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Clinical/Translational Research

MicroRNA-15a and MicroRNA-16 Impair Human Circulating Proangiogenic Cell Functions and Are Increased in the Proangiogenic Cells and Serum of Patients With Critical Limb Ischemia

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Rationale: Circulating proangiogenic cells (PACs) support postischemic neovascularization. Cardiovascular disease and diabetes mellitus impair PAC regenerative capacities via molecular mechanisms that are not fully known. We hypothesize a role for microRNAs (miRs). Circulating miRs are currently investigated as potential diagnostic and prognostic biomarkers.

Objective: The objectives were the following: (1) to profile miR expression in PACs from critical limb ischemia (CLI) patients; (2) to demonstrate that miR-15a and miR-16 regulate PAC functions; and (3) to characterize circulating miR-15a and miR-16 and to investigate their potential biomarker value.

Methods and Results: Twenty-eight miRs potentially able to modulate angiogenesis were measured in PACs from CLI patients with and without diabetes mellitus and controls. miR-15a and miR-16 were further analyzed. CLI-PACs expressed higher level of mature miR-15a and miR-16 and of the primary transcript pri–miR-15a/16-1. miR-15a/16 overexpression impaired healthy PAC survival and migration. Conversely, miR-15a/16 inhibition improved CLI-PAC–defective migration. Vascular endothelial growth factor-A and AKT-3 were validated as direct targets of the 2 miRs, and their protein levels were reduced in miR-15a/16–overexpressing healthy PACs and in CLI-PACs. Transplantation of healthy PACs ex vivo–engineered with anti–miR-15a/16 improved postischemic blood flow recovery and muscular arteriole density in immunodeficient mice. miR-15a and miR-16 were present in human blood, including conjugated to argonaute-2 and in exosomes. Both miRs were increased in the serum of CLI patients and positively correlated with amputation after restenosis at 12 months postrevascularization of CLI type 2 diabetes mellitus patients. Serum miR-15a additionally correlated with restenosis at follow-up.

Conclusions: Ex vivo miR-15a/16 inhibition enhances PAC therapeutic potential, and circulating miR-15a and miR-16 deserves further investigation as a prognostic biomarker in CLI patients undergoing revascularization. (*Circ Res*. 2013;112:335-346.)

Key Words: angiogenesis ■ diabetes mellitus ■ ischemia ■ microRNAs ■ microRNA-15a ■ microRNA-16 ■ proangiogenic cells

Peripheral artery disease can evolve to critical limb ischemia (CLI), a life-threatening condition characterized by pain at rest and tissue loss with ulcer and gangrene.[1] Once CLI occurs, blood flow (BF) must be restored by either percutaneous angioplasty or surgical revascularization. Both options are too often not feasible, and thus amputation may be the only remedy, especially in diabetic patients.[2]

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Therapeutic stimulation of the angiogenesis process represents a novel strategy to support postischemic BF recovery, wound closure, and tissue regeneration. Bone marrow (BM)–derived proangiogenic cells (PACs), which were previously known as early endothelial progenitor cells (EPCs), have been implicated

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Non-standard Abbreviations and Acronyms	
BF	blood flow
BM	bone marrow
Cel-miR-39	Caenorhabditis elegans microRNA-39
CXCR4	CXC chemokine receptor, type 4
CLI	critical limb ischemia
FGF-R1	fibroblast growth factor receptor-1
KDR	kinase insert domain receptor
HUVEC	human umbilical vein endothelial cell
miR	microRNA
PAC	proangiogenic cell
PB	peripheral blood
SDF-1α	stromal cell–derived factor-1α
snU6	small nuclear RNA u6
T2DM	type 2 diabetes mellitus
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell

in both native and therapeutically guided angiogenesis.[3] PACs derive from mononuclear cells by culture enrichment.[4] The real nature, antigenic definition, and proangiogenic mechanisms of PACs are still debated. Notwithstanding, their capacity to promote angiogenesis in ischemic tissue is accepted.[3] The pioneer clinical trial Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) showed initial therapeutic promises of PACs enriched from either the BM or the peripheral blood (PB) of patients with acute myocardial infarct.[5] Furthermore, a recent meta-analysis of 37 clinical trials has revealed that autologous transplantation of BM-derived cells is a feasible, relatively safe, and potentially effective therapeutic strategy for CLI patients.[6] The efficacy of this cell therapy approach needs further consolidation by large, randomized, placebo-controlled, double-blind studies, including the ongoing injection of autologous CD34-positive cells for critical limb ischemia (ACT34-CLI),[7] bone marrow outcome trial in critical limb ischemia (BONMOT-CLI),[8] and rejuvenating endothelial progenitor cells via transcutaneous intra-arterial supplementation (JUVENTAS).[9]

Despite the encouraging evidence from early clinical trials, the regenerative potential of PACs derived from patients with tissue ischemia and diabetes mellitus (DM) is reduced, and the underpinning molecular mechanisms are not fully clarified.[10–12] Here, we aimed to analyze the contribution of microRNAs (miRs) to PAC dysfunction.

miRs are small (21–25 nucleotides in their mature forms) noncoding RNAs capable of posttranscriptionally inhibiting gene expression.[13,14] Importantly, each miR regulates the expression of several target genes, with the possibility to modulate multiple pathways. Several miRs control postischemic angiogenesis acting at different levels.[15] Furthermore, miRs influence the therapeutic potential of human embryonic stem cell–derived endothelial progenitor cells and of pericyte progenitor cells[16,17] on their transplantation in mouse models of peripheral or myocardial ischemia.

In this study, after an initial expressional screening for 28 angiogenesis-related miRs in PACs from CLI patients with and without type 2 DM (T2DM), we focused our attention on miR-15a and miR-16. In *Homo sapiens*, 2 miR-15/16 clusters exist: miR-15a/miR-16-1 and miR-15b/miR-16-2 (at 13q14.2 and 3q25.33, respectively). miR-15a and miR-16 share a portion of their seed sequence (ie, the sequence that binds to the 3′ untranslated region of the targeted mRNAs) with 5 other miRs, including miR-503 and miR-424.[18] We previously showed that miR-503 impairs angiogenesis in the setting of CLI and diabetes mellitus.[19] In addition, Chamorro et al[20] demonstrated that miR-16 and miR-424 inhibit in vitro endothelial function and angiogenesis by modulating the expression of VEGF-A, VEGF kinase insert domain receptor (KDR) and fibroblast growth factor receptor-1 (FGF-R1). Furthermore, Hullinger et al[21] showed that miR-15 inhibition protects against cardiac ischemic injury.

Here, we show that CLI increases miR-15a and miR-16 levels in PACs. Furthermore, the 2 miRs impair PAC survival and migration toward chemoattractants. Conversely, ex vivo inhibition of miR-15a and miR-16 empowers PACs, increasing their therapeutic potential when transplanted in an immunocompromised mouse limb ischemia model. Finally, the levels of circulating miR-15a and miR-16 are increased in CLI patients, and correlates with restenosis and post-restenosis amputation 12 months after revascularization.

Methods
An expanded Methods section is available in the Online Data Supplement.

Clinical Study
The data reported here were produced to meet the secondary objective (namely, to identify new molecular mechanisms responsible for the known functional impairment of PACs in CLI) of the clinical trial Diabetic Foot and Vascular Progenitor Cells (NCT0126958) developed at MultiMedica-Milan with a consecutive series of consented patients. Nonischemic and nondiabetic volunteers were recruited external to this trial to serve as controls. The characteristics of the human populations who donated blood for this study are reported in Online Table I. Import of human samples, storage, and use at the University of Bristol were approved by the UK National Research Ethic Service South West (REC-11/SW/0093).

PAC Culture
PACs were prepared from PB mononuclear cells, as described.[22]

miR Expressional Analyses
Mature miR expression was measured using Taqman-validated polymerase chain reaction primers and normalized to either the small nuclear RNA U6 (snU6) (for cells and exosomes) or the synthetic Caenorhabditis elegans miR-39 (Cel-miR-39) (Qiagen; for serum and plasma). When necessary, standard curves for miR quantification were used.

PAC Transfection
PACs were transfected with pre-miR mimics (pre–miR-15a and pre–miR-16), miR inhibitors (anti–miR-15a and anti–miR-16), or scramble negative control (Applied Biosystems) at a final concentration of 50 nmol/L.

PAC In Vitro Assays
PAC apoptosis was measured by flow cytometry using annexin V and propidium iodide. PAC migration and capacity to support endothelial cell networking on 2-dimensional Matrigel were investigated as described.[22] Three-dimensional angiogenesis assay (with conditioned culture medium [CCM] of PACs and human umbilical vein

endothelial cells [HUVECs]) was performed by Spheroid assay according to the manufacturer's instructions (PromoCell). The effect of miR manipulation on PAC growth was measured counting trypan blue–negative cells at 48 hours after transfection.

miR Target Gene Analysis

miR-15a and miR-16 gene targets were identified using MirWalk (http://ma.uni-heidelberg.de/apps/zmf/mirwalk), which allows searching of several independent prediction software programs. The 3′ untranslated region luciferase activity assays were used to validate vascular endothelial growth factor (VEGF)-A and AKT3 as direct targets of each miR. VEGF and AKT3 mRNA and protein levels in PACs were measured (real-time reverse-transcriptase polymerase chain reaction and Western blotting or enzyme-linked immunosorbent assay [ELISA], respectively).

In Vivo Study

Experiments on mice were approved by the UK Home Office and conducted at the University of Bristol in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources.

PACs from healthy donors (described in the Online Table II) were transfected with pre–miR-15a plus pre–miR-16, anti–miR-15a plus anti–miR-16, or negative control. Unilateral hindlimb ischemia was induced in anesthetized immunocompromised CD1-*Foxn1*nu mice (Charles River, United Kingdom; n=11–14 mice/group).[16] Transfected PACs (1×10^5 cells in 15 μL of culture medium) or fresh cell culture medium was injected in 3 equidistant sites of the ischemic adductor muscle. Postischemic BF recovery was sequentially measured (laser color Doppler). After 2 weeks, mice were euthanized and muscular microvascular density was assessed.[16,19,22]

Statistical Analyses

Continuous variables were compared between cases and controls by using Student *t* test and analysis of variance [ANOVA] or by means of the Kruskal-Wallis test if they had a skewed distribution. Categorical variables were compared by means of the χ^2 test. Correlations were evaluated by Spearman correlation coefficient. To evaluate the association between miR expression and the risk of events, odds ratios (OR) and their 95% confidence intervals (CIs) were calculated from multiple logistic regression after adjustment for age and sex. Continuous variables that were positively skewed were analyzed on the log-2 scale. Results were considered statistically significant at $P<0.05$.

Results

CLI Impacts on PAC miR Expression

PAC identity was assessed as previously described[22] (Online Figure I). Using PACs from small cohorts of healthy donors (n=5) and CLI patients with and without T2DM (n=6 per group) who were randomly selected from larger populations illustrated in Online Table I, we screened the expression of 28 miRs (internal control: snU6) selected because of their potential to modulate angiogenesis (Online Table III). These analyses were performed using the delta delta cycle threshold (2-ddCT) method and without using miR standard curves for quantification. Eighteen of the 28 miRs appeared differentially regulated among groups (Figure 1A). miR-16 and miR-15 have been related to angiogenesis[20,23] and ischemia,[21] respectively. Furthermore, they belong to the extended miR-16 family that interests us.[18,19] Hence, we selected miR-15a and miR-16 for further expressional and functional analyses.

miR-15a and miR-16 Expression in PACs From Healthy and Ischemic Subjects

Relative expression of miR-15a and miR-16 was measured again in PACs prepared from larger cohorts and analyzed using miR standard curves. We confirmed that miR-15a and

miR-16 were increased in CLI-PACs, but without further difference induced by T2DM (Figure 1B). Furthermore, hypoxia, which mimics ischemia in vitro, increased miR-15a and miR-16 in healthy PACs (Online Figure II). Levels of miR-15a and miR-16 in PACs were directly correlated (Spearman correlation coefficient=0.6601; $P<0.0001$). This can be reconciled with the fact that miR-15a and miR-16-1 are clustered and, hence, transcribed together as a primary transcript (pri–miR-15a/16-1). In fact, pri–miR-15a/16-1 expression was increased in CLI-PACs (Figure 1C). The enzymes Drosha and Dicer are essential for miR maturation. Drosha cleaves pri-miRs to pre-miRs (precursor miRs), and Dicer cleaves pre-miRs to mature miRs.[15] We found that PACs express both enzymes at mRNA level, without difference among groups (Figure 1D). The sum of the aforementioned evidence suggests that PACs are able to transcribe and process miR-15a and miR-16. In addition, as shown in Figure 2B, PACs are able to secrete the 2 miRs embedded in exosomes, an emerging class of microvesicles of endosomal origin involved in the transport of protein and mRNA with implications in neovascularization.[24,25] This allows for speculating a role of PAC-derived miR-15a and miR-16 in cell-to-cell communications during angiogenesis, as well as their contribution to the pool of PB circulating miRs.

Expression and Secretion of miR-15a and miR-16 by Different Cell Populations

We additionally measured the expression of miR-15a and miR-16 in several cell populations that are relevant for neovascularization, including unfractionated PB mononuclear cells, CD34^pos mononuclear cells, several endothelial cell lines, vascular smooth muscle cells (VSMCs), and pericytes. Figure 2A shows different levels of miR-15a and miR-16 among these cell types. Interestingly, the 2 miRs and especially miR-15a seemed enriched in the exosomes prepared from the CCM of the tested cell populations (Figure 2B).

