The effects of microvesicles on endothelial progenitor cells are compromised in type 2 diabetic patients via downregulation of the miR-126/VEGFR2 pathway

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Wu K, Yang Y, Zhong Y, Ammar HM, Zhang P, Guo R, Liu H, Cheng C, Koroscil TM, Chen Y, Liu S, Bihl JC. The effects of microvesicles on endothelial progenitor cells are compromised in type 2 diabetic patients via downregulation of the miR-126/VEGFR2 pathway. Am J Physiol Endocrinol Metab 310: E828–E837, 2016. First published March 8, 2016; doi:10.1152/ajpendo.00056.2016.—Our previous study showed that circulating microvesicles (cMVs) of diabetic mice have negative effects on the function of endothelial progenitor cells (EPCs). Whether this is true in diabetic patients deserves further study. In this study, the effects of cMVs and EPC-MVs on EPCs were tested under different conditions. The levels of miR-126 and vascular endothelial growth factor receptor 2 (VEGFR2) were determined in cMVs, EPC-MVs, and/or EPCs to modulate the miR-126 level in EPC-MVs. We found the following: 1) the circulating EPC level was reduced but the circulating EPC-MV level increased in uncontrolled diabetic patients; 2) the cMVs and EPC-MVs of healthy controls had beneficial effects on EPCs (migration, apoptosis, ROS), whereas the effects were reversed in the cMVs and EPC-MVs of uncontrolled diabetic patients; and 3) the cMVs and EPC-MVs of uncontrolled diabetic patients carried less miR-126 and had downregulated VEGFR2 expression in EPCs. Manipulating the miR-126 level in EPC-MVs with inhibitor or mimic changed their function. The effects of cMVs and EPC-MVs are compromised in diabetes due to the reduction of their carried miR-126, which might provide a therapy target for diabetic vascular complications.

DIABETES MELLITUS (DM) is considered one of the major risk factors for various cardiovascular complications. Endothelial dysfunction is a key initiator for vascular disease, which results from increased oxidative stress in the vascular cells (39). Endothelial progenitor cells (EPCs) are known to play important roles in maintaining vascular function and structure by repairing or replacing dysfunctional or injured endothelial cells (ECs) (33). Impaired EPC proliferation, differentiation, adhesion, mobilization, and survival have been reported in DM (1, 26). Our previous study has demonstrated that the circulating EPC level is decreased and the function of EPCs impaired in db/db diabetic mice (8). Moreover, we found that the circulating microvesicles (cMVs) of db/db diabetic mice compromised the functions of EPCs. Investigation on the regulatory effects of cMVs on EPCs in diabetic patients could provide novel therapeutic avenues for vascular complications of diabetes.

Extracellular MVs are submicrometric fragments released from cells in response to activation and apoptosis (22, 37). cMVs are the MVs released from the cells in the blood and from the vascular wall. An elevation of cMV levels has been reported in vascular diseases such as thrombotic diseases, diabetes, and cardiovascular diseases (2, 8, 25). Furthermore, one study has shown that the level of cMVs could predict the severity of vascular diseases (30). MVs released from EPCs (EPC-MVs) have been reported to serve as an index for EPC loss and functional incompetence (27). Moreover, the level of circulating EPC-MVs can predict aortic stiffness in atherosclerotic patients (27). Therefore, circulating MV and EPC-MV levels could serve as biomarkers and predictors for vascular diseases. On the other hand, accumulating evidence suggests that MVs mediate cell-cell communication via transferring proteins, mRNAs, and miRNAs (miRs) from their parent cells to the target cells (5, 17, 29). The functions of MVs are complex and multifactorial, depending on the stimulator and origin. Previously, we demonstrated that cMVs of diabetic mice impair the function of EPCs, whereas cMVs of healthy controls do not have detrimental effects on EPCs (8). However, whether this is true in diabetic patients remains unclear.

