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ORIGINAL RESEARCH

microRNA-29a Regulates ADAM12 Through Direct Interaction With ADAM12 mRNA and Modulates Postischemic Perfusion Recovery

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BACKGROUND: Peripheral artery disease is caused by atherosclerotic occlusion of vessels outside the heart and most commonly affects vessels of the lower extremities. Angiogenesis is a part of the postischemic adaptation involved in restoring blood flow in peripheral artery disease. Previously, in a murine hind limb ischemia model of peripheral artery disease, we identified ADAM12 (a disintegrin and metalloproteinase gene 12) as a key genetic modifier of postischemic perfusion recovery. However, less is known about ADAM12 regulation in ischemia. MicroRNAs are a class of small, noncoding, single-stranded RNAs that regulate gene expression primarily through transcriptional repression of messenger RNA (mRNA). We showed microRNA-29a (miR-29a) modulates ADAM12 expression in the setting of diabetes and ischemia. However, how miR-29a modulates ADAM12 is not known. Moreover, the physiological effects of miR-29a modulation in a nondiabetic setting is not known.

METHODS AND RESULTS: We overexpressed or inhibited miR-29a in ischemic mouse gastrocnemius and tibialis anterior muscles, and quantified the effect on perfusion recovery, ADAM12 expression, angiogenesis, and skeletal muscle regeneration. In addition, using RNA immunoprecipitation–based anti-miR competitive assay, we investigated the interaction of miR-29a and ADAM12 mRNA in mouse microvascular endothelial cell, skeletal muscle, and human endothelial cell lysates. Ectopic expression of miR-29a in ischemic mouse hind limbs decreased ADAM12 mRNA expression, increased skeletal muscle injury, decreased skeletal muscle function, and decreased angiogenesis and perfusion recovery, with no effect on skeletal muscle regeneration and myofiber cross-sectional area following hind limb ischemia. RNA immunoprecipitation–based anti-miR competitive assay studies showed miR-29a antagomir displaced miR-29a and ADAM12 mRNA from the AGO-2 (Argonaut-2) complex in a dose dependent manner.

CONCLUSIONS: Taken together, the data show miR-29a suppresses ADAM12 expression by directly binding to its mRNA, resulting in impaired skeletal muscle function, angiogenesis, and poor perfusion. Hence, elevated levels of miR-29a, as seen in diabetes and aging, likely contribute to vascular pathology, and modulation of miR-29a could be a therapeutic target.

Key Words: ADAM12 ■ angiogenesis ■ hind limb ischemia ■ miR-29a and peripheral artery disease

Peripheral artery disease (PAD), sometimes called peripheral arterial disease, is a common manifestation of atherosclerosis of the lower extremities that is associated with a high risk of morbidity and mortality.[1,2]

Despite the magnitude of PAD in influencing poor quality of life, the disease is often underrecognized in clinical practice until its limb manifestations are severe.[3,4] This makes it difficult to estimate PAD's true prevalence. However, it has been estimated globally

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CLINICAL PERSPECTIVE

What Is New?

- Peripheral artery disease is the result of arteriosclerotic blockage of blood vessels of the lower extremities, and the extent of the severity of the disease varies among people with similar occlusions; this suggests that the individual genetics is mediating the severity of the disease.
- Previously, we identified a gene called *ADAM12* that improves recovery of blood flow after a vessel is blocked and microRNA-29a as a microRNA that regulates ADAM12 (a disintegrin and metalloproteinase gene 12) expression.
- In this study, we assessed whether miR-29a regulation of ADAM12 occurs through direct interaction with ADAM12 messenger RNA or indirectly through secondary effects on the transcriptome. Moreover, we studied the physiologic effects of miR-29a modulation on postischemic adaptation.

What Are the Clinical Implications?

- The mechanism through which miR-29a regulate ADAM12 is not fully understood.

Nonstandard Abbreviations and Acronyms

ADAM12	a disintegrin and metalloproteinase gene 12
AGO-2	Argonaut-2
HLI	hind limb ischemia
TA	tibialis anterior

that ≈7% of men and ≈2% of women aged over 50 years have intermittent claudication.[5]

Using an experimental PAD or hind limb ischemia (HLI) model in mice, we previously identified a genetic locus on mouse chromosome 7 termed Limb Salvage QTL-1 (Lsq-1) in C57BL/6 mice that is associated with reduced ischemic limb injury.[6] Within LSq-1 we identified *ADAM12* as a gene that improves postischemic perfusion recovery through regulating Tie2 activation resulting in endothelial cell proliferation, survival, and angiogenesis.[6,7] Less is known about the regulation of ADAM12 (a disintegrin and metalloproteinase gene 12) in ischemia. We previously showed that a microRNA, microRNA-29a (miR-29a), is involved in posttranscriptional regulation of ADAM12[6,7]; however, whether this occurs through direct interaction with ADAM12 messenger RNA (mRNA) or through an indirect mechanism is not known. Additionally, in experimental PAD, miR-29a expression is downregulated in the ischemic hind limb muscles of nondiabetic mice, suggesting that its downregulation might play a key role in postischemic adaptation.[7,8] However, whether downregulation of miR-29a is required for normal postischemic recovery in experimental PAD is not known. A better understanding of how miR-29a regulates ADAM12 and the physiologic role of miR-29a in postischemic recovery in experimental PAD under nondiabetic conditions will provide useful insight on how ADAM12 and miR-29a could be manipulated to improve PAD outcomes.

In this study, we investigated the functional outcomes of ectopic overexpression and inhibition of miR-29a in ischemic hind limbs of nondiabetic C57BL/6 mice and whether miR-29a regulates ADAM12 through direct interaction with ADAM12 mRNA.

METHODS

Data and Materials Disclosure

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

Animals

All experiments were performed under protocols approved by the University of Iowa Institutional Animal Care and Use Committee. Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, MA; cat. number: 000664) aged between 16 and 20 weeks were used. Mice were fed normal chow diet (Envigo; cat. number: 7913) and used in 4 groups (control+empty vector [EV], miR-29a overexpression, control+saline, and miR-29a inhibitor treated).

In Vivo miR-29a Overexpression

A plasmid with miR-29a expression driven by the cytomegalo virus promoter was purchased from Origene (cat. number SC400963), and was commercially packaged into adeno-associated virus 9 (AAV9) to produce AAV9-miR-29a viral particles by Vigene Biosciences (Rockville, MD). The control vector AAV9-cytomegalo virus-EV-GFP viral particles (cat. number: CV10009) was purchased from Vigene Biosciences. Mice were randomized into 2 groups that received AAV9-miR-29a viral particles (miR-29a) or control AAV9-EV viral particles (cytomegalo virus) injected into both gastrocnemius and tibialis anterior (TA) muscle at day 7 before surgery.

In Vivo miR-29a Inhibition

The miR-29a inhibitor was purchased from ThermoFisher (cat. number: 4464084). The miR-29a inhibitor was mixed with specially formulated lipid

nanoparticles that would disperse and release their nucleic acid load once the target site was treated with ultrasound.[9,10] The lipid nanoparticle/nucleic acid complex was delivered 5 days and 1 day before surgery by intraocular injection followed by an ultrasound-triggered release in the hind limb as previously described.[9,10]

Experimental PAD or HLI Surgery and Perfusion Imaging

Experimentally induced HLI surgery was performed as described previously.[6,7,9,10] Briefly, mice were anesthetized by intraperitoneal injection of a mix of xylazine (5 mg/kg) and ketamine (100 mg/kg), followed by ligation and excision of the femoral artery of the left hind limb. Mice temperatures were kept warm at 37 °C throughout the procedure. The unoperated right hind limb served as nonischemic control, and peripheral blood flow was measured by Laser Speckle Contrast Imaging using PeriCam PSI (Perimed). Data were obtained immediately following surgery (day 0) as well as at day 3, day 7, and day 14 after surgery. The relative changes of blood flow in the hind limbs were expressed as the ratio of the operated to the contralateral hind limb blood flow using the manufacturer's software.

