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

Basak Icli, A.K.M. Wara, Javid Moslehi, Xinghui Sun, Eva Plovie, Meghan Cahill, Julio F. Marchini, Andrew Schissler, Robert F. Padera, Jianru Shi, Hui-Wen Cheng, Srilatha Raghuram, Zoltan Arany, Ronglih Liao, Kevin Croce, Calum MacRae, Mark W. Feinberg

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 p21WAF1/CIP1 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.

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. (Circ Res. 2013;113:1231-1241.)

Key Words: 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 morphogenetic 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 p21WAF/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 3′-untranslated regions (3′-UTRs) of mRNA targets and have been found to regulate a variety of physiological and cellular functions in health 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-p21WAF/CIP/p27 signaling axis to promote an antiangiogenic program in ECs. Furthermore, neutralization of miR-26a rapidly induced an antiangiogenic 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 means±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 CTDSP1, 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 ischemia-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 IV A). 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...
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 p21WAF/CIP1 or p27 in ECs.

We first verified that overexpression of miR-26a decreased Id1 expression and increased p21WAF/CIP1 expression, whereas inhibition of miR-26a increased Id1 and decreased these cell cycle inhibitors in HUVECs (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. 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. 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. Conversely, activation of BMP signaling (by overexpression of an activated BMP type II receptor) induced ectopic sprouts along the axial vein. 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. 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 nonspecific control anti-miRs (NSi; 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

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**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 (NSm), miR-26a mimics (miR-26am), miR inhibitor negative control (NSi), or miR-26a inhibitor (miR-26ai) (B) were subjected to Western analysis using antibodies to Id1, p21, p27, SMAD1, and β-actin (n=3–5 experiments). C, HUVECs transfected with NSm or miR-26am 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 NSm or miR-26am 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 NSm; All data represent mean±SEM.
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 IXC). After systemic delivery of miR-26a or nonspecific miR mimic controls, miR-26a was overexpressed 10-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 p21WAF1/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 p21WAF1/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.2,3 MiR-26a overexpression 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.8,12 Id1, a known SMAD1 target gene, has been implicated in tumor-associated angiogenesis. Id1 knockout mice exhibited reduced angiogenesis and increased p21WAF1/CIP1 expression in several cell types.15 In addition, genetic ablation of p21WAF1/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.17 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 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.
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. Interestingly, inhibition of miR-26a in osteoblasts increased bone marker genes and promoted osteoblast differentiation. In contrast, inhibition of miR-26a decreased skeletal muscle myoblast differentiation. Intriguingly, miR-26a inhibition in vascular smooth muscle cells in vitro 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), 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. In support of this concept, miR-26a expression in liver cancer cells induces cell cycle arrest through direct targeting of cyclins D2 and E2. 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 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...

**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-26ai; 24 mg/kg) or scrambled nonspecific control LNA-anti-miRs (NSi; 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 NSi. Angiogenesis was quantified by CD31 (C) or isolectin staining (D) in sections from the entire LV on day 2. *P<0.05 compared with NSi. Scale bars, 500 μm in (C) and 100 μm in (D). E, LVEF (%) was measured by echocardiography on days 2 and 8. *P<0.05 compared with NSi.
a BAK1 pathway; 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. 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. 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. 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. 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. 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. 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. 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 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.

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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.

References
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Novelty and Significance

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SUPPLEMENTAL MATERIAL

MicroRNA-26a regulates pathological and physiological angiogenesis by targeting BMP/SMAD1 signaling

Basak Icli, MS, PhD¹, Javid Moslehi, MD¹, AKM Wara, PhD¹, Xinghui Sun, MD, PhD¹, Eva Povie, PhD¹, Meghan Cahill, BS¹, Julio F. Marchini, MD¹, Andrew Schissler, MD¹, Robert F. Padera, MD, PhD², Jianru Shi, PhD¹, Hui-Wen Cheng, MD¹, Srilatha Raghuram, MS, PhD³, Zoltan Arany, MD, PhD³, Ronglih Liao, PhD¹, Kevin Croce, MD, PhD¹, Calum MacRae, MD¹, and Mark W. Feinberg, MD*¹

¹Cardiovascular Division, Department of Medicine, and ²Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115. ³Cardiovascular Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115.

