Deficiency of the MicroRNA-31–MicroRNA-720 Pathway in the Plasma and Endothelial Progenitor Cells From Patients With Coronary Artery Disease

Hsei-Wei Wang,* Tse-Shun Huang,* Hung-Hao Lo,* Po-Hsun Huang, Chih-Ching Lin, Shing-Jyh Chang, Ko-Hsun Liao, Chin-Han Tsai, Chia-Hao Chan, Cheng-Fong Tsai, Yi-Chieh Cheng, Ya-Ling Chiu, Tsung-Neng Tsai, Cheng-Chung Cheng, Shu-Meng Cheng

Objective—Defects in angiogenesis/vasculogenesis or vessel repair are major complications of coronary artery disease (CAD). Endothelial progenitor cells (EPCs) play a fundamental role in postnatal vascular repair and CAD. The role of microRNAs in CAD pathogenesis and their potential as biomarkers remain to be elucidated.

Approach and Results—MicroRNA-31 (miR-31) level in both the plasma and EPCs of patients with CAD is found lower. miR-31 regulates EPC activities by targeting FAT atypical cadherin 4 and thromboxane A2 receptor, which show increased expression in CAD EPCs. Overexpressing miR-31 in CAD EPCs rescued their angiogenic and vasculogenic abilities both in vitro and in vivo. When exploring approaches to restore endogenous miR-31, we found that far-infrared treatment enhanced the expression of not only miR-31, but also miR-720 in CAD EPCs. miR-720, which was also decreased in EPCs and the plasma of patients with CAD, stimulated EPC activity by targeting vasohibin 1. The miR720–vasohibin 1 pair was shown to be downstream of FAT atypical cadherin 4, but not of thromboxane A2 receptor. FAT atypical cadherin 4 inhibited miR-720 expression via repression of the planar cell polarity signaling gene four-jointed box 1 (FJX1), which was required for miR-720 expression through a hypoxia-inducible factor 1, α subunit–dependent mechanism. Restoring miR-720 level strengthened activity of CAD EPCs. The miR-31–miR-720 pathway is shown critical to EPC activation and that downregulation of this pathway contributes to CAD pathogenesis. Circulating levels of miR-31, miR-720, and vasohibin 1 have the potential to allow early diagnosis of CAD and to act as prognosis biomarkers for CAD and other EPC-related diseases.

Conclusions—Manipulating the expression of the miR-31–miR-720 pathway in malfunction EPCs should help develop novel therapeutic modalities. (Arterioscler Thromb Vasc Biol. 2014;34:857-869.)

**Key Words:** coronary artery disease • microRNAs

Coronary artery disease (CAD) still ranks as one of the most fatal diseases worldwide despite advances in prevention and treatment. A progressive impairment of endothelial function and integrity starts a cascade of events that leads to microcirculation damage, atherosclerosis, CAD, and other common cardiovascular disorders including myocardial infarction, heart failure, stroke, and peripheral arterial disease. Developing new early diagnostic biomarkers and therapeutic strategies based on the endothelial lineage of cells is therefore of clinical significance and important to pharmaceutical companies.

Adult stem cells fuel the renewal of many tissues. Convincing evidence suggests that neovascularization in adults is not solely the result of proliferation of local endothelial cells (angiogenesis), but also involves bone marrow–derived circulating endothelial progenitor cells (EPCs). EPCs, which are derived from hematopoietic stem cells, are capable of forming new blood vessels (vasculogenesis) even in the absence of a pre-existing vessel network. The number of circulating EPCs in patients has been reported to be negatively correlated with the progression of coronary heart disease, hypertension, diabetes mellitus, and aging. Increased circulating CD31+/annexin V+ apoptotic microparticles and decreased circulating EPC levels have also been detected in hypertensive patients with microalbuminuria. In terms of mechanisms, several studies have reported that inflammation influences the mobilization and differentiation of EPCs. EPCs are currently being coded equally to this article.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.303001/-/DC1. Correspondence to Shu-Meng Cheng, MD, Division of Cardiology, Department of Internal Medicine, Tri-Service General Hospital, National Defense Medical Center, No. 325, Sec. 2, Chenggong Rd, Neihu District, Taipei 114, Taiwan. E-mail chengsm@cm1.hinet.net

© 2014 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.113.303001
miR-31 is a positive regulator of some fundamental endothelial functions. Other experiments have shown that miR-31 targets FAT atypical cadherin 4 (FAT4), a newly identified tumor suppressor, in both blood vessel and lymphatic vessel endothelial cells for inducing endothelial cell migration and invasion.\(^1\)\(^9\)\(^1\) miR-31 also functions as a negative regulator of lymphatic vascular lineage-specific differentiation in vitro and vascular development in vivo by targeting prospero homebox 1 (PROX1), a transcription factor that functions as a master regulator of lymphatic lineage-specific differentiation.\(^2\)\(^1\)\(^5\) Recently, levels of miR-31 have been found to be significantly higher in EPCs with better angiogenic ability: EPCs from cord blood are more active and express more miR-31 compared with those derived from peripheral blood (PB).\(^2\)\(^3\) The pathological role and underlying mechanisms by which miR-31 affects cardiovascular diseases, however, have not been examined.

We hypothesized that miR-31, on top of its known physiological functions, also plays a crucial role in CAD pathogenesis. We examined levels of miR-31 in EPCs from diseased individuals and in the plasma of patients with CAD to evaluate its potential as a biomarker. How miR-31 regulates CAD EPC activity was also explored. Finally, we tested the possibility that restoring CAD EPC function by increasing miR-31 levels in diseased EPCs either by the transfection of oligonucleotide mimics of miR-31 or by far-infrared (FIR) treatment of ex vivo expanded EPCs.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

**Reduced miR-31 Levels in the Plasma and EPCs of Patients With CAD**

Late EPCs from the PB of healthy donors (HDs) and patients with CAD were isolated and characterized as described.\(^2\)\(^4\) Both healthy and diseased late EPCs have a cobblestone-like morphology (Figure IA in the online-only Data Supplement). Both EPCs were confirmed as having endothelial lineage characteristics because both precursor cells were positive for acLDL (acetylated-low density lipoprotein) uptake (Figure IB in the online-only Data Supplement). They also express similar levels of endothelial marker genes and the CD34 precursor gene in the fluorescence-activated cell sorter analysis (Figure IC in the online-only Data Supplement).

Next, the possible involvement of miR-31 in CAD pathogenesis was explored by detecting miR-31 levels in EPCs and the plasma from patients with CAD and from healthy control individuals. EPCs isolated from CAD cases were found to express less miR-31 than those from healthy controls (Figure 1A, left). The levels of circulating miR-31 in plasma from patients with CAD were also significantly lower than in plasma from healthy controls (Figure 1A, right). Overexpressing miR-31 in healthy EPCs increased EPC migration and the ability of EPCs to form microvascular tubes (Figure 1B), whereas knockdown of endogenous miR-31 in healthy EPCs hindered these abilities (Figure 1C). Notably, flow cytometric analysis showed clearly that the CAD EPCs expressed less vascular endothelial growth factor (VEGF) receptor 2/kinase insert domain receptor but more vascular endothelial-cadherin (Figure IC)
miR-31 Regulates EPC Function via the Suppression of FAT4 and Thromboxane A2 Receptor

FAT4, a tumor suppressor, has been previously identified as a direct downstream target of miR-31 in mature lymphatic endothelial cells. We examined whether the same scenario also occurs in EPCs. Increased expression of FAT4 was, as expected, found to be present in CAD EPCs (Figure 2A). FAT4 levels were negatively correlated with those of miR-31 during overexpression and knockdown experiments using Hds’ PB EPCs (Figure 2B). These observations implied that the dysregulation of the miR31–FAT4 axis contributes to defective CAD EPC activity.

To better understand the mechanism underlying the activity of miR-31, we investigated new miR-31 downstream targets. miR-31 is more abundant in cord blood EPCs than in PB EPCs from healthy individuals and is also more abundant in cord blood EPCs than in mature endothelial cells such as human umbilical vein endothelial cell (not shown). We aligned the genes downregulated in cord blood EPCs in comparison with PB EPCs and human umbilical vein endothelial cells to predict miR-31 targets using the miRTar bioinformatics tool. To further narrow down possible miR-31 targets, we also checked whether the target candidates had known antiangiogenic activity. A known antiangiogenic gene, thromboxane A2 receptor (TBXA2R), fulfilled all these criteria (Figure 2C, left). TBXA2R is a G protein–coupled receptor that is involved in platelet aggregation and endothelial cell migration. Furthermore, TBXA2R is an important mediator of isoprostanes, which are the endogenously formed end products of lipid peroxidation and are known to inhibit VEGF-induced angiogenesis. The involvement of TBXA2R in CAD pathogenesis has not been reported previously to our knowledge.

