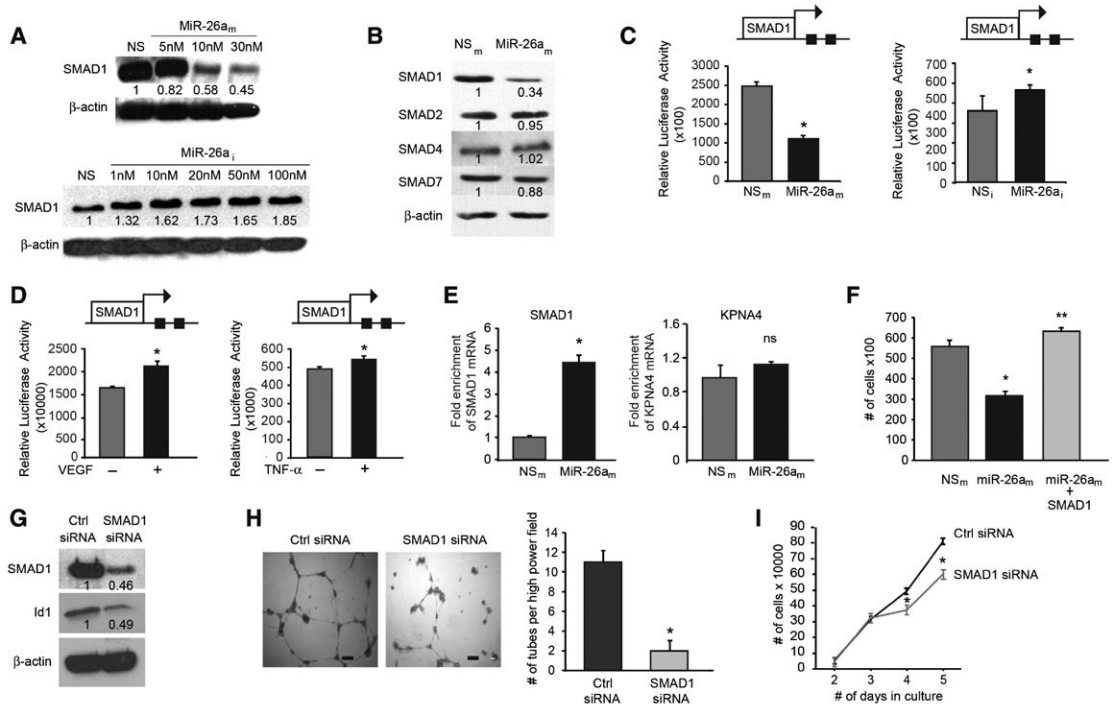

Figure 3. SMAD1 is a bona fide target of microRNA (miR)-26a in ECs

A, Protein expression of SMAD1 was examined by Western blotting after human umbilical vein endothelial cells (HUVECs) were transfected with miR negative control (NS_m), miR-26a mimics (miR-26a$_m$), miR inhibitor negative control (NS_i), or miR-26a inhibitor (miR-26a$_i$). **B**, Protein expression of SMAD family members in HUVECs was determined by Western blotting using antibodies to SMAD1, SMAD2, SMAD4, SMAD7, and β-actin (n=3–5 experiments). **C** and **D**, Luciferase activity of SMAD1-3′-untranslated region (UTR) normalized to β-galactosidase was quantified in HUVECs transfected with NS_m, miR-26a$_m$, NS_i, or miR-26a$_i$ or stimulated with vascular endothelial cell growth factor (VEGF) or tumor necrosis factor (TNF)-α for 6 hours (n=3 experiments). **E**, Microribonucleoprotein immunoprecipitation analysis of enrichment of SMAD1 mRNA in HUVECs transfected with NS_m or miR-26a$_m$. *P<0.01. Real-time quantitative polymerase chain reaction was performed to detect SMAD1 (**left**) or karyopherin alpha 4 (KPNA4) (**right**). **F**, HUVECs transfected with NS_m, miR-26a$_m$, or with miR-26a$_m$ in the absence or in the presence of lentiviral SMAD1 lacking its 3′-UTR were subjected to cell growth assays (n=4 replicates per condition). *P<0.005 compared with NS_m; **P<0.0005 compared with miR-26a$_m$. **G–I**, HUVECs were transfected with siRNA to SMAD1 or scrambled control (Ctrl) siRNA. **G**, Protein expression was determined by Western analysis using antibodies to SMAD1, Id1, and β-actin (n=2 experiments). **H**, Tube-like network formation was quantified in matrigel. *P<0.005 (n=6 replicates per condition). **I**, ECs were subjected to cell growth assays. *P<0.01. Scale bar, 100 μm. Results are representative of n=3 replicates per group and 2 independent experiments. All data represent mean±SEM.