*Corresponding author: Dr. Mark W. Feinberg, Department of Medicine, Cardiovascular Division, Brigham and Women’s Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, NRB-742F, Boston, MA 02115. Email: mfeinberg@rics.bwh.harvard.edu, Tel: (617) 525-4381, Fax: (617) 525-4380
Detailed Methods

Cell Culture, transfection, and lentiviral transduction

Human umbilical vein endothelial cells (HUVECs) passaged less than five times were used for all experiments. For transfection studies, HUVECs were cultured overnight before being transfected with Lipofectamine™ 2000 transfection reagent. HUVECs were transduced with lentivirus expressing GFP or SMAD1 at 10 MOI. For reporter studies, HUVECs were transfected with 800 ng of the indicated reporter constructs and 200 ng β-galactosidase (β-gal) expression plasmids. Each reading of luciferase activity was normalized to the β-gal activity read for the same lysate. Human umbilical vein endothelial cells (HUVECs) (cc-2159; Lonza, Walkersville, MD) were cultured in growth media EGM®-2 (cc-3162; Lonza Walkersville, MD). VEGF-A, TNF-α, and BMP-2 were obtained from R&D Systems. HUVECs were treated with 10 ng/ml TNF-α for 24 h, collected into TRizol, and total RNAs were prepared for miRNA microarray analysis (LC Sciences). For transfection studies, HUVECs were plated at 50,000 cells/well and cultured overnight before being transfected with Lipofectamine™ 2000 transfection reagent (Invitrogen), following manufacturer’s instructions. MiRNA negative controls, miR-26a mimics, and miR-26a inhibitor (AM17100; Ambion, Inc.) were transfected in HUVECs at 30 nM concentration except where indicated. MiR-26a inhibitor and negative control was transfected at 100 nM concentration. Cy™3 labeled-negative control #1 (AM17120; Ambion) was transfected in parallel to assess transfection efficiency which was >90%. HUVECs were transduced with lentivirus expressing GFP(OHS5899-101186481; ThermoScientific) or SMAD1 (PLOHS_100005140; ThermoScientific) at 10 MOI. For reporter studies, HUVECs were plated (50,000/well) in triplicate on a 12-well plate, grown to 70-80% confluency, and transfected with 800 ng of the indicated reporter constructs and 200 ng β-galactosidase (β-gal) expression plasmids. MiR-26a mimics or inhibitors were co-transfected at 30 or 100 nM final concentration where indicated. Transfected cells were collected in 200 µl Reporter Lysis Buffer (Promega). Each reading of luciferase activity was normalized to the β-gal activity read for the same lysate.

Real-time qPCR

HUVECs were suspended in TRIzol® reagent (Invitrogen) and total RNA and microRNA was isolated using Trizol® reagent (Invitrogen) per manufacturers instructions. Reverse transcriptions were performed by using miScript Reverse Transcription Kit from Qiagen (218061). Either QuantiTect SYBR Green RT-PCR Kit (204243) or miScript SYBR Green PCR Kit (218073) from Qiagen was used for quantitative real-time qPCR analysis with the Mx3000P Real-time PCR system (Stratagene) following the manufacturer’s instructions. Gene-specific primers were used to detect mouse SMAD1 (forward primer: TTCCGCAACCTGGGACAAAAT; reverse primer: AGGCTGGAACAACCATGCC) and p21 (forward primer: CCTGGTGATGTCCGACCTG; reverse primer: CCATGAGCGCATCGCAATC). To amplify mature miRNA sequences, miScript primer assays for Hs_RN5S1_1 (MS00007574), Hs_miR-26a_1 (MS00006559), and Hs_miR26b_1 (MS00003234) from Qiagen were used. Samples were normalized to endogenous 5S RNA (human). Fold changes were calculated by △△Ct method.
Tube-like network formation on Matrigel in vitro and in vivo