Microarray data showed that the level of TBXA2R is higher in mature endothelial cells (Figure 2C, middle). We confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) that the expression levels of TBXA2R are higher in both mature endothelial cells (Figure 2C, right) and in CAD EPCs (Figure 2D) compared with healthy EPCs. The RNA and protein levels of TBXA2R were found to be decreased when miR-31 was overexpressed in healthy EPCs, but were increased when endogenous miR-31 was knocked down (Figure 2E). The direct repression of TBXA2R by miR-31 was explored using the luciferase reporter assay, and it was found that miR-31 is able to repress luciferase expression when the construct contained the TBXA2R 3′ untranslated region fused downstream of the luciferase gene (Figure 2F, the wild-type group). Such repression could be reversed by mutating the predicted miR-31–binding site (Figure 2F, the mutant group).

To further clarify the hierarchical relationship between miR-31 and TBXA2R, we compared the results of knocking down miR-31 with those of knocking down TBXA2R, either independently or in combination with miR-31 knockdown (Figure 2G). EPCs with reduced miR-31 showed poorer
migration activity and higher TBXA2R expression, while diminishing TBXA2R in miR-31low EPCs restored cell motility (Figure 2H, left and Figure IIA in the online-only Data Supplement; miR-31 and TBXA2R levels detected by RT-qPCR are presented in Figure 2G). Similar results were found using the tube formation assay (Figure 2H, right and Figure IIB in the online-only Data Supplement). These findings suggested that TBXA2R is also an important direct downstream target of miR-31. Knocking down TBXA2R did not alter miR-31 levels in EPCs (Figure 2G, lanes 1 versus 3), which suggests that regulation of the miR31–TBXA2R pathway is not reciprocal.

### Induction of miR-31, and Its Partner miR-720, in CAD EPCs by FIR Treatment

The aforementioned findings suggest that the restoration of miR-31 levels in diseased EPCs might have potential as a novel therapeutic strategy. Nevertheless, delivering miRNA-related biological drugs, such as synthetic oligonucleotide mimics in vivo, is still a major challenge. Therefore, we sought an alternative approach that might be able to stimulate and restore the expression of endogenous miR-31 in CAD EPCs. It has been reported that FIR treatment is able to boost EPC activity and angiogenesis both in vitro and in vivo.28 We were able to confirm that FIR treatment not only induces cellular migration, microvascular formation, and endothelial NO synthase production in healthy EPCs (Figure 3A and Figure IIIA in the online-only Data Supplement), but also restores migration and tube formation in CAD EPCs (Figure 3B and Figure IIB in the online-only Data Supplement). FIR irradiation was found to result in the upregulation of miR-31 accompanied with the downregulation of the miR-31 targets (FAT4 and TBXA2R) in healthy EPCs (Figure 3C). Treating CAD EPCs with FIR restored the expression of endogenous miR-31 to a level comparable with that of healthy EPCs (Figure 3D). Furthermore, FAT4 and TBXA2R levels were also suppressed by FIR in CAD EPCs (Figure IIIC in the online-only Data Supplement). To clarify whether miR-31 mediates FIR-induced endothelial cell activation, we used healthy EPCs with knocked down miR-31 or FIR (Figure 3E).

To provide further mechanistic insights into the miR-31–mediated FIR functions, partner miRNAs to miR-31 that also respond to FIR treatment were explored. RNA extracted from FIR-treated PB EPCs were subjected into miRNA microarray analysis and 11 more miRNAs (in addition to miR-31) were found to be induced by FIR by ≥2-fold in these EPCs, with miR-720 as the most upregulated (Figure IID in the online-only Data Supplement). The increase in miR-720, miR-301a, and miR-7 levels induced by FIR in healthy EPCs was confirmed by RT-qPCR using independent batches of blood, and again miR-720 was found to be the most upregulated miRNA after FIR treatment (Figure 3F). Similarly, in CAD EPCs, miR-720 was the most significant induced miRNA by FIR (Figure IIE in the online-only Data Supplement).
In the light of the above results, we speculated that there is a cross-talk between miR-31 and miR-720. In healthy EPCs, overexpression of miR-31 increased miR-720 levels (Figure 3G, left), whereas knockdown of endogenous miR-31 resulted in a reduction of endogenous miR-720 (Figure 3G, right). To further elucidate whether miR-31 mediates FIR induction of miR-720 in PB EPCs, we used healthy EPCs with knocked down endogenous miR-31 for the FIR treatment and analyzed levels of miR-720, as well as the transcript levels of FAT4 and TBXA2R. Knocking down of miR-31 abolished FIR-induced miR-720 induction (Figure IIIF in the online-only Data Supplement). Similarly, knocking down of miR-31 abolished FIR-mediated FAT4/TBXA2R repression in healthy EPCs (Figure IIIG in the online-only Data Supplement).

The New Angiogenic miRNA miR-720, Which Is Regulated by miR-31, Contributes to CAD EPC Activity

No report up to the present has suggested that miR-720 contributes to angiogenesis or to CAD pathogenesis. Similar to miR-31, miR-720 levels were found to be lower in both the EPCs and the plasma from patients with CAD (Figure 3H) compared with healthy EPCs. miR-720 levels in CAD EPCs could be restored by FIR to a level comparable with that of healthy EPCs (Figure 4A). Schopman et al.²⁹ showed by in silico sequence alignment that the sequence annotated as miR-720 overlaps with that of tRNA⁷⁶, which is encoded by the mitochondrion. Nevertheless, because of the short sequence that makes up miR-720, it is still possible to map miR-720 to other regions of human genome. We found that both the pre-miR-720 and miR-720 sequences could also be aligned to human chromosome 3 (chr3: 164059155–164059171; Figure IVA in the online-only Data Supplement).

Before checking the angiogenic role and related mechanisms of miR-720, we examined whether miR-720 has the functional characteristics of an miRNA, such as a Dicer1-dependent biogenesis, physical association with Argonaute proteins, and the ability to repress mRNA transcripts in a sequence-specific manner. We first checked the physical association of mature miR-720 with the Argonaute 2 protein by performing RT-qPCR assays on RNA immunoprecipitation (RIP) products brought down by an anti–Argonaute 2 antibody. Mature miR-720 was significantly enriched in anti–Argonaute 2 RIP products (Figure VA in the online-only Data Supplement; miR-31 was included as a positive control). To validate our RIP-qPCR data, we extracted another 19 anti–Argonaute 1/2 immunoprecipitation small RNA sequencing results (RIP-seq, CLIP-seq [cross-linking immunoprecipitation-sequencing], or PAR-CLIP-seq [photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation-sequencing]; Figure IVB in the online-only Data Supplement), which correspond to 10 data sets, from the NCBI GEO database, and analyzed whether miR-720 was also significantly enriched in other anti–Argonaute 1/2 immunoprecipitation experiments. miR-720 signals were found to be enriched in 4 sequencing results, namely those obtained from HeLa cells, the Sup-T1 human T-cell lymphoblastic lymphoma cell line, and the BC-1 B-cell line (Figure IVC in the online-only Data Supplement). The pre–miR-720 sequence and RIP-seq reads that mapped onto matured
miR-720 are shown in Figure IVD in the online-only Data Supplement.

We next examined whether biogenesis of miR-720 occurs in a Dicer1-dependent manner. When DICER1 RNAs were knocked down in healthy EPCs, less mature miR-720 could be detected by RT-qPCR (Figure VB in the online-only Data Supplement). These results suggested that miR-720 maturation, just like other miRNAs recorded in the miRBase, follows the miRNA biogenesis rule set. Collectively, our findings support the hypothesis that miR-720, whether derived from tRNAThr or from other sites on human chromosome 3, has the features of an miRNA.