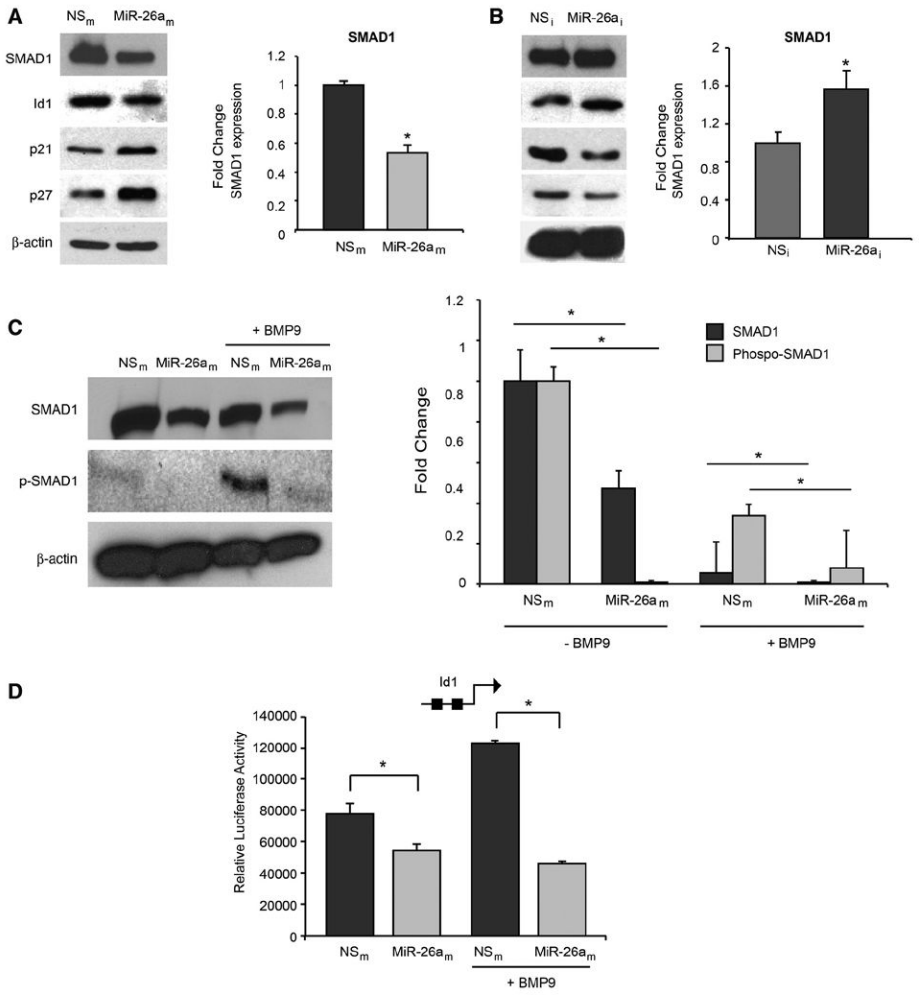

Figure 4. MicroRNA (miR)-26a regulates the expression of downstream bone morphogenic protein (BMP)/SMAD1 signaling in endothelial cells (ECs)

A, Human umbilical vein ECs (HUVECs) transfected with miR negative control (NS_m), miR-26a mimics (miR-26a_m), miR inhibitor negative control (NS_i), or miR-26a inhibitor (miR-26a_i) (**B**) were subjected to Western analysis using antibodies to Id1, p21, p27, SMAD1, and β-actin (n=3–5 experiments). **C**, HUVECs transfected with NS_m or miR-26a_m were treated in the presence or in the absence of BMP9 (0.1 ng/mL) for 2 hours and subjected to Western analysis using antibodies to SMAD1, phosphorylated-SMAD1 (p-SMAD1), and β-actin (n=2 experiments). **D**, HUVECs were cotransfected with the Id1 promoter along with NS_m or miR-26a_m in the presence or in the absence of BMP9 (0.1 ng/mL) and subjected to luciferase reporter assays (n=3 experiments). **P*<0.05 compared with NS_m; All data represent mean±SEM.

