Deregulation of microRNA-503 Contributes to Diabetes Mellitus–Induced Impairment of Endothelial Function and Reparative Angiogenesis After Limb Ischemia

Andrea Caporali, PhD; Marco Meloni, PhD; Christine Völlnkle, PhD; Desiree Bonci, PhD; Graciela B. Sala-Newby, PhD; Roberta Addis, BSc; Gaia Spinetti, PhD; Sergio Losa, MD; Rachel Masson, PhD; Andrew H. Baker, PhD; Reuven Agami, PhD; Carlos le Sage, PhD; Gianluigi Condorelli, MD, PhD; Paolo Madeddu, MD; Fabio Martelli, PhD; Costanza Emanuelli, PhD

Background—Diabetes mellitus impairs endothelial cell (EC) function and postischemic reparative neovascularization by molecular mechanisms that are not fully understood. microRNAs negatively regulate the expression of target genes mainly by interaction in their 3′ untranslated region.

Methods and Results—We found that microRNA-503 (miR-503) expression in ECs is upregulated in culture conditions mimicking diabetes mellitus (high D-glucose) and ischemia-associated starvation (low growth factors). Under normal culture conditions, lentivirus-mediated miR-503–forced expression inhibited EC proliferation, migration, and network formation on Matrigel (comparisons versus lentivirus.GFP control). Conversely, blocking miR-503 activity by either adenovirus-mediated transfer of a miR-503 decoy (Ad.decoymiR-503) or by antimiR-503 (antisense oligonucleotide) improved the functional capacities of ECs cultured under high D-glucose/low growth factors. We identified CCNE1 and cdc25A as direct miR-503 targets which are downregulated by high glucose/low growth factors in ECs. Next, we obtained evidence that miR-503 expression is increased in ischemic limb muscles of streptozotocin-diabetic mice and in ECs enriched from these muscles. Moreover, Ad.decoymiR-503 delivery to the ischemic adductor of diabetic mice corrected diabetes mellitus–induced impairment of postischemic angiogenesis and blood flow recovery. We finally investigated miR-503 and target gene expression in muscular specimens from the amputated ischemic legs of diabetic patients. As controls, calf biopsies of nondiabetic and nonischemic patients undergoing saphenous vein stripping were used. In diabetic muscles, miR-503 expression was remarkably higher, and it inversely correlated with cdc25 protein expression. Plasma miR-503 levels were also elevated in the diabetic individuals.

Conclusions—Our data suggest miR-503 as a possible therapeutic target in diabetic patients with critical limb ischemia. (Circulation. 2011;123:282-291.)

Key Words: microRNAs ▶ angiogenesis ▶ cells ▶ diabetes mellitus ▶ peripheral vascular disease

Ischemic complications represent the leading cause of morbidity and mortality in diabetic patients.1 Early in the course of diabetes, intracellular hyperglycemia causes endothelial dysfunction and microvascular rarefaction.2 The overall result is tissue hypoperfusion, which, in limbs, results in the formation of not-healing ulcers. Moreover, diabetes mellitus impairs endogenous reperfusion accomplished by reparative angiogenesis, thereby worsening the recovery from an ischemic insult.2 In diabetic patients, the ischemic disease follows an inexorable course, and limb amputation is too often the ultimate remedy.2 A better understanding of the molecular mechanisms underpinning diabetes mellitus–associated vascular complications is urgently needed to improve therapeutic options.2

Editorial see p 236

Clinical Perspective on p 291

Because of their incapacity to regulate glucose influx, endothelial cells (ECs) represent an important target for diabetes mellitus–induced damage. In particular, it is well established that ECs cultured in high glucose show delayed replication,3,4 abnormal cell cycling,5 and increased apoptosis.6 Progression through the cell cycle is a tightly regulated process that includes multiple checkpoints. An orderly ex-
pression of cyclin-dependent serine/threonine kinases (cdks), their binding partners, cyclins (CCNs), and associated regulatory proteins modulates entry into each of the cell-cycle phases. In particular, CCNDs activate Cdk4 and/or Cdk6 in early G₁ phase, whereas CCNEs activate Cdk2 in the late G₁ phase, leading to the passage into the S-phase of the cell cycle. CCNA/Cdk2 complexes assure S phase progression, and CCNB/Cdk1 complexes complete mitosis (M). However, among CCNs and CCds, widespread compensations exist. Phosphatases of the cell division cycle 25 family (CDC25s) are critical for timely Cdk1 and Cdk2 activation, thus regulating G₁-S and G₂-M transitions. Regulation of cell-cycle progression through modulation ofcdc25A, CCNE1, CCND1 and CCND2 has been reported in ECs.

miR-503 was highly expressed in both ischemic muscle specimens and the plasma of diabetic patients with critical limb ischemia. Our results suggest that miR-503 could be a novel biomarker and therapeutic target in diabetes mellitus and ischemia. Moreover, miR-503–induced endothelial derangements compared with targeting a specific gene, could have a greater chance of correcting complex molecular derangements compared with targeting a specific gene. Hence, manipulation of miRNAs may open new avenues for molecular therapeutics of complex diseases, like diabetes mellitus and vasculopathies. Recently, some miRNAs have been shown to regulate EC functions implicated in angiogenesis, including EC proliferation, migration, and assembing in branched networks.

The present study is the first to provide evidence for a role of miRNAs in diabetes mellitus–induced endothelial defects contributing to impaired postischemic angiogenesis. In fact, here we show that in vitro culture conditions mimicking diabetes mellitus and ischemia upregulate miR-503 in ECs and that, in vivo, diabetes mellitus increases miR-503 expression in ECs from ischemic limb muscles. We also show that increased miR-503 is responsible for repressed cdc25A and CCNE1 expression in ECs cultured under conditions mimicking diabetes mellitus and ischemia. Moreover, miR-503–forced expression inhibited EC proliferation, migration, and network formation on Matrigel and it additionally reduced vascular smooth muscle cell (VSMC) proliferation and migration, 2 processes that are instrumental for arteriogenesis. Furthermore, adenovirus–mediated local gene transfer with a decoy for miR-503 improved blood flow recovery and angiogenesis in diabetic mice with limb ischemia. Our results suggest that miR-503 could be a novel therapeutic target in diabetic patients with peripheral ischemic complications.

**Methods**

An expanded version of Methods in the online-only Data Supplement includes detailed methods for the following: cells and cell culture; RNA extraction and Taq-Man Quantitative Real Time, lentivirus preparation, luciferase assays, preparation of an adenovirus for decoymiR-503 (Ad.decoymiR-503), Western blot analyses, cell cycle, cell biology assays, mouse limb ischemia and in vivo gene transfer, enrichment of ECs from limb muscles, and flow cytometry.

**Cells and Cell Culture**

Human umbilical vein ECs (HUVECs) and human microvascular ECs (HMVECs) were grown in EGM-2 (EBM-2 added with growth factors and other supplements) with 2% FBS. Human VSMCs were cultured in 10% FBS Dulbecco modified Eagles medium. To mimic hyperglycemia, ECs were incubated in 25 mmol/L D-glucose (high glucose [HG]). D-Mannitol was used as osmotic control (normal glucose condition [NG]). To mimic ischemia-induced tissue starvation, ECs were incubated in EBM-2 with 2% FBS, only (low growth factors [LGF]).

**RNA Extraction and Taq-Man Quantitative Real-Time Analysis**

Total RNA was extracted using TRIzol. Real-time quantification to measure miRNAs was performed with the TaqMan miRNA reverse transcription kit and miRNA assay (Applied Biosystems, Carlsbad, CA) with the DNA Engine Opticon 2 system (Bio-Rad Laboratories, Hercules, CA). miR-503 expression was normalized to the U6 small nuclear RNA (snRU6). Primer identification numbers are: 4373228 for hsa-miR-503 and 4427975 for snRU6 (Applied Biosystems). For mRNA analysis, single-strand complementary DNA was synthesized from 1 μg of total RNA. Complementary DNA was amplified by real-time polymerase chain reaction (PCR) and normalized to 18S ribosomal RNA (endogenous control). Each reaction was performed in triplicate. Quantification was performed by the 2ΔΔCt method. Quantitative reverse transcription PCR (RT-PCR) was used to measure cdc25A, CCNE1, CCND1, CCND2, eGFP and 18S ribosomal RNA. RT-PCR primers are reported in the online-only Data Supplement.

