Expression of Angiogenic MicroRNAs in Endothelial Progenitor Cells From Type 1 Diabetic Patients With and Without Diabetic Retinopathy

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Purpose. MicroRNA (miR) expression in endothelial progenitor cells (EPCs) in type 1 diabetes (DM1) and its relation with different stages of diabetic retinopathy (DR) have not been reported to date. Our aim was to analyze miR-222, miR-221, and miR-126 expression in EPCs from DM1 patients with and without DR.

Methods. We included 41 patients with DR, 35 without DR, and 38 controls. Blood was collected for flow cytometry and EPC culture. Total RNA was extracted and purified and real-time quantitative PCR was performed for miR expression in cultured EPCs. Relative changes in miR expression were analyzed with the $2^{-\Delta\Delta C}{}_T$ method.

Results. Circulating EPCs were reduced and miR-126 expression was increased in DM1 compared to controls (0.030 [interquartile range [IQR], 0.020–0.050] vs. 0.060 [IQR, 0.030–0.110], $P = 0.004$; 1.740 [IQR, 0.890–4.120] vs. 0.990 [IQR, 0.487–3.015], $P = 0.047$ respectively) without differences between patients with and without DR. Patients with DR had higher expression of miR-221 than those without DR (1.405 [IQR, 0.820–2.867] vs. 0.915 [IQR, 0.507–1.292], $P = 0.019$) without differences among degrees of DR. Circulating EPCs were reduced in patients on statins (0.010 [IQR, 0.010–0.050] vs. 0.045 [IQR, 0.020–0.087], $P = 0.008$), and miR-221 expression increased in patients on angiotensin-converting enzyme (ACE) inhibitors/angiotensin receptor blocker (ARB) II (1.430 [IQR, 1.160–2.705] vs. 1.000 [IQR, 0.520–1.330], $P = 0.021$) compared to those without treatment. MicroRNA-126 expression was associated with body mass index (BMI; $\rho = -0.267$, $P = 0.026$) and diastolic blood pressure ($\rho = -0.267$, $P = 0.034$). MicroRNA-221 was associated with triglyceride concentration ($\rho = 0.296$, $P = 0.012$).

Conclusions. Circulating EPCs were reduced and miR-126 expression was increased in DM1 compared to controls. Patients with DR had higher expression of miR-221 than those without DR. The identification of biomarkers of diabetic complications might be useful for monitoring disease progression and potential therapeutic targets.

Keywords: endothelial progenitor cells (EPCs), miR-126, miR-221, miR-222

Diabetic retinopathy (DR), the leading cause of visual impairment in the Western world, results in severe vision loss in the late stages of type 1 diabetes (DM1). Hyperglycemia damages retinal microvasculature, which results in increased permeability, blood and serum leakage to the extravascular space, and progressive decline in retinal blood flow, as well as closure of the retinal microvasculature. When retinopathy progresses to the late stages, chronic hypoxia/nonperfusion leads to compensatory neovascularization, which occurs in an aberrant manner mostly on the surface of the retina. The exact pathophysiological mechanisms that are involved in this process remain to be elucidated.

Endothelial progenitor cells (EPCs) are now recognized as a key cell type responsible for healthy maintenance of the vasculature, including the retina. Since Asahara et al.[1] first described the presence of circulating EPCs in 1997, accumulating evidence has indicated that bone marrow–derived EPCs are involved in angiogenesis of ischemic tissues, including ischemic retina.[2–6] Both type 1 and type 2 diabetes are associated with widespread EPC reduction and dysfunction.[7,8] Several studies have shown that circulating EPC number is reduced in patients with nonproliferative DR (NPDR)[9] but increased in patients with proliferative DR (PDR).[10,11] It is possible to hypothesize that the reduced number and functionality of EPCs found in noncomplicated diabetic patients[8–12] might predispose these patients to the later development of DR. Once the damage is widespread and specific chemokines are produced by the suffering

retina, the bone marrow would respond by increasing the production of EPCs. It has been shown that endothelial colony-forming cells from patients with PDR are mobilized into the circulation but may be unable to migrate and incorporate into and form vascular tubes with human retinal endothelial cells in order to repair damaged capillary endothelium.[13] While there is increasing evidence that EPCs contribute significantly to both normal and pathological angiogenesis, there has been little consensus on the markers that should be used to identify these cells. Circulating EPCs are enumerated by flow cytometry on the basis of the combined expression of surface antigens. Endothelial progenitor cells in peripheral blood have been generally defined as mononuclear cells positive for surface antigens characteristic of hematopoietic stem cells (CD34) and endothelial cells (vascular endothelial growth factor [VEGF] receptor-2 [KDR]), but negative for the common leukocyte antigen CD45.[14] Late EPCs, also called endothelial outgrowth cells and endothelial colony-forming cells, are positive for CD31, CD34, CD144 (VE-cadherin), and KDR and negative for CD45. In vitro assays were also developed to permit identification of EPCs. Cells that adhered immediately to fibronectin-coated dishes and 4 days later displayed distinctive changes in morphology, demonstrated the ability to ingest 1,1-dioctadecyl-3,3,3,3 tetramethylindocarbocyanine-perchlorate (Ac-LDL) and bound the plant lectin *Ulex europaeus* were also defined as early-outgrowth EPCs. Adherent endothelial cobblestone-patterned colonies that appeared from 7 to 21 days after plating are referred as late-outgrowth endothelial EPCs. Early EPCs (hematopoietic proangiogenic cells) secrete proangiogenic molecules and are thought to play paracrine roles in vascular repair and regeneration.[15] Late EPC cells have a high proliferative potential and generate vascular tubes in Matrigel in vitro.[16]