Forced miR-15a/16 Overexpression Impairs PAC Survival and Migration

To investigate the functional relevance of miR-15a and miR-16, PACs from healthy donors were transfected with pre–miR-15a and pre–miR-16 (control: scramble). Efficacy of pre-miR transfection is shown in Online Figure IIIA. PACs overexpressing both miRs showed increased apoptosis (Figure 3A) and reduced capacity to migrate toward fetal bovine serum (unspecific stimulus), stromal cell–derived factor-1α (SDF-1α), basic FGF, and VEGF-A (Figure 3B i–3B iv). However, single overexpression of either miR-15a or miR-16 inhibited migration to basic FGF and VEGF-A only (Figure 3B iii and 3B iv). We assessed the potential regulation of SDF-1α receptor CXC chemokine receptor, type 4 (CXCR4), FGF-R1, and KDR by pre-miR transfection of PACs. Different from what was reported by Chamorro-Jorganes et al[20] in HUVECs, miR-15a/16 overexpression did not affect the expression of these receptors in PACs (Figure 3C and 3D). Next, to study whether miR-15a and miR-16 alter the capacity of PACs to collaborate with ECs in the angiogenic process, we evaluated the impact of PACs transfected with both pre-miRs on the capillary-like network formation from HUVECs seeded on Matrigel. However, we could not observe any significant difference induced by miR-15a/16 overexpression in PACs (Online Figure IVA i
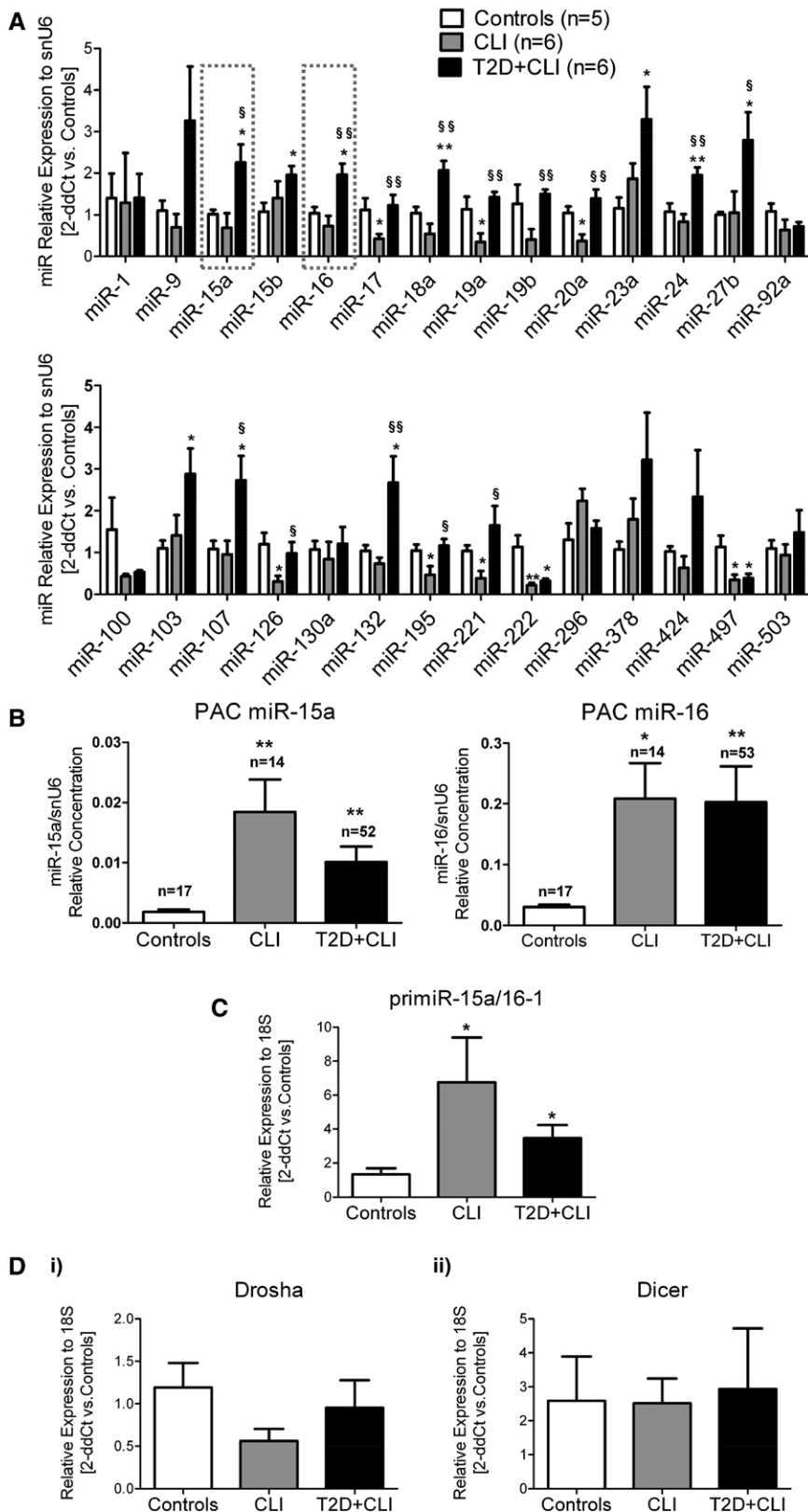
Figure 1. Critical limb ischemia (CLI) affects the expression of microRNAs (miRs) in proangiogenic circulating cells (PACs).
A, Expression of 28 miRs (TaqMan real-time polymerase chain reaction) in PACs from small cohorts of CLI patients with or without type 2 diabetes mellitus (T2D) and in healthy controls. Data were reported to the control group by the 2-ddCt method [internal control: small nuclear RNA u6 (snu6)]. Rectangles indicate miR-15a and miR-16. **B**, miR-15a (**left**) and miR-16 (**right**) relative expression (measured using standard curves for miR and snU6) in PACs prepared from larger cohorts of CLI patients with and without T2D and controls. **C**, Pri–miR-15a/16-1 PAC expression (at least n=6 donors/group). **D**, Drosha and Dicer mRNA expression in PACs (at least n=3 donors/group). Data in (C) and (D) are analyzed vs the control group by the 2-ddCt method (internal control: mRNA18s). All data are mean±SEM. *$P<0.05$ and **$P<0.001$ vs healthy controls. §$P<0.05$ and §§$P<0.001$ vs CLI without T2D.
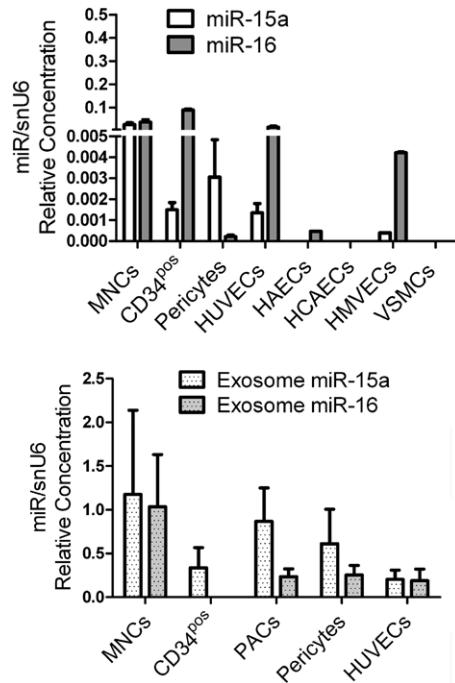
Figure 2. Expression of microRNA (miR)-15a and miR-16 in bone marrow, vascular cells, and exosomes. Bar graphs of relative miR-15a and miR-16 expression by (**A**) circulating nonselected mononuclear cells (MNCs), CD34^{pos} MNCs, and vascular cells, that is, pericytes, human umbilical vein endothelial cells (HUVECs), human microvascular endothelial cells (HMVECs), human coronary artery endothelial cells (HCAECs), and human aorta endothelial cells (HAECs; at least n=3/cell type). **B**, Exosomes purified from corresponding cell-conditioned medium (n=4 cell replicates). Data in (A) and (B) are relative expression (measured using standard curves for miR and snU6).

and IVA ii). The CCM of miR-15a/16–overexpressing PACs also failed to affect HUVEC networking on Matrigel (control: medium of PACs transfected with scramble; data not shown). Similarly, negative results were observed when the CCM from healthy PACs overexpressing pre-miRs or scramble was used to stimulate HUVECs in the 3-dimensional spheroid angiogenesis assay (Online Figure IVB)

Inhibition of miR-15a/16 Rescues the Impaired Migratory Capacity of PACs From CLI-T2DM Patients

Based on the results obtained in healthy PACs by increasing miR-15a/16, we hypothesized that lowering miR-15a and miR-16 levels could improve the functional capabilities of patient-derived PACs. Thus, T2DM-CLI PACs were transfected with anti–miR-15a and anti–miR-16. As controls, we used diseased and healthy PACs transfected with scramble. After confirmation of miR-15a and miR-16 efficient reduction by anti-miRs (Online Figure IIIB), we assayed PAC apoptosis, migratory capacity, and proangiogenic activity. As expected, apoptosis was higher in diseased PACs (Figure 4A). This defect could not be corrected by anti–miR-15a/16 intervention. As expected, diseased PACs showed impaired migration to fetal bovine serum or SDF-1α. Importantly, this could be improved by anti–miR-15a/16 (Figure 4B). CXCR4 mRNA and protein expression were similar in healthy and diseased PACs (Online Figure VA and VB, respectively). Hence, the

decreased capacity of diseased PACs to migrate toward SDF-1α does not depend on changes in receptor expression. As shown in Online Figure IVA iii and IVA iv, T2DM-CLI PACs could not compare with healthy PACs in the support offered to HUVECs in forming endothelial networks on Matrigel. However, anti–miR-15a/16 did not increase the proangiogenic capacity of diseased PACs. Furthermore, the CCM of T2DM-CLI PACs showed impaired capacity to stimulate HUVEC sprouting in the spheroid assay, which could not be rescued by anti–miR-15a/16 (Online Figure IVB). Finally, PAC growth in culture was not affected by disease or forced changes in miR-15a and miR-16 expression (Online Figure VI).

VEGF-A and AKT3 Are Direct Targets of Both miR-15a and miR-16

VEGF-A was predicted as direct target of both miR-15a and miR-16 (by 6/9 prediction software searched using miRwalk). AKT-3 was predicted as direct target of miR-15a (7/9 software) and miR-16 (6/9 software). We validated that VEGF-A and AKT-3 are direct targets of miR-15a and miR-16 using 3′ untranslated region luciferase assays (Figure 5A). The significant decrease in luciferase activity in the presence of miR-15a and miR-16 confirmed the binding to the 3′ untranslated region of VEGF-A and AKT3. Furthermore, this effect was reverted by mutations in the putative miR-binding sites. In control PACs, pre–miR-15a/16 did not change AKT3 and VEGF-A mRNA expression (Figure 5B) but reduced intracellular AKT3 and VEGF-A protein levels (Figure 5C) and secreted VEGF-A (Figure 5D). The active, Ser(473)-phosphorylated, form of AKT was also decreased (Figure 5C), pointing to a deregulation of AKT-associated signaling pathway induced by miR-15a and miR-16. Expression of AKT1 and AKT2 was not affected by pre–miR-15a/16 (data not shown). Next, we sought confirmation of similar miR-15a/16–associated changes in patient-derived PACs. Importantly, PACs from CLI patients with/without T2DM showed lower RNA and protein expression of AKT-3 and VEGF-A. Phospho-AKT was also decreased (Figure 6A and 6B).

miR-15a and miR-16 Modulate the Therapeutic Potential of PACs in a Mouse Model of Limb Ischemia

Next, we sought in vivo confirmation of the in vitro evidence suggesting a role for miR-15a and miR-16 in controlling PAC functions. Control PACs were engineered ex vivo to either increase or reduce the expression of miR-15a and miR-16 and then were transplanted into the ischemic adductor muscles of nude mice. Control groups consisted of mice receiving PACs transfected with scramble or no cells (substituted by the fresh cell medium). As shown in Figure 7, anti–miR-15a/16 PACs increased BF recovery and arteriole density in the ischemic adductor. In contrast, mice transplanted with pre–miR-15a/16 PACs showed impaired BF recovery and reduced muscular capillary number in comparison with mice given scramble-transfected PACs.

