Original Research

Modulation of miR29a improves impaired post-ischemic angiogenesis in hyperglycemia

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Impact statement

Individuals with diabetes are more likely to develop peripheral arterial disease (PAD), and when PAD is present, in those with diabetes, it is more severe and there is currently no effective medical treatment for impaired blood flow which occurs in diabetics with PAD. The current work advances the field by providing an understanding of a molecular mechanism involved in impaired post ischemic angiogenesis in diabetes. It shows for the first time that failure to downregulate miR29a in ischemic diabetic tissues is a major contributing factor to poor perfusion recovery in experimental PAD, and miR29a is elevated in skeletal muscle samples from human diabetics compared with levels in those without diabetes. Knocking down the elevated miR29a in ischemic diabetic mouse hind limbs improved perfusion recovery following experimental PAD. This shows miR29a modulation as a novel therapeutic target for improving blood flow in diabetics with PAD.

Abstract

Individuals with diabetes mellitus suffer from impaired angiogenesis and this contributes to poorer peripheral arterial disease outcomes. In experimental peripheral arterial disease, angiogenesis and perfusion recovery are impaired in mice with diabetes. We recently showed that a disintegrin and metalloproteinase domain-containing protein 12 (ADAM12) is upregulated in ischemic endothelial cells and plays a key role in post-ischemic angiogenesis and perfusion recovery following experimental peripheral arterial disease. Here we investigated the role of miR29a in the regulation of endothelial cell ADAM12 expression in ischemia and how hyperglycemia negatively affects this regulation. We also explored whether modulating miR29a can improve impaired post-ischemic angiogenesis associated with hyperglycemia. Additionally, we tested whether miR29a modulation could improve post ischemic angiogenesis in the setting of impaired vascular endothelial growth factor signaling. We forced miR29a expression in ischemic endothelial cells and assessed ADAM12 expression. We also evaluated whether hyperglycemia in vivo and in vitro impair ischemia-induced ADAM12 upregulation and miR29a downregulation. Lastly, we determined whether modulating endothelial cell miR29a expression in ischemia and hyperglycemia could improve impaired endothelial cell functions. We found under ischemic conditions where ADAM12 is upregulated in endothelial cells, miR29a is downregulated. Forced expression of miR29a in ischemic endothelial cell prevented ADAM12 upregulation. In ischemic hind limbs of mice with type 1 diabetes and in endothelial cells exposed to simulated ischemia plus hyperglycemia, ADAM12 upregulation and miR29a downregulation were blunted while angiogenesis was impaired. Knocking down miR29a with an miR29a inhibitor was sufficient to improve ADAM12 upregulation and angiogenesis in simulated ischemia plus hyperglycemia. It was also sufficient to improve perfusion recovery in type 1 diabetes mellitus mice in vivo and angiogenesis in vitro even when vascular endothelial growth factor signaling was impaired with blocking antibodies. In conclusion, MiR29a regulates endothelial cell ADAM12 upregulation in ischemia and this is impaired in hyperglycemia. Modulating miR29a improves impaired post-ischemic angiogenesis associated with hyperglycemia.

Keywords: MicroRNA, miR29, peripheral arterial disease, hyperglycemia, angiogenesis

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Introduction

Peripheral arterial disease (PAD) refers to atherosclerosis occurring in vascular beds outside the heart, and the lower extremities are the most common sites of PAD[1–3] PAD affects 20% of the population over 55 years of age[2] and diabetes mellitus (DM) is a major risk factor for the development of PAD;[1,3–6] 20–30% of diabetic patients have PAD, and 20–30% of PAD patients have diabetes.[1,3–7]

A preclinical model of PAD, mouse hind limb ischemia (HLI) has been used widely to study the processes involved in vascular adaptation following vessel occlusion.[8,9] Blood flow recovery after HLI in mice with type 1 DM is impaired, but the mechanisms involved remain poorly defined.[7,9] We previously identified a genetic locus on mouse chromosome 7 termed limb salvage QTL-1 (LSq-1), which was associated with a favorable perfusion recovery and tissue preservation when present (e.g. C57BL/6) but associated with poor perfusion and increased tissue loss when absent (e.g. Balb/c).[10,11] We refined this locus and identified a disintegrin and metalloproteinase domain-containing protein 12 (ADAM12) as a gene within the locus that is sufficient to modify PAD severity in mice.[12] We showed that augmentation of ADAM12 expression improved perfusion recovery in Balb/c mice which has poor ADAM12 expression in ischemic hind limbs following HLI.[12] Moreover, knocking down ADAM12 in C57BL/6 mice, which has robust upregulation of ADAM12 in ischemic hind limbs, impaired perfusion recovery. In vitro, augmentation of ADAM12 expression improved endothelial cell (EC) proliferation and angiogenesis in ischemia while knocking down ADAM12 impaired proliferation, survival, and angiogenesis in ischemia.[12] These findings suggest that ADAM12 plays an important role in post-ischemic angiogenesis but less is known about the mechanisms regulating ADAM12 expression in ischemia.

MicroRNAs are small non-coding RNAs, which target the 3′UTR of mRNAs, either destabilizing the mRNA transcript or interfering with its translation into protein to modulate several physiological and pathological processes.[13] Some recent work suggests that in fibroblasts, miR29a can regulate the expression of ADAM12.[14] Whether miR29a is involved in the regulation of ADAM12 expression in EC is not known. Additionally, the role of miR29a in post-ischemic angiogenesis has not been previously described.

Here, we investigated the role of miR29a in the regulation of EC ADAM12 expression in ischemia. Additionally, since hyperglycemia in diabetes is known to impair angiogenesis and perfusion recovery following HLI,[9] we explore whether hyperglycemia negatively impacts miR29a regulation of ADAM12 expression in ischemia and whether this axis can be modulated to improve post ischemic angiogenesis in hyperglycemia.

Methods

Mice, model of type 1 diabetes

All mice (C57BL/6 and C57BL/6J-Ins2Akita) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) either directly or bred internally from parental strains obtained from the Jackson Laboratory. The C57BL/6J-Ins2Akita is the Ins2Akita strain on a C57BL/6 background. The Ins2Akita is a previously described mouse model of type 1 diabetes.[15]

RNA and miRNA isolation, quantitative PCR, and protein analysis

Total RNA was isolated with miRNeasy Mini Kit, following the manufacturer's instructions. Each RNA sample was quantified with a spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, Delaware). To detect the level of miRNA, RT-PCR was performed using the Taqman MicroRNA reverse Transcriptase kit (Applied Biosystems) according to the manufacturer's recommendations and as previously described.[9,12] qPCR was performed using Taqman primers probes purchased from Applied Biosystems (Foster City, CA). qPCR data analysis was performed using the comparative CT method. The generated Ct value of each transcript was normalized by its respective Ct value of internal control (ΔCt) (internal control: HPRT, sno202, RNU44). Each transcript was then further normalized to the average ΔCt value of its corresponding control group (ΔΔCt), i.e. reference group. The final fold expression changes were calculated using the equation $2^{-\Delta\Delta Ct}$. The expression level of ADAM12 protein was analyzed by Western blotting as previously described.[12] ADAM12 antibody was purchased from Abcam (cat#29155, Cambridge, MA, USA). Western blots were analyzed by Odyssey Infrared Imaging System (LI-COR Biosciences, NE) and quantified by Scion Image software (Torrance, CA). In experiments where human miR29a expression was analyzed in human skeletal muscle biopsy, muscle biopsies were randomly selected from a previously described biorepository.[16] Skeletal muscle biopsy procedures were performed as described previously.[16] miRNA was isolated from skeletal muscle lysates as described above.