Muscle Contractile Function

Maximal isometric torque of ankle dorsiflexors, of which the TA muscle is the major agonist, was assessed in control and ischemic limbs using the 1300A 3-in-1 Whole Animal System (Aurora Scientific, Aurora, ON, Canada) as previously described.[8,11] Briefly, mice were anesthetized with 3% isoflurane via a nose cone throughout the measurement process. The tibia was stabilized at the knee, and the foot was immobilized with adhesive tape to a footplate attached to a force transducer. Resting tension and muscle length were iteratively adjusted for each muscle to obtain the optimal twitch contraction force. The ankle dorsiflexors were stimulated by subcutaneous electrodes via the fibular nerve. Proper electrode position was determined by a series of isometric twitches. After a 5-minute equilibration period, maximal isometric tension was evaluated with stimulations of 150 Hz for 300 ms. Data were collected and analyzed to determine muscle torque using the Dynamic Muscle Analysis software (ASI 611A v.5.321; Aurora Scientific).

RNA and TaqMan Quantitative PCR

Total RNA was isolated from cell and tissue lysates using miRNeasy mini kit (Qiagen; cat. number: 2172004), following the manufacturer's instructions and quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). ADAM12 and ADAM17 transcript expression was detected by quantitative real-time–polymerase chain reaction using TaqMan probes and assays (cat. number: 4331182, Adam12 ID: Mm0047519_m1, Adam17 ID: Mm00456428_m1; Applied Biosystems, Foster City, CA), as described previously.[6,7,9] Equal amounts of RNA samples were reverse transcribed using high-capacity RNA to complementary DNA (cDNA) synthesis kit (Applied Biosystems; cat. number: 4388950), and 10 ng of cDNA input was used for quantitative polymerase chase reaction assay as previously described.[6,7,9] Relative change in expression was determined by ΔΔCt method using *Gapdh* (ThermoFisher; cat. number: Mm4351370_g1) expression as the reference gene.

To quantify miR-29a, miR-696, and miR-10a, we used a TaqMan microRNA assay. This method uses the stem-loop quantitative polymerase chase reaction approach for the analysis of mature microRNA. It uses stem-loop Reverse Transcription (RT) primer that binds to a mature microRNA by using microRNA specific RT kit (TaqMan MicroRNA Reverse Transcription Kit, Cat # 4366597, ThermoFisher). The resulting cDNA was polymerase chain reaction amplified with the microRNA-specific forward primer, universal reverse primer, and microRNA-specific TaqMan probe (cat. number: 4427975, assay ID; miR-29a: 002112, miR-696: 001628, miR-10a: 000387; ThermoFisher) according to the manufacturer's recommendations and as previously described.[6,7,9] Relative change in miR-29a and miR696 expression was determined by ΔΔCt method,[6,7,9] using expression of miR-10a as the reference microRNA.[8] miR-10a expression has been previously shown not to change between ischemic and nonischemic TA and gastrocnemius muscle samples.[8] The final fold expression changes were calculated using the equation $2^{-\Delta\Delta Ct}$.

Western Blot Analyses

Total protein from the gastrocnemius muscle was isolated using Radioimmunoprecipitation Assay Lysis and Extraction Buffer (ThermoFisher; cat. number: 89900) according to the manufacturers protocol. Homogenates were separated on 4% to 12% Bis-Tris gels (ThermoFisher; cat. number: NP0326BOX), and transferred to nitrocellulose membranes (BioRad; cat number: 1620094).[6,7,9] ADAM12 protein bands were detected by anti-ADAM12 antibody (1:1000 dilution; cat. number: MBS9128704; MyBio Source), using a secondary antibody conjugated to an infrared dye (1:10 000 dilution IRDye 800CW; LI-COR Biosciences; cat. number: 926–32 211). Signals were recorded using iBright imager (ThermoFisher; Model 1500). Bands from Western blots were quantified using the Image Studio Lite software (LI-COR). Intensity of protein band signals in different lanes were normalized to the total protein content in each lane on Ponceau S–stained membranes. During analysis, we have found

that expression of most housekeeping genes are altered in ischemia (unpublished personal observation), and moreover, studies have shown total protein staining to be more accurate than use of a house keeping protein as a loading control.[12,13]

Immunohistochemistry

During immunohistochemistry analysis, tissue samples were fixed in 2% formalin. Samples were processed overnight as described previously.[6,7,9] Next, 10-μm-thick sections of ischemic and nonischemic TA muscles were subjected to hematoxylin and eosin staining for muscle morphology analysis. Capillaries were identified using a rat anti-mouse CD31 antibody (Novus Biochemicals; cat. number: NB100-64796) at 1:1000 dilution, and muscle regeneration was assessed by using a rabbit anti-heavy chain Myosin/MYH3 antibody (ABCAM; cat. number ab124205) at 1:1000 dilution. All antibodies were diluted in PBS +1% BSA. Controls with only secondary antibodies were included for all staining described in this article. Slides were scanned (at ×20 magnification). All stained cells and muscle fibers in the entire sections were counted, and capillary density expressed as capillaries/fibers. Extent of tissue injury was expressed as the total number of centrally located muscle fiber nuclei to the total number of muscle fibers.[8,14,15] For analysis of myofiber cross-sectional area, tissue samples were fixed in 2% formalin. Samples were processed overnight as described previously. Next, 10-μm-thick sections of ischemic TA muscles were subjected to hematoxylin and eosin. Digital images were obtained at ×20 magnification, and myofiber cross-sectional area (in square micrometers) was quantified by blinded investigators. A minimum of 1000 myofibers per animal was analyzed, using National Institutes of Health's ImageJ image analysis software version 1.43u. The extent of eMyHc staining was expressed as the number of stained myofibers/total number of myofibers.

Cell Line and Culture

Mouse microvascular endothelial cells (from Cell Biologics; cat. number: C57-6220), human umbilical vein endothelial cells (Cell Applications; cat. number: 200pK-05n), and endothelial cells growth medium were obtained from Cell Applications. Cells were grown in standard endothelial cells growth medium supplemented with 10% FBS. In vitro simulation of ischemia was achieved as described previously,[6] with modifications as follows: cells were deprived of nutrients and oxygen by growing in endothelial starvation medium (Cell Applications) and cultured in 2% oxygen in a hypoxic chamber fitted with a ProOx model 110 oxygen controller for continuous monitoring and adjustment of the oxygen content of the chamber (BioSpherix, Lacona, NY).

RNA Immunoprecipitation Based Anti-miR Competitive Assays

RNA immunoprecipitation (RIP) based anti-miR competitive assays were performed as previously described by Ikeda et al.[16] and Androsavich et al.,[17] with some modification. Briefly, cells grown on a 100-mm dish were lysed in 500 μL of radioimmunoprecipitation assay buffer. To preclear the lysate, 20 μL of Protein A/G PLUS-Agarose (Santa Cruz; cat. number; sc-2003) was added and incubated at 4 °C for 30 minutes. The lysate was centrifuges at 900*g* at 4 °C for 30 seconds to remove the pellet. MicroRNA inhibitor competition assay was done by incubating the precleared lysate with specific inhibitors (0, 4, 40, and 400 mmol/L) at 37 °C for 1 hour. Next, 10 μg of AGO-2 (Argonaut-2) antibody was added to the inhibitor treated lysate and incubated at 4 °C for an hour, followed by 20 μL of Protein A/G PLUS-Agarose (Santa Cruz; cat. number: sc-2003) and incubated at 4 °C overnight. Pellets were collected by centrifugation as above. The pellets were washed 4 times with 4 °C radioimmunoprecipitation assay buffer. Total RNA was extracted from the centrifugated pellet using miRNEasy Mini Kit (Qiagen; cat number: 2172004). The amount of mRNA associated with AGO-2 was assessed by real-time–polymerase chain reaction as described above.

Statistical Analysis

All measurements are expressed as mean±SEM. Statistical comparisons between 2 groups at a specific time point were performed using the Student *t* test. For comparison of >2 group means, 1-way or 2-way ANOVA with multiple testing correction was used. In all cases, $P<0.05$ was considered statistically significant.