Previous studies have shown that miR-126 governs vascular integrity (35) and is a biomarker or mediator of vascular diseases (11, 32). Downregulation of miR-126 impairs EPC function (23). EPC-MVs have been shown to improve ischemia-reperfusion injury of hindlimb and kidney through the transfer of miR-126 to target cells (6, 28). In the meantime, miR-126 has been reported to regulate angiogenic process and EC/EPC function by modulatingvascular endothelial growth factor receptor 2 (VEGFR2) (13, 15). Our previous study found that EPC-MVs affect EC functions and apoptosis via their carried miR-126 (34). However, it is unknown whether EPC-MVs, as one type of MVs, would affect EPC functions through the miR-126 and its downstream VEGFR2 pathway. In the present study, we determined the effects of cMVs and EPC-MVs of diabetic patients on EPC survival and functions.
and explored whether miR-126/VEGFR2 is involved in the mechanism.

METHODS

Human subjects. A total of 45 subjects (42–51 yr old) were included in the study with 24 type 2 diabetes patients (14 were well controlled and 10 were uncontrolled) and 21 healthy controls. Patients were recruited from the Endocrinology Department at the Boonshoft School of Medicine, Wright State University, Dayton, OH, and the Affiliated Hospital of Guangdong Medical College, Zhanjiang, China. The protocols were approved by Institutional Review Boards at both Wright State University and Guangdong Medical College. Each patient signed an informed consent form after a thorough explanation. Exclusion criteria included pregnancy, patients with history of myocardial infarction, stroke, unstable angina, renal or heart failure, cancer, chronic alcohol abuse, hypertension requiring more than three antihypertensive medication, and use of any multivitamin supplement, and patients with proliferative diabetic retinopathy or nephropathy or painful diabetic neuropathy requiring chronic narcotic analgesic therapy.

Experimental design. After an 8-h fast, blood samples were taken from patients and healthy controls. Approximately 60 ml of blood was collected from each subject in a tube with a 3.13% citrate buffer. The blood samples were transferred directly to the laboratory and processed within 1 h of collection. The levels of glucose, Hb A1c, and lipid of blood samples were measured. Periphery blood mononuclear cells (PBMCs) isolated from blood samples were used for determining the circulating EPC levels and EPC culture. The plasma was used for isolating circulating MVs. Cross-coculture studies were performed as described in Fig. 3. After 24 h of coculture, EPC migration, apoptosis, and reactive oxygen species (ROS) production were measured. The miR-126 and VEGFR2 expressions were also examined in cMVs and EPCs.

Table 1. Clinical characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy Controls</th>
<th>Well controlled</th>
<th>Uncontrolled</th>
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<tbody>
<tr>
<td>Total nos. (n = 45)</td>
<td>21</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Average age, yr</td>
<td>42 ± 5</td>
<td>44 ± 6</td>
<td>43 ± 8</td>
</tr>
<tr>
<td>Sex ratio (male/female)</td>
<td>12:9</td>
<td>9:5</td>
<td>7:3</td>
</tr>
<tr>
<td>Fasting blood glucose, mg/dl</td>
<td>84.1 ± 11.4</td>
<td>90.4 ± 9.4</td>
<td>165.5 ± 24.5*+</td>
</tr>
<tr>
<td>Hb A1c, %total HGB</td>
<td>4.2 ± 0.3</td>
<td>5.6 ± 0.2</td>
<td>7.8 ± 0.8*+</td>
</tr>
<tr>
<td>Lipid profile, mg/dl</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Total cholesterol</td>
<td>168.8 ± 26.5</td>
<td>175.2 ± 34.2</td>
<td>214.4 ± 12.8</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>98.2 ± 10.7</td>
<td>100.9 ± 22.5</td>
<td>185.2 ± 24.2*+</td>
</tr>
<tr>
<td>HDL</td>
<td>57 ± 7.9</td>
<td>53 ± 8.7</td>
<td>38.8 ± 8.3</td>
</tr>
<tr>
<td>LDL</td>
<td>92 ± 14.1</td>
<td>102 ± 28.1</td>
<td>138.5 ± 12.8*</td>
</tr>
<tr>
<td>VLDL</td>
<td>18.9 ± 3.3</td>
<td>21.2 ± 5.8</td>
<td>32.3 ± 8.4</td>
</tr>
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Data are means ± SE. T2DM, type 2 diabetes mellitus; HGB, glycated hemoglobin. *P < 0.05 vs. healthy controls; +P < 0.05 vs. well-controlled T2DM patients.