Cell line and culture

Pooled human umbilical vein endothelial cells (HUVEC) were purchased from Cell Applications Inc., (San Diego, CA) and grown in standard EC growth medium (Cell Applications Inc.) with 10% fetal bovine serum. HUVECs were exposed to simulated ischemia (2% oxygen, BioSpherix, Lacona, NY and starvation medium, Cell Applications Inc., San Diego, CA) to simulate ischemia in vitro. MiR29a and ADAM12 mRNA levels were determined by qPCR after the cells were exposed to 24 h of simulated ischemia. SiPORT NeoFx transfection agent (AM4511, Thermo Fisher Scientific, Waltham, MA) was used for microRNA and shRNA transfection studies. MiR29a inhibitor (MH12499) and miR29a mimic (MC12499) were from Thermo Fisher Scientific, Waltham, MA. Plasmid vectors delivering shRNA targeting ADAM12 (KH07647N, SABiosciences, a Qiagen company, Frederick, MD) or control (empty vector) were transfected with the Cytofect™ Endothelial Cell Transfection Kit (Cell Applications Inc., San Diego, CA) following the manufacturer's protocol. Cell Proliferation, tube formation, and apoptosis assays were assessed 48 h after transfection.

Cell apoptosis and proliferation assays

HUVECs were plated in a 96-well plate, 24 h post-transfection with miR29a mimic, miR29a inhibitor or control miR

inhibitor (25 nM). Cells were exposed to simulated ischemia (2% oxygen and starvation medium) for 24 h. At the end of incubation, apoptosis in cells was determined using a TUNEL assay (TiterTACS, Trevigen Gaithersburg, MD) as previously described.[17] Cell proliferation was assessed 24 h after exposure to simulated ischemia using tetrazolium dye incorporation (BioVision, Milpitas, CA) as previously described.[17] Each experiment was repeated at least three times.

Tube formation

HUVECs were transfected with control plasmid or shADAM12 plus miR29a inhibitor; 72 h post-transfection HUVECs were plated on matrigel (Cat # 354234, Thomas Scientific, Corning, NY) at a cell density of 30,000 cells/well in a 96-well plate in media containing normal glucose concentration (5 mM), or high D-glucose (25 mM) or L-glucose (25 mM), and then cultured under conditions of simulated ischemia. In experiments assessing the effectiveness of miR29a inhibition to overcome impaired vascular endothelial growth factor (VEGF) signaling, transfected cells or control cells were plated as described above with or without anti-human VEGFR-1 antibody (AF321, 0.5 μg/ml, R&D Systems, Minneapolis, MN), anti-human VEGFR-2 antibody (mab3572, 0.5 μg/ml, R&D Systems, Minneapolis, MN) or both anti-human VEGFR1 and anti-human VEGFR2 (0.5 μg/ml of each antibody). EC tube formation was assessed 4–6 h after plating. Total complete tube numbers were counted and expressed as tube numbers per square cm. Each experiment was repeated at least three times.

Knockdown of miR29a in type 1 diabetic mice post experimental PAD/HLI and perfusion recovery

Twenty Akita (male, 12 weeks old) mice were randomized into two groups, experimental and control. The level of hyperglycemia in the experimental and control groups of mice was determined by measuring hemoglobin A1c (PTS Diagnostics, Indianapolis, IN, USA) as we previously described,[9] and the groups were found to have identical levels of hyperglycemia. LNA™ miR29a inhibitor and negative control (Exiqon, Woburn, MA, USA) were dissolved in PBS (2 mg/ml), then, 8 mg/kg body weight was delivered to each mouse by intramuscular injection into gastrocnemius muscle and tibialis anterior muscle 30 min before experimental PAD or HLI. This was repeated again at days 7 and 14 after HLI.

After anesthesia induction (ketamine 90 mg/kg and xylazine 10 mg/kg), experimental PAD or HLI was achieved by unilateral femoral artery ligation and excision, as described previously.[8–10,18] Blood flow in the ischemic and contralateral, non-ischemic limbs was measured by laser Doppler perfusion imaging (LDPI), as described previously.[10,17,18] All animal experiments were performed under approved protocols by the IACUC at the University of Virginia.

Statistical analysis

All measurements were expressed as mean ± SEM. Statistical comparisons between two groups (e.g. treated vs. untreated) at a specific time point were performed with the independent Student's t-test or χ^2 where appropriate. Comparisons of more than two groups at a time were performed with analysis of variance. In all cases, a P-value of <0.05 was considered statistically significant.

Results

In ischemia, miR29a regulates EC ADAM12 expression

Previously, we showed ECs from ischemic mouse hind limbs upregulated ADAM12 compared to ECs from non-ischemic hind limbs.[18] Prior studies have implicated miR29a in the regulation of ADAM12 in fibroblasts,[14] so we hypothesized that miR29a may be involved in the regulation of ADAM12 expression in ECs. We assessed miR29a expression in ischemic mouse hind limbs and our results show that miR29a expression is downregulated when compared with non-ischemic mouse hind limbs (fold change non-ischemic vs. ischemic $= 0.60 \pm 0.13$ vs. 0.19 ± 0.05, $n = 4$–7, $^{**}P < 0.01$, Figure 1(a)). Next, we analyzed the expression of miR29a in HUVECS exposed to simulated ischemia and found lower miR29a expression in HUVECs in simulated ischemia compared with controls not exposed to simulated ischemia (controls vs. simulated ischemic $= 0.82 \pm 0.07$ vs. 0.58 ± 0.03, $n = 4$, $^{*}P = 0.02$, Figure 1(b)). This shows an inverse relationship exist between ADAM12 mRNA expression and miR29a expression, suggesting that miR29a may be involved in the regulation of ADAM12 expression in ischemia. To determine if miR29a can regulate ADAM12 expression, we transfected miR29a mimic into HUVECs and assessed ADAM12 expression in simulated ischemia (hypoxia and serum starvation). We found that transfection of HUVECs with miR29a mimic (Figure 1(c)) abolished ischemia-induced upregulation of ADAM12 expression (control miRNA vs. miR29a mimic transfected $= 0.98 \pm 0.02$ vs. 0.16 ± 0.01, $n = 4$, $^{**}P < 0.01$, Figure 1(d)). These results show that EC ADAM12 upregulation in ischemia can be regulated by miR29a.

Hyperglycemia impairs ischemia-induced ADAM12 upregulation and miR29a downregulation

ADAM12 plays a key role in perfusion recovery following HLI, and diabetes is associated with impaired perfusion recovery following HLI, so we hypothesized that diabetes may negatively impact ADAM12 expression. Hence, we assessed ADAM12 upregulation in ischemic hind limbs of mice with type 1 DM. In prior studies, we found that perfusion following HLI is similar in day 3 post-ischemic hind limbs of non-diabetic and diabetic mice,[9] and hence we chose day 3 post-ischemic hind limbs for this analysis. We found lower ADAM12 mRNA and protein expression in day 3 post-ischemic hind limbs from mice with DM1 compared with non-diabetic mice (mRNA, non-DM vs. DM $= 0.90 \pm 0.07$ vs. 0.06 ± 0.02, $n = 4$ and $n = 6$, respectively, $^{**}P < 0.01$, Figure 2(a); protein, non-DM