Circulating miR-15a and miR-16

As shown in Figure 8A, both miR-15a and miR-16 were detectable in the PB of nonischemic and nondiabetic subjects, especially in platelet-rich plasma. Furthermore, the 2 miRs also were found in the plasma as part of exosomes (Figure 8B) and
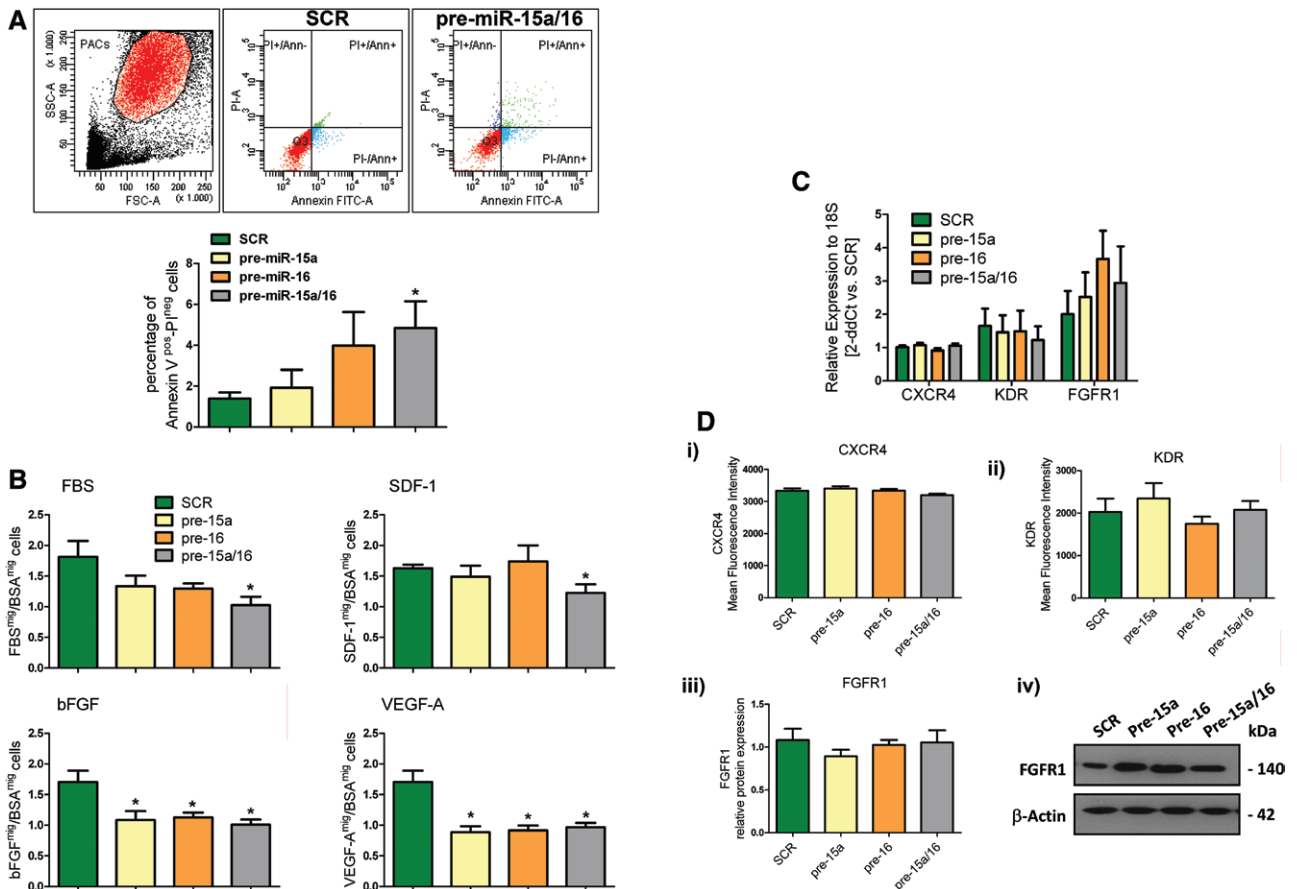
Figure 3. Functional effects of microRNA (miR)-15a and miR-16 overexpression in control proangiogenic circulating cells (PACs). **A**, Apoptosis expressed as percentage of annexin V^{pos}/PI^{neg} cells in healthy PACs (n=5/group). **B**, Migration (in transwell chamber assay) of PACs toward fetal bovine serum (FBS), stromal cell–derived factor-1α (SDF-1α), basic fibroblast growth factor receptor (bFGF), or vascular endothelial growth factor (VEGF)-A (n=5 donors/group assayed in triplicate). **C**, CXC chemokine receptor, type 4 (CXCR4), kinase insert domain receptor (KDR), and FGF receptor-1 (FGFR1) mRNA levels (Taqman real-time reverse-transcriptase polymerase chain reaction) in transfected PACs. **D**, CXCR4, KDR, and FGFR1 protein levels: (**i**) CXCR4 and (**ii**) KDR mean fluorescence intensity (measured by flow cytometry, n=6/group); (**iii** and **iv**) FGFR1 protein levels measured by Western blot (n=9/group); (**iv**) representative Western blot bands. All data are expressed as mean±standard error of the mean. *P<0.05 vs PACs transfected with scramble negative control (SCR).

conjugated to argonaute-2 (Figure 8C), which are regarded as 2 transport systems preserving miR stability. Our aforementioned clinical trial that has supported the PAC study was designed to prepare serum only. Interestingly, miR-15a and miR-16 were increased in the serum of CLI patients with and without T2DM (Figure 8C). No correlation was found between miR-15a or miR-16 levels in PACs and serum (data not shown).

Circulating miR-15a Levels Are Positively Associated With Increased Risk of Adverse Events in T2DM Patients Undergoing Revascularization for CLI

Finally, we conducted statistical analyses to assess the potential value of miR-15a and miR-16 (expressed in either PACs or serum) in predicting adverse events (restenosis and/or amputation) after revascularization. miR expression (both PACs and serum) distribution was similar between CLI cases with and without associated T2DM. Because we could enroll more diabetic than nondiabetic patients in our trial, the T2DM-CLI group (whose characteristics are reported in Online Table III) was used for analysis attempting to correlate miR expression with clinical events. Online Table IV reports the incidence of events recorded

at the 12-month follow-up in our study group. As shown in Online Table V, after the adjustment for the patient age and sex, the risk of an amputation after restenosis positively correlated with relative abundance of serum miR-15a (OR, 2.10; P=0.002) and serum miR-16 (OR, 2.07; P=0.012). Serum miR-15a also was positively associated with post-revascularization restenosis considered as first event (OR, 1.28; P=0.04), but serum miR-16 was not (OR, 0.96; P=0.75). In contrast, miR-15a and miR-16 in PACs were not significantly associated with amputation after restenosis or restenosis alone. Noteworthy, for technical and logistic issues, the PAC samples were less numerous than the serum samples (Figures 1B and 8D show sample sizes). Other potential confounding variables, such as the presence of hypertension, neuropathy, coronary artery disease, smoking, statin, and insulin treatment, were not associated with the risk of restenosis in our population (data not shown), and therefore they were not considered as confounders in multivariable analysis.

miR-15a Does Not Affect VSMC Proliferation and Migration

Restenosis involves increased VSMC proliferation and migration.[26] Because miR-15a levels were associated with the risk of
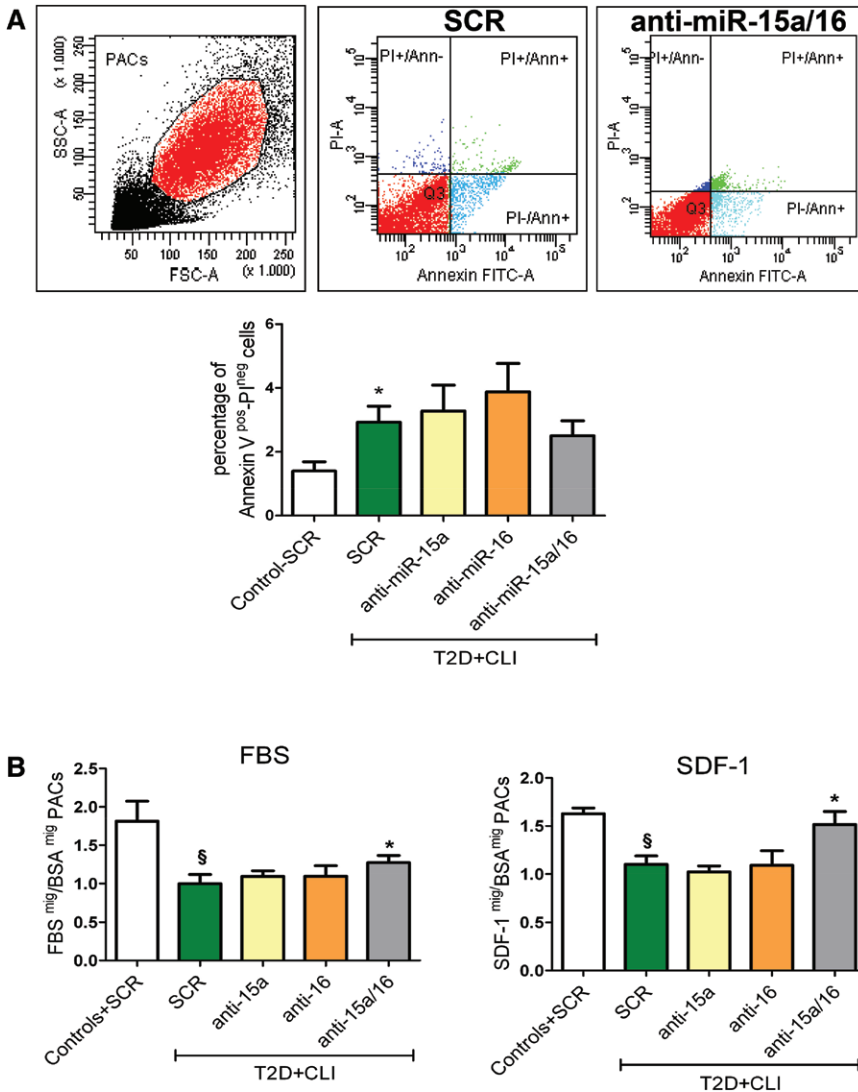
Figure 4. MicroRNA (miR)-15a and miR-16 inhibition improves type 2 diabetes mellitus (T2D) and critical limb ischemia (CLI)- proangiogenic circulating cell (PAC) migration. Untreated T2D-CLI PACs were compared with control healthy PACs for their apoptosis rates and then transfected with anti–miR-15a, anti–miR-16, their combination, or scramble (SCR). SCR healthy PACs served for reference. **A**, PAC apoptosis measured as percentage of annexin V$^+$/PI$^-$ cells (n=4/group); *$P<0.05$ vs SCR healthy PACs. **B**, PAC migration toward fetal bovine serum (FBS) and stromal cell–derived factor-1α (SDF-1α); PACs from n=5 subjects/group, each assayed in triplicate); §$P<0.05$ vs SCR healthy PACs; *$P<0.05$ vs SCR-T2D-CLI PACs. All data are expressed as mean±standard error of the mean.

of restenosis, we characterized the effect of miR-15a over-expression on VSMC proliferation and migration. However, miR-15a inhibited VSMC proliferation and migration (Online Figure VI).

Discussion

This study shows for the first time to our knowledge that several miRs are differently expressed in PACs enriched from the PB of patients with CLI. Furthermore, we have identified miR-15a and miR-16 as critical regulators of in vitro survival and migration and in vivo therapeutic potential of PACs. In addition, we provide preliminary evidence that increased circulating levels of miR-15a and miR-16 are associated with higher risk of limb amputation and restenosis in T2DM patients undergoing percutaneous angioplasty for CLI.

miRs have recently emerged as key regulators of endothelial cell functions and angiogenesis.[15] In addition, miRs are important for both maintaining stem cell pluripotency and inducing stem cell vascular differentiation.[27] Furthermore, we recently showed that miR-132 is essential for therapeutic proangiogenic actions of pericyte progenitor cells.[17] A few

studies highlight the differential expression of a subset of miRs in PACs in patients with heart disease,[28–30] but investigations have not yet been extended to include patients with peripheral vascular diseases, let alone CLI. A recent report shows that miR-126 is downregulated in PACs isolated from diabetic patients without vascular complications.[29] In addition, circulating levels of several miRs are modified by diabetes mellitus.[31] We found that plasma miR-503 increased in diabetic patients with CLI undergoing limb amputation. Notwithstanding, no other miR has been measured in the circulation of CLI patients. These facts outline the novelty and importance of this study.

Increased miR-15a levels in PACs and serum of CLI patients and in PACs cultured under hypoxia are in line with the higher miR-15 expression found in the ischemic pig and mouse myocardium by Hullinger et al.[21] We additionally found pri–miR-15a/16-1 and mature miR-16 expression to be increased by CLI in PACs. Recent studies described an active transcriptional regulation of miR-15a/16-1 and miR-15b/16-2 by E2F1 and E2F3.[32] Interestingly, E2F1 acts as a negative regulator of postischemic angiogenesis by inhibiting VEGF-A expression.[33]
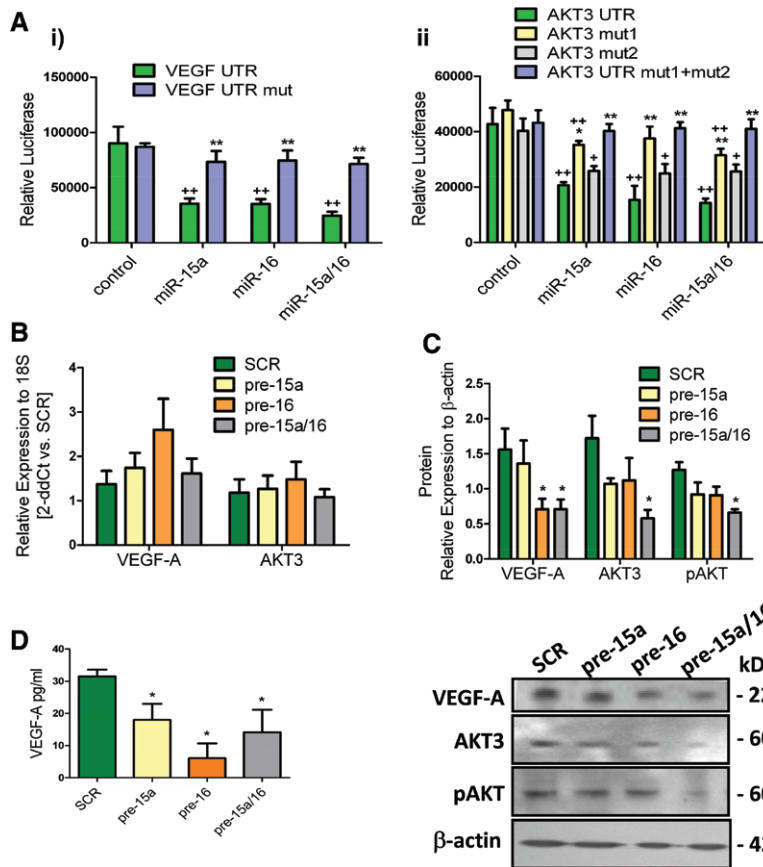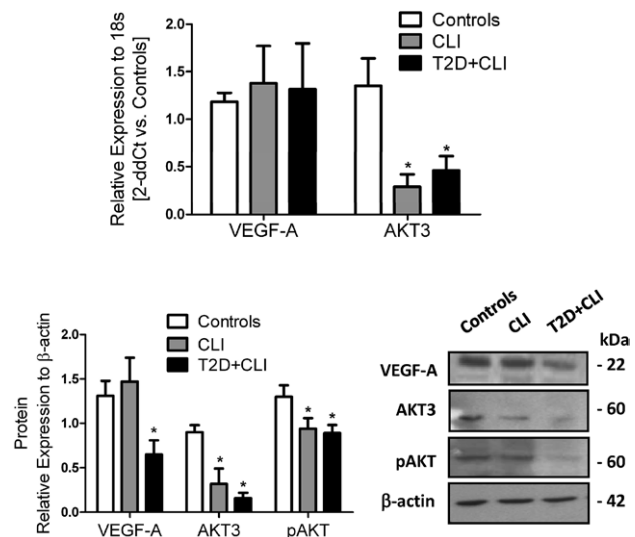
Figure 5. MicroRNA (miR)-15a and miR-16 directly target AKT3 and vascular endothelial growth factor (VEGF)-A. A, Bar graphs showing average luciferase assay data obtained in human embryonic kidney (HEK) 293 cells transfected with plasmid carrying the 3-untranslated region (UTR) sequence for (**i**) VEGF-A wild type or its mutated form (mut) and (**ii**) AKT3 wild type and 2 mutated forms (mut1 and mut2) or their combination (mut1+mut2) (n=4; *$P<0.05$ and **$P<0.01$ vs scramble (SCR) control; +$P<0.05$ and ++$P<0.01$ vs nonmutated corresponding to 3′-UTR). **B**, VEGF-A and AKT3 mRNA levels (Taqman real-time reverse-transcriptase polymerase chain reaction) in healthy proangiogenic circulating cells (PACs) transfected with pre–miR-15a, pre–miR-16, their combination, or SCR; (n=9/group). **C**, Results of VEGF-A, AKT3, and phospho-AKT (pAKT) protein analysis (left) by Western blot (n=4/group) and representative Western blot bands (right). **D**, VEGF-A content (measured by enzyme-linked immunosorbent assay) in the conditioned medium of transfected PACs (n=6/group). Data are mean±standard error of the mean. *$P<0.05$ vs SCR.