**Lentivirus Preparation and In Vitro Infection**

Lentiviral vector-expressing premiR-503 (lenti.miR-503) and lenti.GFP (control) were generated. HUVECs, HMVECs and VSMCs were infected at 25 multiplicity of infection.

**antiRNA Transfection**

A miR-503 inhibitor oligonucleotide (Applied Biosystems, 50 nmol/L) or a scrambled oligonucleotide was transfected into HUVECs, HMVECs and VSMCs were infected at 25 multiplicity of infection.

**Identification of Target mRNAs of miRNA-503**

To determine the gene targets of miR-503, 5 leading miRNA target prediction algorithms (TargetScan 4.1, miRanda, miRBase, Diana microT 3.0, and EJMMo2) were used (online-only Data Supplement Table IA).

**Luciferase Assays**

To investigate whether miR-503 directly regulates cdc25A and CCNE1 expression, portions of the 3'-UTR of these potential target genes were inserted downstream of a luciferase open reading frame. CCNE1 3'-UTR (S204537) and cdc25A 3'-UTR (S213182) vectors were purchased from SwitchGear Genomics (Menlo Park, CA). For controls, we prepared similar vectors in which 5 nucleotide mutations were inserted in the 3'-UTR sequences (cd25a: 675 to 681; cdc25a: 675 to 681; CCNE1: 675 to 681).
CCNE1: 248 to 249 and 486 to 492) complementary to the miR-503 “seed” sequence. For CCNE1, plasmids with single or double mutations in 3′UTR were prepared. Primers and mutation sequences are reported in online-only Data Supplement Table IIA and IIB. The different luciferase constructs were transfected into COS-7 cells together with either premiR-503 or a scrambled oligonucleotide sequence (control). COS-7 cells were chosen for their high efficiency of transfection and for the absence of endogenous miR-503 expression (data not shown). Cells were cultured for 48 hours and assayed with the Dual-Luciferase Reporter Assay System (Promega, Fitchburg Center, WI). Luciferase assay for CCND1 was omitted because previously performed by others.\(^{18}\) Luciferase assay for CCND2 was omitted because we found normal CCND2 protein level in HUVECs engineered to overexpress miRNA-503 (see below).

**Ad.decymiR-503 Preparation and In Vitro Infection**

The decoy for miR-503 was designed using multiple copies of complementary targets. This was intended to optimize the transgene repression in the presence of the miRNA (online-only Data Supplement Methods and Figure IA). ECs were infected with Ad.decymiR-503 or Ad.Null (control) at 250 multiplicity of infection. To study if this titer of adenoviral vector affects EC behavior, HUVECs infected with 250 multiplicity of infection Ad.Null were compared to noninfected (PBS) HUVECs in proliferation and on Matrigel angiogenesis assays.

**Western Blot Analyses**

Western blots for CCND1, CCND2, CCNE1, cdc25A, and β-actin were performed.

**Cell-Cycle Analysis**

HUVECs were infected with lenti.miR-503 or lenti.GFP. After fixation in 70% ethanol and RNase A treatment, cells were stained with propidium iodide. DNA content was analyzed by flow cytometry.

**BrdU Incorporation Assay**

BrdU proliferation assays were performed on HUVECs, HMVECs, and VSMCs infected with lenti.miR-503 or lenti.GFP and then kept in their full media.

**Adhesion Assay**

At 48 hours from lentiviral infection, HUVECs were seeded on fibronectin-coated 96-well plates. One hour later, nonadherent cells were washed out and adherent cells were fixed and stained with DAPI.

**Caspase-Activity Assay**

Caspase-3 activity of HUVECs infected with lenti.miR-503 or lenti.GFP was measured.

**Migration Scratch Assay**

Migration capacities of HUVECs or VSMCs infected with lenti.miR-503 or lenti.GFP and growth arrested were measured using a scratch assay.

**Matrigel Assay**

HUVECs or HMVECs were infected with lentivirus and seeded in 48-well plates coated with growth factors–enriched Matrigel. Endothelial network formation was quantified by morphometry.

**EC Isolation From Mouse Limb Muscles**

At 3 days postischemia induction, adductor muscles of diabetic and nondiabetic mice were harvested and enzymatically digested. ECs were immunomagnetically sorted using a CD146 antibody.\(^{19}\) CD146 positive ECs were immunomagnetically sorted using a CD146 antibody.\(^{19}\) CD146

**Mouse Limb Ischemia and In Vivo Gene Transfer**

The experiments involving mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and with the prior approval of the UK Home Office and the University of Bristol ethics committee.

Type-1 diabetes mellitus was induced by streptozotocin injection in CD1 male mice. Mice at 3 months of diabetes mellitus (without insulin supplementation) and nondiabetic age- and gender-matched controls were anesthetized to induce left limb ischemia. Immediately after, Ad.decymiR-503 or Ad.Null (10⁶ plaque forming units) was delivered to the ischemic adductor muscle of diabetic mice. Nondiabetic mice received Ad.Null only. The superficial blood flow (BF) of the ischemic and contralateral foot was sequentially analyzed (at 30 minutes and at 7, 14, and 21 days) by color laser Doppler.

Values are means ± SEM. \(^*P<0.05\) versus NG; \(^*P<0.05\) versus HG; \(^§P<0.05\) versus LGF. FDR q values: 0.0021 (A) and 0.0158 (B).

---

**Figure 1.** miR-503 expression in ECs is increased by culture in HG and LGF. A and B, Relative expression of miR-503 in HUVECs and HMVECs cultured under NG, HG, LGF, or HG/LGF. miR-503 expression was normalized to snRU6 expression using the comparative Ct method.\(^{17}\) Experiments were performed in triplicate and repeated 3 times. miR-503 expression is reported to expression in the NG group. Values are means ± SEM. \(^*P<0.05\) versus NG; \(^*P<0.05\) versus HG; \(^§P<0.05\) versus LGF. FDR q values: 0.0021 (A) and 0.0158 (B).
is a transmembrane glycoprotein mainly expressed at the intercellular junction of ECs. To verify, EC enrichment by our CD146-positive separation, single cell suspensions were incubated with FITC-conjugated CD146 and APC-conjugated CD31 antibodies or the respective isotype for negative control. Unstained and single-stained controls were performed to define positivity. Flow cytometric analysis revealed that 82.5% of cells isolated from nondiabetic ischemic muscles coexpressed both CD146 and the classic EC marker CD31 and that diabetes mellitus does not significantly change this figure (83.4%, P not significant; online-only Data Supplement Figure II).

Human Tissue Studies

Expressional studies were performed on plasma and on muscular specimens taken from either amputated legs of diabetic patients (n=11) or calf biopsies obtained during saphenous vein stripping surgery in nondiabetic patients (controls, n=11). Patient characteristics are reported in online-only Data Supplement Table III. Studies complied with the ethical principles stated in the Declaration of Helsinki and were covered by ethical approval number 11/2009 from IRCCS-Multimedica. Patients gave written informed consent to sample collection.

Statistical Analysis

Group differences of continuous variables were compared by 1-way ANOVA or Student t test as appropriate. Relationships between variables were determined by the Pearson correlation coefficient. Continuous data are expressed as mean±SEM. A P value <0.05 was considered statistically significant. Analyses were performed with GraphPad Prism 5.0, SigmaStat 3.1 software (San Jose, CA). The overall false discovery rate (FDR) was estimated using the R package LBE.