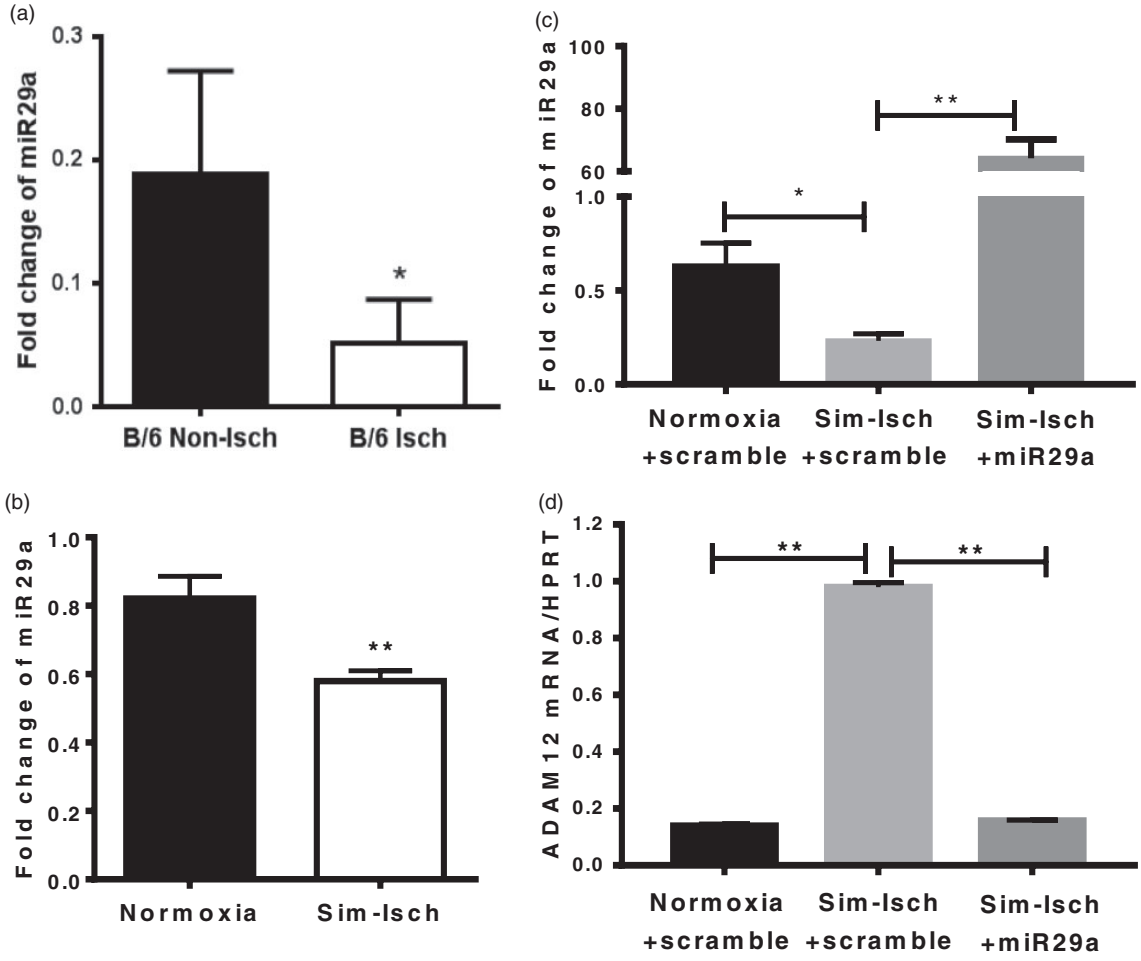

Figure 1 miR29a regulates endothelial cell ADAM12 expression in ischemia. (a) miR29a expression is lower in day 3 post ischemic gastrocnemius (GA) muscle from C57BL/6 mice in comparison with non-ischemic GA ($n = 4$–6, $^{**}P < 0.01$). (b) In HUVECs, miR29a expression is lower in simulated ischemia (HSS: hypoxia and serum starvation) in comparison with non-ischemic condition ($n = 4$, $^{*}P = 0.02$). (c) miR29a mimic transfection into HUVECs yields good miR29a expression in simulated ischemia ($n = 4$, $^{**}P < 0.01$). (d) miR29a mimic inhibits ADAM12 upregulation in simulated ischemia ($n = 4$, $^{**}P < 0.01$)

vs. DM $= 0.62 \pm 0.08$ vs. 0.26 ± 0.07, $n = 3$, $^{*}P < 0.05$, Figure 2(b)). We also compared miR29a expression in the ischemic hind limbs of mice with type 1 DM with that in non-diabetic mice. Our result shows higher expression of miR29a in the ischemic hind limbs of mice with DM1 compared with the non-diabetic mice (0.84 ± 0.06 vs. 0.17 ± 0.02, $n = 4$, $^{**}P < 0.01$, Figure 2(c)). Next, we hypothesized that hyperglycemia in type 1 DM may contribute to altered miR29a expression in ischemic hind limbs. We analyzed miR29a expression in HUVECs cultured in media containing high glucose (25 mM) compared with those cultured in normal glucose (5 mM) with both sets of cells exposed to simulated ischemia. We found miR29a expression in simulated ischemia was higher in HUVECs cultured in high glucose compared to those cultured in normal glucose (0.57 ± 0.07 vs. 0.32 ± 0.02, $^{**}P < 0.01$, $n = 4$–5, Figure 2(d)). Since increased miR29a expression suppresses ADAM12 upregulation in ischemia, these results suggest the lower expression of ADAM12 in ischemic hind limbs of mice with type 1 DM may be due to higher miR29a expression in those tissues. Moreover, the impaired down-regulation of miR29a in ischemia under high glucose may be a mechanism that contributes to poorer EC function in diabetes.

Elevated miR29a expression in ischemia impairs EC function

To understand the impact of elevated miR29a expression on ECs function, we modulated miR29a expression in HUVECs. First, we forced the expression of miR29a in HUVECs in simulated ischemia under normal glucose levels (this is a condition where we previously observed miR29a expression is downregulated when compared to the non-ischemic mock transfected controls). We then measured proliferation, apoptosis and angiogenesis via tube formation assay. We found that forced expression of miR29a mimic resulted in decreased HUVEC proliferation and increased apoptosis in simulated ischemia compared to control mock transfected cells (OD 450 proliferation assay, control vs. miR29a mimic $= 0.55 \pm 0.01$ vs. 0.51 ± 0.01, $^{**}P < 0.01$, $n = 7$, Figure 3(a); OD 450 apoptosis assay, control vs. miR29a mimic $= 0.138 \pm 0.001$ vs. 0.169 ± 0.001, $^{**}P < 0.01$, $n = 5$–7, Figure 3(b)). Next, we assessed EC