We evaluated the proangiogenic role of miR-720 in EPCs from healthy donors (Hds) and patients with CAD. Transwell migration (left) and tube formation (right) assays were conducted when knocking down vasohibin 1 (VASH1) in miR-720–reduced EPCs. All histograms were graphed as means±SD. Numbers above bars indicate the relative fold ratios. Significance levels, *P<0.05; **P<0.01; ***P<0.001. Scr indicates scramble control; UTR, untranslated region; and Vec, vector control.

miR-720 Represses a Major Downstream Target, Vasohibin 1, in a Sequence-Specific Manner

To understand mechanisms related to the activity of miR-720 and to confirm that miR-720 has the characteristics of a functional miRNA, we analyzed the ability of miR-720 to repress mRNA transcripts in a sequence-specific manner. We mined candidate downstream targets of miR-720 by combining bioinformatics prediction with gene expression microarray data and targeting FIR-repressed genes in EPCs. Fifty-nine genes were predicted to be the direct targets of miR-720 (Figure 4E). Among these candidates, 4 genes (SUFU, vasohibin 1 [VASH1], PTK2, and MDM2) are known to be involved in angiogenesis or cell proliferation (Figure 4E). Using a new batch of healthy EPCs as an independent validation cohort, VASH1 was found to be the most significantly affected gene in terms of downregulation by FIR treatment (Figure VE in the online-only Data Supplement). Furthermore, FIR treatment also repressed VASH1 levels in healthy and CAD EPCs (Figure VF in the online-only Data Supplement). VASH1 belongs to a family of N-terminal prolactin fragments that inhibits angiogenesis and vascular functioning.30,31 VASH1 mRNA levels were found to be higher in CAD EPCs than in Hd EPCs. The protein and mRNA expression levels of VASH1 were negatively correlated with
the expression level of miR-720 (Figure 4G). Repression of VASH1 after FIR treatment was relieved by knockdown of miR-31 in Hd EPCs (Figure VG in the online-only Data Supplement), which correlates with the scenario whereby miR-720 levels are reduced in FIR-treated Hd EPCs if endogenous miR-31 is knocked down (Figure IIIF in the online-only Data Supplement).

Bioinformatics analysis using the miRTar webtool indicated a putative miR-720 binding site in the coding region of VASH1 (Figure 4H, left). Luciferase reporter assays were then used to confirm the direct repression of VASH1 by miR-720 (Figure 4H, right). The functional implications of this miR-720–VASH1 pairing with respect to EPC activity were then evaluated. We knocked down miR-720 and VASH1 in healthy EPCs and found that the reduction in miR-720 level in these EPCs led to an increase in cellular VASH1 levels and a reduction in EPC migration and tube formation (Figure 4I and 4J, lane 2). In contrast, a reduction in VASH1 levels in EPCs stimulated cell migration and vasculogenesis (Figure 4I and 4J, lane 3). Most importantly, when VASH1 was further knocked down in EPCs with reduced miR-720 expression, the migration and tube formation ability of EPCs was able to be restored (Figure 4I and 4J, lane 2 versus 4).

Next, we examined to what extent VASH1 contributes to the effects of miR-31. When VASH1 transcripts were knocked down in miR-31–reduced EPCs, the angiogenic-related abilities of EPCs were restored from 15% to 65% (cell migration) and from 11% to 66% (microvascular formation) compared with the controls (Figure VIA and VIIB in the online-only Data Supplement). Similar results were obtained when miR-720 was knocked down in EPCs overexpressing miR-31 (Figure VIIA and VIIB in the online-only Data Supplement). These findings suggest that the miR720–VASH axis contributes ≈40% to 50% of the miR-31–mediated proangiogenic activities. Knockdown of VASH1 was found not to alter miR-31 or miR-720 levels significantly in EPCs (Figure VIA in the online-only Data Supplement and Figure 4A), and a reduction in miR-720 level did not significantly alter the miR-31 level in EPCs (Figure VIIA, left in the online-only Data Supplement). This supports the hypothesis that the regulation of the miR-31–miR-720–VASH1 axis is unidirectional.

miR-31 Regulates miR-720 Expression Through FAT4 and Planar Cell Polarity Signaling, but Not TBXA2R

In EPCs, overexpression of miR-31 affects the miR720–VASH1 pairing by increasing miR-720 expression (Figure 5A). Moreover, knockdown of FAT4, but not TBXA2R, was found to influence the levels of the miR720–VASH1 pair (Figure 5B). Knockdown of FAT4 neither altered miR-31 levels nor changed the level of TBXA2R in EPCs (Figure 5B, left). Similarly, a reduction in TBXA2R level in EPCs did not affect miR-31 or FAT4 expression (Figure 5B, right). Mouse Fat4 genetically interacts with the planar cell polarity (PCP) proteins Vangl2 (VANGL PCP protein 2) and four-jointed box 1 (FJX1). In addition, Atn1 and Atn2l modulate Fat4 activity during vertebral arch fusion and renal tubular elongation, respectively. We knocked down 5 human PCP genes in PB EPCs, these were VANGL2, FJX1, ATN1, DVL1, and DVL2, and then examined the endogenous level of miR-720. Knockdown of FJX1, but not of the other 4 genes, resulted in a reduction in the level of miR-720 (Figure 5C and Figure VIIIC–VIF in the online-only Data Supplement). Moreover, knockdown of FAT4 increased the abundance of FJX1 mRNA and miR-720 (Figure 5D), which indicates that FAT4 is able to repress FJX1 levels and that this consequently leads to an inhibition of miR-720 expression.

FJX1 was shown recently in colorectal carcinoma cells to promote endothelial cell capillary tube formation in a hypoxia-inducible factor 1, α subunit (HIF1α)–dependent manner. We hypothesized that FJX1 in EPCs also regulates miR-31 expression via HIF1α. FIR treatment increased the abundance of HIF1α, as well as those of miR-31/FJX1/miR-720, in EPCs. Nevertheless, knockdown of HIF1α only abolished FIR-induced miR-720 expression (Figure 5E and Figure VIIA in the online-only Data Supplement). Knockdown of FJX1 but meanwhile overexpressing HIF1α in EPCs restored miR-720 levels (Figure 5F and Figure VIIIB in the online-only Data Supplement). We also overexpressed FJX1 in EPCs but at the same time knocked down endogenous HIF1α levels and found that knocking down HIF1α abolished FJX1–mediated miR-720 expression (Figure VIIIC in the online-only Data Supplement), indicating FJX1 regulates miR-720 expression via HIF1α. Overexpression of miR-31 in EPCs induced HIF1α expression, whereas knocking down endogenous miR-31 reduced HIF1α levels (Figure VIIID and VIIIE in the online-only Data Supplement). The functional implications of this miR31–FAT4–miR720 pathway with respect to EPC angiogenesis/vasculogenesis were further evaluated. The effect of knocking down miR-720 within the EPCs overexpressing miR-31 (Figure VIII in the online-only Data Supplement) was then examined, and it was found that, although miR-31 overexpression was able to enhance EPC motility and vasculogenesis, a parallel reduction in miR-720 abolished miR-31–mediated angiogenic activity (Figure VIII–VIIIID in the online-only Data Supplement). Similarly, when FAT4 and miR-720 levels underwent double knockdown in EPCs, the decline in miR-720 expression masked the EPC activation caused by FAT4 reduction (Figure 5G and 5H and Figure VIIIID and VIIIIF in the online-only Data Supplement). Knockdown of miR-720 expression did not alter FAT4 levels in EPCs (Figure 5G, lanes 1 versus 3), suggesting that regulation of the miR31–FAT4–miR720 axis is unidirectional.

miR-31 Promotes Blood Flow Recovery in the Ischemic Limbs of Mice

We also evaluated whether miR-31 may boost the in vivo vasculogenesis/angiogenesis activity of diseased EPCs. Limb ischemia was induced by surgery in nude mice 3 days before EPC injection (Figure 6A). RNA agomirs of miR-31 were transfected into CAD EPCs 24 hours before transplantation, and overexpression of miR-31 in the transplanted EPCs was verified by qPCR before injection (Figure 6B). The CAD EPCs were labeled with the fluorescent marker PKH-26 and then intramuscularly injected locally into the ischemic hindlimb at a site distal to the arterial occlusion site on the third day after surgery. The transfected agomirs still remained in the in vitro cultured EPCs at 14 days after transfection (Figure IXA in
the online-only Data Supplement). The mice were followed for 2 weeks because of the limited lifespan of the transfected miRNA agomirs. As shown in Figure 6C, mice without EPC transplantation (the medium group) showed delayed blood flow recovery after the ischemia surgery compared with the mice that had received EPC therapy using CAD EPCs transplanted with scramble oligonucleotides (the Scr control group); this was determined by laser Doppler imaging. In addition and importantly, compared with both the medium and Scr groups, overexpression of miR-31 in the injected EPCs significantly improved blood flow recovery by 90% in the ischemic limbs of the treated mice (Figure 6C and 6E; n=6 per group).