Results

HG/LGF Upregulates miR-503 Expression in Cultured ECs

miR-503 was increased in both HUVECs and HMVECs cultured in HG/LGF (Figure 1A and 1B). Culture in HG/LGF is intended to mimic advanced diabetes mellitus when hyperglycemia is accompanied with tissue starvation.

miR-503 Impairs EC Functions

To evaluate the functional consequences of high endothelial miR-503 levels, HUVECs were infected with lentil.miR-503.
Successful miR-503 transduction was demonstrated by quantitative RT-PCR analysis (online-only Data Supplement Figure III). As shown in Figure 2A, miR-503–forced expression impaired HUVEC cycle, thus increasing the percentage of cells in G0/G1 and reducing the percentage in S and G2/M phases. The antiproliferative effect of miR-503 on HUVECs was confirmed in a BrdU incorporation assay (Figure 2B). Notably, miR-503–forced expression for 48 hours did not affect EC apoptosis (online-only Data Supplement Figure IVA). The antiangiogenic potential of miR-503 was next tested in a Matrigel assay. HUVECs infected with lenti.miR-503 were impaired in their capacity to form cellular networks (Figure 2C). Furthermore, miR-503–overexpressing HUVECs showed reduced migratory capacity in an in vitro scratch assay (Figure 2D) and decreased adhesiveness to fibronectin (online-only Data Supplement Figure IVB). Forced miR503 expression also inhibited HMVEC proliferation and tube-like structure formation in vitro (online-only Data Supplement Figure VA and VB), as well as VSMC proliferation and migration (online-only Data Supplement Figure VIA and VIB).

miR-503 Directly Targets cdc25A and CCNE1

In line with data from in vitro assays, bioinformatics interrogation of genes modulated by miR-503 confirmed a functional link with expressional control of cell cycle, adhesion, migration, and angiogenesis (online-only Data Supplement Table IB). We next analyzed putative miR-503 targets predicted by defined criteria.21 Four targets were consistently identified by 4 out of 5 prediction softwares: cdc25A, CCND1, CCND2, and CCNE1 (online-only Data Supplement Table IA). CCND1 has been already validated as direct target of miR-503.18 Lenti.miR-503 reduced cdc25A and CCNE1 at mRNA and protein level in HUVECs in comparison with GFP-HUVECs. By contrast, CCND1 was downregulated at protein level only. We decided...
not to validate CCND2 as miR-503 target because our data indicated that its expression is not modulated by miR-503 in ECs (Figure 3A and 3B). As shown in Figure 3C, using luciferase assay, we validated cdc25A and CCNE1 as direct target genes of miR-503. Significant inhibition of the luciferase activity was observed in cells transfected with the constructs bearing an intact miR-503 binding site when compared with pLUC. Mutation of cdc25A target sequence prevented downregulation of luciferase activity by miR-503. For CCNE1, mutation of each conserved target sequence had only a partial effect, and mutation of both sequences fully rescued CCNE1 repression (Figure 3C).

Effect of miR-503 Inhibition in ECs Cultured in HG/LGF

Incubation in HG/LGF for 24 hours decreased HUVEC proliferation (Figure 4A and 4B) and networking capacity on Matrigel (Figure 4C and 4D). In order to understand whether miRNA-503 contributes to these altered functions, we inhibited miR-503 using 2 different approaches: Ad.decoymiR-503 and antimiR503 (antisense oligonucleotide). DecoymiR-503 transcript targeting was preliminarily validated by flow cytometry, which showed a specific reduction of eGFP in COS-7 cells cotransfected with premiR-503. This was consistent with miR-503 binding to the eGFP 3'-UTR (online-only Data Supplement Figure IB and IC). Moreover, reduction of Ad.decoymiR503-associated eGFP in HUVECs cultured in HG/LGF (versus HUVECs cultured in NG) was also revealed by fluorometry (online-only Data Supplement Figure ID). Adenoviral infection of HUVECs did not alter their proliferative and tube-like structure-forming capacities (comparisons: Ad.Null versus PBS; online-only Data Supplement Figure VIIA and VIIIB). Most important, Ad.decoymiR-503 rescued HUVEC proliferation (Figure 4A) and networking capacities (Figure 4C) under HG/LGF. Similar results were obtained with antimiR-503 (Figure 4B and 4D). Furthermore, Ad.decoymiR-503 restored cdc25A and CCNE1 protein expression in HG/LGF-cultured HUVECs (Figure 4E). Because CCND1 expression was not inhibited in HG/LGF-cultured HUVECs (data not shown), this cyclin was not further considered.

Inhibition of miR-503 Normalizes Postischemic BF Recovery and Muscular Neovascularization in Diabetic Mice

miR-503 was remarkably higher in ischemic muscles (3 days postischemia) of diabetic mice in comparison to nondiabetic and/or nonischemic controls (Figure 5A). Importantly, miR-503 was expressed by CD146-positive ECs freshly isolated from murine ischemic limb muscles, with mi-R503 expression being enhanced by diabetes mellitus (Figure 5B). Next, we tested if local miR-503 inhibition improves postischemic reparative neovascularization and blood flow recovery in diabetic mice. The capacity of Ad.decoymiR-503 to transduce limb muscles was preliminarily demonstrated by RT-PCR of the eGFP tag in Ad.decoymiR-503–injected nonischemic limb muscles of nondiabetic mice (online-only Data Supplement Figure VIII).
As expected, postischemic foot BF recovery was impaired in diabetic mice receiving Ad.Null versus nondiabetic mice given Ad.Null (Figure 5C). Importantly, Ad.decoymiR-503 normalized BF recovery in diabetic mice (Figure 5C). Likewise, Ad.decoymiR-503 increased capillary and arteriolar densities in ischemic muscles of diabetic mice (Figure 5D). Moreover, as shown in Figure 5E, at both 3 and 21 days postischemia, cdc25A protein was downregulated by diabetes mellitus but normalized by Ad.decoymiR-503. Diabetes mellitus downregulated CCNE1 at 3 days postischemia, with this response being prevented by Ad.decoymiR-503. CCND1 expression was not modulated by diabetes mellitus (data not shown), and consequently we did not check if Ad.decoymiR-503 could alter CCND1 expression.

**Expression of miR-503 and Target Genes in Human Samples**

miR-503 expression was remarkably higher in limb muscles of diabetic patients undergoing major amputation for critical ischemia in comparison with calf biopsies of nondiabetic and nonischemic control subjects (Figure 6A). Moreover, miR-503 and cdc25A expression were inversely correlated in the diabetic patients (Pearson correlation $r = -0.784$; $P = 0.0025$) (Figure 6C through 6E). By contrast, no correlation was found between miR-503 and either CCND1 or CCNE1 (data not shown). Finally, plasma levels of miR-503 were $\approx 15$ times higher in diabetic patients with critical ischemia compared with control subjects (Figure 6B).

**Discussion**

miRNAs comprise a class of endogenous small noncoding RNAs that control gene expression by acting on target miRNAs for degradation and/or translational repression. Although increasing evidence indicates that miRNAs are key among the nonischemic nondiabetic muscles. C and D, Ad.decoymiR-503 (decoymiR-503) or Ad.Null (Null) was delivered to the ischemic adductors of diabetic mice. Nonisdiabetic mice received intramuscular Ad.Null (Null). C. Line graph shows the time course of postischemic foot BF recovery (calculated as the ratio between ischemic foot BF and contralateral foot BF) in nondiabetic mice given Ad.Null (black dotted line) and in diabetic mice given either Ad.Null (red line) or Ad.decoymiR-503 (yellow line) ($n = 12$ mice per group). Representative color laser Doppler images taken at 21 days postischemia induction are shown. D. Column graph shows expression of cdc25A and CCNE1 in adductors at 3 and 21 days postischemia ($n = 6$ per group). Representative pictures show capillaries stained by isoelectin-B4 (red fluorescence) and arterioles stained by isolectin-B4 and $\alpha$-smooth actin (green fluorescence) (original magnification 500x; scale bar: 500 μm). E. Representative Western blot analyses for cdc25A and CCNE1 in adductors at 3 days and 21 days postischemia ($n = 6$ per group). Bar graphs show relative protein quantification of cdc25A and CCNE1. Relative values are normalized by $\alpha$-actin levels. All values are means±SEM. $+P<0.01$ versus nonischemic muscle of nondiabetic mice; $1P<0.05$ and $11P<0.01$ versus ischemic muscle of diabetic mice; $#P<0.05$ versus nonischemic muscle of diabetic mice; $##P<0.05$ and $##P<0.01$ versus nondiabetic ischemic mice given Ad.Null; $^{*}P<0.05$ and $^{**}P<0.01$ versus diabetic ischemic mice given Ad.Null. FDR q values: 0.0375 (A); 0.0121 (C; Doppler analysis at 14 days); 0.0061 (Doppler analysis at 21 days); 0.0120 (capillary density); and 0.0252 (arteriole density) (D). 0.01921 (cdc25A; 3 days), 0.0106 (cdc25A; 21 days), and 0.0223 (CCNE1; 3 days) (E). Non Diab Isch indicates nondiabetic ischemia; Diab Isch, diabetic ischemia.
players in cardiovascular disease, only minor studies have been carried out on the relationship between miRNAs and diabetes mellitus in the vascular system. One study showed that both miR-503 and miR-320 are upregulated in myocardial ECs of Goto–Kakizaki diabetic rats and that miR-320 impairs angiogenesis in vitro.22 The other investigation reported that HG upregulates miR-221 expression in HUVECs and that miR-221 inhibits HUVEC migration in vitro.23 In our study, we demonstrated that miR-503 is involved in diabetic endothelial dysfunction.