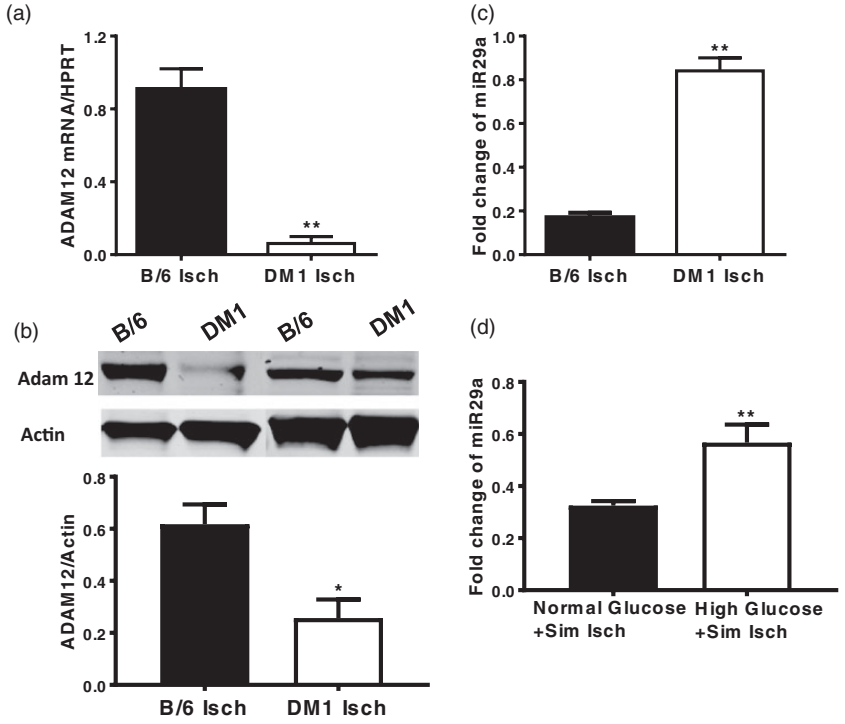

Figure 2 Hyperglycemia is associated with lower ADAM12 and higher miR29a expression in ischemia. (a) Lower levels of ADAM12 mRNA and (b) protein is expressed in day 3 post-ischemic GA tissues from mice with type 1 DM compared with day 3 post-ischemic GA tissues from non-diabetic mice (a: B/6 = non-diabetic C56Bl/6 mice and DM1 = type 1 DM $^{**}P < 0.01$, b; n = 3, $^{*}P < 0.05$). (c) miR29a expression is higher in day 3 post ischemic GA from mice with type 1 DM compared with day 3 post-ischemic GA tissues from non-diabetic mice (n = 5, $^{**}P < 0.01$). (d) In simulated ischemia, higher levels of miR29a is expressed by HUVECs cultured in high glucose (25 mM) compared with those cultured in normal glucose (5 mM) (n = 4–5, $^{**}P < 0.01$)

proliferation, apoptosis and in vitro angiogenesis in simulated ischemia and high glucose condition. This is a condition where we have observed miR29a expression is higher than in normal glucose. We found impaired HUVEC proliferation (OD 450, normal vs. high glucose = 0.299 ± 0.004 vs. 0.258 ± 0.003, $^{**}P < 0.01$, n = 9, Figure 3(c)), increased apoptosis (OD 450, normal vs. high glucose = 0.19 ± 0.01 vs. 0.26 ± 0.01, $^{**}P < 0.01$, n = 4, Figure 3(d)) and decreased angiogenesis (tubes/square cm, normal vs. high glucose = 52.6 ± 2.56 vs. 36.0 ± 2.86, n = 5, $^{**}P < 0.01$, Figure 3(e) to (h)). The effect of high glucose on tube formation was not due to increased osmolality since L-glucose, which cannot be metabolized by ECs did not impair tube formation (normal glucose vs. 25 mM L-glucose = 52.6 ± 2.56 vs. 51.6 ± 2.84, n = 5, NS = P = 0.8, Figure 3(h)). Taken together, these data show that when miR29a levels are elevated in ischemic ECs either through forced expression or high glucose, EC functions involved in angiogenesis are impaired.

Blocking miR29a expression improves EC function in high glucose plus ischemia and this was dependent on ADAM12 expression

Next, we assessed whether blocking miR29a in HUVECs under a condition where it is elevated (simulated ischemia and high glucose) would improve EC function. To block miR29a expression, we transfected HUVECs with an miR29a inhibitor and found this decreased miR29a expression (control vs. miR29a inhibitor = 0.83 ± 0.07 vs. 0.43 ± 0.06, $^{**}P < 0.01$, n = 4, Figure 4(a)). Additionally, we found *that* blocking miR29a in HUVECs was associated with decreased apoptosis (OD 450, control vs. miR29a inhibitor = 0.159 ± 0.011 vs. 0.078 ± 0.009, n = 7, $^{**}P < 0.01$, Figure 4(c)), increased proliferation (OD 450, control vs. miR29a inhibitor = 0.214 ± 0.001 vs. 0.260 ± 0.005, n = 3-5, $^{**}P < 0.01$, Figure 4(d)) and increased angiogenesis (tubes/square cm, control vs. miR29a inhibitor = 26.0 ± 3.07 vs. 45.2 ± 1.43, n = 5, $^{**}P < 0.01$, Figure 4(e) to (g)). Next, we determined whether ADAM12 is involved in the increased angiogenesis observed when miR29a is blocked in HUVECs exposed to simulated ischemia and high glucose. First, we measured ADAM12 mRNA expression in HUVECs treated with miR29a inhibitor and exposed to simulated ischemia and high glucose. We found increased ADAM12 mRNA expression in miR29a inhibitor treated cells compared with control cells treated with inhibitor control (ADAM12/HPRT in control vs. miR29a inhibitor treated = 0.40 ± 0.08 vs. 0.80 ± 0.08, n = 4, $^{**}P < 0.01$, Figure 4(h)). Next, we knocked down ADAM12 expression in HUVECs using shRNA, and cells were then exposed to simulated ischemia and high glucose as described above in Figure 4(a). Our result showed that knocking down ADAM12 was sufficient to abrogate the increased number of tubes formed when HUVECs in simulated ischemia and high glucose are treated with miR29a inhibitor (compare Figure 4(g) to (l)) (tubes/square cm, miR29 inhibitor vs. miR29 inhibitor + shADAM12 = 45.2 ± 1.43 vs. 15.4 ± 3.18, n = 5 and 7, respectively, $^{**}P < 0.01$, Figure 4(k) and (l)).