To further evaluate the effect of miR-31 on the homing and differentiation to endothelial cells of the injected EPCs, as well as neovascularization/angiogenesis in mice, immunofluorescence staining was conducted on mouse tissue sample at 7 days after EPC injection. Capillaries in the ischemic muscles were visualized using anti-CD31 immunostaining (green, Figure 6D), whereas injected human EPCs were detected by PKH-26 fluorescence (red, Figure 6D). Mice that had received the miR-31–transfected EPCs showed the presence of more CD31+/PKH-26+ double-positive cells (white arrowheads) in the capillaries of the ischemic muscle compared with mice receiving control EPCs or FIR-treated, miR-31 knocked down EPCs (Figure 6D in the online-only Data Supplement). Nevertheless, miR31-blocked EPCs, although significantly improved blood flow recovery in the ischemic limbs of the treated mice (Figure 6C in the online-only Data Supplement; quantitative data in Figure XE in the online-only Data Supplement). Immunofluorescence staining conducted on mouse tissue sample after EPC injection also showed that mice receiving FIR-treated EPCs have more CD31+/PKH-26+ double-positive cells (white arrowheads) in the capillaries of the ischemic muscle compared with mice receiving control EPCs or FIR-treated, miR-31 blocked. Laser Doppler imaging showed that mice without EPC transplantation (the medium group) had delayed blood flow recovery in the ischemic limbs of the treated mice (Figure XC in the online-only Data Supplement). Nevertheless, miR31-blocked EPCs, although were treated with FIR, failed to rescue limb ischemia in mice (Figure XC in the online-only Data Supplement; quantitative data in Figure XF in the online-only Data Supplement).

**Discussion**

Endothelial dysfunction is considered the first step in the cascade that leads up to CAD. Repair of defective endothelium at the postnatal stage is mainly the responsibility of EPCs, which are derived from the bone marrow. These cells play an important role in vascular homeostasis and compensatory vasculogenesis/angiogenesis because they show plasticity and are able to differentiate into endothelial cells and act

---

The importance of miR-31 in FIR-enhanced EPC activities was further evaluated by injecting FIR-treated CAD EPCs in which endogenous miR-31 was blocked. Laser Doppler imaging showed that mice without EPC transplantation (the medium group) had delayed blood flow recovery after the ischemia surgery compared with the mice that had received EPC therapy. Pretreating EPCs with FIR significantly improved blood flow recovery in the ischemic limbs of the treated mice (Figure XC in the online-only Data Supplement). Nevertheless, miR31-blocked EPCs, although were treated with FIR, failed to rescue limb ischemia in mice (Figure XC in the online-only Data Supplement; quantitative data in Figure XF in the online-only Data Supplement). Immunofluorescence staining conducted on mouse tissue sample after EPC injection also showed that mice receiving FIR-treated EPCs have more CD31+/PKH-26+ double-positive cells (white arrowheads) in the capillaries of the ischemic muscle compared with mice receiving control EPCs or FIR-treated, miR-31 knocked down EPCs (Figure XD in the online-only Data Supplement). Transwell migration (left) and tube formation (right) assays were conducted (H). Migrated cells...
miR-31–miR-720 in Plasma and CAD EPC

Wang et al

as a source of paracrine proangiogenic factors. The expression profile and functions of miRNAs in healthy EPCs has recently been investigated, yet the roles of miRNAs in diseased EPCs remain to be defined. In this report, we identified that EPCs exploit the miR-31 pathway as a means of regulating their vasculogenesis/angiogenesis-related activities (Figure 6G). This pathway, which is repressed in CAD EPCs, is sufficient for, as well as being critical to, EPC activation, particularly because TBXA2R and FAT4 are direct targets of miR-31. These findings indicate that dysfunction of the miR-31–miR-720 pathway seem to diminish blood vessel repair by impairing vasculogenesis and angiogenesis. This will clearly affect the development and prognosis of cardiovascular disease and other EPC-related syndromes, such as diabetes mellitus–related ischemia.

miR-31 is located in the first intron of a host gene, LOC554202, on human chromosome 9p21.3, and its expression is epigenetically regulated by promoter methylation and CCAAT/enhancer binding protein (C/EBP), beta (C/EBP-β) activation. C/EBP-β does not seem to be involved in the studied events because FIR treatment of PB EPCs did not affect the level of C/EBP-β (Figure XI in the online-only Data Supplement). Furthermore, knockdown of C/EBP-β in healthy EPCs neither affected FIR-mediated miR-31 upregulation nor abolished FIR-stimulated EPC activity (Figure XI in the online-only Data Supplement). Interestingly, miR-31 is critical for not only blood vessel EC activation (by targeting FAT4), but also for lymphatic EC differentiation in vitro and in vivo by targeting the critical transcription factor PROX1. It has also been found that miR-31 plays a crucial role in
tumors in a variety of genetic diseases, such as Duchenne muscular dystrophy, and in the regulation of inflammation mediated by regulatory T cells. miR-31 differential expression is commonly found and has been described as having controversial roles in numerous physiological or pathological conditions. For example, miR-31 acts as a tumor suppressor that inhibits breast cancer metastasis, while it also functions as an oncogenic miRNA in lung cancer. We found miR-31 levels in EPCs and plasma to be closely related to the presence of CAD. miR-31 agonir treatment restores the activity of CAD EPCs to a normal level in vitro and in vivo. If we consider that the restoration of blood supply at an early stage after myocardial infarct to be crucial to patient recovery, these findings may have clinical implications and justify the development to therapies involving inhibition of multiple miRNAs.

Little is known about the expression and the exact function of miR-720. By in silico sequence alignment, it has been suggested that the sequence annotated as miR-720 overlaps that of the mitochondria-encoded tRNAThr. As a result, miR-720 was removed from miRBase from Release 19, 2012 (see also NCBI Gene ID: 100302198, discontinued on August 2, 2012). However, we have proved by wetlab validation that miR-720 has the functional characteristics of an miRNA, including Dicer1-dependent biogenesis, a physical association with Argonaute proteins, and the ability to repress mRNA transcripts in a sequence-specific manner (Figure 4H and Figure IVA and IVB in the online-only Data Supplement). Moreover, pre-miR-720 sequences can also be mapped onto chromosome 3 (Figure IV in the online-only Data Supplement) as well as to the mitochondrion genome. Our findings indicate that miR-720 is still a functional miRNA, and whether it is derived from either tRNAThr (by RNA polymerase III) or human chromosome 3 (chr3: 164059155–164059171) does not affect this. Certain miRNAs are transcribed by RNA polymerase III. We found HIF1α is crucial in inducing miR-720 expression, suggesting that RNA polymerase II is, at least in part, involved in the transcription of the miR-720 locus. The present findings support the existence and importance of miR-720 in vivo. As to the function of miR-720, the level of this miRNA is increased in the serum of pregnant women with fetuses with defect neural tubes. miR-720, together with 2 other serum miRNAs, miR-1308 and miR-1246, have been shown to serve as potential biomarkers for myeloma. Use of miR-720 and miR-1308 together is able to distinguish normal healthy controls, as well as patients with unrelated illnesses, from patients with precancerous myeloma and with myeloma. Here, we show for the first time that miR-720 targets VASH1 for achieving its proangiogenic function.

How miR-31 can regulate miR-720 was also explored in this study. We found that FAT4, but not TBXA2R, is the miR-31 target involved in miR-720 regulation (Figure 5A and 5B). The Fat cadherin protein FAT4 seems to influence both PCP and the Hippo signaling pathways. FAT4 has highest homology to the Drosophila gene atypical cadherin fat (ft), whereas other FAT family members (FAT1–FAT3) are more similar to the second ft-like gene, f2. Loss of Ft4 in mice disrupts PCP signaling and oriented cell division; this leads to cystic kidney disease, which is partly because of the genetic interactions between Fat4 and the PCP proteins Vangl2 and Fjx1. In Drosophila, the transcriptional corepressor atrophin physically interacts with ft and acts as a component of the Fat signaling involved in planar polarity. The mammalian orthologs of atrophin, Atn1 and Atn2l, modulate Fat4 activity during mouse development. When various human PCP genes are knocked down individually in PB EPCs, namely VANGL2, FJX1, ATN1, DVL1 and DVL2, only the FJX1 knockdown showed any alternation in miR-720 level, in this case reduced expression (Figure 5C and Figure VIC and VID in the online-only Data Supplement). We further showed that FJX1 is involved in miR-720 upregulation and FAT4 inhibits miR-720 expression via repressing the level of FJX1 (Figure 5C and 5D). FJX1 is a notch-inducible secreted ligand that is homologous to the Drosophila four-jointed gene. FJX1 promotes angiogenesis and is associated with poor patient survival in colorectal carcinoma. Interestingly, miR-31 and miR-720 are also expressed at significantly higher levels in colon cancer tissues.