Guided migration of PACs from the BM to the PB and then from the PB to the ischemic site is essential for the cells to exert their regenerative functions. Both ischemia and diabetes

Figure 6. AKT3 and vascular endothelial growth factor (VEGF)-A are altered in proangiogenic circulating cells (PACs) from critical limb ischemia (CLI) patients. A, VEGF-A and AKT3 mRNA levels in PACs of patients with CLI with and without type 2 diabetes mellitus (T2D) and healthy PAC control (n=6 donors/group). **B**, Results of VEGF-A, AKT3, and phospho-AKT (pAKT) protein analysis (left) by Western blot (at least n=3/group) and representative Western blot bands (right). Data are expressed as mean±standard error of the mean. **$P<0.001$ and *$P<0.05$ vs healthy PACs.

mellitus compromise PAC migratory capacity.[22,34] Thus, it is significant that miR-15a and miR-16 negatively regulate PAC migration toward a series of chemotactic stimuli, including basic FGF, VEGF-A, and SDF-1α, which are produced by limb muscles in response to ischemia.[35,36] In PACs, miR-15a/16 did not reduce FGF-R1, KDR, or CXCR4 expression, which suggests that the impairment in migration was dictated by post-receptor mechanisms, possibly including deficit in AKT-3 leading to altered phospho-Akt levels.[37] Different from what we observed in PACs, HUVECs overexpressing miR-16 showed reduced levels of both FGF-R1 and KDR.[20] Furthermore, in the same study, HUVEC proliferation was inhibited by pre–miR-16. At difference, PAC growth is not affected by miR-16. These discrepancies can be explained with the differences in cells used in the 2 studies. For example, HUVECs have a higher in vitro proliferative capacity than PACs.[38] Both studies show that pre–miR-16 downregulates VEGF-A. Nonetheless, the paracrine proangiogenic activities of PACs were not influenced by manipulating miR-15a or miR-16. It is possible that the impact of the 2 miRs on VEGF-A alone is not sufficient to alter the proangiogenic property of the PAC secretome, which is composed of a plethora of factors.

Transplantation of PACs ex vivo–manipulated with pre–miR-15a/16 and anti–miR-15a/16 influenced the postischemic recovery of mice with limb ischemia. From the aforementioned in vitro data, we can speculate that the therapeutic benefit achieved by miR-15/16 inhibition in PACs derives from improved survival and migratory capacities of the cells and not from changes in paracrine
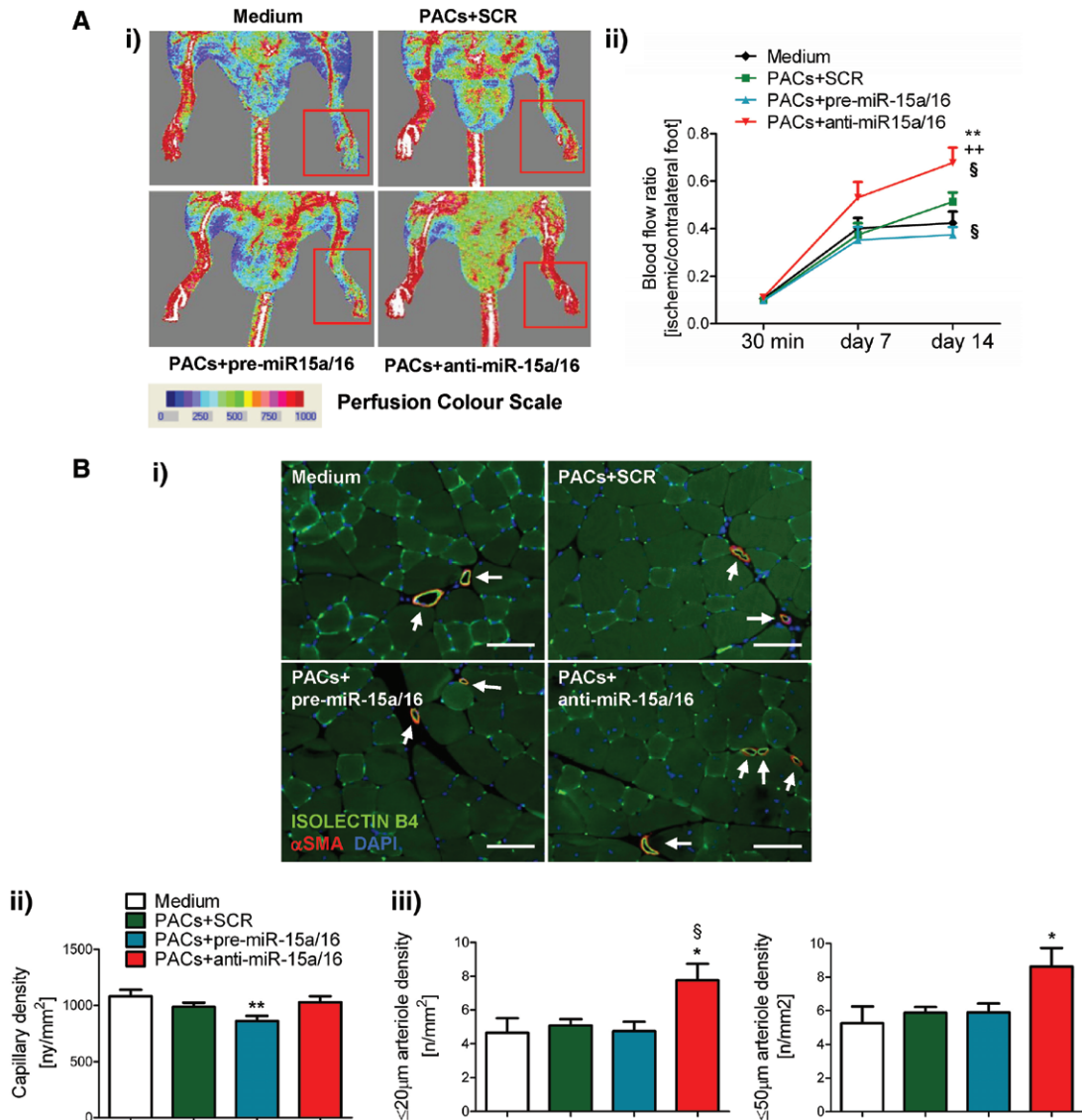
Figure 7. Ex vivo microRNA (miR)-15a/16 manipulation affects the in vivo proangiogenic potential of proangiogenic circulating cells (PACs). Nude mice (n=11–14/group) with unilateral limb ischemia received engineered PACs or fresh cell medium in the ischemic adductor muscle. **A**, Postischemic blood flow (BF) recovery: (**i**) representative color laser Doppler flowmetry images at 14 days from surgery (red squares delimit the area of measurement in the ischemic foot). **ii**, Time course of BF recovery (calculated as the ratio of BF in ischemic to contralateral foot). **B** (**i**), Representative ischemic muscle sections stained with the endothelial marker isolectin B4 (green fluorescent) and an antibody for α-smooth muscle actin (red fluorescent) to identify smooth muscles cells in the arterial wall (scale bars, 100 µm); (**ii**) bar graphs showing capillary and arteriole (≤20 µm and ≤50 µm diameter) density in ischemic adductor muscles at 2 weeks postischemia. Data are expressed as mean±SEM. **P<0.01 and *P<0.05 vs cell medium; §P<0.05 vs scramble PACs; ++P<0.01 vs pre–miR-15a/16 PACs.

actions. Taken together, our PAC data allow proposing that miR therapeutic could be a meaningful strategy to empower PACs before autologous transplantation in patients with CLI. Furthermore, ex vivo miR therapeutics should help in minimizing off-target and side effects, thus facilitating the entrance of small RNA-targeting approaches in the arena of clinical vascular medicine.

A limitation of our study is that the age and sex differences between healthy and diseased donors may introduce a bias in the interpretation of the miR screening data. However, the results we obtained after manipulating miR-15a and miR-16 in PACs and the in vivo confirmation of the crucial role of these miRs in controlling PAC functions strengthen our hypothesis

that they are new potential targets for autologous cell therapy in CLI patients.

The interest toward the use of miRs as diagnostic and prognostic biomarkers is growing. Here, we have found that miR-15a and miR-16 correlate with adverse events at 12-month follow-up in T2DM patients who have undergone percutaneous angioplasty to correct CLI. However, this preliminary finding must be confirmed in larger patient populations, including subjects of different ethnic backgrounds and those affected by several comorbidities. In fact, differences in patient characteristics could alter a series of processes that determine circulating miR values. These processes would include miR transcription,
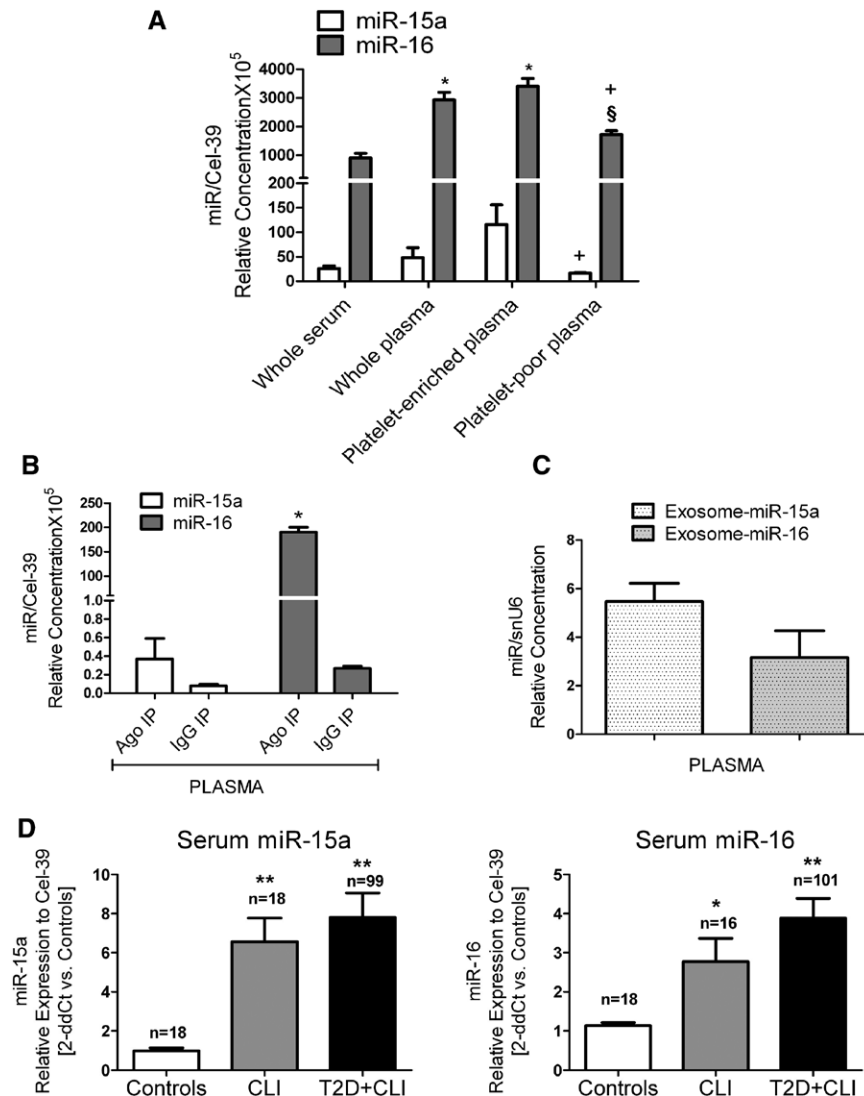
Figure 8. Circulating microRNA (miR)-15a and miR-16. miR-15a and miR-16 relative expression (measured using standard curves for the 2 miRs and Cel-39) in (**A**) serum and fractions of plasma of healthy (n=4; *$P<0.05$ vs whole serum, § vs whole plasma, and + vs platelet-enriched plasma) (**B**) immunoprecipitated (IP) fractions of healthy donor plasma with argonaute-2 (Ago-2) protein and IgG negative control, respectively (n=4; *$P<0.05$ vs IgG negative control). **C**, miR-15a and miR-16 relative expression (measured using standard curves for miR and snU6) in exosomes isolated from healthy control plasma (n=4 donors). **D**, Serum expression of miR-15a (**left**) and miR-16 (**right**) measured in critical limb ischemia (CLI) patients with and without type 2 diabetes mellitus (T2D) and controls. Data were reported to the control group by the 2-ddCt method (internal control: Cel-39). Data are mean±SEM. **$P<0.001$ and *$P<0.05$ vs healthy controls.

maturation, release from cells to the circulation, and uptake from the circulation by other cells, as well as excretion. Furthermore, the data on circulating miR-15a and miR-16 leave several questions open, including their cellular sources, the stimuli triggering and the mechanisms permitting their extracellular release, their form of transportation, and their putative function at local (ischemic muscles?) or at distance communicators.[39] We have attempted to investigate some of these points, and here we provide evidence that miR-15a and miR-16 are differently expressed in BM-derived cells (including PACs) and vascular cell types. In culture, some of these cells release exosomes containing miR-15 and miR-16. Importantly, exosomes prepared from the plasma of healthy donors contain both miRs. Among the 2 miRs, miR-15a seems less expressed in cells, but is enriched in exosomes. This is in line with the level of miR-15 in plasma exosomes. The release of miR-15a–containing and miR-16–containing exosomes from a cell type when cultured in vitro suggests the hypothesis that in vivo cells of the same lineages may contribute to the pool of circulating miR-15a and miR-16.