Fig. 1. The levels of circulating endothelial progenitor cells (EPCs) and EPC-derived microvesicles (EPC-MVs). A and B: representative flow cytometry plots of circulating EPCs (A) and EPC-MVs (B) in healthy controls (HC) and diabetes mellitus (DM) patients. C: summarized data on circulating EPC and EPC-MV levels; n = 21 for HC, 14 for well-controlled DM, and 10 for uncontrolled DM. **P < 0.01 vs. HC; ++P < 0.01 vs. well-controlled DM by 2-way ANOVA.

VEGFR, VEGF receptor; PE, phycoerythrin.
**cMV isolation.** cMVs were isolated as described in our previous paper (8). Platelet-rich plasma was centrifuged (1,500 g, 15 min) at 4°C to get platelet-free plasma (PFP). PFP was divided to numerous tubes (1 ml/tube) and then centrifuged at 30,000 g for 30 min (4°C). The MV pellet was resuspended in 100 μl of PBS for flow cytometric analysis.

Flow cytometric analyses of the levels of circulating EPCs and EPC-MVs. The levels of circulating EPCs and EPC-MVs were determined per our previous study (8). For analysis of circulating EPC level, isolated PBMCs were incubated with 1 μl of FITC-conjugated anti-human CD34 (eBioscience, San Diego, CA) and 5 μl of PE-conjugated anti-human VEGFR2 (BD Bioscience, San Jose, CA) antibodies for 30 min at 4°C in the dark. Isotype-matched (IgG) nonspecific antibodies were used as negative controls. Labeled EPCs were analyzed in a flow cytometer (Accuri Cytometers, Ann Arbor, MI). EPCs were defined as CD34+/VEGFR2+ cells. The numbers of circulating EPCs were described as the CD34+/VEGFR2+ cells per microliter of whole blood.

For analysis the of EPC-MV level, isolated cMVs were incubated with antibodies (CD34 and VEGFR2) under the same conditions as those for EPCs. The size of particles was calibrated using 1- and 2-μm flow cytometry beads (Molecular Probes, Invitrogen, Eugene, OR). MVs were defined as particles with a diameter of <1.5 μm. The numbers of circulating EPC-MVs were determined as CD34+/VEGFR2+ events in the gate of MVs. The data were described as the number of EPC-MVs per microliter of whole blood.

**EPC isolation, culture, and characterization.** EPCs were cultured from peripheral blood, as described in previous reports (4, 21). Peripheral blood taken from patients was diluted in PBS (2 times) and then gently layered over 4 ml of lymphocyte separation liquid (Sigma) for centrifugation (800 g, 30 min at 4°C). The PBMCs in the interface layer were transferred to a new tube and washed with PBS buffer by centrifugation at 400 g for 5 min at 4°C. EPCs were cultured from PBMCs in endothelial cell basal medium-2 (Lonza, Walkersville, MD) on a 24-well plate (5 × 10⁶ cells/well). Cells were cultured continuously for 10 days for coculture studies. The EPCs in the cultures were characterized by assays of double-staining with Di-LDL and BS-lectin according to our previous reports (7–9). In brief, the adherent cells were incubated with PE-labeled Di-LDL (Biomedical Technologies, Stoughton, MA) for 2 h at 37°C. After that, cells were analyzed by flow cytometry.

**Fig. 2.** Migration ability, apoptotic rate, and reactive oxygen species (ROS) production of EPCs from diabetic patients and HC. A: summarized data on migration ability and apoptotic rate. B: representative flow cytometry plots and summarized data of ROS production. P1, EPCs with low ROS; P2, EPCs with high ROS; n = 21 for HC, 14 for well-controlled DM, and 10 for uncontrolled DM. **P < 0.01 vs. HC; **P < 0.01 vs. well-controlled DM by 2-way ANOVA. DHE, dihydroethidium.
EPCs and microvesicles in diabetes

Fixed with 2% paraformaldehyde and then counterstained with FITC-labeled BS-lectin (Sigma, Fairfax, VA). Cells were viewed under an inverted fluorescence microscope (EVOS).

**EPC apoptosis and migration assays.** To determine the effective dose of cMVs for coculture experiments, EPCs were treated with different doses of cMVs (0.25, 0.5, 0.75, and 1 dilution to the total original cMV concentration from plasma of each individual). After 24 h of coculture, apoptotic rate of EPCs was assessed using an apoptosis assay kit (BD Bioscience) according to our previous studies (7–9). The EPC apoptotic rate was analyzed by a flow cytometer. Based on dose-response studies, a dilution of 1:1 was used for coculture experiments, as described in Experimental design section. EPC migration was evaluated using the Boyden chamber system (Millipore, Temecula, CA), as we have reported previously (7–9).