Matrigel (BD Bioscience) basement membrane matrix was added to 96-well culture plates and incubated at 37°C until gelation occurred as described. HUVECs transfected with miR-26a mimic, miR-26a inhibitor, and non-specific negative controls were cultured for 72 hours before being plated on Matrigel at 20,000 cells/well. Network tube formation was assessed 14 hours post-plating and quantitated by counting the number of tubes formed per high power field as described. Six technical replicated were used per condition. For in-vivo angiogenesis assay, HUVECs transfected with miR-26a mimic, miR-26a inhibitor, or non-specific negative controls were cultured for 72 hours before admixed with Matrigel (1x10⁶ cells/ml), bFGF (250 ng per mL, R&D Systems), and heparin (60 units per mL; Hospira, Inc.). Matrigel plugs admixed with transfected HUVECs were implanted subcutaneously into nude mice and collected 7 days post implantation. Angiogenesis in matrigel plugs was analyzed using human CD31 Ab staining of the paraffin embedded matrigel sections.

Aortic Ring Assay
The aortic ring assay was performed in an analogous manner as described. Briefly, aortas were harvested from 8-10 weeks old, C57BL/6 mice (Charles River), cut into 1 mm pieces, and placed in 12-well plates with 1ml medium. Aortas were then transfected with miR-26a mimic, miR-26a inhibitor, or non-specific miR negative control using Lipofectamine™ 2000 transfection reagent for 12 hrs followed by embedding into matrigel (BD354006). Aortic sprouting was observed 4-7 days post embedding into matrigel. Sprouting distance was measured using Image J software (NIH).

Chemotaxis assays
Migration assay was performed using ChemTX multiwell system (Neuro probe Inc, MD) with 5 mm pore size and 96-well format as described. HUVECs transfected with miR-26a mimic, miR-26a inhibitor, or non-specific negative controls were cultured for 72 hours before being plated on the upper compartment of the multiwell plate to assess migration. Lower compartments were filled with EBM-2 medium containing TNF-α, BMP-2 or VEGF (R&D Systems). The number of cells migrating to the lower chamber was counted using a hemocytometer after 5 hours. Three technical replicated were used per condition and studies were validated in at least 2 independent experiments.

Western Blots Analysis
HUVECs transfected with miR-26a mimic, miR-26a inhibitor, or non-specific negative controls were cultured for 72 hours. Total cellular protein was isolated by RIPA buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche). Cell or tissue debris was removed by centrifugation at 12000rpm for 10 min. Lysates were separated by 8% or 10% SDS-PAGE gels, transferred to PVDF membranes (Bio-Rad). Protein quantification was performed using the BCA kit (Thermo Scientific) and cellular lysates were subjected to Western blotting using antibodies against SMAD1 (Cell Signaling), SMAD2 (Abcam), SMAD4 (Cell Signaling), SMAD7 (Abcam), Id1 (Abcam), p21(Cel Signaling), p27 (Cell
Signaling), cyclin D2 (Cell Signaling), cyclin E2 (Cell Signaling), and β-actin (Cell Signaling). HRP-conjugated goat anti-rabbit or mouse antibody (Santacruz) was used at 1:5000 dilution. ECL assay was performed per manufacturers instructions (RPN2132; GE Healthcare).

**BrdU Assay**
HUVECs transfected with miR-26a mimic, miR-26a inhibitor, or non-specific negative controls were cultured for 5 days. Cell proliferation was measured using the BrdU ELISA assay as described by the manufacturer (Roche).

**Argonaute2 (AGO2) micro-ribonucleoprotein IP (miRNP-IP) studies**
MiRNP-IP was performed as we previously described. Briefly, Myc-tagged Ago-2 (a kind gift from G. Hannon, Cold Spring Harbor, NY) was co-transfected with either miR-26a or miRNA negative control in HUVECs. Cells were washed in ice cold PBS, released by scraping, and lysed in buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 100 units/ml of RNasin Plus (Promega) supplemented with 1x protease inhibitor (Roche). The lysed cell solution was adjusted to a final NaCl concentration of 150 mM prior to centrifugation. One-twentieth of the supernatant volume was collected in TRIzol for use as an extract control. The remaining portion of the supernatant was pre-cleared with Protein A/G UltraLink Resin (Pierce), to which 2µg anti-c-myc antibody was added and the mixture allowed to incubate overnight at 4°C; the following day Protein A/G UltraLink Resin was added. After 4h of mechanical rotation at 4°C, the agarose beads were pelleted and washed four times in wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Triton X-100). Finally, 1 ml of TRIzol was added into the beads and RNA was isolated. Total RNA was reverse transcribed into cDNA for real-time qPCR analysis.