Notwithstanding, additional efforts are necessary to track the cell/tissue origin of PB circulating exosomes. At difference with miR-15a, miR-16 is also present in the healthy blood as conjugated to argonaute 2. These preliminary data should foster more research into the mechanisms and significance of in vivo miR release from cells and tissues and the impact of diseases. Noteworthy, miR-16 was found to be prevalently expressed in plasma, which could have influenced the evaluation of its biomarker potential in our patients (of whom only serum was available). This additionally suggests the convenience of simultaneous collection of different blood derivates when studying miR potential as novel biomarkers.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Proangiogenic circulating cells (PACs) can contribute to postischemic vascular repair.
- MicroRNAs (miRs) inhibit the expression of their target genes by acting at the posttranscriptional level.
- miRs play essential roles in vascular development and repair.
- Hypoxia and ischemia regulate the expression of some miRs (including miR-15a).

What New Information Does This Article Contribute?

- miR-15a and miR-16 impair PAC survival and migration in vitro. Ex vivo inhibition of the 2 miRs enhances the therapeutic potential of human PACs in a mouse model of limb ischemia.
- miR-15a and miR-16 levels are increased in PACs and serum prepared from patients with critical limb ischemia.
- Serum levels of miR-15a and miR-16 are positively associated with postrevascularization restenosis and amputation in patients with critical limb ischemia.

New therapeutic approaches for critical limb ischemia are urgently needed. Cell therapies have been tested in clinical studies with promising results, but disease-related molecular defects might limit the success of cell transplantation. Here, we show that critical limb ischemia affects the expression of several angiogenesis-related miRs in PACs, including miR-15a and miR-16 that were found to be upregulated. Importantly, both miRs diminish PAC survival and function, possibly by reducing the expression of their target genes vascular endothelial growth factor-A and AKT-3. Furthermore, we found that nonviral delivery of anti–miR-15a and anti–miR-16 oligonucleotides improves the capacity of human PACs to promote blood flow recovery and angiogenesis in ischemic limb muscles. Finally, we provide evidence that serum miR-15a and miR-16 levels are increased by critical limb ischemia, and they are positively associated with adverse events after revascularization in a cohort of 122 patients. Although validation in a larger cohort of patients is needed, the findings of this study suggest that miRs could be biomarkers for the prediction of clinical outcomes in patients with critical limb ischemia.

Spinetti *et al.*

MicroRNA-15a and microRNA-16 Impair Human Circulating Pro-Angiogenic Cell (PAC) Functions and are increased in the PACs and Serum of Patients with Critical Limb Ischemia

Online Supplements

Detailed Methods

Clinical study
ClinicalTrials.gov: NCT01269580, Title: Diabetic Foot and Vascular Progenitor Cells. The study was conducted on patients with critical limb ischemia (CLI) with or without type 2 diabetes mellitus (T2D) enrolled at the time of percutaneous angioplasty (PTA) for CLI. CLI was defined according to TASC criteria (2007). Exclusion criteria were drug-induced diabetes, liver failure or dialysis due to renal failure, cancer, chemotherapic treatment, pregnancy, lack of consent to participate to the study. CLI patients were visited at a 12 month follow up to monitor for the occurrence of selected events (i.e. mortality, major amputation, and restenosis in treated limb). The primary endpoint of the trial is defining the potential prognostic value of the altered number and migratory ability of antigenically characterized PACs for the evolution of major cardiovascular endpoints at 12 month follow up (June 2012). The main results of the study will be the subject of a separate manuscript.

miR transfection
PACs were transfected with 50 nmol/L pre-miR mimics (pre-miR-15a and pre-miR-16) or with 50 nmol/L miR inhibitors (anti-miR-15a and anti-miR-16), or negative control (a non-targeting sequence, also identified as scramble, SCR, throughout the manuscript) (all from Applied Biosystems) using GeneSilencer (Dharmacon) following the manufacturer's instructions. Using the same protocol, vascular smooth muscle cells (VSMCs) were transfected with 50 nmol/L pre-miR-15a or SCR. When pre- or anti-miR15a and -16 were transfected together in PACs, 25 nmol/L each was used to reach the final 50 nmol/L concentration. Mimic and inhibitor concentrations were selected based on pilot concentration-response experiments, in which the changes in relative miR-15a and -16 expression were measured by TaqMan PCR (*vide infra*). In parallel experiments, we assessed the efficiency of PAC transfection by transfecting fluorescently-labeled miR-mimic (miR-mimic-Pe-Cy3) (Applied Biosystems). The percentage of transfected PACs was greater than 95%.

Cell culture
To prepare PACs, PB (35ml) was withdrawn from forearm vein puncture and MNCs were separated on Ficoll-Paque PLUS (Amersham Biosciences) gradient at 400g. To ensure PACs enrichment, MNCs ($1x10^7$/well) were plated on fibronectin (Sigma)-coated 6-well plates (BD Falcon) and cultured in EBM-2, supplemented with EGM-2 MV SingleQuots and 10% FBS (all from Cambrex) for 4 days. Pericyetes were prepared from human vena saphena as previously described[1]; human vascular smooth muscle cells (VSMCs), human umbilical vein endothelial cells (HUVECs), human microvascular ECs (HMVECs), human coronary artery EC (HCAECs) and human aorta ECs (HAECs) were all purchased by Lonza and cultured accordingly to Lonza protocols.

Exosome isolation
Cell conditioned medium (CCM) or plasma were processed for exosome collection and ultrapurification, as described[2]. For CCM, cells were removed by centrifugation ($500g$, 5 min), then CCM or plasma were clarified by centrifugation ($2000g$, 30 min followed by $12000g$, 45 min at 4℃). Exosomes were collected by ultracentrifugation ($110000g$, 2 hours),

washed in PBS and pelleted. The purified exosome fraction was re-suspended in PBS for use. Exosome purity was confirmed by flow cytometric analysis. Briefly, exosomes were conjugated to 4µm latex beads/aldehyde sulphate for easy detection as described previously[3]. Exosome-coupled beads were washed in PBS/BSA 0.5%, stained with AnnexinV (FITC) and anti-CD63 (APC) as exosomes markers for 15min at room temperature. Stained beads-conjugated exosomes were analyzed in a FACSCanto flow cytometer using the FACSDiva software (both from BD Biosciences).

RNA extraction and TaqMan quantitative Real Time PCR

RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The concentration of total RNA was determined using the Nanodrop ND1000 Spectrophotometer (Thermo Scientific) and the size and integrity of RNAs was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies).

For serum and plasma analyses, 10 mL of peripheral venous blood was collected. Half of this was placed in a citrate tube (BD) containing anticoagulants. The remainder was kept in plain tubes without an anticoagulant. 'Whole plasma' was obtained by centrifugation of the citrated blood (1500 rpm, 15 min, 4°C). Some of this plasma (1 mL) underwent a second centrifugation (14000 rpm, 15 min, 4°C) to form a platelet pellet. The top 850 µL was removed ('platelet-poor plasma') and the remaining fraction (including the platelet pellet) was also saved ('platelet-enriched plasma'). Serum was collected by allowing the blood in the plain tube to coagulate at room temperature for 30 min, followed by centrifugation (1500 rpm, 15 min, 4°C). RNA was extracted using 100 µl of input fluid and the miRNeasy kit (Qiagen), with 25 fmol of the synthetic C.elegans-miR-39 (cel-39) spiked-in as a normalizer, as described by Kroh E *et al.* 2010[4].

Argonaute-2 (Ago-2) immunoprecipitation from whole plasma was performed using the method described above by Arroyo *et al*[5]. In brief, a monoclonal antibody to Ago-2 (Abcam) (or non-immune mouse IgG as control) was conjugated with magnetic anti-mouse IgG beads. A 1:1 dilution of plasma and PBS (100 µL each) was incubated with the conjugated antibody at 4°C overnight. Following this, the beads were washed and the RNA extracted as described above.

RNA reverse transcription to measure miRs was performed with the TaqMan miR reverse transcription kit following the manufacturer's instructions (Applied Biosystems). miR expression was analyzed by the Applied 7900 Real Time PCR System and normalized to the U6 small nucleolar RNA (snRU6) for PACs and/or synthetic cel-miR-39 (Qiagen) for serum and plasma. For gene expression analyses, single-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using TaqMan Reverse Transcription reagents (Applied Biosystems). Quantitative RT-PCR was performed with the Applied 7900 Real Time PCR System (Applied Biosystems) using the following primers: 18s rRNA (forward 5'-CGCAGCTAGGAATAATGGAATAGG-3'; reverse 5'-CATGGCCTCAGTTCCGAAA-3'), AKT3 (forward: 5'-GCAGAGGCAAGAAGAGGAGA-3'; reverse: 5'-ACTTGCCTTCTCTCGAACCA-3'), FGFR1 (forward 5'-CCTCTATGTGGGCATGGTTT-3'; reverse 5' TACAGGAAGGACGATCTGGG-3'), VEGF-A (forward: 5'-CCCACTGAGGAGTCCAACAT-3'; reverse: 5'-AAATGCTTTCTCCGCTCTGA-3'), CXCR4 (forward: 5'-GGTGGTCTATGTTGGCGTCT-3'; reverse: 5'-TGGAGTGTGACAGCTTGGAG-3'); KDR (forward: 5'-GTGACCAACATGGAGTCGTG-3'; reverse: 5'-TGCTTCACAGAAGACCATGC-3').3') Dicer (forward: 5'-ACCAAGTTGCATTTCGGTAT-3', reverse: 5'-AGGAAATTTCGAGCACATGA-3'), Drosha (forward: 5'-CACCTGTTCTAGCAGCTCAGAC-3', reverse: 5'-CTCCTCCCACTGAAGCATATTG-3'), Pri-mir15a-16-1 (forward: 5'-AAGGTGCAGGCCATATTGTG-3', reverse: 5'- AAGGCACTGCTGACATTGC-3'). Data were normalized to 18S ribosomal RNA as an endogenous control.

For both miR and gene expression, each PCR reaction was performed in triplicate and analyzes were performed by either the 2–ddCt method[6, 7] or after obtaining relative miR abundance, using a standard curve built on serial dilutions of synthetic mature double-stranded miR templates (Ambion). In the latter case, data were expressed as mean relative

quantity versus internal control (namely snU6 for cells and exosomes or cel-miR-39 for serum and plasma). Concentrations of each miR were calculated from the standard curve linear regression line using the following formula: $10^{-(Ct-Y\ intercept)/slope\ value)}$, where Ct represents the threshold cycle value[8, 9]. Values were then normalized to internal control using [miR]/[control].

Luciferase assays
To investigate whether VEGF-A, and AKT-3 are direct targets of miR-15a and miR-16, the 3'-UTR of the potentials target genes were inserted downstream of a luciferase open reading frame (pLUC). VEGF-A 3'-UTR (SC217121) vector was purchased from Origene and AKT3 3'-UTR (S811011) from SwitchGear Genomics.
Conserved binding sites in VEGF-A and AKT-3 3'UTR were identified using TargetScan 6.2 (http://www.targetscan.org). Binding sites are: VEGF-A position 292-299; AKT-3 positions 235-242 and 3041-3048. For controls, we prepared similar vectors in which five nucleotide mutations were inserted in the 3'-UTR sequences complementary to the miR-15a/16 binding sequences. For AKT-3, plasmids with a single or double mutation in the 3'-UTR were prepared. Primers for mutation are reported in **Online Table VI**. HPLC-purified oligonucleotides (Sigma) were used for mutagenesis, performed with Pfu enzyme following the *in vitro* mutagenesis kit protocol (Invitrogen). The different luciferase constructs were transfected into HEK293 cells together with pre-miR-15a or pre-miR-16 or both or a scrambled oligonucleotide sequence (control). Cells were cultured for 48 hours and assayed with the Dual-Luciferase Reporter Assay System (Promega). Values were normalized using Renilla expression level.

Western Blot analyses
PAC proteins were extracted by incubation with lysis buffer containing 50 mmol/L Hepes, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 25 mmol/L NaF, 5 mmol/L NaPPi, 1%Triton, 1% NP40, 1mmol/L Na_3VO_4, 0.25% sodium deoxycholate, 0.5 mmol/L Na-orthovanadate, 1 mmol/L benzamidine and 0.1 mmol/L phenylmethylsulfonyl fluoride. Thirty micrograms of protein were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (PVDF, GE Healthcare, Slough, UK) to be probed with the following antibodies: AKT3 and pAKT (both 1:1000), VEGF-A (1:500), KDR (1:1000), FGFR1 (1:1000), Phospo-eNOS (ser1177) (1:1000) (all from Cell Signaling), eNOS (1:1000, Santa Cruz), BCL2 (1:1000, Dako) and β-actin (1:5000, Santa Cruz Biotechnology) (used as loading control). For detection, goat anti-rabbit or goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (GE Healthcare, 1:2000) were used. Detection was developed by a chemiluminescence reaction (ECL, GE Healthcare).