Downstream of miR-720 is VASH1, which has been identified as a VEGF-inducible secreted protein in endothelial cells by cDNA microarray analysis and has also been found to belong to a family of N-terminal prolactin fragments that inhibits angiogenesis and vascular functioning. VASH1 induces prollyl hydroxylase–mediated degradation of HIF1α via the induction of prolyl hydroxylase. Expression of VASH1 is low in proliferating endothelial cells at the sprouting front but is high in nonproliferating endothelial cells in newly formed blood vessels that are formed behind the sprouting front, which is where angiogenesis terminates. The presence of VASH1 in endothelial cells is also evident in various cancers, atherosclerotic lesions, age-dependent macular degeneration, diabetic retinopathy, rheumatoid arthritis, and arterial re-endothelialization after denudation. Cancer cell–released VEGF stimulation in cancer-associated endothelial cells leads to increased expression of EZH2 (enhancer of zeste homolog 2 [Drosophila]), a transcriptional repressor, which thereby causes VASH1 silencing by promoter methylation; this subsequently leads to an increase in angiogenesis. As a result of the proteins antiangiogenic potential, VASH1 has been reported to be a potential biomarker or a possible therapeutic molecule for diseases associated with angiogenesis. Because circulating miRNAs are being considered as novel biomarkers, not only for cardiovascular diseases but also for cancers, four serum levels of miR-720, miR-31, and VASH1 may have clinical potential and are an attractive biomarker panel. Their use would allow early diagnosis of relevant diseases and also allow the monitoring of the effectiveness of a relevant therapeutic intervention, such as Avastin as a tumor therapy or statins as a lipid-lowering therapy.

miR-31 also achieves its angiogenic role by targeting the receptor of TBXA2R. TBXA2, which is a prostanoid, has received great attention because of its involvement in platelet function and the pathogenesis of many cardiovascular diseases, including atherosclerosis and hypertension, via interaction with its specific receptor TBXA2R, a G protein–coupled receptor on endothelial cells. TBXA2R signaling is able to inhibit VEGF-induced endothelial cell differentiation and migration. Therefore, remains a possible mechanism involved in miR-31 angiogenic functioning.
(Figure 6G). However, when miR-31 is overexpressed in healthy EPCs, the levels of VEGF transcript and protein were not changed significantly (not shown). TBX2AR in smooth muscle promotes angiotensin II–induced hypertension, vascular remodeling, and sudden death. Recent experimental findings have indicated that synthetic TBX2AR/prostaglandin receptor inhibitors seem to have potent antiplatelet activity with an antiatherosclerotic effect. Such activity has important implications especially when they are used to target clinical conditions associated with an increased production of prostanoids, such as CAD and diabetes mellitus. In the present study we found that miR-31 is an endogenous inhibitor of TBX2AR. Recently, a phase I trial using an miRNA mimic of the tumor suppressor miR-34 (licensed from Marina Biotech) was initiated. This was delivered using a liposomal formulation and in a trial involving patients with unresectable primary liver cancer or metastatic cancer with liver involvement (http://clinicaltrials.gov/ct2/show/NCT01829971). This application of an miR-31 mimic as a nucleic acid drug for therapeutic purposes is an attractive approach and should see further development.

In this study, we have shown that one of the approaches that can be used with CAD EPCs to restore endogenous miR-31 levels, and therefore angiogenic activity, is FIR irradiation (Figure 3). FIR treatment represents a noninvasive and convenient therapeutic modality that helps to improve blood flow, endothelial functioning, and EPC activity. FIR therapy has therefore been suggested as a treatment for ischemic lesions and necrosis in skin tissue as a way of improving arteriogenous fistula access flow in hemodialysis patients and as a means of reducing cardiovascular disease frequency. Yet, the molecular mechanisms by which FIR acts remain to be elucidated. It is known that FIR therapy inhibits vascular endothelial inflammation via the induction of heme oxygenase-1. The MEK (mitogen-activated protein kinase)extracellular signal–regulated kinase signaling pathway, but not the VEGF/Akt/endothelial NO synthase–dependent pathway, has been shown to be involved in FIR-mediated angiogenesis. In vascular smooth muscle cells, the expression of miR-31, which has a proproliferative effect on vascular smooth muscle cells, is mediated via the mitogen-activated protein kinase/extracellular signal–regulated kinase pathway. Whether the mitogen-activated protein kinase/extracellular signal–regulated kinase pathway is suppressed in CAD EPCs, thereby resulting in the reduction of miR-31 levels in patients, is under investigation. Another intriguing possibility is that there may be cellular photosensor(s) by which FIR affects intracellular signaling and changes miRNA expression. It has been shown that a green light-emitting diode irradiation is able to activate directional motility in human orbital fat stem cells via activation of the extracellular signal–regulated kinase/MAP kinase/p38 signaling pathway. Two nonvisual opsins, encephalopsin and short-wave–sensitive opsin 1, serve as the photoreceptors that respond to green light-emitting diode irradiation. Thus, elucidation of the levels and activities of various opsins in healthy and CAD EPCs, as well as roles of opsins in FIR-induced miR-31 expression, EPC migration, and microvascular formation, will help to clarify FIR’s mechanisms and its possible applications.

In summary, our research has revealed the existence of a new pathogenesis pathway that regulates EPC self-renewal potential and angiogenic capability in patients with CAD. With these findings, it will be possible to develop not only novel therapeutic modalities that enhance angiogenesis during cardiovascular repair, but also new ways to inhibit angiogenesis when treating cancer. Finally, it should be possible to design new diagnostic panels based on circulating miR-31, miR-720, and VASH1 levels that can help early identification of relevant diseases.

Acknowledgments

We thank the C6 RNAi Core, which is supported by the National Science Council, at the Academia Sinica for providing us the short hairpin RNA clones.

Sources of Funding

This work is supported by National Science Council (NSC: NSC101-2320-B-010-059-MY3, NSC101-2627-B-010-003, NSC102-2622-B-010-002, and NSC101-2321-B-010-011), Tri-Service General Hospital (TSGH-C102-027), Veterans General Hospitals University System of Taiwan (VGHUST) Joint Research Program, Tsou’s Foundation (VGHUST102-G7–3–2), National Health Research Institutes (NHRI-EX102-10254SI), UST-UCSD International Center for Excellence in Advanced Biotechnology sponsored by the Taiwan NSC I-RICE Program (NSC101-2911-I-009-101), and in part a grant from National Yang-Ming University (Ministry of Education, Aim for the Top University Plan).

Disclosures

None.

References

miRTar: an integrated system for identifying miRNA-target interactions in growth factor-induced endothelial cell migration, tube formation, and cancer.

Steenpass A, Ergün S, Böger RH. Isoprostanes inhibit vascular endothelial growth factor-induced angiogenesis in diabetic mice and restores high glucose-suppressed endothelial progenitor cell activation.


Zhang Y, Kang S, Lahtinen MJ. Dysregulation of angiogenesis-related miRNAs in endothelial progenitor cells from patients with coronary artery disease.


Huang PH, Chen JW, Chang YC, Chang ST, Wang HW. The M type K15 protein of Kaposi’s sarcoma-associated herpesvirus regulates miRNA expression via its SH2-binding motif to induce cell migration and invasion.


Sadeqzadeh E, de Bock CE, Thorne RF. Sleeping giants: emerging roles for the fat cathedrins in health and disease.


Cottonham CL, Kaneko S, Xu L. miR-21 and miR-31 converge on TIM1 to regulate migration and invasion of colon carcinoma cells.


Abe M, Sato Y. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells.


Safai Y. The vasohibin family: a novel family for angiogenesis regulation.


Tijssen AJ, Pinto YM, Creemers EE. Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases.


van Emmerik VP, De Windt LJ, da Costa Martins PA. Circulating miRNAs: reflecting or affecting cardiovascular disease?

We found that microRNA (miR)-31 was critical for endothelial progenitor cell (EPC) functions and was repressed in diseased EPCs. Important downstream members of miR-31 (including FAT atypical cadherin 4, four-jointed box 1, hypoxia-inducible factor 1, α subunit, miR-720, vasohibin 1, and thromboxane A2 receptor) were identified and investigated extensively for their hierarchical relationships. Expression levels of the miR-31 pathway components were also abnormal in coronary artery disease EPCs, while manual reverse of such abnormalities repaired the defects of coronary artery disease EPCs. Far-infrared irradiation was yet another promising noninvasive way to restore the miR-31 pathway in diseased EPCs, thereby rescuing the functions of impaired EPCs. Our results also indicated that miR-31 and miR-720 served well as biomarkers in coronary artery disease plasma. The administration of miR-31, either via EPC cell therapy or direct injection to blood stream, is an attractive approach of treatment to vascular defects.