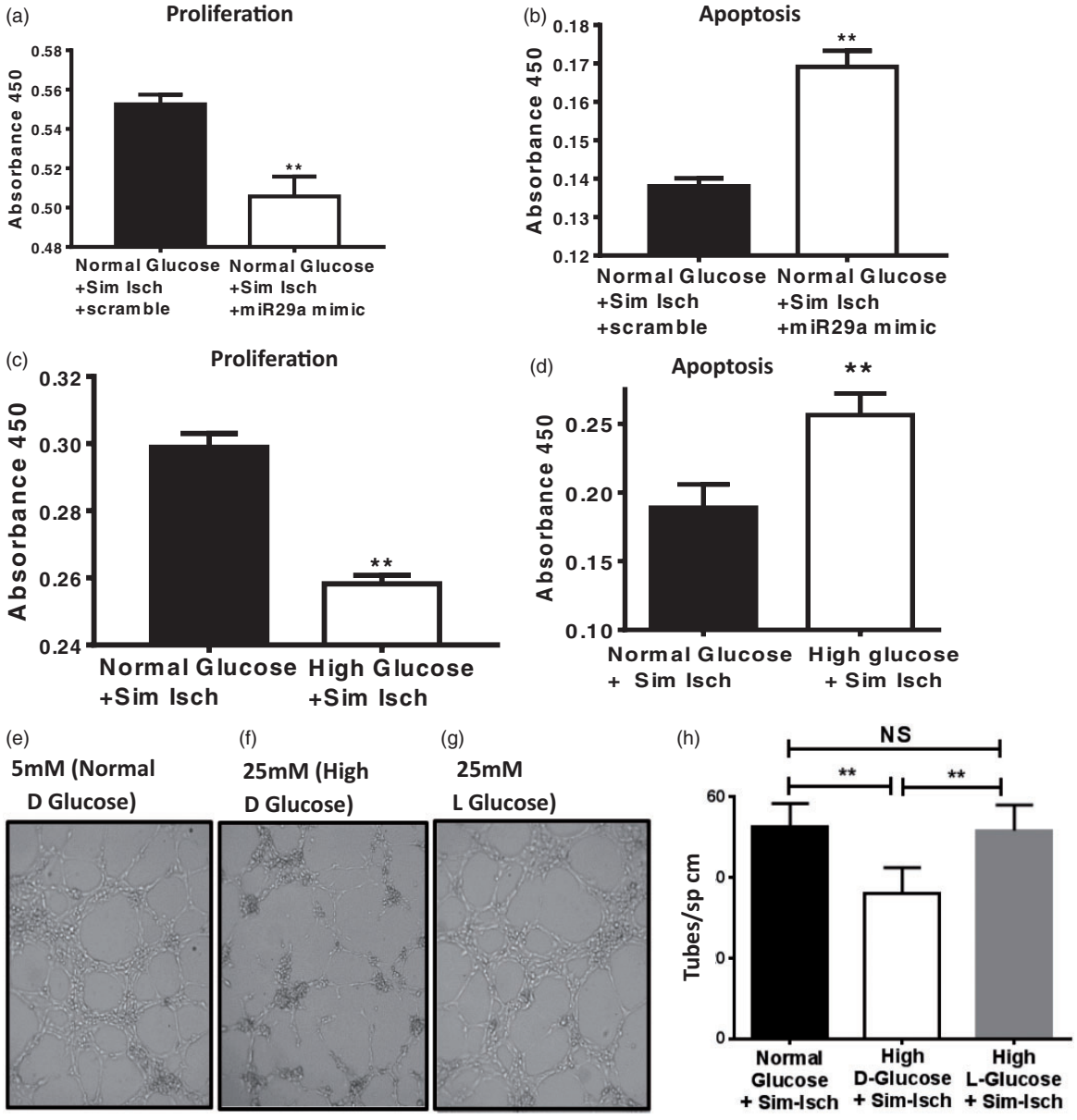

Figure 3 Effect of miR29a modulation on endothelial cell function. (a) Forced expression of miR29a with miR29a mimic resulted in less HUVECs proliferation ($n = 7$, $^{**}P < 0.01$) and (b) increased apoptosis ($n = 5$–7, $^{**}P < 0.01$) in simulated ischemia compared to HUVECs of controls in non-ischemic conditions. (c) HUVEC proliferation is impaired in high glucose and simulated ischemia ($n = 9$, $^{**}P < 0.01$) and (d) HUVECs apoptosis is increased in high glucose and simulated ischemia ($n = 4$, $^{**}P < 0.01$). Representative pictures of tubes formed by HUVECs exposed to simulated ischemia and normal growth media (e, 5 mM), high glucose (f, 25 mM) or L-glucose (g, 25 mM). (h) Number of tubes per square cm in (e), (f) and (g) ($n = 5$, $^{**}P < 0.01$)

Inhibition of miR29a in vivo improves perfusion recovery in mice with type 1 diabetes

In this set of experiments, we explored whether inhibiting the elevated miR29a we observed in ischemic hind limbs of mice with type 1 DM (Figure 2(c)) would improve perfusion recovery following HLI. We observed that mice with type 1 DM treated with miR29a inhibitor showed better perfusion recovery following HLI when compared with untreated control type 1 DM mice (day 21 percent perfusion recovery, untreated vs. treated $= 67 \pm 4.0$ vs. 88.4 ± 3.2, Figure 5(a) and (b)). Collectively, our data show that blocking miR29a expression improves impaired EC functions in hyperglycemia and simulated ischemia. It also improves perfusion recovery in mice with type 1 DM.

Inhibition of miR29a is sufficient to improve angiogenesis in conditions where VEGF signaling is impaired

VEGF is a pro-angiogenic agent that achieves its angiogenic functions by signaling through its receptors VEGFR1 and VEGFR2.[9,19] Our laboratory and others have previously shown that in mice with diabetes, VEGF signaling in ischemic hind limbs is impaired and contributes to impaired