Flow Cytometry
PACs ($5x10^5$) were stained with appropriate fluorescent conjugated antibodies: CD34 (PE-Cy7), CD45 (APC-H7), CD14 (FITC), CXCR4 (APC) all from BD Biosciences, and KDR (PE) from R&D. After 15 min incubation at room temperature in the dark, cells were washed, resuspended in PBS and analyzed. For each test, $1x10^5$ to $5x10^5$ total events were analyzed using a FACSCanto flow cytometer with the FACSDiva software (both BD Biosciences).

Migration assay
For PACs migration assay 5 μm pore-size filter-equipped transwell chambers (Corning) coated with fibronectin were used. Cells ($7.5x10^4$) were placed in the upper chamber and allowed to migrate toward SDF-1a (R&D) (100 ng/mL), FBS (10% in EBM medium), VEGF-A (100 ng/mL), bFGF (100 ng/mL), or BSA (control) for 16 hours at 37°C. The cells on the upper part of the filter were scraped away before fixing the filter. The lower side of the filter (containing the migrated cells) was mounted with Vectashield containing DAPI. For each chamber, migrated cells were counted in 5 random fields at 20X magnification. Migration data are expressed as the number of cells migrated toward the specific chemoattractant vs. the number of cells migrated in the absence of stimulus (i.e. cells migrated to BSA).

Vascular smooth muscle cell (VSMC) migration was evaluated by scratch assay. VSMCs were transfected with premiR-15a or scramble (each at 50 nmol/L). A spatula was used to make a scratch in the cell monolayer. Cells were treated with hydroxyurea (2 mmol/L, Sigma) to arrest cell proliferation and incubated with 10% FBS/DMEM. Pictures were taken immediately after scratching and 6, 12 and 24 hours thereafter. Gap closure was quantified using captured microscopic fields (magnification 4X). Experiments were repeated 4X.

VSMC proliferation by BrdU incorporation assay
After 24 hours of transfection, VSMCs over-expressing miR-15a or scramble were seeded in a 96-well plate (3×10^3 cells per well) and treated for 12 hours with 0.5% FBS/DMEM. The medium was then replaced by 10% FBS/DMEM with BrdU (10 µmol/L). BrdU incorporation was measured by the BrdU ELISA assay kit (Roche) after 6, 12 and 24 hours of stimulation with high FBS. Experiments were repeated 4X.

In vitro angiogenesis
Capillary-like network formation Assay: 5×10^4 PHK67 (Sigma) stained, FITC-labelled PACs were added to 8-well chamber slides pre-coated with 150 µL Matrigel (Becton Dickinson), together with 50×10^3 PKH26 red-stained (Sigma) HUVECs in a total volume of 150 µL EBM-2 with 0.1% BSA. After 16 hours incubation at 37°C, floating cells were removed by gentle washing and the adherent cells were fixed in 2% paraformaldehyde and treated with DAPI containing PBS. The assays were performed in duplicate wells. PACs effect on network formation from HUVECs was measured by counting the 1) number of intersection points, 2) average and total tube length, and 3) percentage of adherent PACs, in 5 microphotographs of random view fields (magnification 20X). Fluorescence was visualized and captured using an AXIO OBSERVER A1 microscope equipped with digital image processing software (AxioVision Imaging System), both from Zeiss. Similar assays were repeated adding the conditioned medium of PACs to the HUVECs.
Spheroid assay: 3D angiogenesis assay (with CCM of PACs and HUVECs) was performed according manufacturer's instructions (PromoCell).

Apoptosis assay
Apoptosis was measured by quantifying the percentage of Annexin V[pos]/PI[neg] PACs by flow cytometry. In brief, transfected PACs (2×10^5 cells) were stained with 5 µL of Annexin V (BD Biosciences) and 1 µL of propidium iodide (PI) in 200 µL binding buffer for 15 min at room temperature in the dark. After incubation, cells were suspended in 150 µL of Annexin Binding Buffer and the percentage of Annexin V[pos]/PI[neg] PACs was assessed using FACS Canto flow cytometer and FACS Diva Software (both from BD Biosciences).

ELISA
VEGF protein levels were measured in PACs conditioned to a medium level using a commercially available ELISA kit (R&D). Briefly, transfected PACs were cultured for 24 hours in EBM-2 medium in the absence of serum, then CM was harvested, centrifuged at 2000 rpm for 10 min at RT, and stored at -20°C until used. CM (200 µL) was assayed for VEGF concentration according the manufacturer's instructions.

Analyses of post-ischemic blood flow recovery and muscular microvessel density in nude mice with limb ischemia
Immunocompromised CD1-*Foxn1*nu mice (Charles River, UK, n=11 to 14 mice/group) underwent unilateral limb ischemia as previously reported[10] and were immediately transplanted with engineered PACs in their ischemic adductor muscle. Post-ischemic foot blood flow recovery was measured at 30 minutes, 7 days and 14 days after ischemia by using a high resolution laser Doppler imaging system (MoorLDI2, Moor Instruments, Axminster, UK). At 14 days post-surgery, the limbs of terminally anesthetized mice were perfusion-fixed and ischemic adductor muscles harvested for histological analyses. Evaluation of capillary and arteriolar density was performed in transverse muscular sections

(5 µm thick) after fluorescent immunohistochemical staining for α-smooth muscle actin (α-SMA, Sigma) to identify muscularized blood vessels (and hence arterioles) and with fluorescent isolectin-B4 (Invitrogen), which binds to endothelial cells. High power fields were captured (at 200X) using a fluorescent microscope. Arterioles were recognized as vessels with one or more continuous layers of α-SMA-positive vascular smooth muscle cells and an isolectin-B4 positive lumen. According to their luminal diameter, arterioles were also stratified as ≤20 µm and ≤ 50µm. The number of capillaries per mm^2 was evaluated in the same sections by counting the number of isolectin-B4-positive and α-SMA-negative microvessels.

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Online Table I. Characteristics of the human populations who donated their blood for this study

	Controls N=43	CLI N=20	T2D+CLI N=122	p-value
Age (years)	36.7 (7.3)	77.7 (9.7)	71.2 (9.3)	<0.0001
Gender (% males)	38	60	67	0.01

Quantitative data are expressed as mean and standard deviation (SD).
CLI=critical limb ischemia, T2D= type2 diabetes

Online Table II. Characteristics of the healthy human sub-population who donated blood for testing the effect of engineered PACs in mice with limb ischemia

	Healthy Donors (n=10)
Age (years)	35.2 (8.9)
Gender (% males)	60

Quantitative data are expressed as mean and standard deviation (SD).

Online Table III. miR and angiogenesis: evidences supporting the selection of 28 miRs screened in human PACs

miR	Expressed by EC or EPC	miR-16 family membership	Target genes with angiogeneis regulation potential	Induced by angiogenesis stimuli or inhibitors	Ref
miR-1	YES		HSP60, HSP70, KLF4	Myocardial infarction Cardiac ischemia preconditioning	He et al. 2011[11], Elia et al. 2009[12], Kane et al. 2010[10], Duan et al 2012[13]
miR-9	YES		MMP-14		Zhang et al 2012[14], Zhuang et al 2012[15]
miR-15a	YES	YES	BCL-2, VEGF, FGF2	Cardiac ischemia	Bonci et al. 2008[16], Yin et al. 2012[17], Caporali et al. 2012[18], Finnerty et al. 2012[19]
miR-15b	YES	YES	Pdk4, Sgk1	Cardiac ischemia	Liu et al 2012[20], Hullinger et al. 2012[21], Caporali et al. 2012[18], Finnerty et al. 2012[19]
miR-16	YES	YES	VEGF, KDR, FGFR	VEGF, bFGF	Chamorro-Jorganes et al. 2011[22], Poliseno et al. 2006[23], Caporali et al. 2012[18], Finnerty et al. 2012[19]
miR-17	YES		Tsp-1, TIMP-1	VEGF	Dews et al. 2006[24],

				Otsuka et al. 2008[25], Suarez et al. 2008[26], Doebele et al. 2010[27]
miR-18a	YES		VEGF	Doebele et al. 2010[27], Suarez et al. 2008[26]
miR-19a	YES		ischemia	Doebele et al. 2010[27]
miR-19b	YES	FGFR2	ischemia	Yin et al. 2012[28], Doebele et al. 2010[27]
miR-20a	YES	VEGF	VEGF	Hua et al. 2006[29], Suarez et al 2008[26] Doebele et al. 2010[27]
miR-23a	YES	Sprouty/Sema 6A		Polisenso et al. 2006[23], Larsson et al. 2009[30], Zhou et al. 2011[31]
miR-24	YES	GATA2, PAK4	Cardiac ischemia	Polisenso et al. 2006[23], Larsson et al. 2009[30], Fiedler et al. 2011[32]
miR-27	YES	Sprouty/Sema 6A		Zhou et al. 2011[31], Urbich et al. 2011[33]
miR-92a	YES	ITGB5	Limb and cardiac ischemia	Bonauer et al. 2009[34], Zhang et al. 2011[35], Doebele et al. 2010[27]

miR-100	YES		mTOR	Limb ischemia	Grundmann et al. 2011[36]
miR-103	YES	YES	Caveolin-1	T2D	Polisenso et al. 2006[23], Trajkovski et al. 2011[37], Caporali et al. 2012[18], Finnerty et al. 2012[19]
miR-107	?	YES	CDK6, Caveolin-1	T2D	Feng et al. 2012[38], Trajkovski et al. 2011[37], Caporali et al. 2012[18], Finnerty et al. 2012[19]
miR-126	YES		Spred1/PIIG3 R2		Fish et al. 2008[39], Zhang et al. 2011[35], Meng et al 2012[40], Larsson et al. 2009[30]
miR-130a	YES		HOXA5, GAX	VEGF, bFGF	Zhang et al. 2011[35], Chen et al. 2008[41]
miR-132	YES			VEGF, bFGF	Anand et al. 2010[42], Katare et al 2011[43]
miR-195	?	YES	CDK4, ELN, Col1a1, Col1a2, Check1	Aortic and heart development	Ott et al. 2011[44], Porrello et al. 2011[45], Caporali et al. 2012[18], Finnerty et al. 2012[19]

miR-221	YES		c-kit		Polisenso et al[23]. 2006, Zhang et al. 2011[35], Kuehbacher et al. 2007[46]
miR-222	YES		c-kit		Polisenso et al[23]. 2006, Zhang et al. 2011[35], Kuehbacher et al. 2007[46]
miR-296	YES		HGF	VEGF, EGF	Wurdinger et al. 2008[47]
miR-378	YES		Sufu, Fus-1		Lee et al. 2004[48]
miR-424	YES	YES	VEGFR2, FGF-R1, VEGF, MEK, CCNE1	Hypoxia, Ischemia, Hemangioma, VEGFA, bFGF	Ghosh et al. 2010, Chamorro-Jorganes et al. 2011[22], Nakashima et al. 2010[49], Caporali et al. 2012[18], Finnerty et al. 2012[19]
miR-497	?	YES	ELN, Col1a1, Col1a2	Aortic development	Ott et al. 2011[44], Caporali et al. 2012[18], Finnerty et al. 2012[19]
miR-503	YES	YES	CCNE1, cdc25A	T2D+CLI	Caporali et al.2011[7], Caporali et al. 2012[18], Finnerty et al. 2012[19]

Online Table III. Age, gender and clinical characteristics of type-2 diabetic patients at the moment they underwent angioplasty for critical limb ischemia

	T2D+CLI (n=122)
Age (years)	71.2 (9.3)
Gender (% males)	67
CAD (%)	48
Hypertension (%)	65
Neuropathy (%)	21
Retinopathy (%)	20
Ictus (%)	12
Active smoker (%)	13
HbA1c (%Hb)	7.8±1.5
Oral anti-diabetic drugs (%)	36
Insulin therapy (%)	64
Diet (%)	22
Aspirin therapy (%)	67
Clopidogrel therapy (%)	7
Anticoagulant therapy (%)	20
Statin therapy (%)	41

Quantitative data are expressed as mean and standard deviation (SD).
T2D= type2 diabetes, CLI=critical limb ischemia, CAD=coronary artery disease.

Online Table IV. Incidence of adverse events at one year follow up after angioplasty in type 2 diabetic patients described in Online Table IV (Total patients: N=122).