Constitutively low miR-503 expression in human ECs is remarkably upregulated by culture in HG/LGF to mimic advanced diabetes mellitus when hyperglycemia is accompanied by tissue starvation. Moreover, we found that mouse ECs freshly isolated from ischemic limb muscles express miR-503 and that diabetes mellitus upregulates miR-503 in these ECs. In addition, miR-503 expression was remarkably increased in ischemic muscles of both type-1 diabetic mice and diabetic patients undergoing foot amputation for critical ischemia. Plasma miR-503 was also elevated in the diabetic and ischemic patients. We have shown that miR-503–forced expression impairs EC proliferation, migration, adhesion, and network formation capacities. miR-503 also reduced VSMC proliferation and migration, which are instrumental for arteriogenesis.16 Conversely, miR-503 inhibition restored normal EC proliferation and in vitro angiogenesis under HG/LGF, without affecting EC function under normal culture conditions. Thus, miR-503 might be considered a suppressor of postischemic neovascularization in diabetes mellitus and a potential therapeutic target for improving healing of diabetic ischemic tissues. To gain further insights into this issue, we employed a gene therapy approach based on the adenovirus-mediated local delivery of a 3'UTR decoy for miR-503 to downregulate miR-503 activity in ischemic muscles. Decoys, at least when delivered by viral vectors, can stably antagonize a miRNA without requiring multiple administrations.24 Moreover, local injection of a decoy vector should minimize off-target effects that could follow the systemic administration of other miRNA inhibitors.24 Decoys have not been previously employed in angiogenesis studies. By contrast, miRNA antisense oligonucleotides, which include cholesterol-conjugated antagonirs, have been already used to demonstrate the role of miR-92 and miR-126 in the postischemic neovascularization process in nondiabetic mice.25,26 Our data show that adenoviral-mediated local delivery of the miR-503 decoy normalizes postischemic muscular neovascularization and blood flow recovery in diabetic mice. To the best of our knowledge, this is the first demonstration of a therapeutic approach targeting a specific miRNA and resulting in relevant benefit for healing of limb ischemia in diabetes mellitus.

Each miRNA is believed to directly bind to and regulate the translation or stability of many target mRNAs. Recognizing these gene targets is essential before interfering with miRNA expression for therapeutic purposes. Using a Web-based computational tool developed to identify molecular pathways potentially altered by miRNAs,27 we identified cell cycle as the main function affected by miR-503. To find the genes directly regulated by miR-503, we performed an in
silico analysis using 5 target prediction software programs and considering targets that were identified by at least 4 algorithms. Experimental evidence identified cdc25A, CCND1, and CCNE1 as direct targets of miR-503. However, CCND1 expression was not modulated by either HG/LGF in HUVECs or by diabetes mellitus and ischemia in vivo. Notably, miR-503 inhibition prevented cdc25A and CCNE1 downregulation in ECs cultured in HG/LGF. Similarly, Ad.decoymiR-503 normalized cdc25A and CCNE1 protein levels in the ischemic limb muscles of diabetic animals.

One of the most striking findings in this study was the dramatic increase in miR-503 expression in limb muscle of diabetic patients. Interestingly, in these human samples, miR-503 and cdc25 expression were inversely correlated. By contrast, miR-503 and CCNE1 expression did not correlate, possibly because CCNE1 is under the control of divergent transcriptional commands. The high level expression of miR-503 is compatible with the advanced vasculopathy of these patients. Our data suggest that miR-503 and cdc25A may represent therapeutic targets for diabetic patients who are developing muscular ischemia.

miRNAs are protected from RNase and stable in the blood. Therefore, their stability makes miRNAs concentrations well suited for being tested in patient samples as potential biomarkers of different pathological conditions. In line with this insight, the plasma levels of miR-503 were dramatically increased in diabetic patients with critical limb ischemia in comparison with controls. Further studies are required to investigate miR-503 as a potential circulating biomarker of ongoing ischemia in diabetic subjects.

In conclusion, we have demonstrated that miR-503 is antiangiogenic in the context of diabetes mellitus. miR-503 is upregulated by diabetes mellitus and ischemia, and it plays a pathogenic role in the diabetes-induced impairment of reparative angiogenesis. The findings of this study highlight important clinical implications of miR-503 in diabetes-associated vascular complications.

Acknowledgments

Dr Ben Carter and Dr Alessandro Cardinali (Bristol Heart Institute Clinical Trial Office) performed some statistic analyses. We thank Orazio Fortunato, BSc, and Daniela Cordella, BSc (IRCCS-Multimedica), for technical assistance.

Sources of Funding

This work was funded by Medical Research Foundation grant G0901764, British Heart Foundation grants BS/05/001, PG/06/146/21946, and RG/09/005/27915, and European Community integrated project RESOLVE! FP7 HEALTH-F4–2008. Dr Emanuelli is a British Heart Foundation Basic Science Senior Research Fellow.

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

MicroRNAs (miRNAs) are post-transcriptional inhibitory regulators of gene expression that bind to complementary messenger RNA transcripts. After initial studies in developmental biology and cancer, miRNAs have recently come into focus of cardiovascular diagnostics and therapeutics. Because each miRNA can repress many target mRNAs, it is possible that dysregulation of a single miRNA might account, at least in part, for complex pathological situations. Here, we report for the first time the importance of miR-503 in diabetes mellitus–associated ischemic disease, which currently represents a major cause of morbidity and mortality in diabetic patients. In vitro, the combination of high glucose and starvation remarkably enhances the expression of miR-503 in human endothelial cells, and so does diabetes mellitus in endothelial cells extracted from murine ischemic limb muscles. In vitro experiments showed that forced expression of miR-503 inhibits endothelial cell proliferation and endothelial network formation. Because miR-503 represses cell cycle–associated genes, we investigated whether miR-503 activation may impinge on postischemic reparative angiogenesis. In a diabetic mouse model of limb ischemia, local inhibition of miR-503 activity accelerated vascular healing and blood flow recovery. Importantly, miR-503 was found up-regulated in muscular biopsies and peripheral blood–derived plasma of diabetic patients with critical limb ischemia. From a therapeutic perspective, manipulation of miR-503 may represent a novel molecular means to foster reparative angiogenesis in diabetic patients. In the diagnostic context, more studies are necessary to determine if miR-503 could be exploited as a biomarker of progressive vascular disease.
Deregulation of microRNA-503 Contributes to Diabetes Mellitus–Induced Impairment of Endothelial Function and Reparative Angiogenesis After Limb Ischemia
Andrea Caporali, Marco Meloni, Christine Völlenkle, Desiree Bonci, Graciela B. Sala-Newby, Roberta Addis, Gaia Spinetti, Sergio Losa, Rachel Masson, Andrew H. Baker, Reuven Agami, Carlos le Sage, Gianluigi Condorelli, Paolo Madeddu, Fabio Martelli and Costanza Emanueli