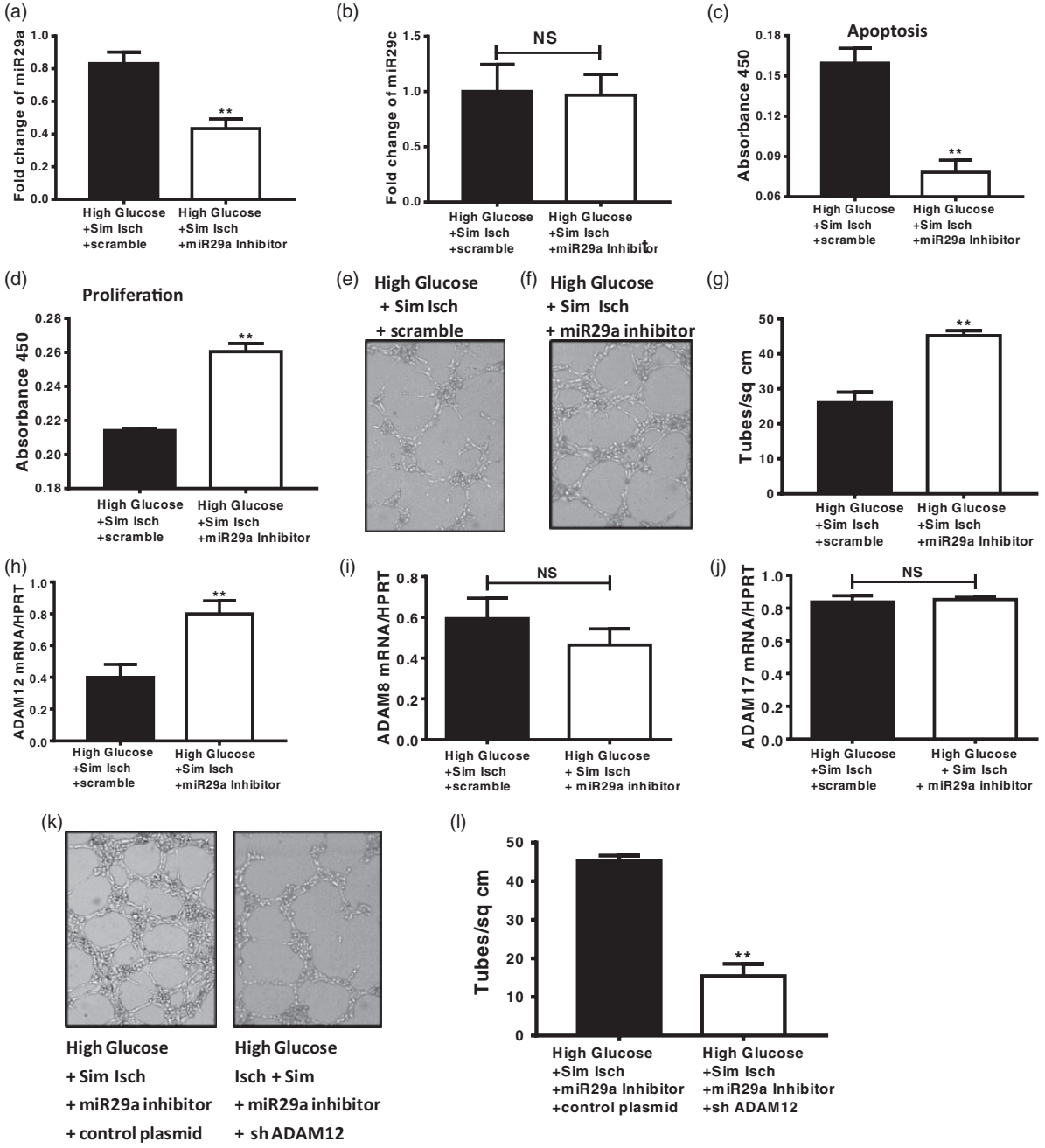

Figure 4 Blocking miR29a improves EC function and angiogenesis but shADAM12 abrogates the effect. miR29a inhibitor treatment decreased HUVEC miR29a expression in simulated ischemia and high glucose (a: $n=4$, $^{**}P<0.01$), without altering expression of miR29c (b: $n=4$, NS $P=0.91$), decreased apoptosis (c: $n=7$, $^{**}P<0.01$), increased proliferation (d: $n=3$–5, $^{**}P<0.01$) and tube formation (e, f and g: $n=5$, $^{**}P<0.01$). miR29a inhibitor treatment increased ADAM12 mRNA expression in HUVECs under simulated ischemia and high glucose (h: $n=4$ $^{**}P<0.01$) without altering expression of ADAM8 and ADAM17 (i: $n=4$, NS $P=0.43$, j: $n=4$, NS $P=0.49$). Knocking down ADAM12 expression with shRNA in miR29a inhibitor treated HUVECs abrogates the improved tube formation observed with miR29a inhibitor treatment (k and l: $n=5$–7, $^{**}P<0.01$)

angiogenesis and perfusion recovery.[9,20,21] We therefore tested whether inhibition of miR29a expression is sufficient to improve angiogenesis when VEGF signaling is impaired using VEGFR blocking antibodies in simulated ischemia and high glucose. In HUVECs exposed to simulated ischemia and high glucose, we assessed in vitro tube formation when cells were treated with blocking antibodies against VEGFR1, VEGFR2, and both VEGFR1 and VEGFR2 with and without miR29a inhibitor. We found that antibody-treated HUVECs exposed to simulated ischemia and high glucose showed poorer tube formation compared with control cells in simulated ischemia plus normal glucose or simulated ischemia plus high glucose (tubes/square cm in no antibody + normal glucose vs. no antibody + high glucose vs. anti-VEGFR1 + high glucose $= 29.4 \pm 2.50$ vs. 18.4 ± 1.75 vs. 11.25 ± 2.78, $n=4$–5, $^{*}P<0.05$, $^{**}P<0.01$, Figure 6(a), (b), (c), and (e)). Inhibition of miR29a was sufficient to improve tube

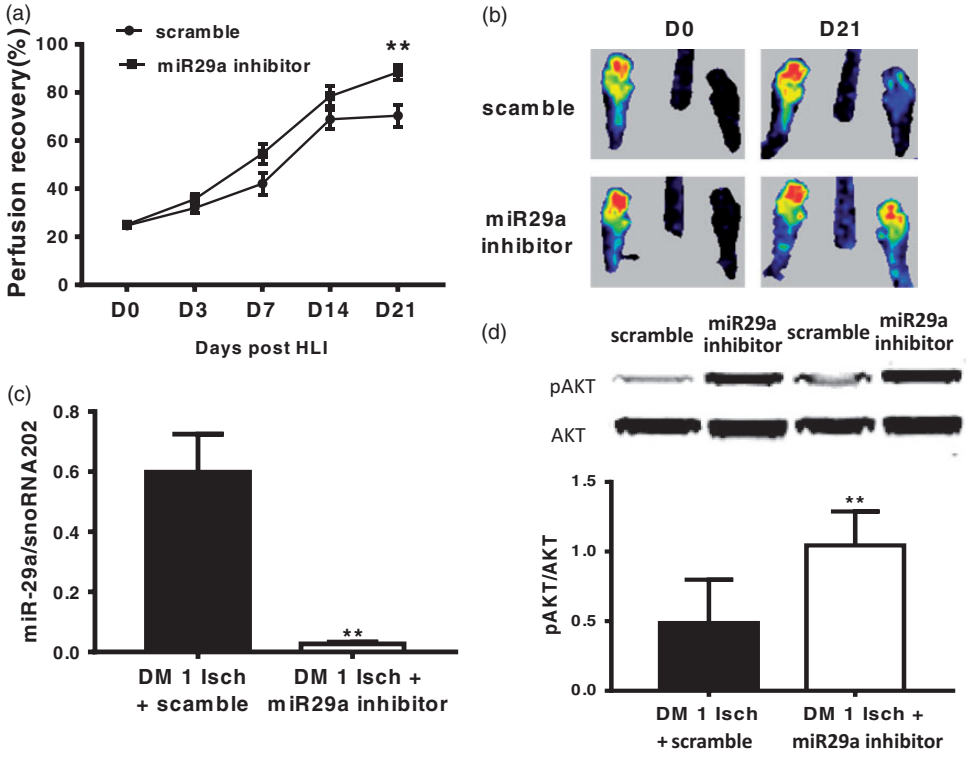

Figure 5 Blocking miR29a expression improved perfusion recovery in diabetic C57BL/6 mice. (a) Following weekly injection of miR29a inhibitor, mouse GA show knock down of miR29a expression is sustained at 21 days post HLI and seven days after three weekly injection ($n = 6$, $^{**}P < 0.01$). (b) Type 1 diabetic mice treated with miR29a inhibitor showing significantly improved perfusion recovery following HLI compared with control type 1 diabetic treated with control miRNA inhibitor ($n = 7$–10, $^{**}P < 0.01$). (c) Representative Laser Doppler perfusion imaging (LDPI) images showing perfusion recovery after HLI in type 1 diabetic mice treated with miR29a inhibitor compared to control mice. (d) The ratio of pAKT/AKT is increased in the GA muscle of type 1 DM mice treated with miR29a inhibitor compared with control type 1 DM mice treated with control miR29a inhibitor ($n = 5$, $^{**}P < 0.01$)

formation in anti-VEGFR1-treated HUVECs (tubes/square cm in anti-VEGFR1 vs. anti-VEGFR1 + miR29a inhibitor = 11.25 ± 2.78 vs. 30.25 ± 3.22, $n = 4$, $^{**}P < 0.01$ Figure 6(c) to (e)). We had similar findings when HUVECs were treated with anti-VEGFR2 (tubes/square cm in no antibody + normal glucose vs. no antibody + high glucose vs. anti-VEGFR2 + high glucose = 29.4 ± 2.50 vs. 18.4 ± 1.75 vs. 10.25 ± 2.98, $n = 4$–5, $^{**}P < 0.01$, Figure 7(a), (b), (c) and (e)). Inhibition of miR29a was again sufficient to improve tube formation in anti-VEGFR2-treated HUVECs (tubes/square cm in anti-VEGFR2 vs. anti-VEGFR2 + miR29a inhibitor = 10.25 ± 2.98 vs. 25.2 ± 2.13, $n = 4$–5, $^{**}P < 0.01$, Figure 7(c) to (e)). Inhibition of miR29a was also sufficient to improve tube formation in HUVECs treated with both anti-VEGFR1 and anti-VEGFR2 (tubes/square cm in anti-VEGFR1 + anti-VEGFR2 vs. anti-VEGFR1 + anti-VEGFR2 + miR29a inhibitor = 3.00 ± 1.23 vs. 13.60 ± 1.72, $n = 4$ and 5, respectively, $^{**}P < 0.01$), Figure 7(f) to (h)).