**Intracellular ROS production assay.** Intracellular ROS production was determined by dihydroethidium (DHE; Sigma-Aldrich, St. Louis, MO) staining (10, 14). Cells were incubated with the DHE working solution (1 μM) at 37°C for 2 h in dark. Then, the cells were observed under an inverted fluorescence microscope (EVOS), and the percentage of DHE-positive cells was analyzed using a flow cytometer.

**Gene expression analysis.** miRNA from cMVs was extracted using a mirVana miRNA isolation kit (Ambion) by following the manufacturer’s instructions. cDNA was synthesized using a miScript reverse transcription kit (Qiagen) according to miScript SYBR Green PCR Kit (Qiagen) on a real-time PCR system (Bio-Rad). Small nuclear RNA U6 was used as an internal control. Relative expression of miR126 was calculated using the 2−ΔΔCT method (34).

**Transfection of EPCs.** To generate EPCmiR-126-MVs, EPCmiR-126in-MVs, and EPCmiR-126m-MVs, EPCs were transfected with miR-126 control (1 nM; Applied Biosystems), inhibitor (cat. no. 4464084), or mimic (cat. no. 4464066) using lipofectamine 2000 for 16 h and exposed to 1 nM VEGFR2 (1:1,000; Cell Signaling Technology). β-Actin (1:4,000; Sigma) was used to normalize protein loading. After being washed thoroughly, membranes were incubated with horseradish peroxidase-conjugated IgG (1:40,000; The Jackson Laboratory) for 1 h at RT. Blots were then developed with enhanced chemiluminescence-developing solutions and quantified.

**Statistical analysis.** All data are expressed as means ± SE. Comparisons for two groups were performed by Student’s t-test. Multiple comparisons of the data, including the ethic factor, were analyzed by two-way ANOVA, followed by a Tukey post hoc test (SPSS version 19.0; SPSS, Chicago, IL). For all tests, a P value of <0.05 was considered significant.

**RESULTS**

**Study subject characteristics.** The study subject characteristics are summarized in Table 1. There were no significant differences in average age between healthy controls, well-controlled patients, and uncontrolled diabetic patients (P > 0.05). The Hb A1c and glucose levels were significantly higher in uncontrolled diabetic patients compared with healthy controls and well-controlled diabetic patients (P < 0.05). Also, the levels of triglycerides and very low-density lipoprotein were significantly higher in uncontrolled diabetic patients (P < 0.05). However, there were no significant differences between diabetes and healthy controls on the levels of total cholesterol, HDL, or LDL (P > 0.05).

The level of circulating EPCs was decreased and the level of EPC-MVs increased in uncontrolled diabetic patients. By using flow cytometric analysis, we found that the numbers of circulating EPCs were significantly decreased in uncontrolled diabetic patients compared with healthy controls and well-controlled diabetic patients (Fig. 1, A and C). However, the numbers of circulating EPC-MVs were significantly in-

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**Protocol 1**

Healthy subjects (n=21)

- **EPCs**
- **cMVs**

Well-controlled patients (n=14)

- **EPCs**
- **cMVs**

Uncontrolled patients (n=10)