	N (%)
Any event	61 (50%)
Death (only)	17
Restenosis (only)	20
Amputation (only)	2
Restenosis and death	8
Restenosis and amputation	13
Restenosis and amputation and death	1
No event	61 (50%)

Online Table V. Association between miR expression and adverse events (restenosis and amputation)

Event	miR (2-ddCT)	OR*	95% CI	P-value
Restenosis (first event)	circulating miR-15a	1.28	1.01-1.61	0.04
	circulating miR-16	0.96	0.73-1.26	0.75
	PAC miR-15a	1.26	0.72-2.19	0.42
	PAC miR-16	0.79	0.51-1.23	0.30
Restenosis plus amputation	circulating miR-15a	2.10	1.32-3.36	0.002
	circulating miR-16	2.07	1.17-3.63	0.012
	PAC miR-15a	1.74	0.70-4.30	0.229
	PAC miR-16	0.70	0.35-1.41	0.315

*for 1 unit increase in log2

Online Table VI. Primers for VEGFA and AKT3 mutation

| |
|---|
| AKT3 mut1
Forward: 5'-AGTCTAAGGTCTCATGCTGTatttaattCTGTCTTACT -3'
Reverse: 5'-ACAGCATGAGACCTTAGACTGAGATACAAT-3' |
| AKT3mut2
Forward: 5'-AAGTGCTGCGATTATAGACGatttaatCTGCACCTGG-3'
Reverse: 5'-CGTCTATAATCGCAGCACTTTGGGAGGCCGA-3' |
| VEGFAmut
Forward: 5'-ATTCGCCATTTTATTTTTCTatttaattAATCACCGAG-3'
Reverse: 5'-AGAAAAATAAAATGGCGAATCCAATTCCAA-3' |

Supplemental Figures and Figure legends

Online Figure I.
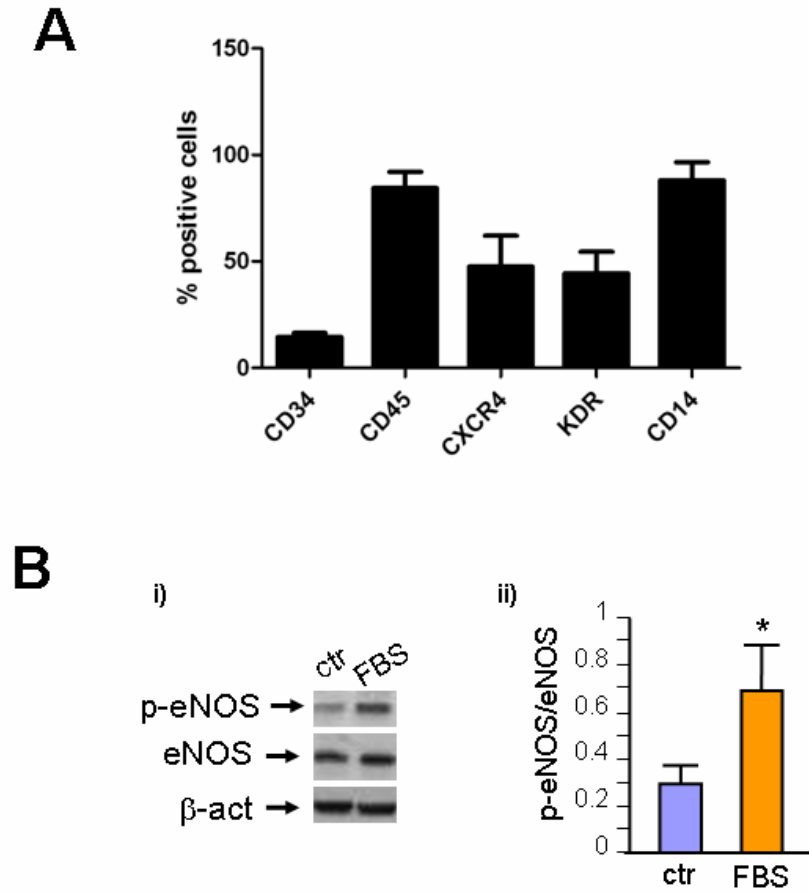
Online Figure I. Characterization of culture-selected PACs. A) Flow cytometric characterization of human PACs originated by culture selection from peripheral blood mononuclear cells (MNCs). Percentage cells positive for specific antigens are shown (n=8 donors). **B**) i) bar graph showing results of eNOS and phospho eNOS (p-eNOS) protein analysis by Western blot (n=3 PAC donor); ii) representative Western blot bands. All data are expressed as mean ±SEM.

Online Figure II.
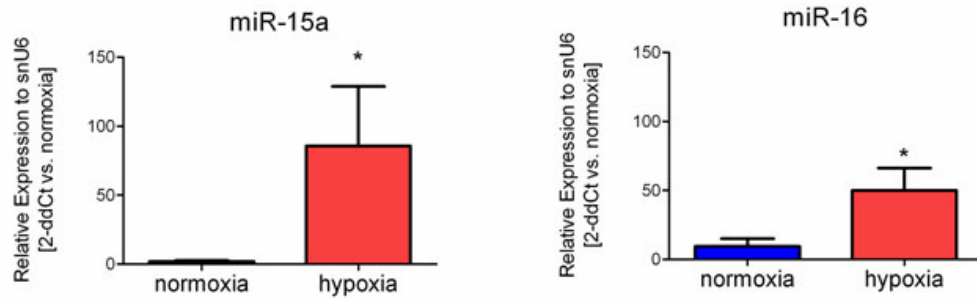
Online Figure II. Hypoxia increases miR-15a and miR-16 relative expression in healthy control PACs. PACs from healthy controls (n=10) were submitted to 48 hours hypoxia (2% oxygen) or kept under normal normoxic condition. miR-15a (left) and miR-16 (right) were measured by real time PCR using standard curves for miR-15a, miR-16 and Snu6. Data were normalized to snU6 and the relative expression of each of the two miR was quantified using the 2-ddCt method using as reference results obtained in PACs cultured in normoxia.. *p<0.05 vs. normoxia.
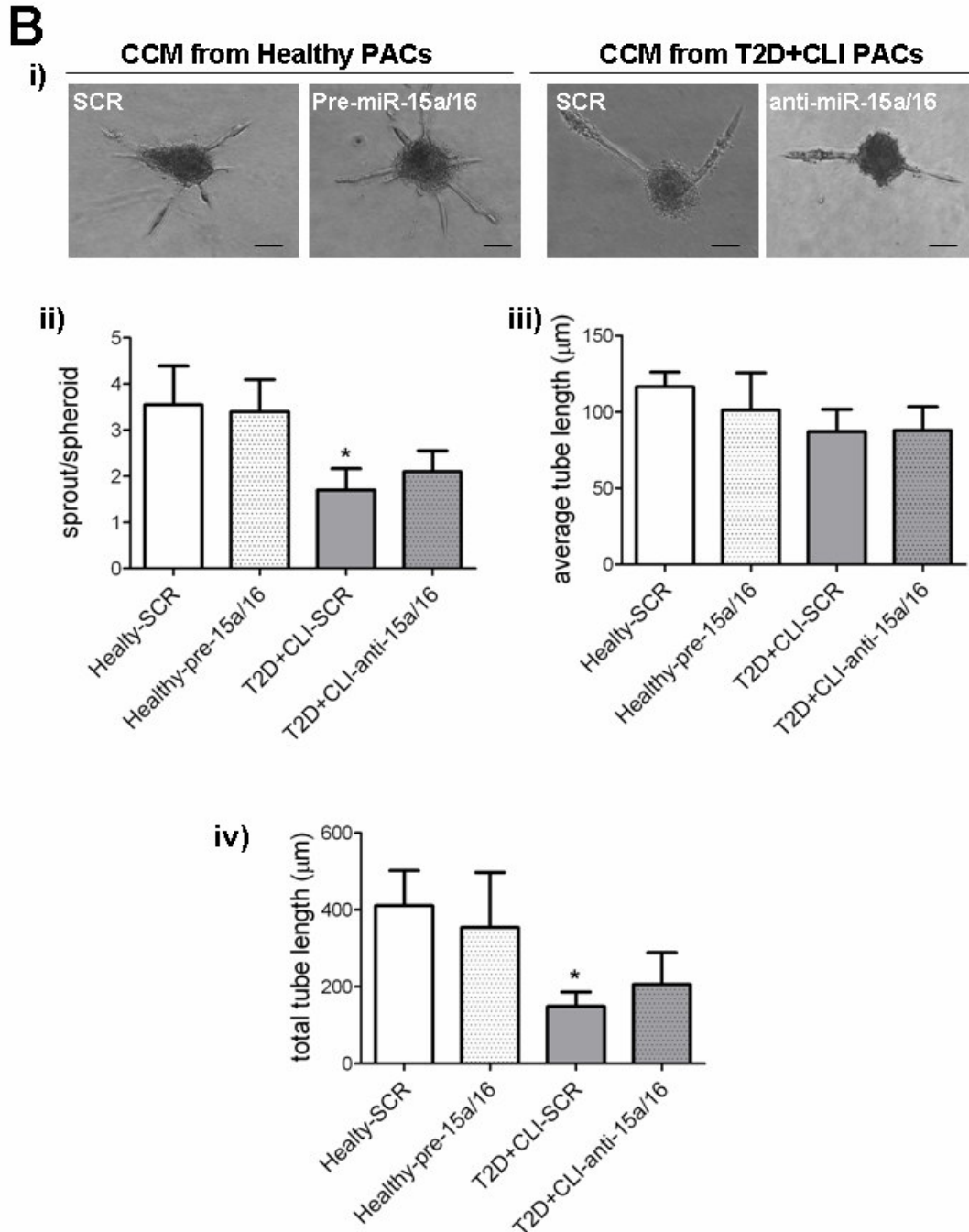
Online Figure III.
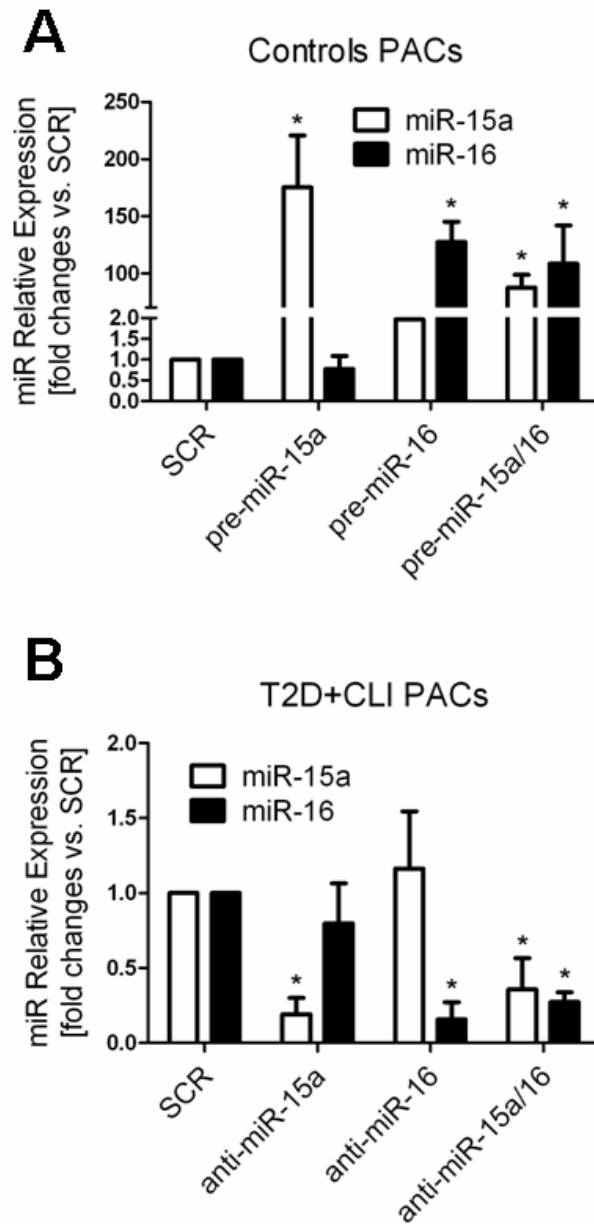
Online Figure III. miR-15a and miR-16 efficient expressional manipulation in human PACs. Relative expression (TaqMan PCR) of miR-15a and miR-16 after transfection with premiRs, antimiRs or scramble (SCR) control. **A**) Healthy (controls) PACs pre-miRs overexpression. **B**) T2D+CLI anti-miRs inhibition (both n=3 patients/group, *p<0.05 vs. SCR). All data are expressed as mean±SEM.