_Circulation_. 2011;123:282-291; originally published online January 10, 2011;
doi: 10.1161/CIRCULATIONAHA.110.952325
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/123/3/282

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2010/12/09/CIRCULATIONAHA.110.952325.DC1
http://circ.ahajournals.org/content/suppl/2011/01/06/CIRCULATIONAHA.110.952325.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/
Supplemental Methods

Cells and cell culture
Human umbilical vein ECs (HUVECs) and human microvascular ECs (HMVECs) (both from Cambrex) were grown in EGM-2 (EBM-2 added with growth factors and other supplements) with 2% FBS (Cambrex). Human VSMCs (Cambrex) cells were cultured in 10% FBS D-MEM. To mimic hyperglycemia, ECs were incubated in 25 mM D-glucose (high glucose, HG). D-Mannitol was used as osmotic control (normal glucose condition, NG). To mimic ischemia-induced tissue starvation, ECs were incubated in EBM-2 with 2% FBS, only (low growth factors, LGF). HUVECs and HMVECs were used at P2 to P5. COS-7 cells (ATCC®, CRL-1651) were cultured in 10% FBS D-MEM.

RNA extraction and Taq-Man Quantitative Real Time analysis
Total RNA was extracted using TRIzol (Invitrogen, Paisley, UK), according to the manufacturer’s instructions. Real-time quantification to measure miRNAs was performed with the TaqMan miRNA reverse transcription kit. miRNA assay was performed according to the manufacturer’s protocol (Applied Biosystems, UK) with the DNA Engine Opticon 2 system (Bio-Rad Laboratories, UK). Expression was normalized to the U6 small nucleolar RNA (snRU6). Total RNA for miR-503 analysis in plasma was obtained using TRIzol (Invitrogen) with a slight modification to the manufacturer’s protocol. Briefly, 1ml of TRIzol was used for 400ul of plasma mixed and centrifuged after addition of 200µl chloroform. RNA precipitation consisted into the addition of 2µl of 15 µg/µl glicogen (Applied Biosystems) and 1 ml of isopropanol to the previously collected aqueous phase followed by a centrifugation step. Primer identification numbers were as follows: hsa-miR-503: 4373228; snRU6: 4427975. (Applied Biosystems, UK)

For gene expression analysis, single-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using Sensiscript RT kit (Qiagen, Crawley, UK). Quantitative PCR was performed in a LightCycler (Roche, Burgess Hill, UK) using the following primers:

18s rRNA (forward: 5’- TTAGGGACAAAGTGGCGTTC -3’, reverse: 5’- TGTACAAAGGGCAGGGCTT-3’)

1
Cdc25A forward: 5’-TAAGACCTGTATCTCGGCTG-3’; reverse: 5’-
CCCTGGTTCACTGATCTCT-3’;
CCNE1 forward: 5’-GAGCCAGCCTTGGGACAAATAA-3’; reverse: 5’-
GCACGTTGAGTTGGAATTACC-3’;
CCND1 forward: 5’-GTGCTGCGAAGTGGAAACA-3’; reverse: 5’-
ATCCAGGCGGACGATCT-3’;
CCND2 forward: 5’-CTACCTTCCGCAGCTCTCTA-3’; reverse: 5’-
CCCAGCCAAAGAAACGGGTCC-3’;
eGFP forward: 5’-CATGGGCTCCTGCTGGAGT-3’; reverse: 5’-
CGTCGCCGTCCAGCTGACCAG-3’;
18S rRNA forward: 5’-TAGGGGACAAAGTGCTGC-3’; reverse: 5’-
TGTACAAGGGGCGGACT-3’

Each reaction was performed in triplicate, and analysis was performed by the \(2^{-\Delta\Delta CT}\) method as described previously\(^1\). Data were normalized to 18S ribosomal RNA, as an endogenous control.

**Lentivirus preparation and *in vitro* lentivirus-mediated cell infection *in vitro***

Lentiviral vector expressing pre-miR-503 plasmid (*lenti.miR-503*) and *lenti.GFP* (control) were generated by removal of GFP from pRRLsinpptCMV.GFP.WPRE by *BamH*1/*Sal*1 digestion and ligation of pre-miR-503 sequence. *Lenti.GFP* served as control. Lentiviral vectors were produced by co-transfection of 293-T cells and the virus harvested by ultracentrifugation of the supernatant. Viral p24 concentration was determined by immunocapture (Alliance, Perkin Elmer Life Sciences Inc.) and transducing activity quantified. Cells were plated 24h prior to infection, incubated with fresh media containing the required multiplicity of infection per cell (m.o.i.) of virus, left for 1h, washed and maintained until harvesting. All lentivirus infections were performed in the presence of 8 µg/ml polybrene (Sigma, Poole, UK)\(^2\). HUVECs, HMVECs and VSMCs were infected at 25 m.o.i.

**miR-503 inhibition *in vitro***

Applied Biosystem anti-miR™ miR-503 Inhibitor (catalog # AM10378) and Cy™3 dye-labeled Anti-miR™ Negative Control (AM17011) were used. A decoy for miR-503 was also used (*vide infra*).
Preparation of Ad.decoymiR-503 and in vitro infection with adenoviral vectors

Ad.decoymiR-503 was prepared to be used both in vitro and in vivo. For decoymiR-503 vector generation, tandem sequences complementary to miR-503 separated by a 18 bp unrelated spacer (decoy503 sequence: 5’-CTGCAGAACTGTTCCCGCTGCTAgagaactagtaactgttCTGCAGAACTGTTCCCGCTGCTATC-3’) were synthesized as oligonucleotides (Invitrogen) and inserted into XhoI/XbaI multicloning site in a TW3’UTR vector. After sequencing, the CMV-eGFP decoymiR-503 fragment was inserted into an adenoviral vector, pDC515 (Microbix), for adenovirus production. The vector produces a stable eGFP transgene under CMV promoter. This design, using multiple copies of complementary targets, was intended to optimize the transgene repression in the presence of the miRNA

Replication-deficient adenoviruses were generated by site-specific FLP-mediated recombination of the cotransfected shuttle and genomic plasmids in 293 cells. Viral stocks were amplified, CsCl banded, and titrated as previously described. Cells were plated 24h prior to infection, incubated with fresh media containing the required multiplicity of infection per cell (m.o.i.) of virus, left for 6h, washed and maintained until harvesting.

Software programs for miRNAs target genes prediction

To determine the gene targets of miR-503, we used five leading miRNA target prediction algorithms: TargetScan 4.1 (http://www.targetscan.org/)7, miRanda (http://microrna.sanger.ac.uk/sequences/), miRbase (http://www.mirbase.org), Diana microT 3.0 (http://diana.cslab.ece.ntua.gr)10 and EIMMo2 (http://www.mirz.unibas.ch/EIMMo2)11. To determine genes that are similarly identified by 2 or more of these algorithms, the online program Matchminer (http://discover.nci.nih.gov/matchminer) was used. Analysis of molecular pathway for miR-503 target genes was performed with Diana mirPath12.

Luciferase assays

To investigate whether miR-503 directly regulates cdc25A, and CCNE1 expression, portions of the 3’-UTR of these potentials target genes were inserted downstream of a luciferase open reading frame (pLUC). CCNE1 3’-UTR (S204537) and cdc25A 3’-UTR (S213182) vectors were purchased from SwitchGear Genomics. For controls, we prepared similar vectors in which five nucleotide mutations were inserted in the 3’-UTR sequences (cdc25a: 675-681; CCNE1: 248-254 and 486-492) complementary to the miR-503 “seed”
sequences. For CCNE1, plasmids with single or double mutation in 3'-UTR were prepared. Primers and mutation sequences are reported in Supplement Table IIA and IIB. 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 COS-7 cells together with pre-miR-503 or a scrambled oligonucleotide sequence (control). COS-7 cells were chosen for their high efficiency of transfection and for the absence of endogenous miR-503 expression (data not shown). Cells were cultured for 48h and assayed with the Dual-Luciferase Reporter Assay System (Promega).