**ELISA**
After acute myocardial infarction, plasma from mice injected with LNA-anti-miR-26a or NS ctrl were collected after 24 hrs for ELISA analysis by means of SearchLight Multiplex Immunoassay Kit (Aushon BioSystems, Inc) as we previously described.

**In vivo miR-26a inhibition or over-expression and mouse experiments**
Animal protocols were approved by the Laboratory Animal Care at Harvard Medical School. For acute myocardial infarction, male, 8-10 weeks old, C57BL/6 mice (Charles River) were used to tail-vein inject either scrambled control LNA-anti-miR or LNA-anti-miR-26a (Exiqon, Inc) at 24 mg/kg. Twenty-four hrs later, mice underwent 45 minutes of ischemia and 5 min into ischemia, 50 µl of fluorescent microspheres (10-µm FluoSpheres, Molecular Probes, Inc (F8834) were injected into the LV cavity. After 45 min, the LAD ligature was released, and reperfusion visually confirmed. To delineate the size of infarct, the heart was sliced into 2-mm sections. Each slice was used to quantify the area at risk (AAR) and the infarct area. To delineate the infarct, sections were incubated in 1% (wt/vol) triphenyltetrazolium chloride (TTC, Sigma) in PBS (pH 7.4) at 37°C for 20 minutes. For each section, the AAR and infarct area were measured from enlarged digital micrographs with NIH Image (ImageJ). Percent myocardial infarction (%MI) was calculated as the total infarction area divided by the total AAR for that heart.
as previously reported.\(^9\) Angiogenesis in heart was analyzed by mouse CD31 staining and isolectin B4 (B-1205; Vector Laboratories, Inc) of the paraffin embedded heart sections. Myocardial apoptosis and necrosis was quantified using Annexin V (LS Bio C171652) and C5B9 antibodies (Abcam 55811). Smooth muscle α- actin (SMA) expression in the heart was detected using SMA antibodies (Abcam 7817). Fluorescent images were acquired by Olympus Fluoview FV1000 confocal microscope. For exercise studies, C57BL/6, 8-10 weeks old mice were tail vein injected with a mixture of lipofectamine™ 2000 and miR-26a mimics or non-specific negative control mimics at the indicated time points as we previously described.\(^7\) Mice underwent nocturnal running on wheels in a single cage over a period of nine days. The quadriceps muscles were harvested for confocal immunofluorescent staining for Abs specific for SMAD1(Cell Signaling), CD31(DIA-310; Dianova, Inc), Ki67(RM-9106-S; ThermoScientific, Inc), and DAPI (H21492; Invitrogen, Inc).

**Left ventricular function by echocardiography**

Echocardiography was performed on conscious mice using a 28 MHz linear array transducer connected to a digital ultrasound console (Vevo2100 Visualsonics, Toronto, ON). All echocardiograms were performed at baseline (1 day before initial surgery), 1 or 7 days following surgery. For measurements of LV function and geometry, M-mode images were recorded from parasternal short axis images at the mid-papillary level. Fractional shortening, ejection fraction, and LV dimensions were measured from M-mode images. LV mass was calculated from parasternal long axis tracings using a Modified Simpson’s formula.

**Zebrafish studies**

Wild-type and flk:EGFP Tg (Flk1:EGFP) zebrafish embryos were maintained using standard methods. Embryos were staged according to morphological criteria (somite number) or by hours post-fertilization. For miR-26a overexpression studies, mimics were suspended in sterile water to a concentration of 1nM and diluted to 0.01 nM with 1×Danieau’s solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO\(_4\), 0.6 mM Ca(NO\(_3\))\(_2\), 0.5 mM Hepes, pH 7.6]. The mimics were injected at the single-cell stage in a volume of 1-2 nl. An equal amount of scrambled non-specific mimic was used as a negative control. For SMAD1 overexpression studies, 1nl of the 100 ng/µl \textit{in vitro} transcribed capped mRNA stock was used.