- **EPCs**
- **cMVs**

**Cross co-culture of EPCs with cMVs**

24 hrs

Migration

Apoptosis

ROS production

**Protocol 2**

Healthy subjects

- **EPCs**
- **EPCmiR-126cd-MVs**
- **EPCmiR-126cm-MVs**

Well-controlled diabetic patients

- **EPCs**
- **EPCmiR-126cd-MVs**
- **EPCmiR-126cm-MVs**

Uncontrolled diabetic patients

- **EPCs**
- **EPCmiR-126cd-MVs**
- **EPCmiR-126cm-MVs**

**Cross co-culture of EPCs with EPC-MVs**

24 hrs

Gene expression

Migration

Apoptosis

ROS production

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Fig. 3. Diagram depicts the design of cross-coculture experiments for determining the effects of circulating MVs (cMVs) and EPC-MVs on EPCs. In protocol 1, EPCs from HC were cocultured with cMVs from well-controlled or uncontrolled diabetic patients, whereas EPCs from uncontrolled diabetic patients were cocultured with cMVs from HC. In protocol 2, EPCs from healthy controls were cocultured with EPC-MVs released from EPCs of diabetic patients transfected with miR-126c or miR-126m, whereas EPCs from uncontrolled diabetic patients were cocultured with EPC-MVs released from EPCs of diabetic patients transfected with miR-126c or miR-126m. After coculture, the expressions of miR-126 and VEGFR2 and migration, apoptosis, and ROS production of EPCs were measured. EPCmiR-126cd-MVs, EPCmiR-126cm-MVs, and EPCmiR-126m-MVs, microvesicles released from EPCs transfected with miR-126 control, inhibitor, and mimic, respectively.
There were two ROS production populations, high ROS production population and low ROS production population. The percentage of high ROS production of EPCs from uncontrolled diabetic patients was significantly higher than that of EPCs from healthy controls and well-controlled diabetic patients. However, the percentage of low ROS production population of EPCs from uncontrolled diabetic patients was significantly decreased. There were no significant differences in ROS production between EPCs from healthy controls and well-controlled diabetic patients.

**The detrimental effects of cMVs from uncontrolled diabetes on the migration, apoptosis, and ROS production of EPCs from healthy controls.** To determine the effective dose of cMVs for coculture, EPCs were treated with different doses of cMVs for 24 h. As seen in Fig. 4A, cMVs from uncontrolled diabetes dose-dependently increased the apoptotic rate of EPCs from healthy controls. Based on these data, we chose dilution 1:1 for the coculture experiments (Fig. 3). Moreover, we found that co-incubation with cMVs from uncontrolled diabetes decreased the migration ability but increased apoptotic rate of EPCs from healthy controls (P < 0.01 vs. vehicle, P < 0.05 or 0.01 vs. cMVs from well-controlled diabetes; Fig. 4B). These changes were accompanied with the increase of high ROS production population (P < 0.01) and decrease of low ROS production population (P < 0.01; Fig. 4B). However, the cMVs from well-controlled diabetes did not have effects on healthy EPC migration, apoptosis, or ROS production. These data indicate that cMVs from uncontrolled diabetes could impair the function and survival of healthy EPCs by increasing oxidative stress.
The protective effects of cMVs from healthy controls on the migration, apoptosis, and ROS production of EPCs from uncontrolled diabetes. As shown in Fig. 5, coculture with cMVs from healthy controls significantly improved the migration ability and decreased the apoptotic rate of EPCs from uncontrolled diabetes (P < 0.01 vs. vehicle; Fig. 5, A and B). These changes were accompanied with the increase of the high ROS production (P < 0.05) and the increase of the low ROS production (P < 0.05 vs. vehicle; Fig. 5C). These data indicate that cMVs from healthy controls could improve the function and survival of EPCs from uncontrolled diabetes by decreasing oxidative stress.

The expressions of miR-126 in cMVs and EPC-MVs. The miR-126 expression was decreased in the cMVs from uncontrolled diabetes (Fig. 6A). However, there was no significant difference in miR-126 expression between cMVs from healthy controls and well-controlled diabetes (Fig. 6A). Moreover, we isolated EPC-MVs from EPCs cultured from healthy controls or diabetic patients and found that the miR-126 level was decreased in EPC-MVs released from EPCs of uncontrolled diabetes (Fig. 6B). miR-126 inhibitor transfection on healthy EPCs downregulated the miR-126 levels in their released EPC-MVs, whereas the miR-126 levels mimic transfection on EPCs from uncontrolled diabetes upregulated the miR-126 level in their released EPC-MVs.

The expressions of miR-126 and VEGFR2 in EPCs. EPCs were cultured from healthy controls, well-controlled patients, and uncontrolled diabetic patients to generate EPC-MVs and used for coculture. Results showed that both miR-126 and VEGFR2 levels were decreased in healthy EPCs after coculture with EPC-MVs released from EPCs of uncontrolled diabetes (Fig. 7A). On the contrary, the miR-126 and VEGFR2 levels were increased in uncontrolled diabetic EPCs after coculture with EPC-MVs released from EPCs of healthy controls (Fig. 7B). Coincubation with EPC-MVs released from EPCs of well-controlled diabetes did not change the levels of miR-126 or VEGFR2 in healthy EPCs. Moreover, EPC\textsuperscript{miR-126\_i-MVs} from uncontrolled diabetes were able to increase the miR-126 and VEGFR2 levels in healthy EPCs, whereas EPC\textsuperscript{miR-126\_m-MVs} from healthy controls were able to decrease the miR-126 and VEGFR2 levels in EPCs from uncontrolled diabetes.