**Significance**

We found that microRNA (miR)-31 was critical for endothelial progenitor cell (EPC) functions and was repressed in diseased EPCs. Important downstream members of miR-31 (including FAT atypical cadherin 4, four-jointed box 1, hypoxia-inducible factor 1, α subunit, miR-720, vasohibin 1, and thromboxane A2 receptor) were identified and investigated extensively for their hierarchical relationships. Expression levels of the miR-31 pathway components were also abnormal in coronary artery disease EPCs, while manual reverse of such abnormalities repaired the defects of coronary artery disease EPCs. Far-infrared irradiation was yet another promising noninvasive way to restore the miR-31 pathway in diseased EPCs, thereby rescuing the functions of impaired EPCs. Our results also indicated that miR-31 and miR-720 served well as biomarkers in coronary artery disease plasma. The administration of miR-31, either via EPC cell therapy or direct injection to blood stream, is an attractive approach of treatment to vascular defects.
Deficiency of the MicroRNA-31–MicroRNA-720 Pathway in the Plasma and Endothelial Progenitor Cells From Patients With Coronary Artery Disease

Hsei-Wei Wang, Tse-Shun Huang, Hung-Hao Lo, Po-Hsun Huang, Chih-Ching Lin, Shing-Jyh Chang, Ko-Hsun Liao, Chin-Han Tsai, Chia-Hao Chan, Cheng-Fong Tsai, Yi-Chieh Cheng, Ya-Ling Chiu, Tsung-Neng Tsai, Cheng-Chung Cheng and Shu-Meng Cheng

Arterioscler Thromb Vasc Biol. 2014;34:857-869; originally published online February 20, 2014;
doi: 10.1161/ATVBAHA.113.303001

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/4/857

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/02/20/ATVBAHA.113.303001.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology 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 Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

Late EPC isolation, cultivation, and characterization

Late EPC isolation and characterization were done as described previously with minor modification. All patients gave informed consent, and the study was approved by the local Institutional Research Board (IRB). The protocols of this study complied with the ethical guidelines of the 1975 Helsinki Declaration. Peripheral blood samples were obtained from healthy donors (Hd) or CAD patients. Mononuclear cells (MNCs) (5×10⁶) were plated in 2 ml endothelial growth medium-2 (Lonza Ltd, Basel, Switzerland) with complete supplements and seeded onto fibronectin-coated six-well for cultivation. After 4 days of culturing, the medium was changed and non-adherent cells were removed; attached early EPCs appeared to be elongated with spindle shapes. A certain number of early EPCs were allowed to grow into ECFCs (endothelial colonies forming cells), which emerged 2–4 weeks after the start of the MNC culture. The ECFCs, or called late EPCs, exhibited a cobblestone morphology and monolayer growth pattern typical of mature endothelial cells at confluence.

These late EPCs/ECFCs were positive for endothelial cell and hematopoietic stem cell surface markers as described. The antibodies used in FACS to characterize the adherent cell population were kinase insert domain receptor (KDR)/VEGF receptor 2 (R&D system, Minneapolis, MN USA), CD34 (BD Pharmingen, Franklin Lakes, NJ USA), VE-cadherin (Cell Signaling Technology, Inc.), platelet–endothelial cell adhesion molecule-1 (CD31; Santa Cruz Biotechnology, Inc.) and CD45 (Biolegend, San Diego, CA USA). Flow cytometry was performed using a FACSCanto flow cytometer (BD Pharmingen, Franklin Lakes, NJ USA).

Late EPCs were also assessed for endothelial and progenitor features by direct fluorescent staining with 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (DiI-acLDL; Molecular Probes, Invitrogen, Carlsbad, California, USA). Briefly, the adherent cells were first incubated with 2.4 ug/ml DiI-acLDL at 37°C for 4 hours, and then fixed in 4% paraformaldehyde. The images were recorded using a fluorescent microscope.

Transfection of microRNA oligonucleotides and plasmids

A plasmid expression miR-31 precursor sequence were described previously and used in Figures 1-4, where the control for miR-31 were vector controls (Vec) rather than scramble oligonucleotide (Scr) ones. Both microON™ agomir and microOFF™ antagonim (RiboBio Co., Guangzhou, China) for miR-31 and miR-720 are commercial synthetic RNA molecules with several chemical modifications for direct transfection without transfect reagents. To over-express or knock down miR-31
and miR-720 in EPCs, agomir or antagonir were added into culture medium at a concentration of 50 nM at 70% to 80% cell confluence. Both agomir and antagonir are stable in EPC for at least 14 days for further in vitro or in vivo studying (Supplementary Figure VIII). The shRNAs, including those against FAT4, TBXA2R, VASH1, Dicer1, FJX1, VANGL2, ATN1, DVL1, DVL2, and control clones, were designed by the The RNAi Consortium, and were purchased from the RNAi Core Facility in the Academia Sinica, Taipei, Taiwan. The FJX1 cDNA expression plasmid was distributed from Addgene (Plasmid #46931). The HIF1α expression plasmid was a kind gift from Dr. Muh-Hwa Yang, National Yang-Ming University, Taiwan. The Lipofectamine® 2000 Reagent (Life Technologies, Inc., CA, USA) was used for transfection of shRNA for knockdown experiments in this study. All protocols followed manufacturer’s instructions faithfully. The expression level of transfected genes and microRNAs were monitored and measured by quantitative RT-PCR.

**EPC Matrigel tube formation and Transwell migration assay**

*In vitro* tube formation assay was performed on EPCs for assessing the capacity of neovascularization. Thawed Basement Membrane Extract (BME, 3433-005-01, Trevigen Inc.) was plated in 96-well at 37°C for up to 1 hour to form a reconstituted basement membrane. EPCs were harvested by trypsin/EDTA, and 1x10^4 cells in 100 µl medium were seeded on Matrigel then incubated at 37°C for 3 to 6 hours. Tube structures were inspected under an inverted light microscope (100 X). To evaluate the tube formation capacity, five representative fields were captured and analyzed by calculating total tube length in each group. For easily interpret the significance of the experiments, the total tube length were further normalized to control group and presented into relative tube length.

For Transwell cell migration assay, 600 µl medium with 10% FBS were added to the lower chamber, while 5x10^4 EPCs in 100 µl medium were subjected to upper chamber of Costar Transwell Polycarbonate Permeable Supports (Corning, NY, USA). After 3 hours incubation at 37°C, cell suspensions were removed from upper chamber and the 8 µm permeable membranes were fixed with 4% paraformaldehyde for at least 15 minutes at room temperature. Migrated cells were then stained with Hochest 33342 reagents (Sigma-Aldrich) for 30 minutes and counted under fluorescent microscope by five representative fields.

**Far-infrared (FIR) treatment**

FIR treatment on cultured late EPCs were done as described previously with minor modifications. Briefly, FIR radiation was administered using a WSTM TY101N emitter (WS Far Infrared Medical Technology Co., Ltd., Taipei, Taiwan).
The electrified ceramic plates of the emitter generate the electromagnetic waves with the wavelengths in the range between 3 and 25 μm (a peak between 5 and 6 μm). The top radiator was set at a height of 30 cm above the bottom of the tissue culture plates and the cells were exposed to FIR radiation for 30 minutes. FIR treated EPCs were then incubated one hour at 37°C for further experiments.

**RNA extraction and quantitative RT-PCR**

EPCs were immersed in TRIzol® reagent and fresh plasma in TRIzol® LS reagent (Life Technologies, Inc., Carlsbad, CA, USA) for the extraction of total RNA, which including the small RNA fraction. All protocols followed manufacturer’s instructions with minor modifications. Total RNA ranging from 100 ng to 1 μg of total RNA was used to perform reverse transcription (RT) using the RevertAid™ Reverse transcriptase kit (Fermentas, Glen Burnie, Maryland, USA) according to manufacturer’s protocols. Real-time PCR reactions were performed using Maxima™ SYBR Green qPCR Master Mix (Fermentas), and the specific products were detected and analyzed using the StepOne™ sequence detector (Life Technologies). The expression level of each microRNA was normalized to that of U6 small nuclear RNA, while the expression level of each gene was normalized to GAPDH expression. Expression level of plasma miRNAs was measured and normalized to miR-16a in plasma, which has been documented as a stable small RNA in CAD patient’s plasma for normalization. All the primer sequences are listed in Supplementary Table I online.