Over the last few years, small noncoding RNA molecules termed microRNAs (miRs) have emerged as critical regulators of signalling pathways in multiple cell types including endothelial and perivascular cells. MicroRNAs regulate gene expression at both the mRNA and protein levels by either degrading or translationally repressing specific target mRNAs by imperfect pairing of the 5′-proximal "seed" region in the miRs with the 3′-untranslated region of the mRNA,[17] and thereby altering cellular signalling responses to specific stimuli. MicroRNAs have been shown to alter specific signalling pathways that affect proliferation, differentiation, migration, and cell survival in endothelial cells and smooth muscle cells. Therefore, miRs can either function in a proangiogenic manner and potentate angiogenesis or act as antiangiogenic miRs by enhancing cell death and decreasing endothelial proliferation.[18] MicroRNA-221 and miR-222 are highly expressed in vascular endothelial cells where they exert antiproliferation, antimigration, and proapoptosis effects.[19] MicroRNA-221 seems to play a role in diabetes-induced endothelial dysfunction. MicroRNA-221 affects expression of c-kit, the receptor for stem cell factor, which plays a key role in EPC migration and homing.[20] Under hyperglycemic conditions, miR-221 is induced in human umbilical vein endothelial cells (HUVECs), which consequently triggers inhibition of c-kit and impairment of HUVEC migration.[21] MicroRNA-126 has been identified as proangiogenic and increases colony formation, proliferation, and migration of EPCs from patients with type 2 diabetes mellitus,[22] but its expression is significantly reduced in diabetic patients.[22,23]

However, the expression of miRs in EPCs from patients with DM1 and its relation with different stages of DR have not been reported to date. Therefore, the aim of the present study was to analyze the expression of antiangiogenic miR-222 and miR-221 and proangiogenic miR-126 in EPC from patients with DM1 with and without DR.

Materials and Methods

Patients and Blood Samples

This cross-sectional study was performed with the approval of the Hospital Ethics Committee of Hospital Clínico San Carlos. We enrolled 41 DM1 patients with DR, 35 DM1 without DR, and 38 nondiabetic healthy controls. Patients with DM1, diagnosed at least 6 months before entering the study, were recruited consecutively from the diabetes outpatient clinic and the ophthalmology outpatient clinic from January 2012 to June 2014. Controls were recruited among the health staff from the hospital who decided to participate voluntarily. Informed consent was obtained from all patients and controls who agreed to participate in the study in accordance with the Declaration of Helsinki. Exclusion criteria were pregnancy, hepatic insufficiency (hepatic enzymes more than double the upper limit of normality), renal insufficiency (estimated glomerular filtration rate less than 30 mL/min), overt macrovascular disease (ischemic heart disease, cerebrovascular disease, peripheral vascular disease) in the previous 6 months, or other severe diseases. Clinical history, analytical parameters, and medications were evaluated.

Diabetic retinopathy was defined by a complete eye examination with a 66 Volk Slit Lamp Biomicroscopy Lens (Volk Optical, Inc., Mentor, OH, USA). Early Treatment Diabetic Retinopathy Study (ETDRS) seven standard field stereoscopic color photographs of each eye were used to diagnose and grade retinopathy.[24] The retinopathy grade assigned to the individual patient was that for the eye with the more advanced level of retinopathy.

After overnight fasting of at least 12 hours, 40 mL venous blood was collected into five sodium citrate + Ficoll tubes (362782; BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for EPC culture within 3 hours after blood drawing. Additionally, 2 mL blood was collected into a EDTA tube (no. 05.1167; Sarstedt AG & Co., Nümbrecht, Germany) and stored at 4°C to obtain mononuclear cells for flow cytometry analysis.

Isolation, Culture, and Characterization of Cultured EPCs

Peripheral blood mononuclear cells (PBMNCs) were isolated from 40 mL peripheral blood by Ficoll gradient centrifugation, washed three times with PBS (Dulbecco's phosphate buffer, X0515; Biowest, Nuaillé, France), and plated at a density of 106 cells/cm^2 on human fibronectin-coated flasks (0172003; Tebubio, Le-Perray-en-Yvelines, France). Cells were cultured in EGM-2MV (C-22022; PromoCell, Heidelberg, Germany) containing vascular endothelial growth factor (VEGF), fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor, ascorbic acid, hydrocortisone, and gentamycin, supplemented with 10% fetal calf serum. Cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere. After 3 days of culture, nonadherent cells were washed off with PBS, and fresh medium was added. After 7 days of culture, flasks were washed with PBS and samples were incubated with accutase (enzyme cell detachment, L11-007; PAA Laboratories, Pasching, Austria) at 37°C for 5 minutes to detach EPCs, after which we confirmed under the microscope that the cells had been removed. Cells were placed in a polystyrene round-bottom snap cap tube (Eppendorf, (Eppendorf, Hamburg, Germany), washed with PBS, and divided in two pellets for RNA extraction and flow cytometry to confirm EPC phenotype.