The detrimental effects of EPC-MVs released from EPCs of uncontrolled diabetes on the migration, apoptosis, and ROS production of EPCs from healthy controls. Coculture with EPC-MVs from uncontrolled diabetes decreased the migration ability but increased apoptotic rate of healthy EPCs (P < 0.01 vs. vehicle, P < 0.05 or 0.01 vs. EPC-MVs from well-controlled diabetes; Fig. 8). These changes were accompanied with the increase in high ROS production population and decrease in low ROS production population (Fig. 8C). However, the EPC-MVs released from EPCs of well-controlled diabetes did not have effects on healthy EPC migration, apoptosis, or ROS production (Fig. 8). These data indicate that EPC-MVs released from EPCs of uncontrolled diabetes could impair the function and survival of healthy EPCs by increasing ROS production. Interestingly, EPC\textsuperscript{miR-126\_m-MVs} from uncontrolled diabetes with miR-126 upregulation in EPC-MVs significantly decreased their detrimental effects on healthy EPCs (Fig. 8).

The protective effects of EPC-MVs released from EPCs of healthy controls on the migration, apoptosis, and ROS production of EPCs from uncontrolled diabetes. As shown in Fig. 9, coculture with EPC-MVs released from EPCs of healthy controls significantly improved the migration ability and decreased the apoptotic rate of EPCs from uncontrolled diabetes compared with vehicle (Fig. 9, A and B). These changes were accompanied with the decrease in high ROS production population and the increase in low ROS production population (Fig. 9C). These data indicate that EPC-MVs released from EPCs of healthy controls could improve the function and survival of EPCs from uncontrolled diabetes by increasing ROS production. More importantly, EPC\textsuperscript{miR-126\_m-MVs} from healthy controls with miR-126 downregulation in EPC-MVs could diminish their protective effects on EPCs from uncontrolled diabetes. These data suggest that EPC-MVs could be the modulator for EPC function, apoptosis, and oxidative stress via their carried miR-126.

**DISCUSSION**

EPCs play an important role in maintaining endothelium hemostasis and integrity. Our previous studies found that circulating EPCs are decreased and bone marrow-derived EPCs dysfunctional in \( db/db \) type 2 diabetic mice (7, 8). In the
In the current study, we conducted a cross study to investigate the role of cMVs in modulating the EPC function in diabetic patients. We cocultured EPCs from uncontrolled diabetes with cMVs from healthy controls and healthy EPCs with cMVs from well-controlled or uncontrolled diabetes. Interestingly, we found that cMVs from uncontrolled diabetes have negative effects on healthy EPCs by decreasing migration and increasing apoptosis and ROS production, (Figs. 4 and 5). This is in agreement with our previous study in a db/db mouse. The present study extends the investigation to human subjects. Moreover, we also found that cMVs from uncontrolled diabetes have negative effects on healthy EPCs by decreasing migration and increasing apoptosis and ROS production, (Figs. 4 and 5). This is in agreement with our previous study in a db/db mouse. The present study extends the investigation to human subjects.

**Fig. 7. Expressions of miR-126 and VEGFR2 in EPCs after coculture with EPC-MVs.** A: expressions of miR-126 and VEGFR2 in EPCs from HC after coculture with EPC-MVs released from EPCs of well-controlled or uncontrolled diabetes. Data are expressed as a fold change of vehicle; \( n = 6 \), * \( P < 0.05 \) vs. vehicle; + \( P < 0.05 \) vs. EPCmiR-126c-MVs from well-controlled diabetes; ## \( P < 0.01 \) vs. EPCmiR-126c-MVs from uncontrolled diabetes. B: EPC-MVs released from EPCs of HC increase the expression of miR-126 and VEGFR2 in EPCs from uncontrolled diabetes, which can be reversed by miR-126i transfection. Data are expressed as a fold change of vehicle; \( n = 6 \), ** \( P < 0.01 \) vs. vehicle; ## \( P < 0.01 \) vs. vehicle.