**Circulating miR-26a levels in patients with acute coronary syndromes**

Patient plasma samples (21 healthy controls and 14 individuals with acute coronary syndrome) were collected as part of a prospectively enrolled cohort of patients that underwent cardiac catheterization in accordance with the Institutional Review Board-approved protocol at Brigham and Women’s Hospital. Written informed consent was obtained from all participants or their appropriate surrogates. Plasma was isolated from whole blood. Control patients were defined as without clinically significant coronary atherosclerosis (<20% stenosis in any epicardial coronary artery determined by angiography) and had no elevation of cardiac biomarkers. Patients with acute coronary syndromes (ACS) were defined as acute atherothrombotic coronary artery occlusion resulting in either an NSTEMI (with >70% occlusion of an epicardial artery) or an STEMI
(complete occlusion of an epicardial coronary artery determined by angiography) with elevation of cardiac biomarkers. Anonymized plasma samples were generated from blood collected in EDTA-containing tubes at the time of the procedure and stored at −80°C. Plasma was isolated from whole blood at 1500g for 15 minutes at room temperature. Total RNA was isolated from plasma by using total RNA purification kit from Norgen Biotek Corporation and reverse transcription and real time qPCR was performed as described in the Section of “Real-time qPCR”.
REFERENCES (for Detailed Methods)


Online Figure I. Expression of miR-26a, miR-26b, and CTDSP2 in response to pro-angiogenic stimuli. Real-time qPCR was performed from RNA extracted from HUVECs treated in the presence or absence of TNF-α (10 ng/mL, 24 hrs) or VEGF-A (50 ng/mL, 6 hrs). TNF-α reduced miR-26a (A) and miR-26b (B) expression and VEGF reduced miR-26a (C) and CTDSP2 (D) expression. * P < 0.001 compared to Ctrl. All data represent means ± s.e.m.
Online Figure II. Expression of miR-26a after acute MI in the heart. Mice underwent acute myocardial infarction consisting of 45 minutes of ischemia and reperfusion of the left anterior descending artery (LAD). Heart tissues were harvested at 1, 6 and 24 hrs later for miR-26a expression by qPCR. P < 0.05 compared to Sham.
Online Figure III. Effect of miR-26a overexpression or inhibition on EC proliferation or apoptosis. HUVECs transfected with miR-26a mimic or inhibitor were cultured for 24 hrs and harvested for (A) cell proliferation by BrdU, * P < 0.05; or (B) caspase-3 or (C) Annexin V levels by FACS (n=3 experiments). NS, non-significant. All data represent means ± s.e.m.
Online Figure IV. Mir-26a targets SMAD1 and not cyclins D2 and E2 in ECs. (A) HUVECs were transfected with SMAD1-3’-UTR-MUT containing 2 mutated SMAD1 binding sites and stimulated with TNF-α for 24 hrs. Luciferase activity of the SMAD1-3’-UTR-MUT normalized to β-gal is shown (n = 3 experiments). NS, non-significant. (B-C) Quantification of Western analyses of Id1, p21 and p27 (normalized to β-actin) from HUVECs transfected with (B) miR negative control (NSm) or miR-26a mimics (miR-26am) or (C) miR inhibitor negative control (NSi), or miR-26a inhibitor (miR-26ai) are shown above (n = 3 experiments). *P < 0.05 compared to NSm or NSi with 2 tailed t-test, #P < 0.05 compared to NSm or NSi with 1 tailed t-test. All data represent means ± s.e.m. (D) HUVECs were transfected with miR-26am in parallel with the non-specific (NSm) scrambled control. Protein expression in HUVECs was determined by Western blotting using antibodies to cyclin D2, cyclin E2, and β-actin (n = 3 experiments).
Online Figure V, Overexpression of miR-26a in zebrafish embryos. Zebrafish Tg(flk:eGFP) embryos injected with miR-26a_m or scrambled control were harvested 48 hrs post-fertilization for RNA extraction and miR-26a overexpression was verified by RT-qPCR (n=10 embryos). * P < 0.01. Data represents mean ± s.e.m.
Online Figure VI: Delivery of LNA-miR-26a reduced miR-26a expression in vivo