RESULTS

Treatment of Ischemic Mouse Hind Limbs With AAV9-miR-29a Impairs Ischemia-Induced ADAM12 Upregulation

In prior studies we observed an inverse correlation between miR-29a and *ADAM12* gene expression such that lower expression of miR-29a was associated with higher ADAM12 protein expression in ischemic nondiabetic mouse hind limbs.[6] In diabetic mouse hind limbs, we observed impaired downregulation of miR-29a was associated with impaired upregulation of ADAM12.[9] However, whether miR-29a downregulation is required for normal postischemic adaptation following experimental PAD has not been explored, and the physiologic implication of elevated miR-29a expression in ischemic mouse hind limbs is

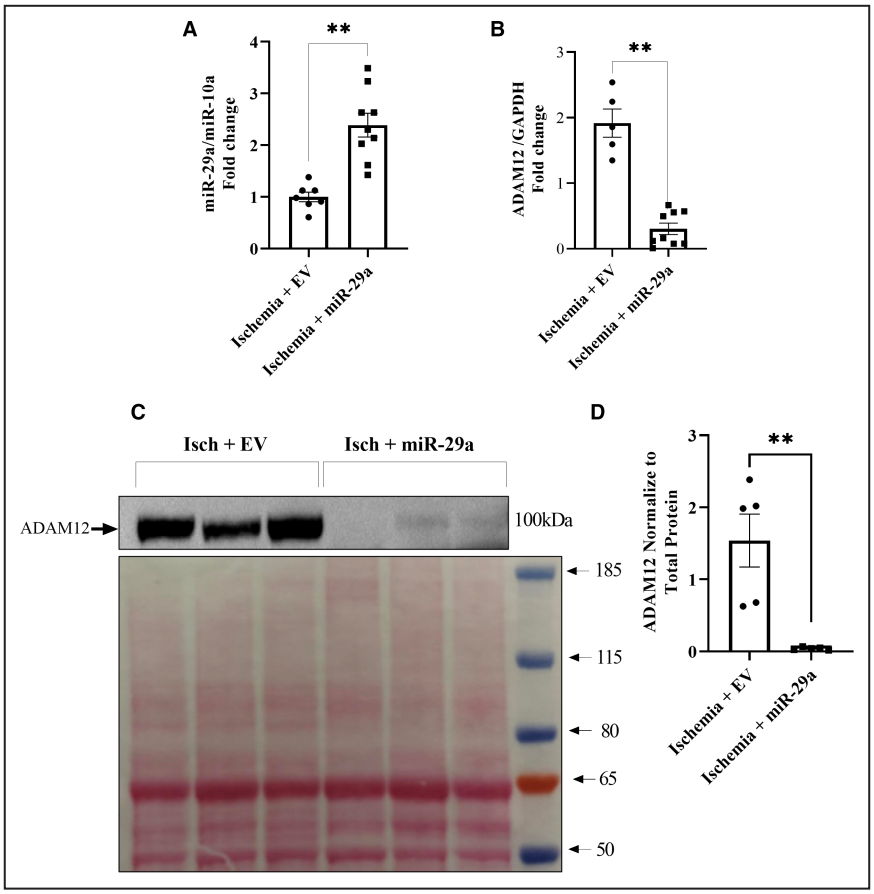

Figure 1. Treatment of ischemic mouse hind limbs with microRNA-29a (miR-29a) impairs ischemia-induced ADAM12 (a disintegrin and metalloproteinase gene 12) upregulation. **A**, Higher miR-29a is expressed in the ischemic gastrocnemius of mice treated with miR-29a compared with the Ischemia+Empty Vector (EV) group (Ischemia+EV: n=5 and Ischemia+miR-29a: n=9, ***P*<0.005). **B**, Lower ADAM12 messenger RNA is expressed in the ischemic gastrocnemius of mice treated with miR-29a compared with controls (Ischemia+miR-29aEV: n=5 and Ischemia+miR-29a: n=9, ***P*<0.005). **C**, Western blotting showing lower ADAM12 protein expression in gastrocnemius lysates from mouse hindlimbs that were treated with miR-29a compared with controls. **D**, quantification of Western blot bands (Ischemia+EV: n=5 and Ischemia+miR-29a: n=5, ***P*<0.005). All analysis were performed using samples from day 3 postischemic gastrocnemius muscles. GAPDH, indicates glyceraldehyde-3-phosphate dehydrogenase.

not clear. In this study, we forced miR-29a expression in ischemic nondiabetic mouse hind limbs (Figure 1A: Ischemia+EV: 1.0±0.09 versus Ischemia+miR-29a: 2.39±0.23, ***P*<0.005). At day 3 following induction of limb ischemia, we assessed the impact of ectopic miR-29a over expression on ADAM12 expression and postischemic perfusion recovery. The day 3 time point was chosen because at this time point, we previously found miR-29a expression is significantly downregulated and ADAM12 expression is significantly upregulated.[6,7,9] miR-10a was used as a loading control because it has been shown to be stable in ischemic and nonischemic conditions.[8] Our data revealed that ectopic overexpression of miR-29a leads to a significant impairment of ischemia-induced ADAM12 mRNA (Figure 1B: Ischemia+EV: 1.0±0.11 versus Ischemia+miR-29a: 0.15±0.04, ***P*<0.005, n=5–9) and protein (Figure 1C and 1D: Ischemia+EV: 1.0±0.24 versus Ischemia miR-29a: 0.02±0.005, ***P*<0.005, n=5/group) expression.

miR-29a Inhibition in Ischemic Gastrocnemius Muscle of C57BL/6 Mice Did Not Increase ADAM12 Expression

Given that we observed that miR-29a overexpression inhibit ischemia-induced ADAM12 upregulation in the ischemic gastrocnemius muscle of C57BL/6 mice, we investigated the effect of miR-29a inhibition in the ischemic muscle of C57BL/6 mice. Our results show