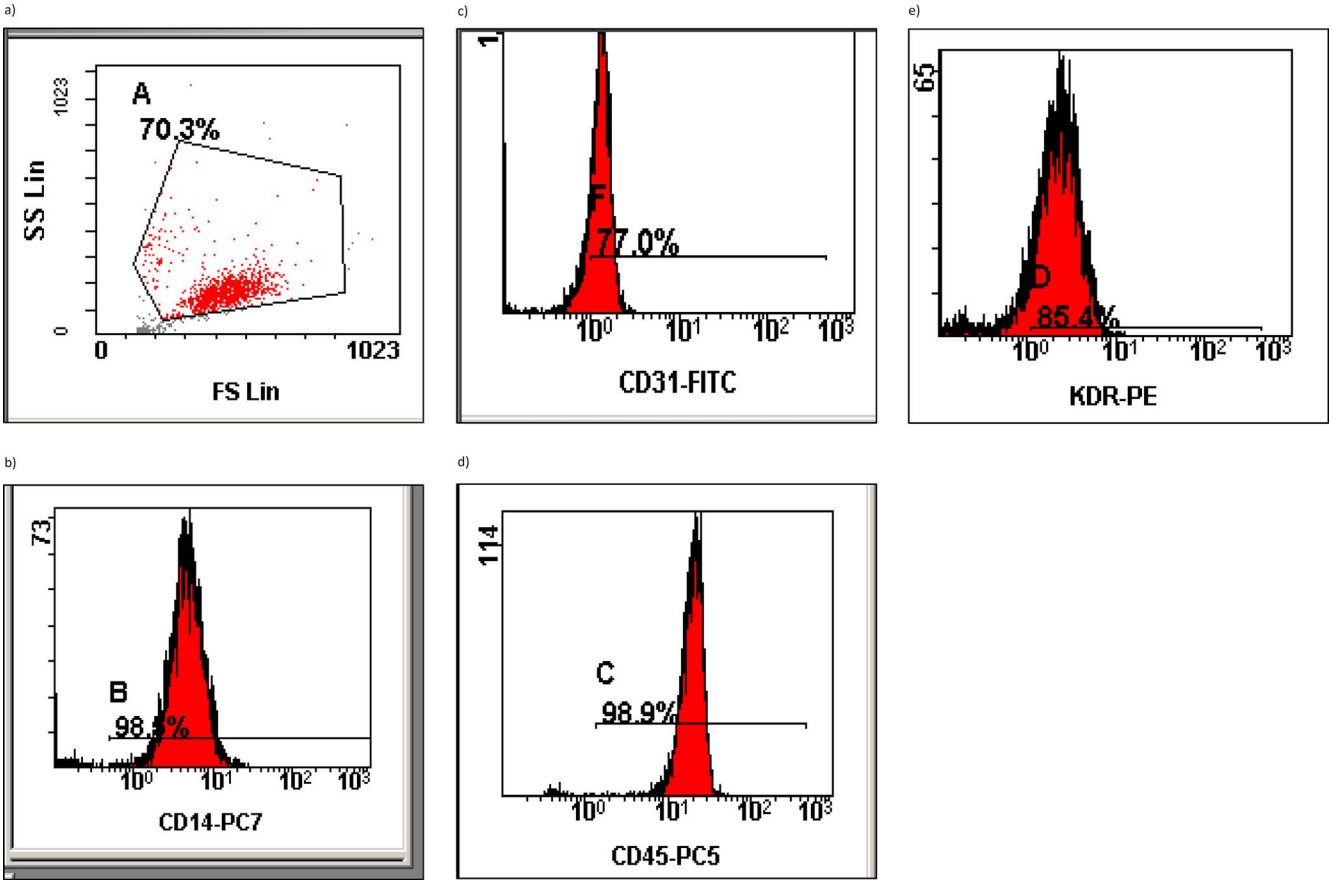

Figure 1. Cultured EPC surface markers analyzed by flow cytometry. (**a**) Uniform live culture. (**b**) Positivity for CD14. (**c**) Positivity for CD31. (**d**) Positivity for CD45. (**e**) Positivity for KDR.

To confirm that the cells used for RNA extraction corresponded to EPCs, 100 μL suspended cells in PBS was divided among five 1.5 mL Eppendorf tubes and incubated with the following monoclonal antibodies: CD34-FITC (IM1870; Beckman Coulter, Brea, CA, USA), CD45-PC5 (A07785, Beckman Coulter), CD14-PC7 (A22331, Beckman Coulter), CD31-FITC (IM1431U, Beckman Coulter), anti-human VE-cadherin/CD144-PE (FAB9381P; R&D Systems, Minneapolis, MN, USA), and anti-human VEGF R2/KDR-PE (FAB357P; R&D Systems). Isotype-matched antibodies were used as negative controls: IgG1-PC5 (A07798, Beckman Coulter), IgG1-FITC (A07795, Beckman Coulter), IgG2a-PE (A09142, Beckman Coulter), IgG2a-PC7 (A12692, Beckman Coulter), and IgG1-PE (A07796, Beckman Coulter). Sample tubes were incubated for 30 minutes on ice and in the dark. Cytometric analysis was performed within the next 24 hours. Endothelial progenitor cell surface markers were analyzed by flow cytometry: uniform live culture (Fig. 1a), positivity for CD14 (Fig. 1b), CD31 (Fig. 1c), CD45 (Fig. 1d), and KDR (Fig. 1e).