**Western-Blot analyses**

Proteins were extracted from cultured cells or muscles by using ice-cold buffer A (50mM Hepes, 150mM NaCl, 1mM EDTA, 1mM EGTA, 25mM NaF, 5mM NaPi, 1% Triton, 1% NP40, 1 mM Na3VO4, 0.25% sodium deoxycholate, 0.5mM Na-orthovanadate, 1 mM benzamidine, 0.1mM phenylmethylsulfonyl fluoride). Protein concentration was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, UK). Detection of proteins by Western blot analyses was done following separation of whole cell extracts (50µg) on SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, GE Healthcare, Slough, UK) and probed with the following antibodies: CCNE1 (Biolengend, San Diego, CA, USA; 1:1000), cdc25A (SantaCruz Biotechnology, Santa Cruz, CA, USA; 1:1000), CCND1 (MBL, MA, USA; 1:1000), CCND2 (MBL, MA, USA; 1:1000), and β-actin (Santa Cruz Biotechnology, 1:2000) (used as loading control). For detection, we used secondary antibodies which were goat anti-rabbit or rabbit anti-goat. Secondary antibodies were conjugated to horseradish peroxidase (GE Healthcare, 1:5000). Detection was developed by chemiluminescence reaction (ECL, GE Healthcare).

**Cell cycle analysis**

HUVECs were infected with lenti.miR-503 or lenti.GFP. After fixation in 70% ethanol and RNase A treatment, cells were stained with propium iodide (PI). DNA content was analyzed by flow cytometry (BD FACScan). Data quantification was performed using the software Modfit LT for MAC version 2.0.
BrdU incorporation assay
HUVECs (5x10^3 cells/well), HMVECs (5x10^3 cells/well) or VSMCs (3.5x10^3 cells/well) were seeded in a 96-well plate and infected with lenti.miR-503 or lenti.GFP. Then, the medium was replaced with a complete medium added with BrdU (10µM) for 24h. BrdU incorporation was measured by the BrdU ELISA assay kit (Roche)^1.

Adhesion assay
At 48h from infection with lenti.miR-503 or lenti.GFP, HUVECs (15x10^3/well) were seeded on fibronectin coated 96-well plates. One hour later, non-adherent cells were discarded by washing and adherent cells were fixed and stained with DAPI^1.

Caspase-activity assay
To measure apoptosis, HUVECs (7x10^3/well) were seeded in flat bottomed 96-well plates and infected with lenti.miR-503 or lenti.GFP. Caspase-3 activity was measured by using a luminescent cell death detection kit (Caspase-Glo Assay, Promega)^1.

Migration Scratch Assay
Confluent HUVECs or VSMCs were infected with lenti.miR-503 or lenti.GFP. After 5h, a cell scratch spatula was used to make a scratch in the cell monolayer. Cells were incubated with EGM-2 containing 2mM of hydroxyurea (Sigma) to arrest cell proliferation. Pictures were taken immediately after scratching and 24h thereafter. The migration distance (in µm) as the reduction of the width of the open area was calculated^13.

Matrigel assay
HUVECs or HMVECs (4X10^5 cell/well) were infected with lenti.miR-503 or lenti.GFP and seeded in 48-well plates coated with a growth factors-enriched Matrigel. Endothelial network formation was quantified in randomly captured microscopic fields (magnification 100x) by counting the number of intersection points and calculating the length of cellular network^1.

In vivo mouse studies
The experiments involving mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal
Resources and with the prior approval of the UK Home Office and the University of Bristol ethic committee.

Type-1 diabetes was induced by streptozotocin (STZ) injection in CD1 mice (Charles River, Margate, UK), as previously described\(^1^4\). Briefly, STZ (Sigma-Aldrich) was delivered \textit{i.p.} for 5 consecutive days (40mg/Kg in citrate buffer per each day). Non-diabetic (injected with the STZ buffer) CD1 male mice were used as controls. Fourteen days after the first STZ injection, glycaemia at fast and glycosuria were measured and only those mice with glycaemia above 200 mg/dL and overt glycosuria entered the protocol. Absence of hyperglycemia and glycosuria in buffer-injected non-diabetic mice was also verified.

Following anesthesia (tribromoethanol, 880 mmol/kg \textit{i.p.}, Sigma-Aldrich), unilateral limb ischemia was induced in mice with 12 weeks of diabetes and in age-matched non-diabetic control mice using a refined procedure which consists of ligation (with a 7-0 silk suture) in 2 points and electrocoagulation of the upper part of the left femoral artery, but leaving the femoral vein and nerve untouched\(^1^5\). Immediately after, \textit{Ad.decoymiR-503} or \textit{Ad.Null} (10\(^9\) p.f.u. in 10 µl total volume) was delivered to the ischemic adductor muscle of diabetic mice in three equidistant sites along the projection of the femoral artery, as we previously described\(^1^5\). Non-diabetic mice received \textit{Ad.Null}, only. Foot blood flow was measured at 30 min, 7 days, 14 days and 21 days after ischemia by using a perfusion image system (Lisca colour laser Doppler, Perimed, Sweden) (n=at least 12 mice per group)\(^1^5\). The ischemic/non-ischemic foot blood flow ratio was calculated as an index of perfusion recovery\(^1^5\). At 3 and 21 days post-ischemia, n=6 mice per group were sacrificed for molecular biology analyses. At 21 days post-ischemia, adductor muscles from terminally anesthetized mice (n=6) were \textit{in situ} perfused with heparinased PBS for 1 min and then with 10% buffered formalin for 5 min \textit{via} a cannula inserted into the abdominal aorta in the direction of limbs. Ischemic and contralateral muscles were then removed, kept in 4% buffered formalin for 24 h and processed for paraffin embedding\(^1^5\). Muscular sections were stained as previously described\(^1\). Briefly, sections were incubated overnight at 4 °C with Alexa 568-conjugated isolectinB4 (1:100 red fluorescence, Molecular Probes) to identify ECs and FITC-conjugated \(\alpha\)-vascular smooth actin (1:100 green fluorescence, Sigma Aldrich) to recognize VSMCs (which are part of the arteriole walls). Slides were observed under a fluorescence microscope (Olympus CX41, Olympus, Southend-on-Sea, UK). High power fields were captured (at 400X) and the number of capillaries and arterioles per field were counted. At least 30 randomly chosen fields were evaluated. Arterioles were recognized from venules by their morphology. In fact, arterioles have a lumen which is
circular or elliptical and well opened by the perfusion/fixation. Moreover, arterioles have one or more continuous layers of VSMCs in the tunica media. Capillaries can be also identified by this staining as being the small (not more than 8 µm in diameter) vessels composed by 1 or 2 ECs stained by lectin, but missing VSMCs. Capillary and arteriole densities were expressed as number of vessels per mm$^2$ of muscular sections.

Isolation of endothelial cells from mouse ischemic limb muscles and flow cytometric analysis
Adductor muscles at 3 days post-ischemia induction in diabetic and non-diabetic mice were rinsed and digested with collagenase II (Worthington) plus DNase I (Sigma) using gentleMACS™ Dissociator, following the manufacture’s protocol. Next, ECs were immunomagnetic sorted using a CD146 antibody (clone ME-9F1) (Miltenyi Biotech)$^{16}$. CD146 is a transmembrane glycoprotein mainly expressed at the intercellular junction of ECs$^{17}$. To verify, EC enrichment by our CD146-positive separation, single cell suspensions were incubated with FITC-conjugated CD146 and APC-conjugated CD31 antibodies or the respective isotype for negative control. Unstained and single stained controls were performed to define positivity. Fluorescence was analysed in a Canto II flow cytometer using the Diva software (BD).