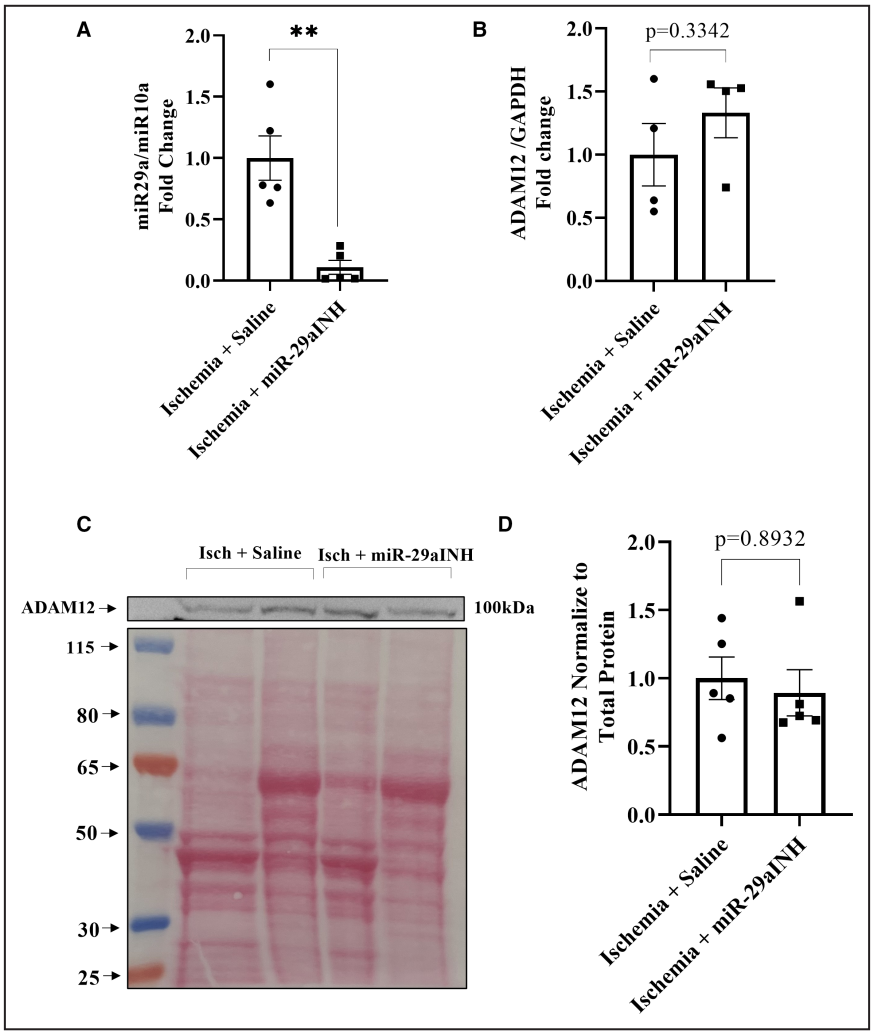

Figure 2. Treatment of ischemic mouse hind limbs with microRNA-29a (miR-29a) inhibition did not increase ischemia-induced ADAM12 (a disintegrin and metalloproteinase gene 12) upregulation.
A, Lower miR-29a is expressed in the ischemic gastrocnemius of mice treated with miR-29a inhibition (miR-29aINH) compared with the ischemic+saline group (Ischemia+Saline: n=5 and Ischemia+miR-29aINH: n=5, $^{**}P<0.005$). **B**, Equal ADAM12 messenger RNA is expressed in ischemic gastrocnemii of mice treated with miR- 29aINH compared with the Ischemia+Saline-treated group (Ischemia+Saline: n=4 and Ischemia+miR-29aINH: n=4, $P=0.32$). **C**, Western blotting showing equal ADAM12 protein expression in gastrocnemius lysates from mouse hindlimbs that were treated with Ischemia+Saline compared with Ischemia+miR-29aINH. **D**, quantification of Western blot bands (Ischemia+Saline: n=5 and Ischemia+miR-29aINH: n=5, $P=0.65$). All analysis were performed using samples from day 3 postischemic gastrocnemius muscles.

that miR-29a inhibition resulted in a significant reduction of miR-29a in ischemic gastrocnemius muscle of C57BL/6 mice (Figure 2A; Ischemia+Saline: 1.0±0.18 versus Ischemia+miR-29aINH: 0.11±0.06, $P<0.05$, n=5/group). However, miR-29a inhibition did not increase ADAM12 mRNA (Figure 2B; Ischemia+Saline: 1.0±0.25 versus Ischemia+miR-29aINH: 1.33±0.20, $P=0.32$, n=5/group) and protein expression (Figure 2C and 2D; Ischemia+Saline: 1.0±0.16 versus Ischemia+miR-29aINH: 0.89±0. 17, $P=0.65$, n=5/group) in C57BL/6 mice.

Overexpression of miR-29a in Ischemic Mouse Hind Limbs Impairs Postischemic Perfusion Recovery and Capillary Density in Experimental PAD

We have previously shown that ADAM12 plays a key role in perfusion recovery following experimental PAD in C57BL/6 mice.[6] We therefore hypothesized that impaired ADAM12 expression in ischemic hind limbs where miR-29a was overexpressed may lead to impaired perfusion recovery and reduced capillary

density in ischemic mouse hind limbs. Hence, we investigated perfusion recovery and capillary density in ischemic mouse hind limbs in which miR- 29a was overexpressed. We found miR-29a–treated mice (ischemia+miR-29a) showed decreased perfusion recovery at day 14 compared with noninjected control mice (Ischemia+Control) and control virus treated mice (Ischemia+EV) (Figure 3A and 3B: Ischemia+Control: 90.95±2.15, n=5, Ischemia+EV: 101.91±5.55, n=6 versus Ischemia+miR-29a: 66.13±12.39, n=5, *P<0.05). We also performed immunohistochemistry for CD31$^+$ cells to quantify capillary density in the day 14 postischemic samples and found a significant decrease in the number of CD31+ cells per muscle fibers (Figure 4A through 4C; Ischemia+EV: 1.0±0.07 versus Ischemia+miR-29a: 0.66±0.05, **P<0.005).

Overexpression of miR-29a in Ischemic Mouse Hind Limbs Increases the Extent of Skeletal Muscle Injury, Decreases Skeletal Muscle Function, But Did Not Affect Myofiber Cross-Sectional Area and Skeletal Muscle Regeneration in Experimental PAD

We next investigated the effect of miR-29a overexpression on the extent of ischemic muscle injury and muscle function following experimental PAD. miR-29a was overexpressed as described above and in methods. Treated and control mice were subjected to experimental PAD, and at day 14 following HLI, ischemic gastrocnemius muscles were harvested, and sections were stained with hematoxylin and eosin. Centrally located nuclei are known indications

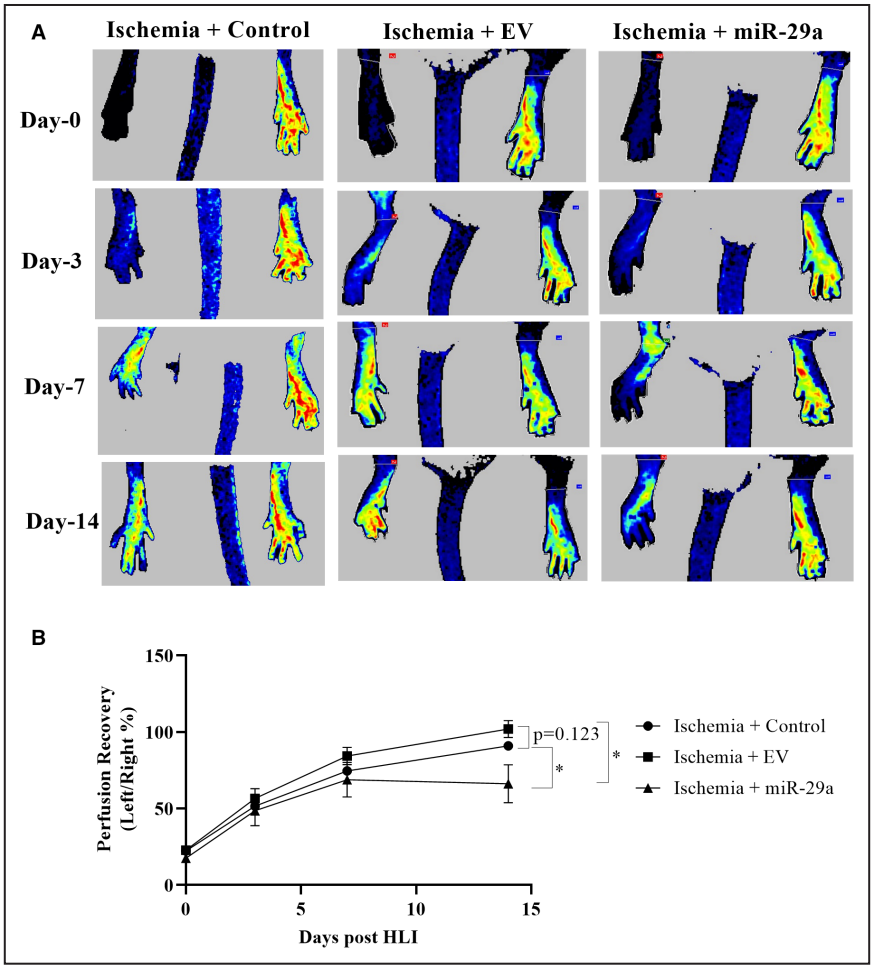

Figure 3. Overexpression of microRNA-29a (miR-29a) in ischemic mouse hind limbs impairs postischemic perfusion recovery.
A, Representative laser speckle contrast imaging illustrating decreased blood perfusion in postischemic hindlimbs of mice treated with miR-29a. **B**, Quantification of perfusion recovery following hind limb ischemia (HLI) at various time points. Vertical axis shows extent of perfusion in the ischemic limb relative to the nonischemic limb. Horizontal axis shows days following HLI (day 14, Ischemia+Control: n=5, Ischemia+EV: n=7 and Ischemia+miR-29a: n=8, *P<0.05). EV indicates empty vector.