(A) MiR-26a (blue) was detected in sham heart by in-situ hybridization. (B) Cardiac myocytes and non-myocytes were harvested from hearts (n=4-6) and miR-26a expression levels were detected by RT-qPCR. * P < 0.05. (C) After a single tail vein injection in mice of LNA-anti-miR-26a (MIR-26a) (24mg/kg) or scrambled non-specific control LNA-antimiRs (NSi) (n = 11-12 per group) on day 0, mice underwent acute myocardial infarction consisting of 45 minutes of ischemia and reperfusion of the left anterior descending artery (LAD) on day 1 followed by measurement of miR-26a expression by RT-qPCR in heart (left) and circulating plasma (right) and in cardiac myocytes and non-cardiac myocytes (D). * P < 0.05. (E) SMAD1 expression was examined by Western analysis in response to miR-26a inhibition from heart extracts where SMAD1 expression increased by 42% (n=3 mice/group). (F-H) Annexin V, C5B9, and SM-a-actin (SMA) staining was performed and quantified in the entire left ventricle on day 2. * P < 0.05. NS, non-significant. Scale bars, 50μm (F-H). All data represent means ± s.e.m.
Online Figure VII. Expression of inflammatory cytokines following miR-26a inhibiton in acute MI. Plasma samples were collected on day 2 from mice injected with NS or miR-26a inhibitors and underwent acute myocardial infarction (MI). Expression for ICAM-1 (A), VCAM-1 (B), TNF-α (C) and E-selectin (D) were measured by ELISA. NS, non-significant. All data represent means ± s.e.m.
Online Figure VIII. Effect of miR-26a inhibition on angiogenesis. After a single tail vein injection in mice of LNA-anti-miR-26a (MiR-26a) (24mg/kg) or scrambled non-specific control LNA-antimiRs (NS) (n = 3 per group) on day 0, mice underwent acute myocardial infarction consisting of 45 minutes of ischemia and reperfusion of the left anterior descending artery (LAD) on day 1. Angiogenesis was quantified by CD31 (A) or isolectin staining (B) in sections from the entire left ventricle on day 8. Scale bars, 100μm (A) and (B). NS, non-significant. All data represent mean ± s.e.m.
Online Figure IX. Overexpression of miR-26a in quadriceps muscles. (A) Representative immunofluorescent staining of CD31 (green) in the quadriceps muscles of exercised mice. The number of cells staining for CD31 were quantified in sedentary and exercised mice. * P < 0.05. (B) Expression of miR-26a in response to the systemic delivery of non-specific scrambled control or miR-26a mimics (1 n mole) was verified by RT-qPCR analysis in the quadriceps muscle on day 9. * P < 0.05. Scale bar, 100μm. (C) Representative immunofluorescent staining of CD31 (green) and isolectin (red) in the quadriceps muscles of exercised mice. Scale bar, 20μm.
Online Table I

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Online Table I. Inhibition of miR-26a improves left ventricular function 24 hours post acute MI. After a single tail vein injection in mice of LNA-anti-miR-26a (MiR-26a) (24mg/kg) or scrambled non-specific control LNA-antimiRs (NS_i) (n = 6-8 per group) on day 0, Mice underwent acute myocardial infarction consisting of 45 minutes of ischemia and reperfusion of the left anterior descending artery (LAD). Echocardiography was performed on day 2. * P < 0.05 compared to NS_i. All data represent mean ± s.e.m.

Online Table II

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Online Table II. Inhibition of miR-26a improves left ventricular function 8 days post acute MI. After a single tail vein injection in mice of LNA-anti-miR-26a (MiR-26a) (24mg/kg) or scrambled non-specific control LNA-antimiRs (NS_i) (n = 6-8 per group) on day 0, Mice underwent acute myocardial infarction consisting of 45 minutes of ischemia and reperfusion of the left anterior descending artery (LAD). Echocardiography was performed on day 7. * P < 0.05 by 2 tailed t-test compared to NS_i. #P < 0.05 by 1 tailed t-test compared to NS_i. All data represent means ± s.e.m.