**Supplemental References**


**Supplemental Tables**

### A

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Targetscan</th>
<th>mirRANDA</th>
<th>miRbase</th>
<th>Diana</th>
<th>EIMMO</th>
<th>Databases</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNE1</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>5/5</td>
</tr>
<tr>
<td>cdc25A</td>
<td>√</td>
<td></td>
<td></td>
<td>no</td>
<td>√</td>
<td>4/5</td>
</tr>
<tr>
<td>CCND1</td>
<td>√</td>
<td>√</td>
<td>no</td>
<td>√</td>
<td>√</td>
<td>4/5</td>
</tr>
<tr>
<td>CCND2</td>
<td>√</td>
<td>√</td>
<td>no</td>
<td>√</td>
<td>√</td>
<td>4/5</td>
</tr>
<tr>
<td>VEGFA</td>
<td>√</td>
<td></td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>3/5</td>
</tr>
<tr>
<td>FGFR</td>
<td>√</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>3/5</td>
</tr>
<tr>
<td>E2F3</td>
<td>√</td>
<td></td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>3/5</td>
</tr>
<tr>
<td>FOSL</td>
<td>√</td>
<td></td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>3/5</td>
</tr>
<tr>
<td>BTRC</td>
<td>√</td>
<td></td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>3/5</td>
</tr>
<tr>
<td>WEE1</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>3/5</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>2/5</td>
</tr>
<tr>
<td>CHEK1</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>2/5</td>
</tr>
<tr>
<td>FGFR2</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>√</td>
<td>2/5</td>
</tr>
<tr>
<td>RAF1</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>2/5</td>
</tr>
<tr>
<td>MAP2K1</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>2/5</td>
</tr>
<tr>
<td>CCND3</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>2/5</td>
</tr>
<tr>
<td>BTRC</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>√</td>
<td>3/5</td>
</tr>
<tr>
<td>BCL2</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>2/5</td>
</tr>
<tr>
<td>EIF4E</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>2/5</td>
</tr>
<tr>
<td>WNT3A</td>
<td>√</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>√</td>
<td>1/5</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>KEGG pathway</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Cycle</td>
<td>CCNE1, E2F3, WEE1, CHEK1, CCND2, CCND3, CCND1, CDC25A</td>
</tr>
<tr>
<td>Wnt signaling pathway</td>
<td>WNT3A, FOSL, BTRC</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>BCL2, MAP2K1, PIK3R1</td>
</tr>
<tr>
<td>mTOR signaling pathway</td>
<td>PIK3R1, AKT3, EIF4E</td>
</tr>
<tr>
<td>VEGF signaling pathway</td>
<td>MAP2K1, PIK3R1, VEGFA, RAF1</td>
</tr>
<tr>
<td>Regulation of actin cytoskeleton</td>
<td>MAP2K1, PIK3R1, FGF7, FGF2</td>
</tr>
</tbody>
</table>

**Supplementary Table I**

**A**, Bioinformatic prediction of the miR-503 target genes. An *in silico* analysis search of potential targets was performed using TargetScan, miRANDA, miRBASE, Diana microT, and EIMMo algorithms. cdc25A, CCNE1, CCND1 and CCND2 were predicted as miR-503 targets by 5/5 or 4/5 software programs. **B**, Analysis of molecular pathways for miR-503 target genes performed with Diana mirPath.
Supplementary Table II

A, Sequences (in red) and introduced mutations (in blue) of the miR-503 binding sites.
B, List of primers used for in vitro mutagenesis assay.
### Diabetic Patients Undergoing Ischemic Limb Amputation

<table>
<thead>
<tr>
<th>Patients</th>
<th>1D</th>
<th>2D</th>
<th>3D</th>
<th>4D</th>
<th>5D</th>
<th>6D</th>
<th>7D</th>
<th>8D</th>
<th>9D</th>
<th>10D</th>
<th>11D</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>65</td>
<td>70</td>
<td>69</td>
<td>66</td>
<td>68</td>
<td>66</td>
<td>59</td>
<td>84</td>
<td>58</td>
<td>81</td>
<td>75</td>
<td>68.09±9.06</td>
</tr>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>10/11 M</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>11/11 non smoker</td>
<td></td>
</tr>
<tr>
<td>HbA1c(%Hb)</td>
<td>7.6</td>
<td>9.2</td>
<td>6.9</td>
<td>7.2</td>
<td>6.8</td>
<td>12.1</td>
<td>7</td>
<td>9.6</td>
<td>6.8</td>
<td>8</td>
<td>7.7</td>
<td>8.08±1.63</td>
</tr>
<tr>
<td>Type1 Diabetes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>1/11 T1</td>
</tr>
<tr>
<td>Type2 Diabetes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>10/11 T2</td>
</tr>
<tr>
<td>Insulin Therapy</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>9/11 receiving insulin</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>5/11 with retinopathy</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>2/11 with nephropathy</td>
</tr>
<tr>
<td>CAD</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>8/11 with CAD</td>
</tr>
</tbody>
</table>

### Non-Diabetic Patients Undergoing Vena Saphena stripping (Controls)

<table>
<thead>
<tr>
<th>Patients</th>
<th>1 ND</th>
<th>2 ND</th>
<th>3 ND</th>
<th>4 ND</th>
<th>5 ND</th>
<th>6 ND</th>
<th>7 ND</th>
<th>8 ND</th>
<th>9 ND</th>
<th>10 ND</th>
<th>11 ND</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>69</td>
<td>51</td>
<td>54</td>
<td>64</td>
<td>69</td>
<td>56</td>
<td>68</td>
<td>62</td>
<td>57</td>
<td>51</td>
<td>80</td>
<td>61.9±9.1</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>5/11 M</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>11/11 non smoker</td>
</tr>
<tr>
<td>CAD</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>0/11 with CAD</td>
</tr>
</tbody>
</table>

**Supplementary Table III.**
Clinical characteristic of diabetic and non-diabetic patients. CAD=coronary artery disease.
Supplementary Figure I. Validation of decoy503 vector

A, Schematic description of the functional elements in the 3'UTR decoymiR-503 (decoy503) vector. CMV: cytomegalovirus immediate early promoter; eGFP: enhanced green fluorescent protein. B and C, Percentage of eGFP positive cells and relative mean fluorescence (measured by flow cytometry) of COS-7 cells transfected with decoy-503 vector together with pre-miR503 or scrambled oligos (miR scr). Values are means±SEM. *P<0.05 and **P<0.01 vs decoy503+miR scrambled. D, eGFP relative fluorescence (measured by fluorometry) in HUVECs infected with an adenovirus carrying decoy503 (Ad.decoy503, 250 m.o.i.) and cultured in HG-LGF (high glucose and low growth factor) or NG (normal glucose and normal conditions) for 24h. Values are means±SEM. **P<0.01 vs Ad.decoy503+NG.
Supplementary Figure II. Flow cytometric analysis of magnetically isolated CD146<sup>pos</sup> cells. CD146<sup>pos</sup> isolated cells were stained with FITC-conjugated CD146, APC-conjugated CD31 antibodies or the respective isotype for negative control. A, percentage of CD146<sup>pos</sup> cells and B, percentage of double positive CD31<sup>pos</sup>/CD146<sup>pos</sup> cells after isolation from ischemic diabetic limb muscles and ischemic non-diabetic limb muscles.
Supplementary Figure III. Infection of human umbilical vein endothelial cells (HUVECs) with \textit{lenti.miR-503} or \textit{lenti.GFP}.

HUVECs were infected with 25 m.o.i. of lentiviral vectors expressing pre-miR-503 (\textit{lenti.miR-503}) or GFP (\textit{lenti.GFP}). (A) Fluorescent and phase-contrast images of HUVECs expressing GFP or miR-503. Scale bars 250 $\mu$m. (B) After 24h, 48h and 72h of lentivirus infection, miRNA expression was analyzed using the TaqMan Real Time PCR assay. Relative expression was calculated using the comparative Ct methods\textsuperscript{18}. Samples were normalized to snRU6 expression. Experiments were performed in quadruplicate. Values are means±SEM (n=3). **$p<0.01$ vs GFP.
Supplementary Figure IV. Effect of miR-503 forced expression on HUVEC apoptosis and adhesion to fibronectin.