**Reporter assays**

For luciferase reporter plasmids, the predicted microRNA binding sites were cloned into the XbaI site of the pGL3-Basic plasmid (Promega, Wisconsin, USA) with the following primers: TBXA2R-UTR-F, TgCTCTAgAGCATgCTCTTTgACCCAgA; TBXA2R-UTR-R, AgCTCTAgACACACAgATTCAtggTggTTT; VASH1-CDS-F, TAgTCTAgACCgCCTACCCCTgTCC; VASH1-CDS-R, TAgTCTAgACATCCTCTTgTTgTCC. The luciferase reporter plasmids containing TBXA2R or VASH1 mutant binding site were created using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, USA). For 3’UTR reporter assays, miRNA agomirs and reporter plasmids were co-transfected by the lipofectamine 2000 reagent (Life Technologies) into 293T cells and analyzed by the measurement of ratio between firefly and rellina luciferase activities.

**RNA immunoprecipitation (RIP) and small RNA sequencing data analysis**

To characterize microRNAs in our study, we used EZ-Magna RIP™
RNA-Binding Protein Immunoprecipitation Kit (#17-701, Merck Millipore) for validating microRNA features. RNA-binding protein immunoprecipitation experiments were performed according to the protocol provided with the manufacturer by using Human AGO2 antibody (Cell Signaling Technology, Beverly, MA). Immunoprecipitated RNA were further measured by qRT-PCR and normalized to input by means of percentage of input (% Input).

Small RNA sequencing (smRNA-seq) raw data pre-processing was performed according to a pipeline we constructed\(^6\). Short reads that align using the bwa\(^7\) aligner to pre-miR-720 sequence are extracted using samtools\(^8\).

**Western Blot analysis**

EPCs were harvested by trypsin/EDTA and lysed in protease inhibitor containing RIPA buffers. After centrifugation for 15 minutes at 14000 × g (4°C), equal amounts of protein sample were separated on SDS-polyacrylamide gels and blotted onto PVDF membrane (Immobilon\(^®\), Millipore). Antibodies against human VASH1 (H00022846-B01P, Abnova), TBXA2R (ab92883, Abcam), HIF1α (#07-628, Merck Millipore) and β-Actin (MAB1501R, Merck Millipore) are used to recognize specific proteins according to manufacturer's instruction. Specific bands were quantified by the ImageJ software package.

**Mouse ischemic hindlimb model and EPC transplantation**

Nude mice ranging from 6 to 8 weeks were purchased from the National Laboratory Animal Center (Taiwan) and kept in microisolator cages on a 12-h day/night cycle for 2 weeks before operation. After two-week stabilization, mice received right femoral artery excision for inducing unilateral hindlimb ischemia as previously described\(^9\). Briefly, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Both proximal and distal portion of right femoral artery were ligated, as well as distal portion of saphenous artery.

Mice were randomly allocated to several groups (n = 6) with different treatments: EGM2 medium, CAD-EPC, CAD-EPC with miR-31 agomirs/antagomirs and FIR treatment. CAD-EPCs from same donors were pre-stained with PKH26 (Sigma-Aldrich), a tracking dye for staining cell membrane, before transplantation. After 72 hours, a total volume of 200 µl medium with 1 × 10^5 EPCs were injected intramuscularly at six different sites of ischemic limb distal to the arterial occlusion site as described\(^2\).

Blood perfusion was monitored by Laser Doppler Perfusion Imager (LDPI) system (Moor Instruments Limited, Devon, UK) before and after the surgery, and was then measured weekly. To prevent individual difference, the results were indicated as
the ratio of perfusion in the ischemic (right) versus non-ischemic (left) limb.

All experimental procedures and protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University and in compliance with the ARRIVE guidelines\(^\text{10}\).

**MicroRNA targets prediction**

To predict and determine the possible target genes of miR-31 or miR-720, we conducted to miRTar, an online microRNA target prediction tool (http://mirtar.mbc.nctu.edu.tw/human/), to investigate the possible regulatory mechanisms of miR-31 and miR-720. MicroRNAs were subjected to the prediction tool and all three criteria: “3’-UTR”, “CDS”, and “5’-UTR” were both selected for prediction. Possible candidates were further studied by literature search and expression level determination to elect as a possible target gene.

**Microarrays and data analysis**

*Gene expression microarrays*

Total RNA collection, cRNA probe preparation, array hybridization and data analysis were done as described previously\(^6\). RMA log expression units were calculated from Affymetrix\(^\text{TM}\) HG-U133 Plus 2.0 whole genome array data using the ‘affy’ package included in the Bioconductor (http://www.bioconductor.org/) suite of software for the R statistical programming language (http://www.r-project.org/). The default RMA settings were used to background correct, normalize and summarize all expression values. Significant differences between the sample groups was identified using the ‘limma’ (Linear Models for Microarray Analysis) package of the Bioconductor suite, and an empirical Bayesian moderated t-statistic hypothesis test between the two specified phenotypic groups was performed. To control for multiple testing errors, we then applied a false discovery rate algorithm to these p values in order to calculate a set of q values, thresholds of the expected proportion of false positives, or false rejections of the null hypothesis. Heat maps were created by the dChip software: http://www.dchip.org/.

*MicroRNA microarray*

The Agilent Human miRNA Microarray Kit V2 (Agilent, Foster City, CA, USA) containing probes for 723 human microRNAs from the Sanger database v10.1 was used. Processing and differential expression analysis of Agilent microRNA chips was done by using the “AgiMicroRna” Bioconductor package\(^11\). To predict the downstream mRNA targets of the miRNAs, the miRTar web tool (http://mirtar.mbc.nctu.edu.tw/human/) was used.
Statistical analyses

All experiments were carried out by more than three independent manipulations and the standard deviation of each assays were further calculated. All data were presented as mean ± the standard deviation as indicated in each graph. The results were statistically analyzed using the software GraphPad Prism 5®. To examine the effects of molecular manipulation, in control and experimental groups, Student’s T test was applied to compare and determine the significant difference (such as gene overexpression, knockdown, FIR, etc). One-way ANOVA test followed by Tukey’s post-hoc test was applied to determine the significance among each group of luciferase reporter assay and all double manipulation assay with more than three sample groups (such as miR-31-TBXA2R, miR-720-VASH1, miR-31-VASH1, FAT4-FJX1 and FAT4-miR-720 double knockdown, FIR-anti-miR-31, miR-31-anti-miR-720, FIR-HIF1α and FJX-HIF1α double manipulation). Mann-Whitney U test was used to analyze the significance of gene expression and circulating microRNAs level in healthy donors or CAD patients in EPCs and plasma, respectively. The rescuing effects of miR-31, miR-720 and FIR treatment to the angiogenic activities of CAD-EPC were evaluated by a one-way ANOVA test followed by Tukey’s post-hoc test. Recovery of blood flow, capillary densities and CD31 / PKH-26 double positive cell counts were conducted to a one-way ANOVA test followed by Tukey’s post-hoc test. A p-value that is lower than 0.05 was considered statistically significant.