Furthermore, the inhibition of miR29a expression restored the upregulation of ADAM12 expression in hyperglycemia and simulated ischemia (similar to Figure 4(h)) even in the presence of impaired VEGFRs signaling in HUVECs (Figure 7(i)). This result is consistent with the notion (see Figures 4 and 7) that blocking miR29a improves ECs functions in conditions of hyperglycemia and simulated ischemia via upregulation of ADAM12 expression, and that ADAM12 signaling in EC functions is downstream of both VEGFRs and miR29a signaling.

MiR29a expression is elevated in human skeletal muscle from individuals with diabetes compared with non-diabetic individuals

Next, we determined whether miR29a expression is elevated in humans with diabetes. We analyzed miR29a expression in skeletal muscle biopsy from humans with diabetes compared with humans without diabetes. Consistent with the findings in our pre-clinical model, we found higher levels of miR29a expression in samples from individuals with DM compared with samples from individuals without DM (miR29a/RNU44 in DM vs. non DM = 3.571 ± 0.7 vs. 1.01 ± 0.6 $n = 8$, $^{**}P < 0.001$, Figure 8).

Discussion

It is well known that individuals with diabetes are at increased risk of developing vascular complications and suffer from diseases characterized by impaired angiogenesis such as PAD and impaired wound healing.[22,23] Prior studies in preclinical models of diabetes have shown impaired angiogenesis and poor perfusion recovery following HLI in mice with diabetes.[9,20] EC are particularly vulnerable to hyperglycemia-induced damages due to the inability of these cells to effectively reduce their internal concentration of glucose when exposed to high glucose.[24] A number of mechanism have been postulated including hyperglycemia-induced generation of reactive oxygen species (ROS) resulting in decreased expression of nitric oxide (NO) synthase and increased degradation of NO.[25–27]

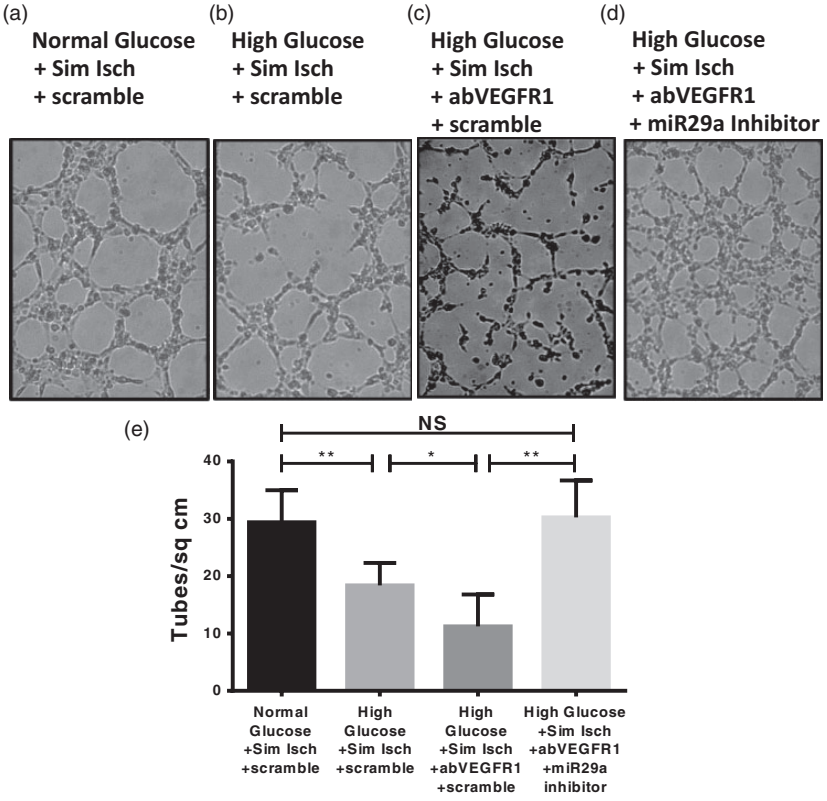

Figure 6 Blocking VEGFR1 signaling further impairs in vitro angiogenesis in ischemia and hyperglycemia and this is rescued by miR29a inhibitor treatment. Representative images of formed tubes by HUVECs cultured in normal glucose and simulated ischemia (a: n = 5), high glucose and simulated ischemia (b: n = 5), anti-VEGFR1 plus high glucose and simulated ischemia (c: n = 4), miR29a inhibitor plus anti-VEGFR1 and high glucose plus simulated ischemia (d: n = 4). Quantification of the number of tubes per square cm shows miR29a inhibition improves angiogenesis in VEGFR1 blocking antibody treated ECs (e: *P < 0.05, **P < 0.01, NS = P = 0.8)

This process can lead to a number of downstream mechanisms including, increased generation of advanced glycation end (AGE) products, increased activation of protein kinase C (PKC) isoforms and increased polyol pathway flux.[24,28,29] Our lab and others have shown impaired expression of pro-angiogenic growth factors and growth factor receptors in diabetes also contribute to the impaired angiogenesis in diabetes.[9,20,30,31] Additionally, our lab and others have shown members of the ADAMs family of metalloprotease are also involved in regulation of angiogenesis Dokun et al.[12,32,33] However, to our knowledge this is the first study to demonstrate that impaired regulation of a member of this family of metalloprotease contributes to impaired angiogenesis in diabetes.

Our understanding of the role of miRNA in biological and pathological conditions has been steadily growing over the last decade.[13,34,35] Several studies have demonstrated miRNAs play a key role in regulating the expression of many EC genes including those involved in angiogenesis.[17,34,36] However, much less is known about the impact of hyperglycemia on EC miRNA expression and how this may impact angiogenesis in diabetes. Additionally, much less is known about whether modulation of microRNAs can be employed as a therapeutic tool to improve the impaired angiogenesis associated with diabetes. Prior studies by Li et al. showed hyperglycemia can upregulate miR221 in HUVECs and this was associated with impaired HUVEC migration.[37] However, whether modulation of miR221 could improve impaired post ischemic angiogenesis associated with hyperglycemia is not known. We are aware of only one study in which modulation of a microRNA was found to improve impaired post ischemic angiogenesis associated with diabetes.[38]

We previously identified ADAM12 as a modifier of PAD outcomes in mice.[12] We showed ADAM12 is upregulated in ischemia and plays an important role in perfusion recovery, through a series of gain of function and loss of function studies.[12] Here, we have identified miR29a as an miRNA involved in regulation of EC ADAM12 expression in simulated ischemia. First, we show that miR29a expression in ischemic mouse hind limbs and EC inversely correlates with that of ADAM12 where ADAM12 is upregulated, while miR29a is downregulated. Forced expression of miR29a in EC exposed to simulated ischemia prevented upregulation of ADAM12. Moreover, in a mouse model of type 1 DM, we found upregulation of ADAM12 in ischemic hind limbs is impaired and this was associated with elevated miR29a expression. Consistent with the findings in mice, lysates from skeletal muscle biopsy from humans with diabetes also show elevated miR29a when compared to individuals without diabetes. Similarly, in vitro, ECs in simulated ischemia and high glucose have impaired proliferation and increased apoptosis with decreased ADAM12 expression and elevated miR29a expression. To understand