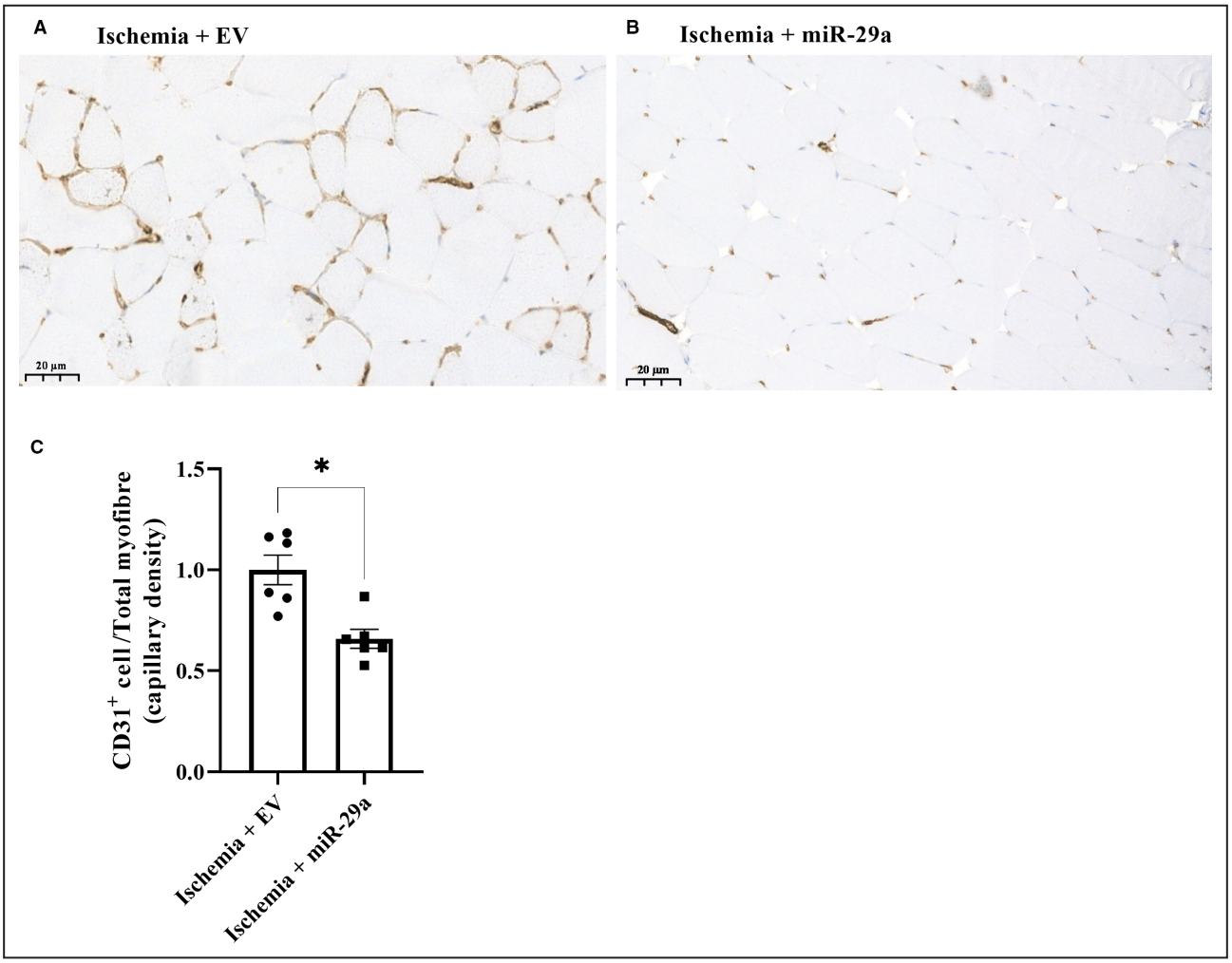

Figure 4. Overexpression of microRNA-29a (miR-29a) in ischemic mouse hind limbs leads to decrease in capillary density in experimental peripheral artery disease.
Tibialis anterior sections from day 14 postischemic hind limbs from empty vector (EV) (**A**) or miR-29a–treated (**B**) animals were subjected to immunohistostaining for CD31 to identify capillaries. Representative images of stained sections are shown (scale bar=20 µm). **C**, Quantification of CD31+ cells per muscle fiber (Ischemia+EV: n=7 and Ischemia+miR-29a: n=8, **P*<0.05).

of skeletal muscle injury, hence we assessed the number of muscle fibers with centralized nuclei by counting the total number of centrally located nuclei divided by the total number of myofibers in the gastrocnemius muscle.[14] Our results showed increased number of muscle fibers with centrally located nuclei in the TA muscle sections from miR-29a–treated mice compared with controls (Figure 5A through 5D; Ischemia+Control: 46.6±3.2%, Ischemia+EV: 43.18±13.0% and Ischemia+miR-29a: 102.05±12.0%, **P*<0.05). Additionally, we investigated the extent of muscle regeneration in the ischemic TA muscle of treated and control mice as defined by eMyHC+ staining (expressed as the percentage of eMyHC+ myofibers/total myofibers). Our result reveals no difference in eMyHC+ staining at day14 following HLI, (Ischemia+EV: 15.0±1.0 versus Ischemia+miR-29a: 15.0±0.01, *P*=0.62, Figure 6A through 6C). We also assessed myofiber cross-sectional areas, and the results reveal that miR-29a treatment did not affect ischemic myofiber cross-sectional area in day 14 following HLI samples (Ischemia+EV: 2240.51±294.29 versus Ischemia+miR-29a: 2333.63±201.01, *P*=0.80, Figure 6D). Lastly, we investigated if maximal isometric torque, a measure of muscle force production, was affected by miR-29a overexpression in ischemic mouse hind limb. Our data showed at day 14 following induction of experimental PAD, miR-29a–treated limbs produced less force in the postischemic TA (Ischemia+EV: 1.10±0.05 and Ischemia+miR-29a: 0.68±0.12, **P*<0.05, Figure 6E) when compared with control.