In addition, identification of EPCs was performed after 7 days by dual positive staining for 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-perchlorate (Dil-Ac-LDL, 42364; Sigma-Aldrich Corp., St. Louis, MO, USA) and lectin (Ulex Europaeus UEA, L9006; Sigma-Aldrich Corp.) and examined by fluorescence microscopy.

Flow Cytometry Analysis for Blood EPCs

For analysis of circulating EPCs, we used the following fluorescence-conjugated mouse anti-human monoclonal antibodies: CD45-PC5 (A07785; Beckman Coulter), CD34-FITC (IM1870; Beckman Coulter), anti-human VE-cadherin/CD144-PE (FAB9381P; R&D Systems), and anti-human VEGF R2/KDR-PE (FAB357P; R&D Systems). For the fluorescence-minus-one (FMO) control, CD45-PC5 and CD34-FITC were pipetted into other polystyrene round-bottom snap cap tubes. One hundred microliters of well-mixed blood was added to each tube by reverse pipetting. Sample tubes were incubated for 30 minutes on ice and in the dark. Red blood cells were lysed by adding 900 μL 1× lysing solution (no. 349202; BD Biosciences) to each tube and incubated for 10 minutes at room temperature in the dark. Afterward, samples were washed with PBS to remove any particulate fraction including apoptotic bodies until the supernatant was clear. After this, tubes were placed immediately on ice and protected from light until ready to measure. Cytometric analysis was performed within the next 24 hours.

Flow cytometry was performed as previously published[25] with some modifications. Sample acquisition was done within 1 hour of red cell lysis on a FC 500 flow cytometer (2-laser, 5-color analysis) with FC 500 CXP software (Beckman Coulter). The flow cytometer was used at least 30 minutes after fluidics startup to allow reliable stabilization and warming up of the system. Isotype-matched antibodies were used as negative controls: IgG1-PC5 (A07798; Beckman Coulter), IgG1-FITC (A07795; Beckman Coulter), IgG2a-PE (A09142; Beckman Coulter), IgG2a-PC7 (A12692; Beckman Coulter), and IgG1-PE (A07796; Beckman Coulter) and the respective antibodies. Only voltages for forward scatter (FSC) and side scatter (SSC) were additionally fine-tuned in the current experiment. The FSC threshold was not adjusted. Amplifier settings for FSC and SSC were used in linear mode, and those for fluorescence channels were used in logarithmic mode. Sample tubes were