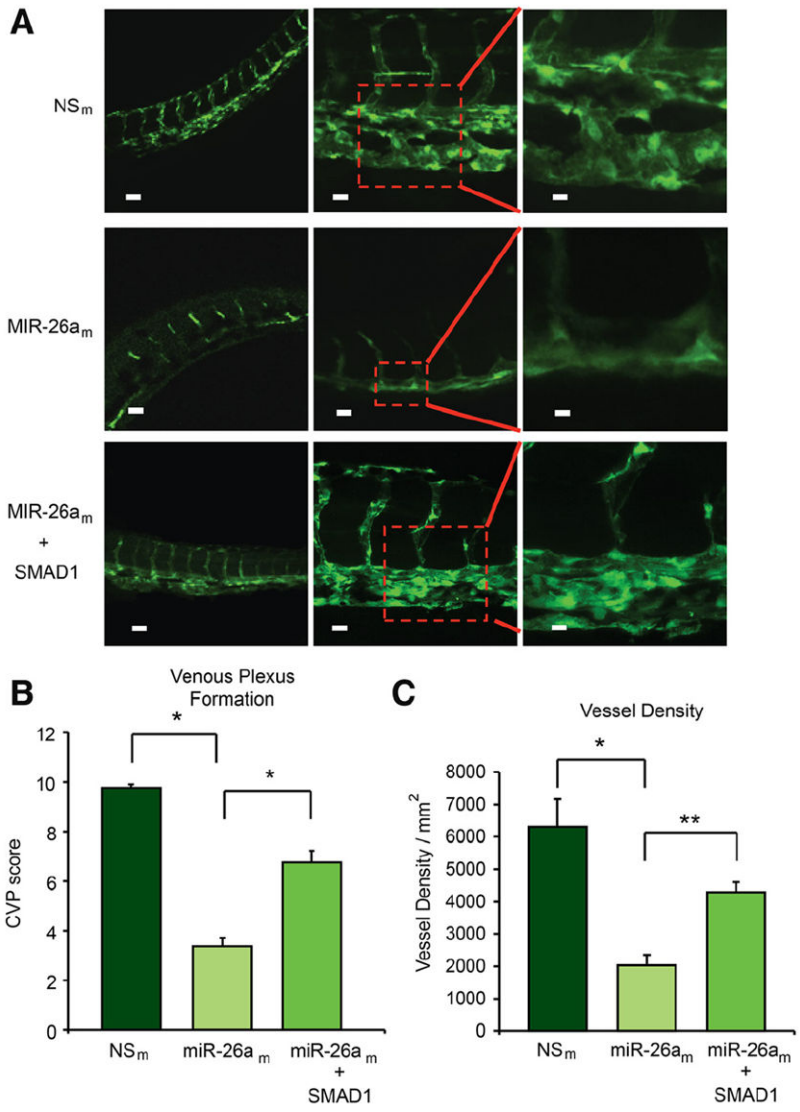

Figure 5. MicroRNA (miR)-26a regulates caudal vein plexus formation, a bone morphogenic protein (BMP)-responsive process, in zebrafish

Tg(Flk1:EGFP) zebrafish embryos were injected with miR negative control (NS_m) or miR-26a mimics (miR-$26a_m$) in the presence or in the absence of SMAD1. **A**, Vasculature of Tg(flk:EGFP) zebrafish embryos was imaged by immunofluorescence confocal microscopy. Inset highlights region of interest for caudal vein plexus. Scale bars, first panel 20 μm, second panel 10 μm, third panel 5 μm. **B**, The formation of the caudal vein plexus, a BMP-responsive region, was quantified 48 hours after fertilization on a scale of 1 to 10 (n=13 per group). * $P<0.001$ and (**C**) vessel density was quantified using ImageJ 1.41. *$P<0.005$ compared with NS_m; ** $P<0.001$ compared with miR-$26a_m$. All data represent mean ±SEM.