Online Figure IV.
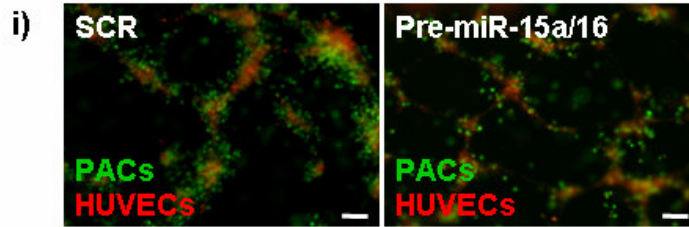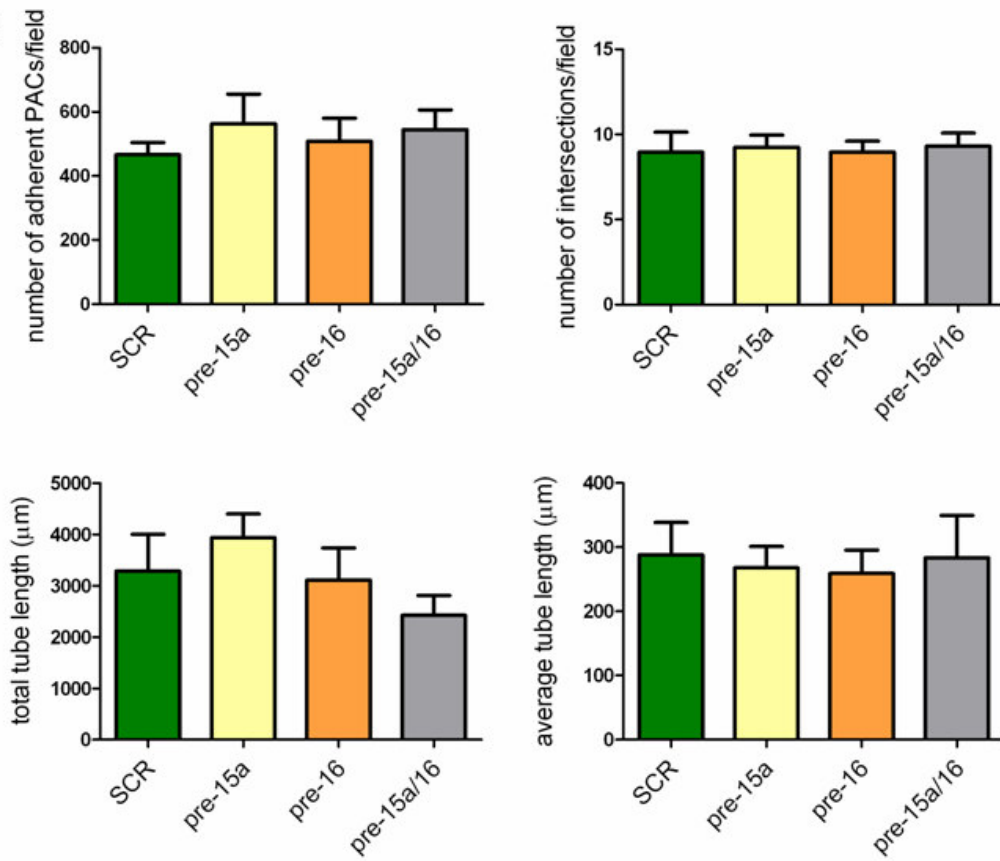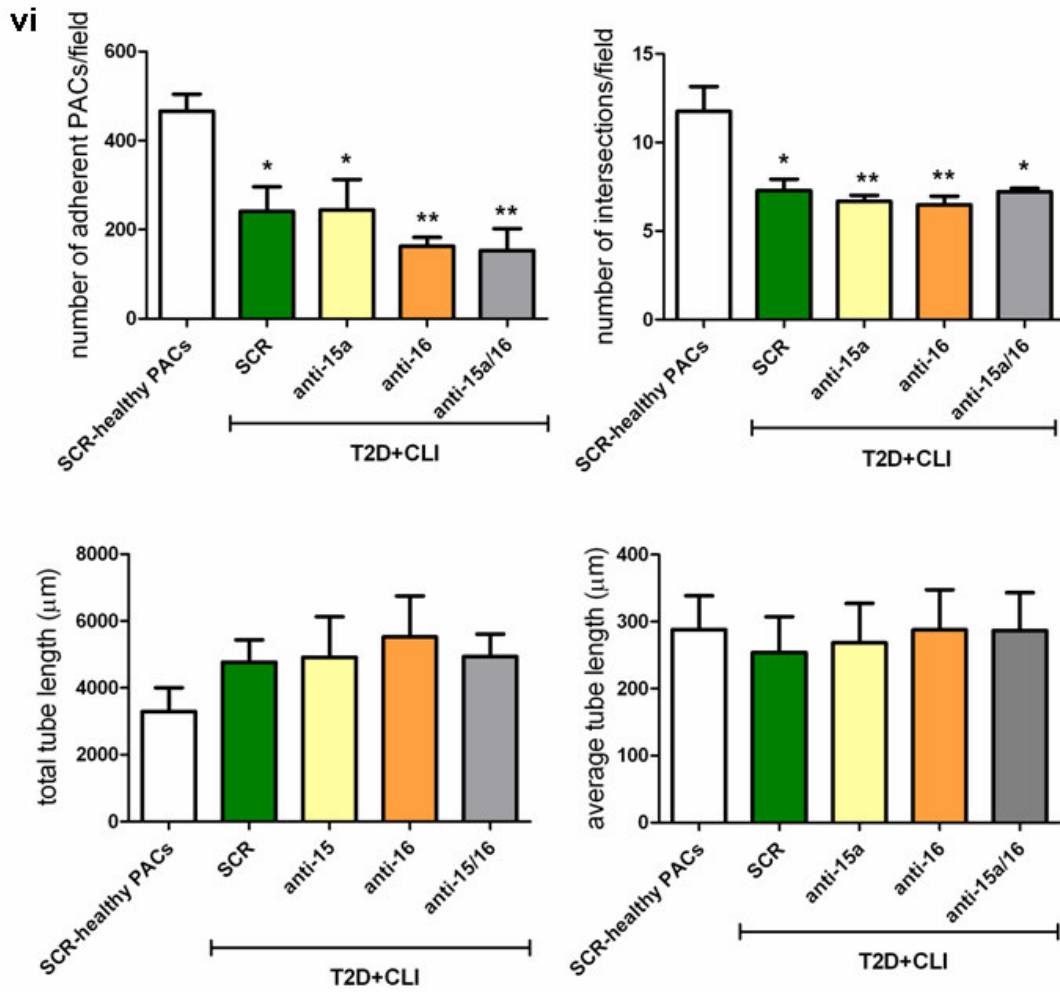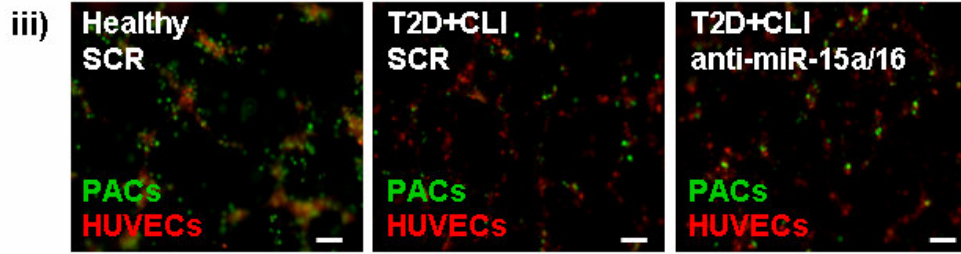
Online Figure IV. miR-15a/-16 manipulation in human PACs does not affect the ability of PACs to support EC networking. A) Capillary-like structure formation from HUVECs seeded on Matrigel and stimulated with: **i, ii**) control (healthy) PACs or **iii, iv**) T2D+CLI-PACs. Upper panels (**i, iii**): representative photomicrographs. Lower panels (**ii, iv**): bar graphs with data expressed as mean ±SEM (n=4 to n=10 subjects assayed in duplicate, p=NS). **B**) Spheroid assay using HUVECs and CCM of engineered PACs from healthy donors and from patients with CLI and T2D: (n=4 donors per group; each CCM was assayed in duplicate) i) representative photomicrographs, and measurements of ii) number of sprout per spheroid, iii) average tube length, and iv) total tube length. Data expressed as mean ±SEM. Scale bar: 100μm.
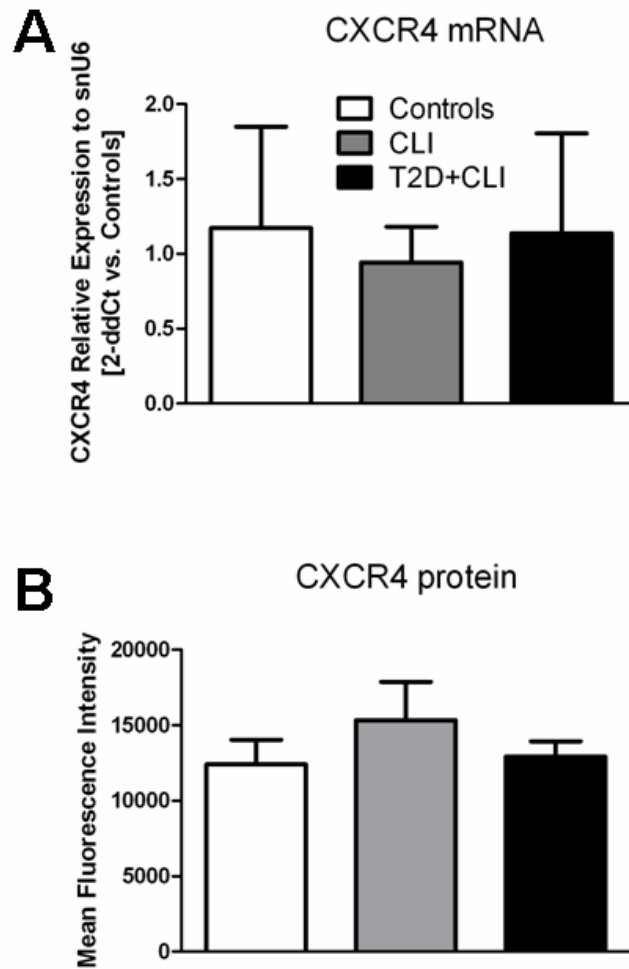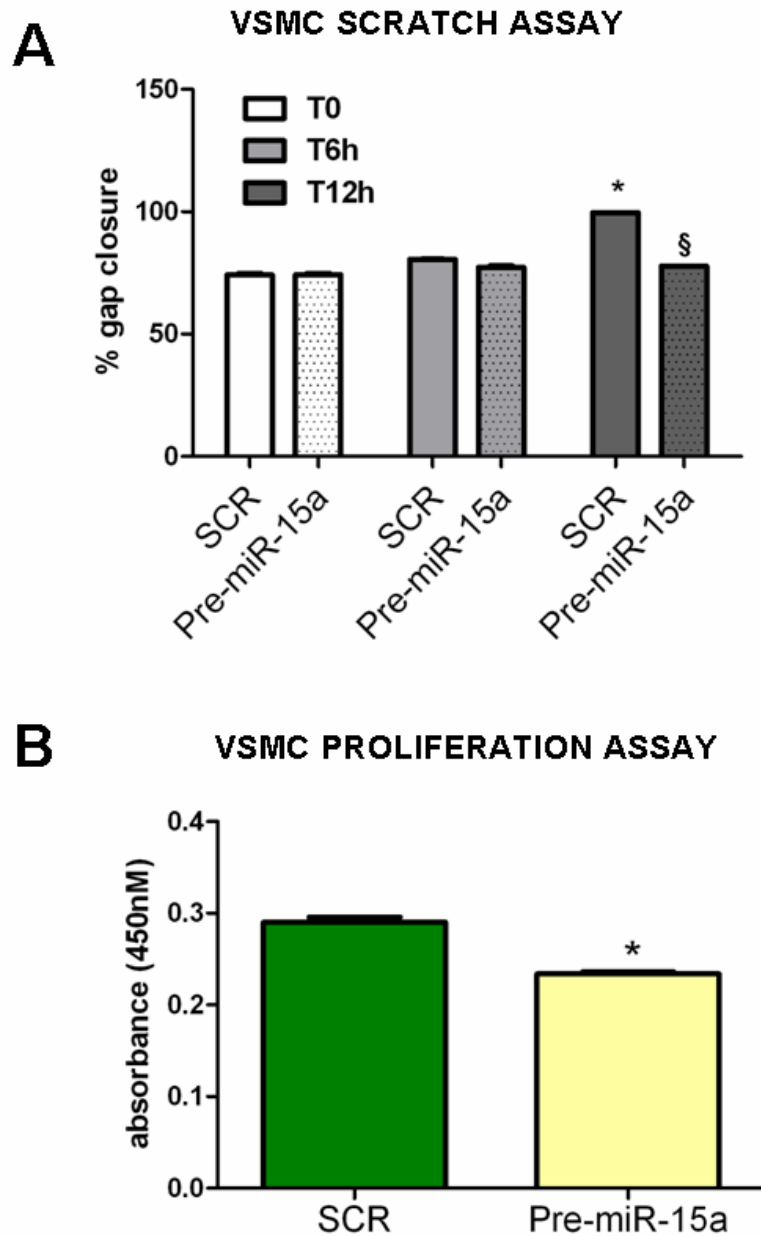
Online Figure V.

Online Figure V. CLI and diabetes do not induce CXCR4 expressional changes. Bar graphs showing CXCR4 expression: **A**) CXCR4 mRNA expression levels (TaqMan real time PCR) in PACs of the 3 groups of subjects enrolled to the study (n=6 subject per group); **B**) flowcytometric data of CXCR4 mean fluorescence intensity in circulating MNCs expressing PACs antigens (CD34^{pos}/CD45^{dim}/CXCR4^{pos}/KDR^{pos}/CD14^{pos}) is shown (controls: n=23, CLI: n=19, T2D+CLI: n=24). All data are expressed as mean±SEM.

Online Figure VI.

Control PACs
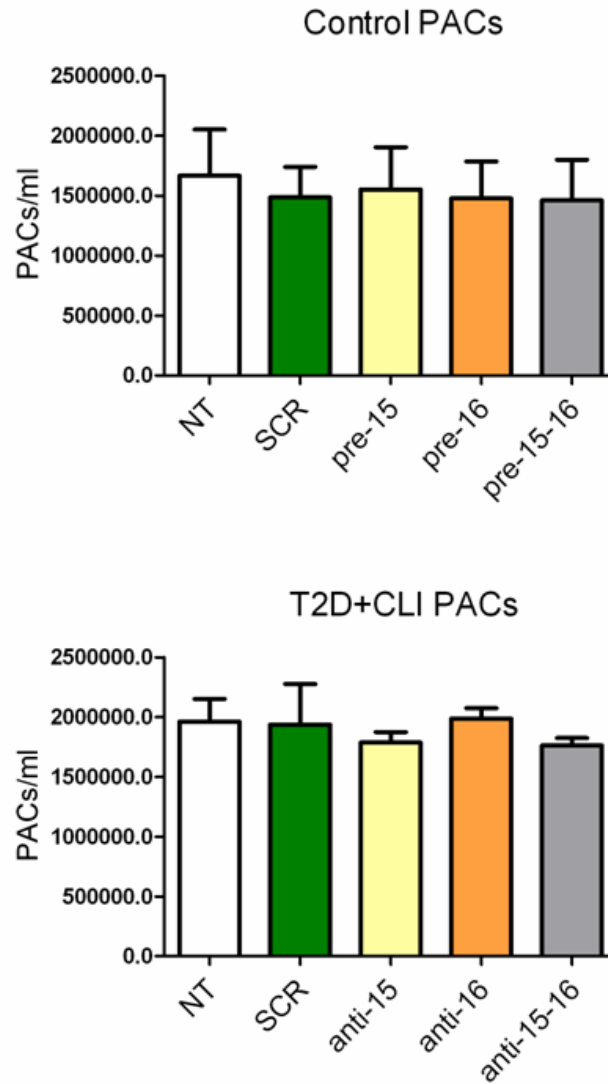
T2D+CLI PACs

Online Figure VI. PACs cell number is not affected by either pre-miRs or anti-miRs for miR-15a or miR-16. Bar graph showing number of healthy control PACs (upper panel) or T2D+CLI-PACs (lower panel) after 48 hour transfection (n=3/group). Cell number was assayed by counting alive, trypan blue-negative PACs.

Online Figure VII.

A VSMC SCRATCH ASSAY

B VSMC PROLIFERATION ASSAY

Online Figure VII. miR-15a overexpression negatively affects the ability human Vascular Smooth Muscle Cell (VSMC) to migrate and proliferate. A) VSMC migration was studied by scratch assay after 6 and 12 hours of scratching the cell monolayer. n=3 in quadruplicate, **p<0.001 vs. T0, §p<0.05 vs. SCR control. **B**) VSMC proliferation was assayed by BrDU incorporation, n=8 in quadruplicate, *p<0.05 vs. SCR control.