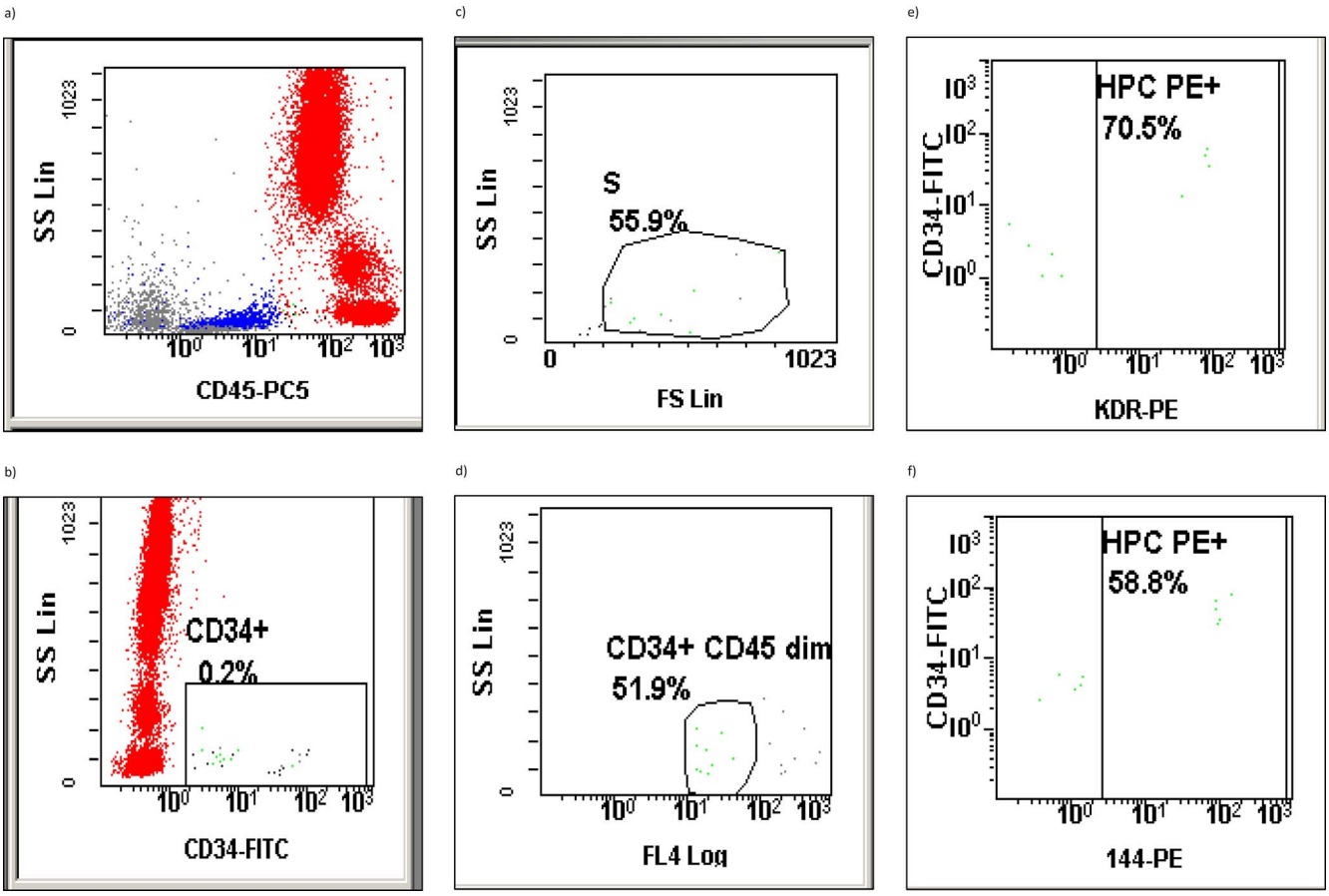

Figure 2. Flow cytometry analysis of circulating EPCs. (**a**) $CD45^+$ events. (**b**) $CD34^+$ events. (**c**) Lymph-blast scatter region. (**d**) $CD45^{dim}$ cells. (**e**) Cutoff for KDR^+ events assessed by the FMO control. (**f**) Cutoff for $CD144^+$ events assessed by the FMO control.

acquired at a medium flow rate of <10,000 events/s by setting acquisition gates on the $CD45^+$ cells. At least 150,000 $CD45^+$ events were recorded.

For analysis of circulating EPCs, all leukocytes were first gated on a FSC/SSC dot plot. This population was shown on a SSC/CD45 dot plot, and the $CD45^+$ events were gated as P1 (Fig. 2a). Then, the cell debris was eliminated, and the P1 cell set was shown on a SSC/CD34 dot plot following gating of the $CD34^+$ events as P2 (Fig. 2b). The events of P2 were subsequently presented on a FSC/SSC dot plot and gated as P3 in order to confirm the lymph-blast (Fig. 2c) scatter region and to remove residual debris. The events of P3 were then shown on a SSC/CD45 dot plot, and only the $CD45^{dim}$ cells were gated as P4 (Fig. 2d). Gating of the FSC/SSC lymph-blast region and backscattering of $CD34^+$ events to a SSC/CD45 dot plot are essential steps to confirm cell size and clustering of bona fide $CD45^{dim}CD34^+$ cells. Finally, the $CD45^{dim}CD34^+$ events were shown on a CD34/KDR dot plot, and the cutoff for KDR^+ events (P5, Fig. 2e) was assessed by the FMO control. The $CD45^{dim}CD34^+$ events were shown on a CD34/CD144 dot plot, and the cutoff for $CD144^+$ events (P5, Fig. 2f) was assessed by the FMO control.

Endothelial progenitor cells ($CD45^{dim}CD34^+KDR^+$ cells and $CD45^{dim}CD34^+CD144^+$ cells) were expressed as percentage of total CD45+ cells.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted and purified using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated RNA purity and concentration were assessed by a NanoDropND-100 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) obtaining ratios between 1.8 and 2, and RNA quality was evaluated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the Agilent RNA 6000 Pico kit, obtaining a RNA integrity number (RIN) between 8 and 10. A Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) was used for reverse transcription (RT). Real-time PCR was performed using miRCURY LNA Universal RT micro RNA PCR SYBR Green Master mix (Exiqon, Denmark) with the following miR-LNA PCR primers (Exiqon, Denmark): hsa-miR-126; hsa-miR-221, and hsa-miR-222. The U6 small nucleolar RNA was used as the housekeeping small RNA reference gene. The reaction was carried out in Mastercycler ep realplex 4 (Eppendorf) and analyzed using realplex T software. The amplification step consisted of denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and then annealing at 60°C for 1 minute. Each reaction was performed in triplicate, and analysis was performed by the $2^{-\Delta\Delta C_T}$ method as described previously.[26] Each set of PCR reactions included a no-template control and an RT-minus control.

Statistical Analysis

Variables are presented with their frequency distribution. Quantitative variables are summarized by their mean and standard deviation (±SD). Quantitative variables that show asymmetrical distribution are summarized by their median interquartile range (IQR).

Table 1. Clinical Characteristics, Circulating EPCs, and miR Expression in Healthy Controls and DM1 Patients

	Controls, $n = 38$	DM1 Patients, $n = 76$	P
Sex %, male/female	42.1/57.9	55.3/44.7	0.234
Age*	42.84 ± 14.42	43.56 ± 11.67	0.789
BMI*	24.05 ± 4.46	25.14 ± 3.92	0.228
Smoking %, yes/no	24/76	19.4/80.6	0.775
$CD45^{dim}CD34^{+}CD144^{+}$†	0.060 [0.030–0.110]	0.030 [0.020–0.050]	**0.004**
$CD45^{dim}CD34^{+}KDR^{+}$†	0.030 [0.010–0.080]	0.035 [0.010–0.070]	0.870
miR-126†	0.990 [0.487–3.015]	1.740 [0.890–4.120]	**0.047**
miR-221†	0.970 [0.350–2.215]	1.190 [0.670–1.935]	0.357
miR-222†	1.022 [0.606–2.363]	1.340 [0.690–2.590]	0.415

Bold values are statistically significant.
* Mean and standard deviation.
† Median [IQR].