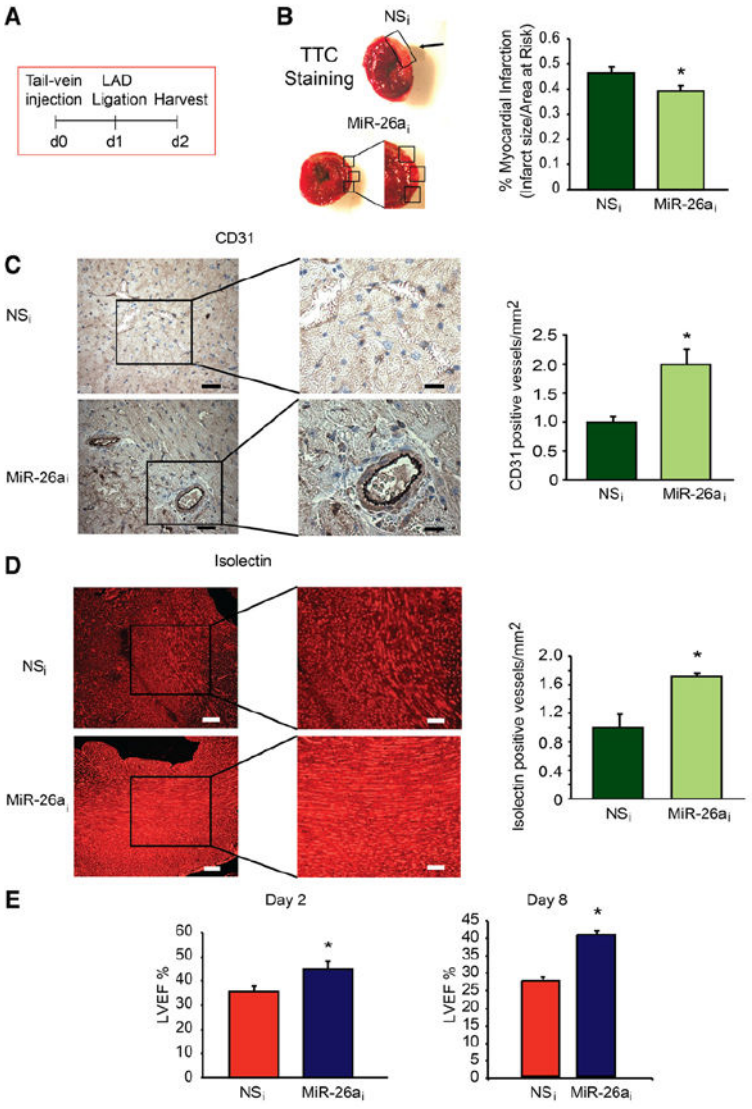

Figure 6. Inhibition of MicroRNA (miR)-26a increases angiogenesis, decreases infarct size, and improves left ventricular (LV) function in a mouse model of acute myocardial infarction (MI)

A, After a single tail-vein injection in mice of LNA-anti–miR-26a (miR-26a$_i$; 24 mg/kg) or scrambled nonspecific control LNA-anti-miRs (NS$_i$; n=11–12 per group) on day 0, mice underwent acute MI consisting of 45 minutes of ischemia and reperfusion of the left anterior descending artery (LAD) and infusion of fluorescent microbubbles on day 1. **B**, 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining (**top**) demonstrates areas of infarct in the left ventricle. MI size was normalized to the area at risk. **P* <0.05 compared with NS$_i$. Angiogenesis was quantified by CD31 (**C**) or isolectin staining (**D**) in sections from the entire LV on day 2. **P*<0.05 compared with NS$_i$. Scale bars, 500 μm in (**C**) and 100 μm in (**D**). **E**, LVEF (LVEF) was measured by echocardiography on days 2 and 8. **P*<0.05 compared with NS$_i$.