**Fig. 8. Effects of EPC-MVs released from EPCs of uncontrolled diabetes on the EPC migration and apoptosis.** A: effects of EPC-MVs released from EPCs of uncontrolled diabetes on the migration of EPCs from HC. B: effects of EPC-MVs released from EPCs of uncontrolled diabetes on the apoptotic rate of EPCs from HC. C: effects of EPC-MVs released from EPCs of uncontrolled diabetes on the ROS production of EPCs from HC; \( n = 6 \), * \( P < 0.05 \) and ** \( P < 0.01 \) vs. vehicle; + \( P < 0.05 \) and ++ \( P < 0.01 \) vs. EPCmiR-126c-MVs from well-controlled diabetes; # \( P < 0.05 \) and ## \( P < 0.01 \) vs. EPCmiR-126c-MVs from uncontrolled diabetes.
EPCs from uncontrolled diabetes; cMVs and EPC-MVs from uncontrolled diabetes decreased EPC survival and functions; in contrast, cMVs and EPC-MVs from healthy controls improved the survival and functions of EPCs from uncontrolled diabetic patients. These data suggest that the origin of MVs and the environment involved in their release are considered the most critical factors that determine the function of MVs. We believe that the different functions might be due to the different contents they carried. Our recent work shows the expression of miR-126 in EPC-MVs, and its high expression is accompanied by the protective effects of EPC-MVs on ECs (34). In the present study, we found that the expression of miR-126 is decreased in cMVs from uncontrolled diabetes and EPC-MVs released from EPCs of uncontrolled diabetes (Fig. 6). EPC-MVs have been reported to promote angiogenesis through the transfer of miR-126 to target cells (6, 28), miR-126 has been reported to regulate angiogenic process and EC/EPC function by targeting VEGFR2-related signal transduction (13, 15). In the present study, we found that the miR-126 and VEGFR2 expressions are increased in EPCs from uncontrolled diabetes after coinubcation with EPC-MVs released from EPCs of healthy controls, whereas they are decreased in healthy EPCs after coinubcation with EPC-MVs released from EPCs of uncontrolled diabetes (Fig. 7), suggesting the role of EPC-MVs in the transfer of miR-126 and activation of the downstream VEGFR2 pathway in EPCs. A recent study reported that modulation of miR-126 by anti-miR-126 or miR-mimic-126 treatment resulted in significant loss of or an increase in proangiogenic effects of CD34(+) PBMCs in diabetes (24). To further confirm the role of miR-126 in mediating the function of EPC-MVs, we modified the EPC-MVs by transfecting EPCs with miR-126 mimic or inhibitor to generate miR-126-upregulated or -downregulated EPC-MVs. A functional study found that the miR-126 mimic diminished the detrimental effects of EPC-MVs released from EPCs of uncontrolled diabetes on healthy EPCs, whereas the miR-126 inhibitor decreased the protective effects of EPC-MVs released from EPCs of healthy controls on EPCs from uncontrolled diabetes (Figs. 8 and 9). For exploring the mechanisms responsible for these effects, we observed the ROS production of EPCs after different MV treatment. It has become widely accepted that enhanced oxidative stress is responsible for EPC number and function reduction in diabetes and cardiovascular diseases (16, 31). MVs could be the link in this process; they might be involved in the regulation of ROS production. Burger and Touyz (3) reported that MVs are able to modulate ROS production; both the stimulator and the origin of MV release play crucial roles in this process. Moreover, miR-126 has been reported to have implications in oxidative stress. Overexpression of miR-126 decreased ROS production and apoptosis in ECs (36). These data suggest that the function of EPC-MVs on EPCs is mediated by their carried miR-126 and that oxidative stress might be one of the mechanisms responsible for EPC function changes.

Our studies indicate that the cMVs and EPC-MVs of uncontrolled diabetic patients may be the factors to induce the reduction of EPC numbers and functions, which is due to the reduction of their carried miR-126. On the contrary, cMVs and EPC-MVs from healthy controls may improve survival and function of EPC from uncontrolled diabetic patients. This may lead to a novel therapeutic agent capable of reducing diabetic vascular complications.
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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