Comparisons between groups for qualitative variables were evaluated by the χ^2 test or Fisher's exact test. For quantitative variables, measures were compared through Student's *t*-test or the nonparametric Mann-Whitney *U* test if the quantitative variables would not adjust to a normal distribution. Receiver operator characteristic (ROC) analysis was calculated to assess the utility of the expression of miRs in EPCs to distinguish DR. Area under the curve (AUC) and its 95% confidence intervals (95% CI) were calculated. In order to evaluate the role of the miR-221 in the association with DR, a multiple logistic regression analysis was fitted.

The correlation between quantitative variables was evaluated with the nonparametric Spearman rank correlation coefficient (ρ).

The null hypothesis was rejected by a type I error < 0.05 ($\alpha < 0.05$). The data were processed with the STATA V.12.0 (STATA Corp. LP, College Station, TX, USA) statistical package.

Results

Diabetic patients and healthy controls did not differ in sex distribution, age, body mass index (BMI), or smoking status (Table 1). Diabetic patients with DR had a longer duration of the disease than those without DR, presented with other diabetic microvascular complications more frequently, smoked more, and received statin and angiotensin-converting enzyme (ACE) inhibitor/angiotensin receptor blocker (ARB) treatment more often, but did not differ in the degree of metabolic control and other clinical characteristics (Table 2).

The EPCs originally isolated from PBMNCs were round and small. After 3 days in culture, cell clusters appeared (Fig. 3a). In 7 days, a number of spindle-shaped cells sprouted from the edges of the cell cluster (Fig. 3b). At day 7, adherent cells were identified by costaining with lectin and Dil-ac-LDL. We observed double-positive cells by fluorescence microscopy (Fig. 4).

Table 2. Clinical Characteristics, Circulating EPCs, and miR Expression in DM1 Patients With and Without DR

	No RD, $n = 35$	RD, $n = 41$	P
$CD45^{dim}CD34^{+}CD144^{+}$*	0.030 [0.010–0.050]	0.030 [0.020–0.050]	0.660
$CD45^{dim}CD34^{+}KDR^{+}$*	0.030 [0.010–0.085]	0.040 [0.010–0.060]	0.664
miR-126*	1.490 [0.690–3.950]	2.050 [1.157–4.225]	0.256
miR-221*	0.915 [0.507–1.292]	1.405 [0.820–2.867]	**0.019**
miR-222*	1.340 [0.630–2.220]	1.315 [0.757–2.672]	0.800
HbA1c % (mmol/mol)†	7.74 (61.1) ± 1.01	7.89 (62.7) ± 1.27	0.591
DM progression, y†	16.86 ± 10.80	30.29 ± 10.29	**0.000**
Systolic blood pressure, mm Hg†	119.46 ± 9.06	123.38 ± 14.16	0.168
Diastolic blood pressure, mm Hg†	74.37 ± 8.52	72.08 ± 8.48	0.278
BMI†	24.50 ± 4.29	25.68 ± 3.53	0.201
Albuminuria, mg/g Cr†	2.50 [1.53–3.45]	3.20 [1.80–8.40]	**0.036**
eGFR, mL/min/1.73 m^2†	93.30 ± 30.36	81.96 ± 19.36	0.053
Total cholesterol, mg/dL†	175.80 ± 25.623	176.71 ± 35.31	0.900
HDL cholesterol, mg/dL†	60.06 ± 10.20	65.34 ± 19.08	0.130
LDL cholesterol, mg/dL†	100.21 ± 21.23	96.88 ± 26.57	0.553
Triglyceride, mg/dL*	70.00 [58.00–86.00]	77.00 [64.00–91.50]	0.156
Smoking %, yes/no	9.1/90.9	28.2/71.8	**0.041**
Regular exercise %, yes/no	17.6/82.4	30.0/70.0	0.217
Macrovascular disease %, yes/no	14.3/85.7	12.5/87.5	1.000
Diabetic neuropathy %, yes/no	0/100	22.5/77.5	**0.003**
Statins %, yes/no	37.1/62.9	61.0/39.0	**0.041**
ACE inhibitors/ARB %, yes/no	15.2/84.8	41.0/59.0	**0.016**
Antiagregation %, yes/no	9.1/90.9	15.4/84.6	0.494

eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Cr, creatnin. Bold values are statistically significant.
* Median [IQR].
† Mean and standard deviation.