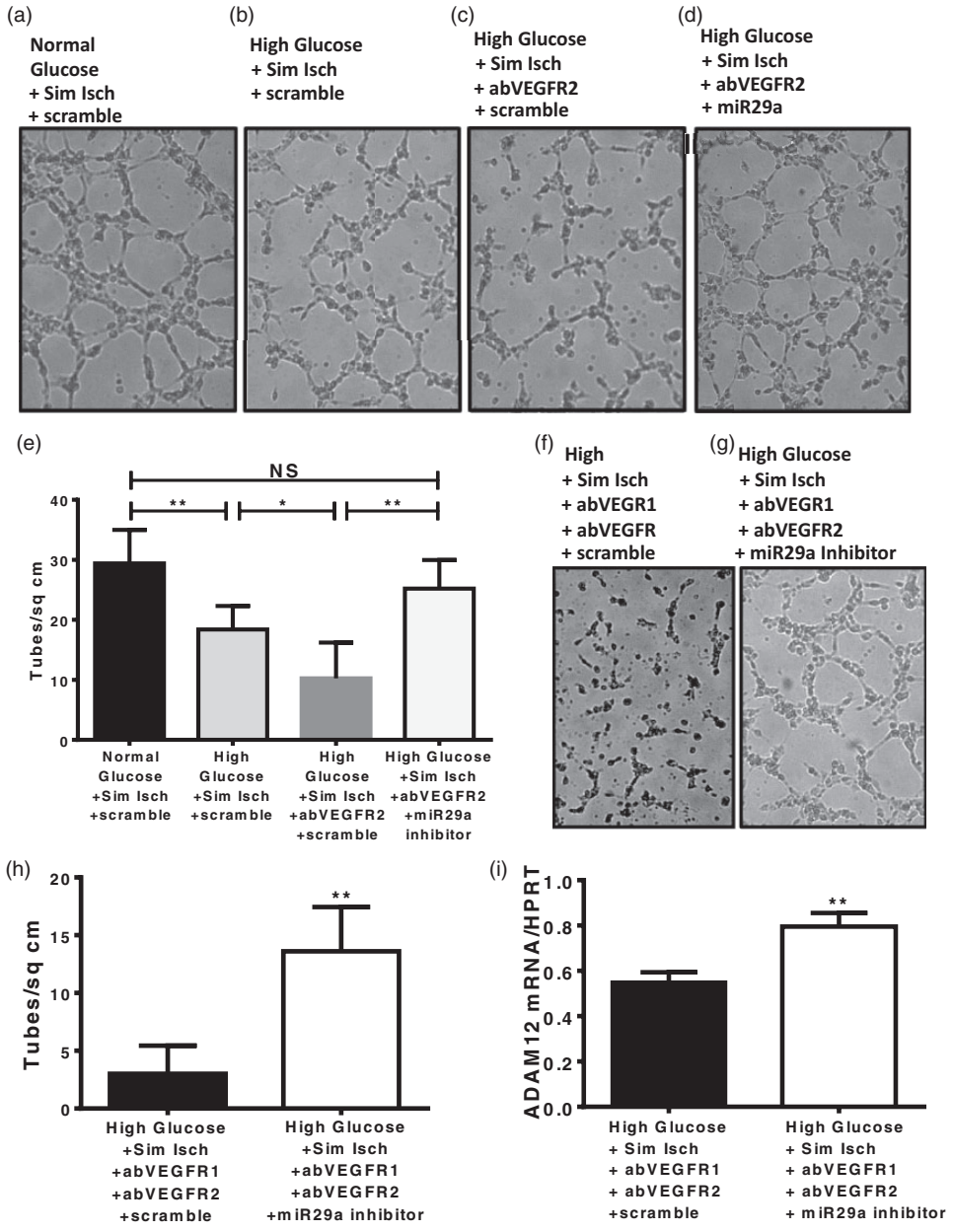

Figure 7 Blocking VEGFR1 and VEGFR2 signaling further impairs in vitro angiogenesis in ischemia and hyperglycemia and this is improved by miR29a inhibitor treatment. Representative images of formed tubes by HUVECs cultured in normal glucose and simulated ischemia (a: n = 5), high glucose and simulated ischemia (b: n = 5), anti-VEGFR2 plus high glucose and simulated ischemia (c: n = 4), miR29a inhibitor plus anti-VEGFR2 and high glucose plus simulated ischemia (d: n = 5). (e) quantification of the number of tubes per square cm in (a) to (d) (*P < 0.05, **P < 0.01, NS = P = 0.2). Anti-VEGFR1 plus anti-VEGFR2 and high glucose plus simulated ischemia (f: n = 4), miR29a inhibitor plus anti-VEGFR1 plus anti-VEGFR1 and high glucose plus simulated ischemia (g: n = 5). Quantification of the number of tubes per square cm in f and g (h: n = 4 and 5, **P < 0.01). Treatment of HUVECs with miR29a inhibitor is associated with upregulation of ADAM12 mRNA expression even in the presence of impaired VEGFRs signaling (i: n = 5, **P < 0.01)

the physiologic significance of EC downregulation of miR29a in simulated ischemia, we forced the expression of miR29a in EC exposed to simulate ischemia and found this resulted in impaired EC proliferation and increased apoptosis. In vitro, blocking the elevated miR29a in EC exposed to simulated ischemia and high glucose with miR29a inhibitor increased expression of ADAM12, decreased apoptosis, increased proliferation, and improved angiogenesis. The improvement in angiogenesis associated with blocking miR29a required ADAM12 since knocking down ADAM12 abolished the increased angiogenesis. Consistent with the in vitro results, the inhibition of miR29a in vivo in the ischemic hind limb of mice with type 1 diabetes significantly improved perfusion recovery. Additionally, since prior studies have shown VEGF signaling is impaired in diabetes/hyperglycemia, we assessed whether miR29a inhibition could improve angiogenesis in the setting of impaired VEGF signaling. Our result showed blocking miR29a was sufficient to improve angiogenesis under conditions where VEGF signaling is impaired. In conclusion, hyperglycemia results in impaired upregulation of

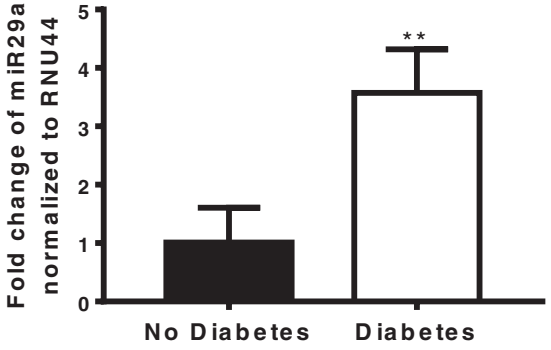

Figure 8 miR29a expression is elevated in skeletal muscle biopsy from humans with DM compared with those with no DM. miR29a levels in lysates from skeletal muscle biopsy samples from individuals with DM is about three-fold higher than levels in individuals without diabetes ($n = 8$, $^{**}P < 0.001$).

ADAM12 in ischemia due to elevated miR29a expression and appears to be a novel mechanism contributing to impaired ischemia-induced angiogenesis in diabetes. The inhibition of miR29a improved EC function and angiogenesis in ischemia and hyperglycemia even when VEGF signaling is impaired. Our finding may have major implication for the treatment of diabetes vascular complications where impaired angiogenesis contributes to poor outcomes. Blocking miR29a shows promise as a potential novel therapy to improve impaired angiogenesis in such disease states as wound healing and PAD.

The molecular mechanisms by which hyperglycemia impairs miR29 expression is not known. However, given the known role of hyperglycemia-induced generation of ROS in endothelial dysfunction, we speculate that hyperglycemia may impair miR29 expression through a mechanism involving generation of ROS.

Authors' contributions: AOD conception and design of research; LC, DA, LS and DW performed experiments; AOD and LC analyzed data; AOD interpreted results of experiments; AOD, LC and EO prepared figures; AOD drafted manuscript; AOD and EO edited manuscript; AOD, LC, EO, DA, LS and DW approved final version of manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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