References

4. Al-Greene NT, Means AL, Lu P, Jiang A, Schmidt CR, Chakravarthy AB,


Supplemental Figure I. Cultivation and characterization of healthy and disease late EPCs. (A) Morphology of healthy and disease EPCs isolated from peripheral blood. (B) Both late EPCs endocytose Dil-acLDL (acetylated low density lipoprotein; red) and were counterstained with DAPI to show the nucleus (blue). (C) Expression of indicated molecules on healthy donor (Hd) and disease (CAD) EPCs by flow cytometric analysis. Scale bars represent 200 µm.
Supplemental Figure II. Knock down of TBX2A2R levels in miR31-reduced EPCs restores cell activities. Transwell migration (A) and tube formation (B) assays were conducted on EPCs transfected with the indicated anti-miRs and/or shRNAs. Scale bars represent 200 µm.
Supplemental Figure III. Influences of FIR irradiation on healthy and CAD EPCs. (A) The influence of FIR treatment on eNOS gene expression in healthy EPCs. mRNA levels were detected by RT-qPCR at indicated time points after FIR treatment. (B) FIR irradiation restored in vitro mobilization and vasculogenesis of CAD EPCs. Pictures were taken 6 hours (migration assays) or 24 hours (tube formation assays) after cells were subjected into cell migration (upper) or tube formation (lower) assays. (C) Levels of FAT4 and TBXA2R, which are both miR-31 targets, were restored in CAD EPCs after FIR treatment. (D) Microarray analysis reveals the up-regulated miRNAs in cord blood EPCs treated with FIR. A dashed line indicates a 2-fold threshold. (E) Differential expression of miR-720, miR-7, and miR-301a in FIR-treated CAD EPCs. (F-G) miR-31 is responsible for FIR-induced miR-720 upregulation and miR-31 targets repression. FIR treatment and RT-qPCR assays on indicated transcripts was performed on healthy EPCs with or without miR-31 knocked down. All histograms were graphed as mean ± standard deviation. Numbers above bars indicate the relative fold ratios. Significance levels, *p < 0.05, **p < 0.01, ***p < 0.001.
Supplemental Figure IV. Characterization of miR-720. (A) The pre-miR-720 (indicated by a purple line) and mature miR-720 (red) sequences could be aligned onto human chromosome 3 (chr3: 164059155 – 164059171). (B) A table summarized the anti-Ago1/2 immunoprecipitation (IP) small RNA sequencing (smRNA-seq) datasets used in the miR-720 enrichment analysis. All raw data were downloaded from the NCBI GEO database. Datasets with miR-720 enrichment after IP are indicated by arrows. RIP: RNA IP; CLIP: cross-linking immunoprecipitation; PAR-CLIP: photoactivatable-ribonucleoside-enhanced CLIP. (C) miR-720 enrichment in several anti-Ago2 RIP-seq datasets generated from different labs using 3 different cell lines. (D) Pre-miR-720 sequences and sequence reads alignment. smRNA-seq reads that mapped to matured miR-720 were highlighted with a red box. The mature miR-720 region is indicated.
Supplemental Figure V. (A) RT-qPCR on anti-Ago2 RNA immunoprecipitation (RIP) products. (B) Pre-miR-720 and miR-720 levels in healthy EPCs with DICER1 knocked down. N.D., not detectable. (C) miR-720 level in healthy EPC transfected with indicated oligonucleotides. (D) Reconstituting miR-720 expression in CAD EPCs restore their functionalities. Scale bars represent 200 µm. (E) mRNA level of indicated genes are evaluated by RT-qPCR in healthy EPCs with FIR treatment. (F) FIR treatment suppressed VASH1 mRNA levels in healthy (left) or CAD EPCs (right) (G) VASH1 levels in PB EPCs with or without miR-31 knocked down/FIR treatment down. All histograms were graphed as mean ± standard deviation. Numbers above bars indicate the relative fold ratios. Significance levels, *p < 0.05, **p < 0.01, ***p < 0.001.
Supplemental Figure VI. (A-B) The extent that VASH1 contributes to the effects of miR-31.
(A) Expression levels of miR-31 and VASH1 in PB EPCs transfected with indicated shRNA or antagomirs. (B) Transwell migration (left) and tube formation (right) assays conducted on transfected PB EPCs. (C-F) miR-720 levels in healthy EPCs knocking down with 4 PCP genes. Healthy EPCs were transfected with indicated shRNAs, and RT-qPCR was performed 2 days after transfection. All histograms were graphed as mean ± standard deviation. Numbers above bars indicate the relative fold ratios. Significance levels, *p < 0.05, **p < 0.01, ***p < 0.001.
Supplemental Figure VII. (A) Knocking down HIF1α in FIR-treated EPCs did not change miR-31 or FAT4 levels. mRNA and miRNA levels of indicated gene were measured by RT-qPCR. Significance levels. (B) Overexpression of HIF1α in EPCs in which endogenous FJX1 was blocked did not change miR-31 or FAT4 levels. (C) Knocking down HIF1α in FJX1(+) EPCs abolished FJX1-induced miR-720 expression. (D) Overexpression of miR-31 in EPCs induced HIF1α expression. (E) Knocking down endogenous miR-31 reduced HIF1α levels in EPCs. Significance levels, *p <0.05, **p < 0.01, ***p < 0.001. (F) Fig. 5E-F were repeated using a new batch of EPCs, and the full length and uncropped gel of HIF1α western blotting data are shown. (G) Supplemental Figure VIIC-E were repeated, and the full length and uncropped gel of HIF1A western blotting data are shown.
**Supplemental Figure VIII.** (A) Knocking down miR-720 in EPCs overexpressing miR-31. mRNA and miRNA levels of indicated gene were measured by RT-qPCR. (B-D) Knocking down miR-720 in miR31(+) EPCs abolished miR-31-induced cellular activities. Transwell migration (C) and tube formation (D) assays were conducted. Migrated cells or tube length was quantified and shown in (B). (E-F) Microscopy images of transwell migration (E) and tube formation (F) assays when knocking down miR-720 in FAT4-reduced EPCs. All histograms are expressed as the mean ± standard deviation. Numbers above bars indicate the relative fold ratios. Significance levels, *p < 0.05, **p < 0.01, ***p < 0.001. Scale bars represent 200 µm.
Supplemental Figure IX. (A) Stability of transfected miR-31 agomirs in EPCs. (Upper) miR-31 over-expression in transfected peripheral blood EPCs remain detectable at 14th day after treatment, as detected by RT-qPCR. The histograms are expressed as the mean ± standard deviation. (Lower) Schematic representation of experimental design.
A. Anti-miR-31 FIR treatment transfection (1 hour before injection)

-3 → -2 → 0 → 7 → 14

Hindlimb Ischemia

PKH26+ CAD EPC Injection

Laser Doppler Scan & IFA

Laser Doppler Scan

(Days)

B. miR-31

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Flow Ratio</th>
<th>***</th>
<th>***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scr</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Scr + FIR</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Anti-31 + FIR</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. (Pre-op) -3

-3 Day (Pre-Op)

-3 Day (Post-Op)

0 Day

7 Day

14 Day

D. Capillary Densities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Capillary / mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium</td>
<td>100</td>
</tr>
<tr>
<td>Scr</td>
<td>200</td>
</tr>
<tr>
<td>Scr + FIR</td>
<td>300</td>
</tr>
<tr>
<td>Anti-31 + FIR</td>
<td>400</td>
</tr>
</tbody>
</table>

E. Cell Counts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD31+ PKH26+ cells / HPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium</td>
<td>N.D.</td>
</tr>
<tr>
<td>Scr</td>
<td>5</td>
</tr>
<tr>
<td>Scr + FIR</td>
<td>10</td>
</tr>
<tr>
<td>Anti-31 + FIR</td>
<td>15</td>
</tr>
</tbody>
</table>
Supplemental Figure X. FIR-treated EPCs in which miR-31 was blocked failed to improve blood perfusion in the ischemic hindlimb. (A) Schematic representation of experimental design. (B) miR-31 levels in transfected EPCs were determined by qPCR and shown as mean ± standard deviation. (C) Representative images of hindlimb blood flow measured by laser Doppler before operation (Pre-Op), immediately after hindlimb ischemia surgery (Post-Op), and up to 2 weeks after intramuscular injection of endothelial culture medium EGM2 (medium), CAD-EPC transfected with scramble control (Scr), or miR-31 antagonirs (Anti-miR-31). Arrows indicate the functional blood flow at hindlimb before surgery and after EPC treatment. (D) Immunofluorescence staining on nude mice tissues 7 days after injection with PKH-26-labeled EPCs. Capillaries in the ischemic muscles were visualized by CD31 immunostaining (green), and injected human EPCs were monitored by PKH-26 fluorescence (red). Mice receiving FIR-treated EPCs had more CD31+/PKH-26+ double-positive cells (white arrowheads) in ischemic muscle than the miR-31 KD, FIR-treated (Anti-31 + FIR) group or the no-FIR (Scr) control. DAPI: nuclear staining of live cells (blue). Scale bars represent 200 µm. (E) Quantitative analysis of capillary densities and CD31+/PKH-26+ double-positive cells in ischemic muscle of mice hindlimb ischemia surgery. HPF: high power field; N.D.: not detectable; Significance levels, **p < 0.01, ***p < 0.001. (F) Quantitative analysis of blood flow expressed as perfusion ratio of the ischemic to the contralateral (non-operated) hindlimb. **p < 0.01, ***p < 0.001
Supplemental Figure XI. C/EBP-β did not involved in the miR-31 expression induced by FIR. (A) FIR treatment on healthy PB-EPCs did not change C/EBP-β levels. (B-D) Knocking down C/EBP-β levels in PB EPCs did not decrease FIR-induced cell migration (left panel in B; C) or microvascular formation (right panel in B; C) activities.