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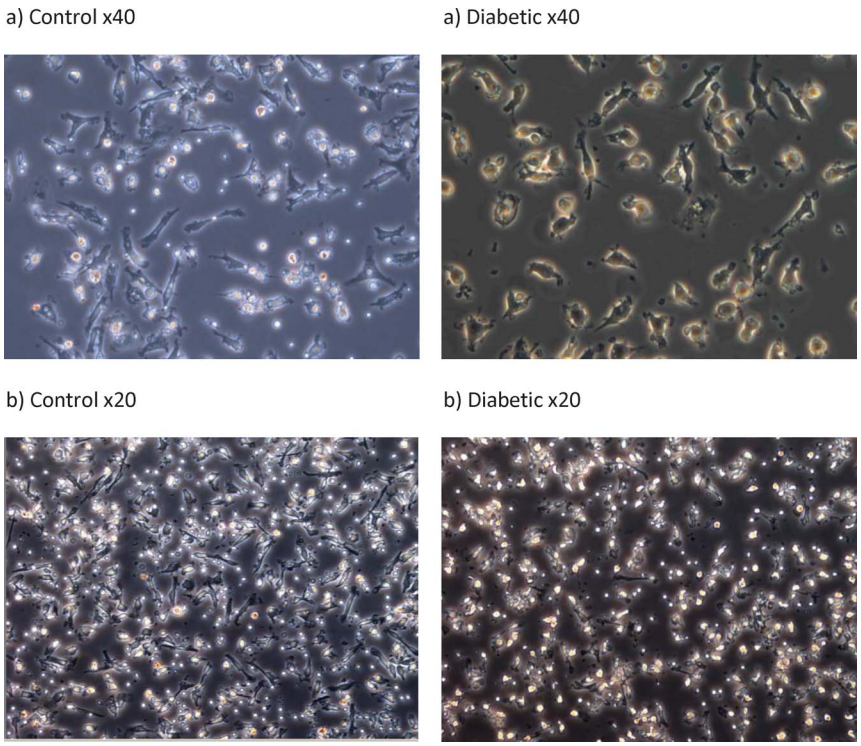

FIGURE 3. EPC culture. (**a**) Three-day culture. Control ×40, diabetic ×40. (**b**) Seven-day culture. Control ×20, diabetic ×20.

The median number of circulating $CD45^{dim}CD34^{+}CD144^{+}$ cells was significantly reduced in patients with DM1 compared to healthy controls (0.030 [IQR, 0.020–0.050] vs. 0.060 [IQR, 0.030–0.110], $P = 0.004$). In addition, there was an increased expression of miR-126 in DM1 patients compared to controls (1.740 [IQR, 0.890–4.120] vs. 0.990 [IQR, 0.487–3.015], $P = 0.047$) (Table 1).

When we analyzed the circulating levels of EPCs and the expression of miRs in EPCs in relation to different stages of DR, we found that DM1 patients with DR had a significantly increased expression of miR-221 compared to patients without DR (1.405 [IQR, 0.820–2.867] vs. 0.915 [IQR, 0.507–1.292], $P = 0.019$) (Table 2). Nevertheless, we did not find any difference among different degrees of DR progression (data not shown). Healthy controls presented a very similar distribution of miR-221 expression to DM1 patients without DR ($P = 0.718$) but lower than in DM1 patients with DR (0.970 [0.350–2.215] vs. 1.405 [IQR, 0.820–2.867] $P = 0.049$).

We performed a ROC analysis for miR expression and found an AUC of 0.696 (95% CI: 0.568–0.823, $P = 0.005$) for miR-221. Considering miR-221 expression as a marker of DR, a cutoff point of 1.14 established according to our data would have a 0.72 sensitivity and a 0.60 specificity (Fig. 5).

To evaluate the independent relationship between miR-221 as a marker for DR, a multiple logistic regression analysis was performed. The model was adjusted by glycated hemoglobin (HbA1c) levels, smoking status, use of statin and ACE inhibitors/ARB treatment, and triglyceride concentration.The association between miR-221 and DR remained statistically significant (odds ratio [OR]: 1.63; CI 95%: 1.02–2.60; $P = 0.040$).

When other clinical characteristics were considered, the median number of circulating $CD45^{dim}CD34^{+}KDR^{+}$ cells was significantly reduced in patients treated with statins compared with those without treatment (0.010 [IQR, 0.010–0.050] vs. 0.045 [IQR, 0.020–0.087], $P = 0.008$), and miR-221 expression was increased in patients treated with ACE inhibitors or ARB II compared with those without treatment (1.430 [IQR, 1.160–2.705] vs. 1.000 [IQR, 0.520–1.330], $P = 0.021$). In addition, miR-126 expression was significantly associated with BMI ($\rho = -0.267$, $P = 0.026$) and diastolic blood pressure ($\rho = -0.267$, $P = 0.034$), and miR-221 was significantly associated with triglyceride concentration ($\rho = 0.296$, $P = 0.012$) in DM1 patients.