A, Apoptosis was assessed by measuring caspase-3 activity assay in HUVECs previously transduced with miR-503 or GFP. B, Adhesion to fibronectin of GFP-HUVECs and miR503-HUVECs. Cell adhesion was quantified by counting the number of adherent cells following washing out of non adherent cells and DAPI staining. Experiments were performed in triplicate and repeated 3 times. In bar graph, values are expressed as means±SEM. *P<0.05 vs GFP.
Supplementary Figure V. Effect of miR-503 forced expression on human microvascular endothelial cell (HMVEC) proliferation and tube-like structure formation on Matrigel.

A, HMVECs were infected with either lenti.GFP (GFP) or lenti.miR-503 (miR503) (25 m.o.i). Proliferation was measured by BrdU incorporation at 48h from infection. B, Cell network formation on Matrigel. In bar graphs, the quantification of endothelial cell network formation at 24h from HMVEC seeding is expressed as the total length of tube-like structures per field (left graph) and as number of branching points per field (right graph). Experiments were performed in triplicate and repeated 3 times. Values are means±SEM. *P<0.05 vs GFP. Panels above show representative photomicrographs (original magnification 100x).
Supplementary Figure VI. Effect of miR-503 on human vascular smooth muscle cells (hVSMC) proliferation and migration

A, hVSMCs were infected with either lenti.GFP (GFP) or lenti.miR-503 (miR503) (25 m.o.i). hVSMC proliferation was measured by BrdU incorporation at 48h from infection; B, hVSMCs migration was assessed in a scratch assay. Cells monolayers were scratched and images acquired immediately after and at 24h later. Gap closure was calculated. Experiments were performed in triplicate and repeated 3 times. All values are means±SEM. *P<0.05 vs. GFP.
Supplementary Figure VII. Effect of adenoviral infection on ECs proliferation and *in vitro* angiogenesis

HUVECs were infected with *Ad.Null* or non-infected (and given PBS) and cultured in HG-LGF or NG for 24h. **A**, HUVEC proliferation under normal culture conditions (NG) or under high glucose and low growth factor culture conditions (HG-LGF) was measured by BrdU incorporation. **B**, Network formation on Matrigel was quantified at 24h from cell seeding and expressed as the total length of tube-like structures. Panels above show representative microphotographs (original magnification 100x; scale bar: 100 µm). Experiments were performed in triplicate and repeated 3 times. All values are means±SEM. *P<0.05 vs NG (PBS); +P<0.05 vs NG (*Ad.Null*). In none of the above tests, significant differences amongst *Ad.Null* and PBS were observed.
Supplementary Figure VIII. Transduction capacity of **Ad.decoymiR-503** in non-ischemic muscles of non-diabetic mice

**Ad.decoymiR-503** or **Ad.Null** ($10^9$ p.f.u. in 10 µl total volume) was delivered to the ischemic adductor muscle of non-diabetic mice. Image shows RT-PCR for eGFP expression at 3, 7 days, 14 days, and 21 days from the injection. 18s ribosomal RNA (18s) was used as loading control.
microRNA는 당뇨병에서 나타나는 혈관재생장애의 주범이다

한 기 존 교수 서울대산병원 심장내과

Summary

배경
당뇨병은 혈관내피세포의 기능을 저하시키고 혈관 이외의
복구를 위한 혈관신생을 저해시킨다. 하지만, 그 기전에
대해서는 잘 연구된 바 없다. 일반적으로 microRNA는 표
적 유전자들의 3'-untranslated region과의 상호작용을 통
하여 유전자들의 발현을 억제한다.

방법 및 결과
본 연구에서는 microRNA-503(miR-503)은 혈관내피세포
에 발현되었을 때까지 당뇨병 상태의 유지하며(high-
D-glucose), 혈관생장과의 근거현상(low growth
factors)의 환경에서 투여하였을 때 나타난다. 정상적인 배양
상태에서는 lentivirus-배양성 miR-503의 강제 발현이
lentivirus-GFP 대조군과 비교하였을 때 나타난다. 또한,
세포이동도 Matrigel에서의 혈관내피세포의 형성 등
을 저해함을 나타낸다. 이러한 배양성 miR-503의 기능을
adenovirus-배양성 miR-503 decoy(Ad.decoymiR-503)
의 발현 또는 antimiR-503(anti sense oligonucleotide)
의 발현으로 증가시켰을 때 혈관내피세포의 high-
D-glucose/low growth factors의 가능성이 상대적 예방
능을 나타낸다. CCNE1과 cdc2SA와 같은 유전자를 miR-
503의 직접적인 표적 유전자로 밝혀졌으며, 이들은 실
질적으로 고혈당/저혈당인자 상태에서 내피세포에 발
현 증가한다. 또한, streptozotocin-diabetic mice의 혈관
유발시기 근육에서 miR-503의 발현도가 증가하며, 여기
에 AddecoymiR-503을 전달하였을 때 당뇨병 상태에
서 유발되는 혈관 주위 신장성 장애 또는 질환의 회
복가능성이 증가된다는 것을 증명하였다. 마지막으로, miR-503
과 표적 유전자의 발현이 당뇨병 환자로부터 얻어진 혈
액에서의 정량적 근육조직에서 유발되는지를 연구하였
으며, 당뇨병 환자 비혈관성 근육과 비교하였을 때 당뇨병
상태의 근육에서는 miR-503 발현도가 매우 특이하게 높
았으며 이는 cdc25 단백발현도에 반영되었다. 또한,
혈중 miR-503 수준이 각각의 당뇨병 환자에서 높게 나타
났다.

결론
본 연구결과는 miR-503이 심각한 사지혈관 상태의 당뇨
병 환자에서 지료적인 표적으로 가능함을 시사한다.
본 연구에서 간단히 제시된 바와 같이 세포의 증식 및 사멸기전의 on-off는 매우 복잡한 요소들로 되어 있다. 즉 세포가 증식시키며 접히는 데에는 많은 cross check point가 존재할 것으로 보이며, 본 연구에서 언급된 표적 유전자인 CNE1과 cdc25A 등과 같은 물질은 cdk의 활성을 유도함으로써 혈관내피세포의 증식을 유발하는 인자이다. 또한 microRNA는 놀라게도 매우 작은 크기의 물질임에도 불구하고 일부의 miRNA와의 상호작용을 통해 많은 종류의 세포의 복잡한 셈체의 역할을 미침으로써 조절하게 된다. 비록 이론을 대한 연구가 있기도 하지만, 한편으로는 단일 특정물질의 존재에 의한 효과가 지지부진한 복잡한 다자원체를 가진(당뇨병,고혈압 등) 질환의 조절을 위해서는 상당한 강점을 잃지 않고 있는 것이 사실이다.

1. 본 논문은 in vitro에서 in vivo까지의 일련의 체계적인 실험을 통하여, 그리고 miRNA에 의한 기능체제를 소위 부가적인 rescue 실험을 시행하여 다시 극복실험으로써 그 신뢰도를 높인 우수한 논문이라고 할 수 있다.

2. 몇 가지 유의하여야 할 점 중 하나는 과연 miR503과 관련된 주요인이 당뇨병이나 아니면 환산이나 하는 점이다. Figure 10에서 보듯이 고혈압 상태보다는 저혈압인자로 대표되는 혈압상태가 좀 더 miR503의 발현을 유도하는 경향을 보였다는 점이다. 하지만 이후의 실험에서는 이러한 구분을 하고 실험한 결과는 없다. 그러나 혈압상태가 아니라라도 miR503의 과 발현에 의하여 matrigel의 네트워크형성이 역에되는 점 miR503의 발현에 의하여 cdc25 및 CNE1의 발현이 특이적으로 조절되는 점을 보이며 miR503의 혈압상태와는 무관하게 그 발현도 따라 cdc25 및 CNE1의 조절을 통하여 혈관내피세포의 증식 응력에 영향을 미치는 것은 확실한 것으로 판단된다.

3. 마우스를 사용한 시험에서 혈관내피에서 만성병 상태에서의 혈관내피 세포의 유도는 실험이 발생하였을 때 역시 miR503이 상승하는 것이 예측되는 결과이지만, 담당병이 없는 상태에서의 혈관내피 세포의 증식을 유발하는 인자이다.