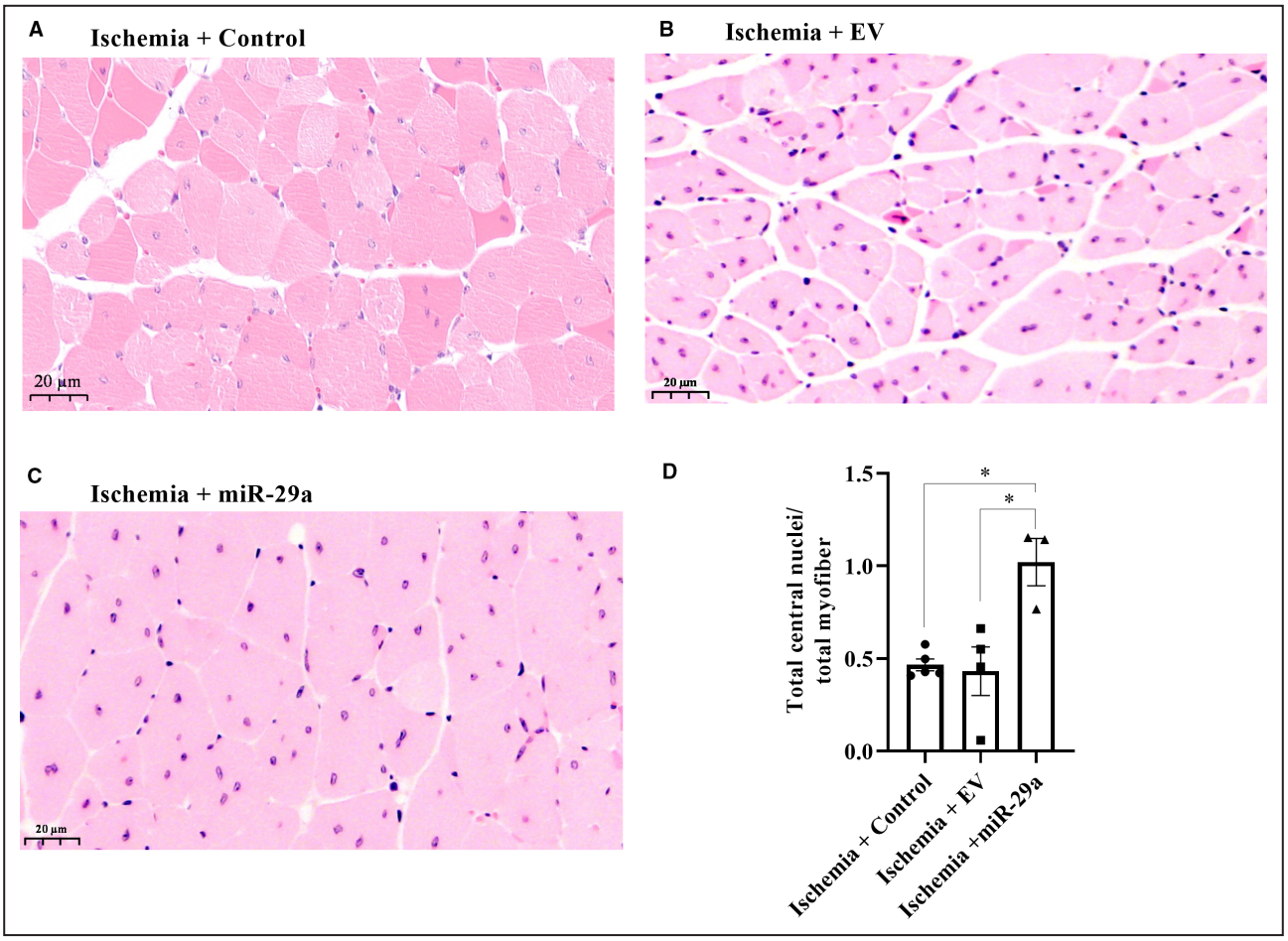

Figure 5. microRNA-29a (miR-29a) overexpression increased the extent of skeletal muscle injury in experimental peripheral artery disease.
Day 14 postischemic tibialis anterior from treatment control as well as sections treated with control, empty vector (EV), or miR-29a were subjected to analysis. **A** through **C**, Representative images of hematoxylin and eosin–stained sections showing centrally located nuclei in muscle fibers. **D**, Quantification of skeletal muscle injury as determined by the percentage of total number of muscle fibers with centrally located nuclei (Ischemia+Control: n=5, Ischemia+EV: n=4 and Ischemia+miR-29a: n=3, **P*<0.05, scale bar=20 μm).

miR-29a Regulates ADAM12 Through Direct Interaction With ADAM12 mRNA

As shown above (Figure 1A through 1D) overexpression of miR-29a in ischemic mouse hind limbs results in downregulation of ADAM12 protein and mRNA expression. However, whether miR-29a regulates ADAM12 through direct interaction with ADAM12 mRNA or indirectly through secondary effects on the transcriptome is not known. We therefore sought to determine if miR-29a targets ADAM12 through direct interaction.

The RNA-induced silencing complex contains members of the AGO protein family bound to microRNAs and their target mRNAs.[18] One of the key proteins in this complex is the AGO-2 protein.[19] Anti-miRs are synthetic oligonucleotides that bind specific microRNAs and can selectively displace the microRNA's binding to its target mRNA transcript.[20,21] We therefore performed RNA immunoprecipitation based anti-miR competitive assays that has been previously used to define direct interaction between microRNAs and their direct target mRNAs.[16,17] The RIP competitive assay involves titrating different concentrations of an anti-miR into cell or tissue lysates followed by immunoprecipitation of AGO-2 protein and analysis of the mRNAs associated with the protein. The addition of anti-miRs results in displacement of the mRNAs that directly interact with the microRNA.[22] Following anti-miR titration, a dose-dependent reduction in the amount of an mRNA transcript associated with immunoprecipitated AGO-2 strongly suggest the mRNA directly interacts with the microRNA.[22] Given our prior data that showed ADAM12 is expressed primarily in the endothelial cells in the ischemic mouse hind limbs,[6] we used lysates from mouse skeletal microvascular endothelial cells to perform RIP based anti-miR competitive assay. We used 10-fold increment doses of anti-miR-29a (4, 40, 400 nmol/L), and the amount of associated mRNA was assessed by quantitative

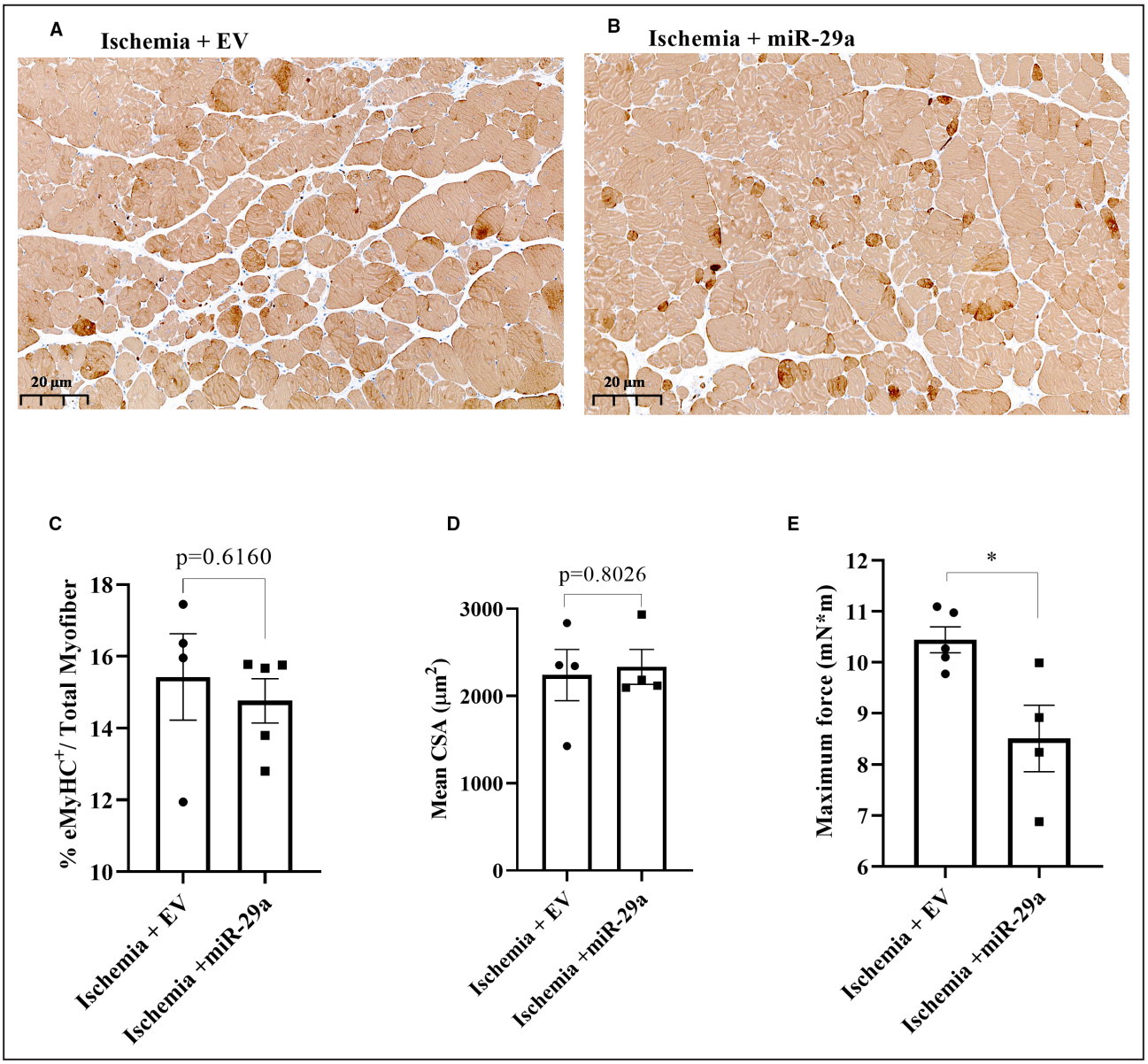

Figure 6. microRNA-29a (iR-29a) overexpression did not affect skeletal muscle regeneration, myofiber cross-sectional area (CSA), but decreased skeletal muscle function in experimental peripheral artery disease.
Day 14 postischemic tibialis anterior (TA) sections treated with empty vector (EV) or miR-29a were subjected to analysis. **A** and **B**, Representative images of eMyHC-stained sections showing regenerating muscle fibers (Ischemia+EV: n=4 and Ischemia+miR-29a: n=5, scale bar=20 μm). **C**, eMyHC+myofiber as an index of myofiber regeneration (Ischemia+EV: n=4 and Ischemia+miR-29a: n=4, *P*=0.62). **D**, Mean myofiber CSA (Ischemia+EV: n=4 and Ischemia+miR-29a: n=4, *P*=0.80). **E**, Maximal isometric torque of ankle dorsiflexors of the TA at day 14 following ischemia (Ischemia+EV: n=5 and Ischemia+miR-29a: n=4, **P*<0.05).