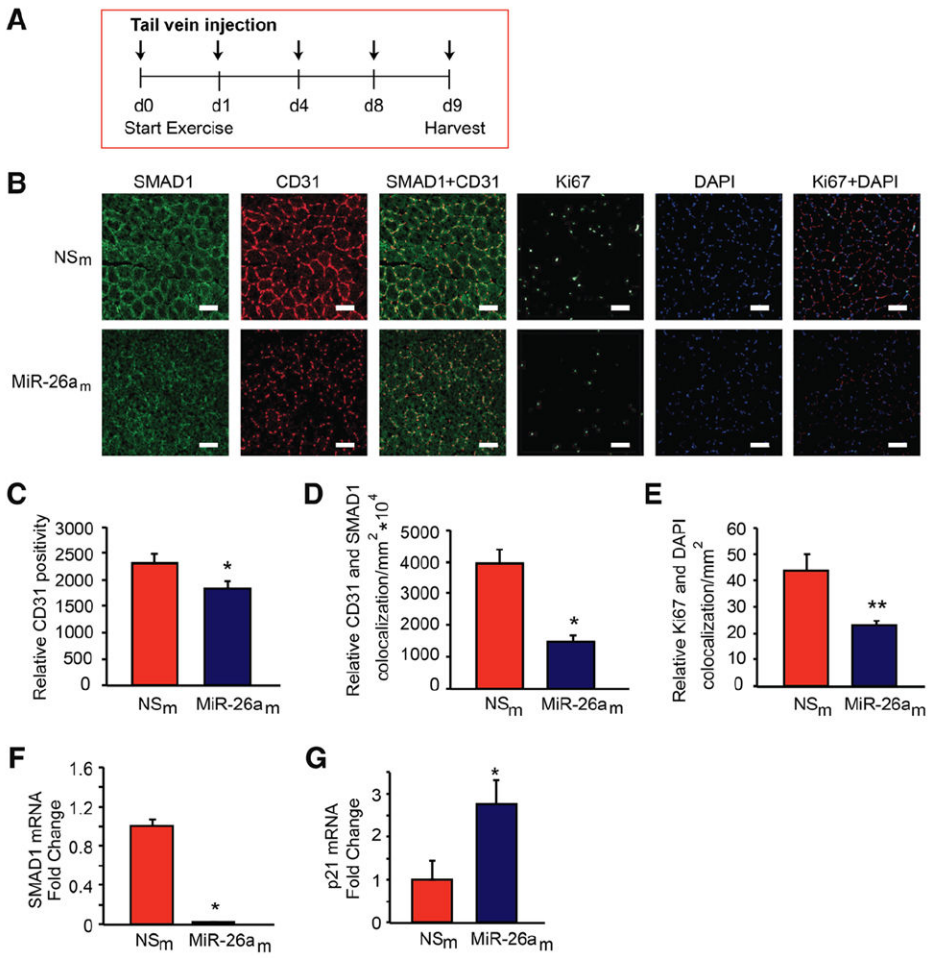

Figure 7. Overexpression of microRNA (miR)-26a inhibits exercise-induced angiogenesis in mice

A, Mice were tail-vein injected with nonspecific scrambled control (NS_m) or miR-$26a_m$ (1 nmol) as indicated for the course of 8 days of defined exercise (wheel-running). **B**, Representative immunofluorescent staining of CD31 (in red), SMAD1 (in green), Ki67 (in light green), and 4′,6-diamidino-2-phenylindole (DAPI) (in blue) of the quadricep muscles are shown. Scale bars, 50 μm. Quantification of the number of cells staining for CD31 (**C**), SMAD1 colocalized with CD31 (yellow; **D**), and Ki67 colocalized with DAPI (**E**) are shown. * P <0.05 compared with NS_m; **P<0.01 compared with NS_m. **F** and **G**, RNA from the quadriceps muscle was harvested for quantitating the expression of miR-26a (Online Figure VIB), SMAD1 (**F**), and p21 (**G**) by real-time quantitative polymerase chain reaction. *P<0.05 compared with NS_m. All data represent mean±SEM.