real-time–polymerase chain reaction. Anti-miR696 was used as a control because it is not predicted to target ADAM12. Additionally, the levels of *ADAM17*, a closely related gene to *ADAM12* but not predicted to be regulated by miR-29a, was also assessed in the RIP based assay as control for the specificity of the anti-miR to displace only miR-29 targeted mRNA.

Our result reveals that levels of miR-29a and ADAM12 mRNA in the AGO-2 complex of Mouse microvascular endothelial cells decreased in samples treated with anti-miR-29a in a dose-dependent manner (Figure 7A and 7B: miR-29a: 0.87, 0.68, 0.44, and ADAM12: 0.89, 0.78, 0.67, fold **P*<0.05), but no dose dependent change in the ADAM17 mRNA, which is a member of the ADAMS family but not a target of miR-29a (Figure 7C). Consistent with these results samples treated with anti-miR-696, a microRNA that does not target ADAM12 did not show a dose-dependent change in ADAM12 mRNA (Figure 7D and 7E: miR-29a: 0.84, 0.84, 0.87, fold, and ADAM12: 1.11, 1.12, 1.10, fold,

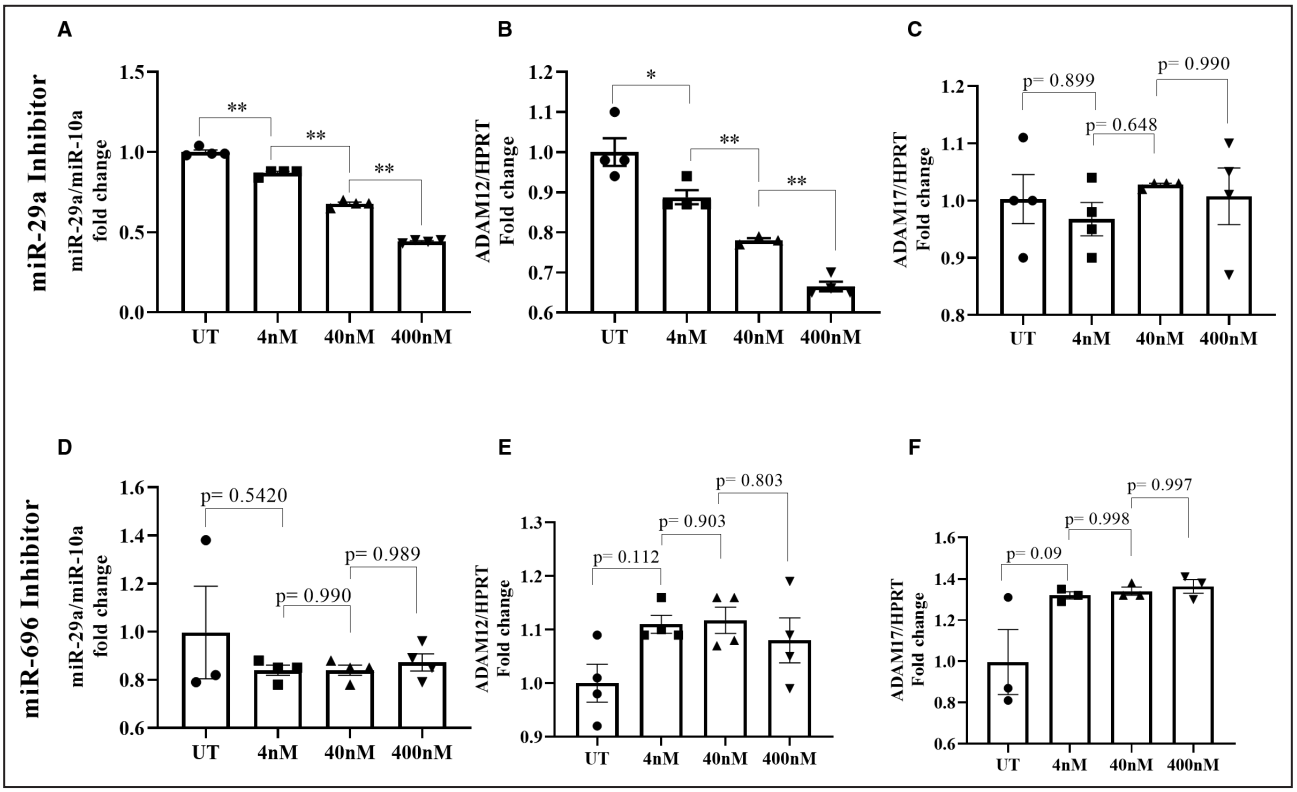

Figure 7. RNA immunoprecipitation–based anti-miR competitive assay reveals dose-dependent displacement of ADAM12 (a disintegrin and metalloproteinase gene 12) messenger RNA (mRNA) from the AGO-2 (Argonaut-2) complex in mouse skeletal microvascular endothelial cells (msMVEC).

msMVEC cell lysates were treated with different concentrations of anti-miRs (0, 4, 40, 400 nmol/L) followed by immunoprecipitation with AGO-2 antibody, and the microRNA and mRNA associated with the AGO-2 complex was analyzed by real time–quantitative polymerase chain reaction. microRNA levels were normalized to miR-10a.[8] **A** through **C**, microRNA-29a (miR-29a), ADAM12, and ADAM17 (a disintegrin and metalloproteinase gene 17) mRNA levels in anti-miR-29a–treated lysates. (**D** through **F**) miR-29a, ADAM12, and ADAM17 mRNA levels in control anti-miR-696–treated lysates (n=3–4 per group, $^{**}P<0.005$ and $^{*}P<0.05$). UT indicates untreated; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

$^{*}P<0.05$) and ADAM17 mRNA. Similar findings were observed when using lysates from human endothelial cells and mouse skeletal muscles (Figures S1 and S2A through S2F respectively). Taken together, our data are consistent with miR-29a directly binding to ADAM12 mRNA.

DISCUSSION

Ischemic complications are the leading cause of morbidity and mortality in patients with PAD.[23,24] An improved understanding of the molecular mechanisms involved in limb ischemia could provide insight to improve therapeutic options in patients with PAD. In prior work, our laboratory identified *ADAM12* as a gene that played a key role in perfusion recovery following experimental PAD.[6] ADAM12 is upregulated in ischemic mouse gastrocnemii and TAs, and plays a key role in postischemic perfusion recovery.[6] Fractionation of cells within the limb skeletal muscles showed ADAM12 upregulation was occurring primarily within endothelial cells in the ischemic tissue.[6] Impaired perfusion recovery in mice with type 1 diabetes was associated with impaired ADAM12 upregulation. In humans, a single nucleotide polymorphism in ADAM12 was linked with critical limb ischemia.[25] However, much less is known about ADAM12 regulation in ischemia. We previously found inverse correlation in miR-29a and ADAM12 expression in ischemic mouse hind limbs, and in vitro overexpression of miR-29a was associated with impaired upregulation of ADAM12, suggesting miR-29a suppresses ADAM12 upregulation.[7–9] However, whether downregulation of miR-29a was required for postischemic perfusion recovery was not known. Higher levels of miR-29a have been observed in skeletal muscle biopsy samples from humans with diabetes.[26] Moreover, miR-29a levels were also elevated in ischemic skeletal muscles of mice with type 1 and type 2 diabetes as well as aged mice.[7,8,27] These findings suggest elevated miR-29a may have pathologic effects

on skeletal muscles, and thus targeting this may be of therapeutic interest. To better understand the physiologic implication of elevated miR-29a in these various conditions, we explored the effect of overexpression of miR-29a on postischemic perfusion recovery and skeletal muscle function in experimental PAD. Our result show overexpression of miR-29a was associated with impaired ADAM12 upregulation, postischemic perfusion recovery, angiogenesis, skeletal muscle function, and worsened severity of skeletal muscle injury. However, overexpression of miR-29a did not alter skeletal muscle myofiber diameter and extent of muscle regeneration. In the nondiabetic mouse, ischemic hind limbs induces miR-29a downregulation and upregulation of ADAM12.[8] Here we explored whether inhibition of miR-29a will further augment ADAM12 expression. We found, although miR-29a inhibitor treatment reduced miR-29a expression, ADAM12 expression was not augmented. This result suggests that in the nondiabetic mice, ischemia-induced downregulation of miR-29a is sufficient to achieve maximal upregulation of ADAM12, such that further lowering miR-29a expression does not achieve addition upregulation of ADAM12 expression.

It is interesting that in our prior studies, ischemic diabetic hind limbs had poor downregulation of miR-29 when compared with nondiabetic controls, and inhibition of miR-29a in the diabetic hind limbs resulted in upregulation of ADAM12, and improved perfusion recovery, angiogenesis, skeletal muscle function, and extent of skeletal muscle injury.[8,9] Taken together, these results suggests downregulation of miR-29a in ischemic mouse hind limbs is important for postischemic adaption, and this occurs sufficiently in nondiabetic ischemic limbs. Moreover, it is interesting that inhibition of miR-29a in the ischemic hind limbs of nondiabetic mice, where miR-29a is already downregulated and ADAM12 expression is already upregulated,[7] did not result in further augmentation of ADAM12 expression. This suggests that there is a threshold of miR-29a reduction in ischemic hind limbs that is needed to yield maximum ADAM12 upregulation, and further reduction in miR-29a beyond that threshold does not yield additional upregulation of its expression.

Although multiple gene targets of miR-29a have been described,[28,29] we are not aware of any study that has provided strong evidence of miR-29a directly interacting with any of the target mRNAs. The 3′ untranslated region of ADAM12 has a target sequence for miR-29a,[30] suggesting miR-29a may directly interact with ADAM12. Here we showed for the first time data strongly supporting miR-29a directly interacting with ADAM12 mRNA and regulating its expression. Our findings provide insight not only on the physiologic significance of miR-29a elevation in ischemic hind limbs but also provide critical foundational knowledge necessary for possible use of microRNA inhibitors to modulate miR-29a and regulate ADAM12 expression to improve postischemic adaption. Hence, the translation of miR-29a inhibition to improve PAD outcomes holds promise.

ARTICLE INFORMATION

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Author contributions: A.O.D.: Conception and design of research. A.O.D., V.L., T.W., and J.V. performed experiments. A.K. and V.A.L. performed and quantified muscle function measurements. V.L. and O.S.D. analyzed data. A.O.D., V.L., and T.W. interpreted the results of experiments. V.L. prepared figures and drafted the article. A.O.D. and V.L. edited and revised article. A.O.D., V.L., T.W., J.V., A.K., and V.A.L. approved final version of article.

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Disclosures

None.

Supplemental Material

Figures S1–S2

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SUPPLEMENTAL MATERIAL

Figure S1. RIP based anti-miR competitive assay reveals dose dependent displacement of ADAM12 mRNA from AGO-2 complex in HUVECS: Lysed HUVECS cell extracts were treated with different concentrations of anti-miRs (0nM, 4nM, 40nM, 400nM) followed by immunoprecipitation with AGO-2 antibody and analyzes by RT-QPCR. miRNA levels were normalized to miR-10a. **A**: miR-29a. **B**: ADAM12 and **C**: ADAM17 or incubated with control anti-miR-696 inhibitor and analyzed by RT-QPCR for **E**: miR-29a. **F**: ADAM12 and **G**: ADAM17, n=4/group, **p<0.005 and * p<0.05

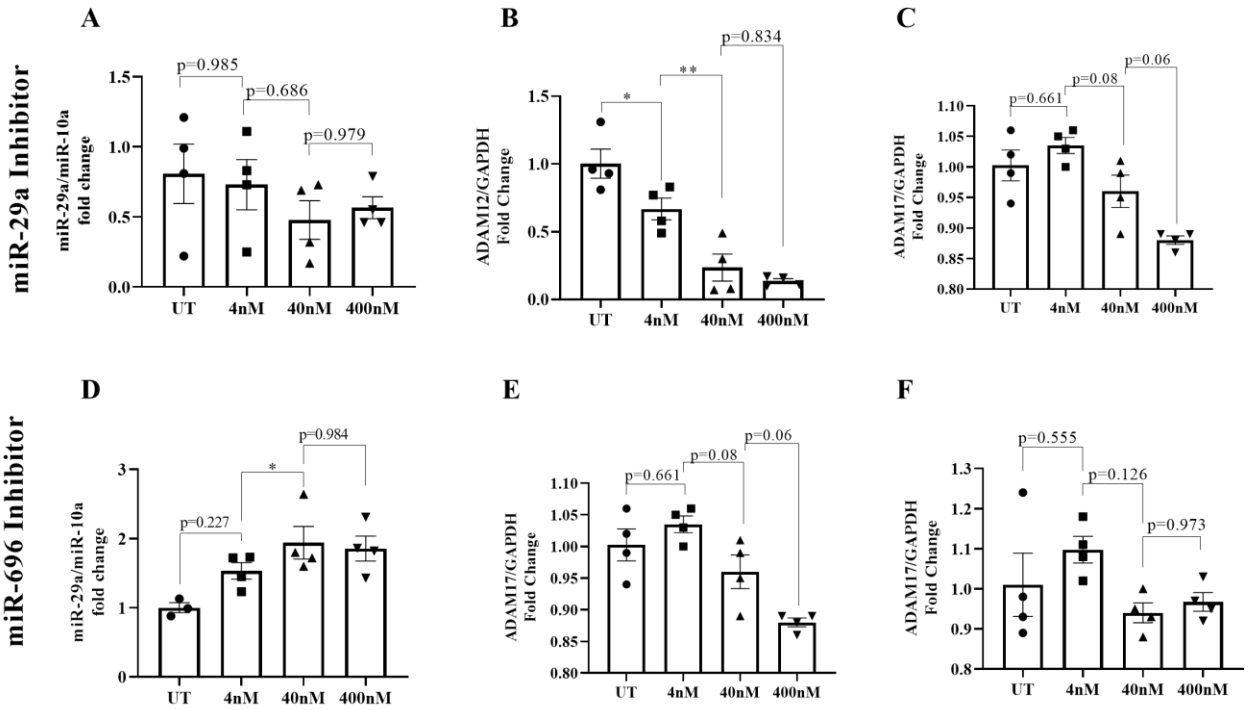

Figure S2. RIP based anti-miR competitive assay reveals dose dependent displacement of ADAM12 mRNA from AGO2 complex in mouse non-ischemic skeletal muscle: gastrocnemius muscle lysates were treated with different concentrations of anti-miRs (0nM, 4nM, 40nM, 400nM) followed by immunoprecipitation with AGO2 antibody and analyzes by RT-QPCR. miRNA levels were normalized to miR-10a. **A**: miR-29a. **B**: ADAM12 and **C**: ADAM17 or incubated with control anti-miR-696 inhibitor and analyzed by RT-QPCR for **E**: miR-29a. **F**: ADAM12 and **G**: ADAM17, n=3-4/group, **p<0.005