DISCUSSION

We have analyzed circulating levels of both early ($CD45^{dim}CD34^{+}KDR^{+}$) and late ($CD45^{dim}CD34^{+}CD144^{+}$) EPCs. We found a significantly reduced circulating level of late EPCs in patients with DM1 compared to healthy controls, in agreement with previous studies,[27,28] but no differences between patients with and without DR. Brunner et al.[11] found a correlation of circulating EPCs to stages of DR in DM1. In NPDR, a reduction of EPCs was observed compared with values in patients without DR; and in PR, a dramatic increase of mature EPCs was observed. In contrast, a recent study including many more patients with DM1 did not find a relation between circulating EPCs and DR.[29]

We studied miR expression in early-outgrowth EPCs. The EPCs obtained in our culture expressed CD45 and the typical myeloid marker CD14 in a high proportion, in accordance with the definition of early EPCs.[30] In addition, early-outgrowth EPCs were identified by costaining with lectin and Dil-ac-LDL. These cells have been shown to secrete proangiogenic molecules and are thought to play paracrine roles in vascular repair and regeneration. Late-outgrowth EPCS show the capacity to form new blood vessels autonomously in vivo.[15] We chose to study early-outgrowth EPCs because vascular repair processes are likely to be present from early stages of DR, while generation of vascular tubes is a process that appears in late stages of DR progression.

Some published studies carried out systematic profiling of a large number of miRNAs, whereas we have focused on a small

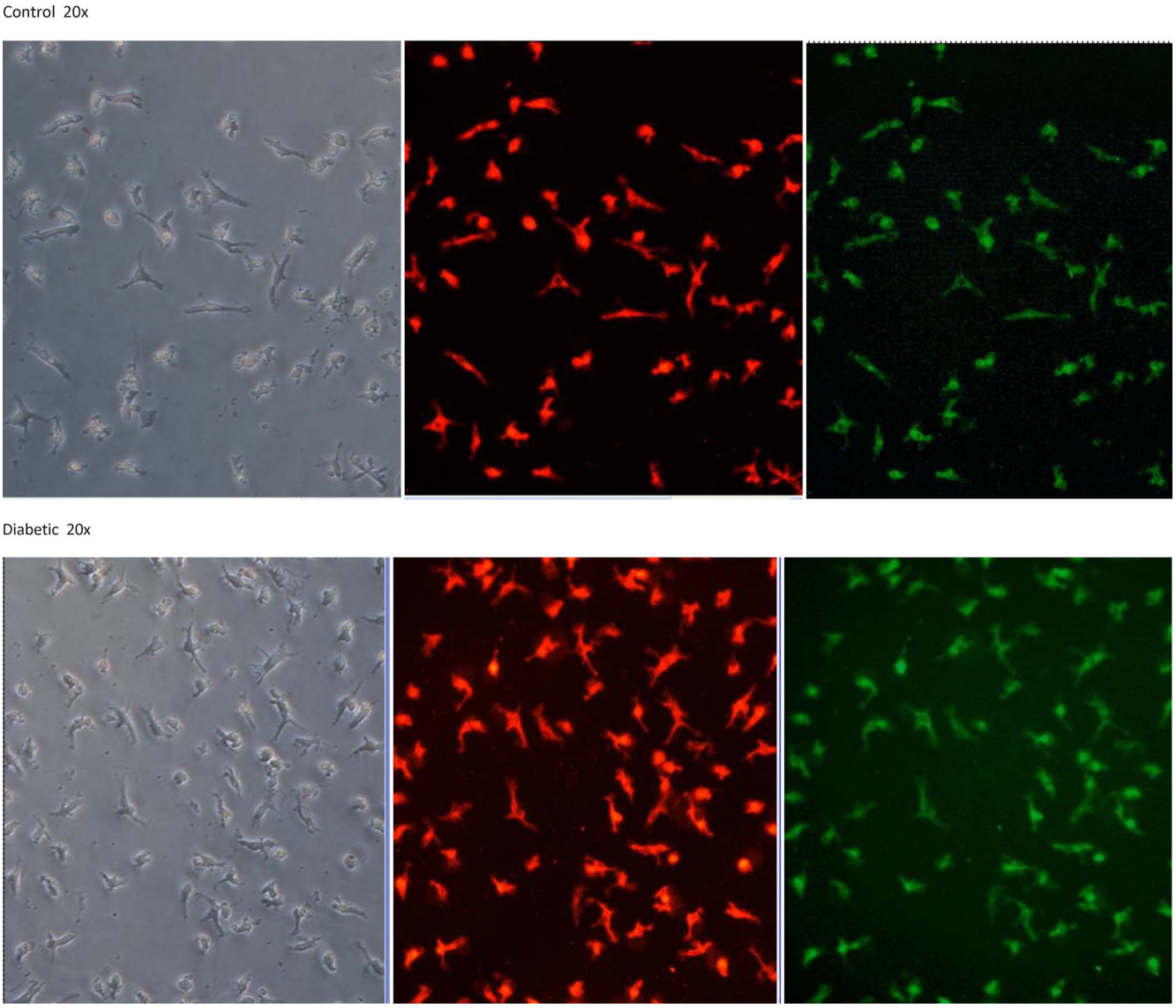

FIGURE 4. Seven-day culture, Dil-Ac-LDL staining (*red*) and lectin staining (*green*). All the cells stain for both markers. Control ×20, diabetic ×20.

group of selected miRNAs that were supposedly most likely to be affected in patients with DR. In fact, we also analyzed the expression of miR-92a, miR-130a, and miR-150 in an initial subgroup of patients and controls, but the expression was very similar between groups (miR-92a, miR-150) or we observed underexpression (miR-130a) and therefore did not carry on with the analysis (data not shown).

It has been shown that miR-126 promotes EPC proliferation and migration and inhibits EPC apoptosis ability via its target Spred-1 and through Ras/ERK/VEGF and PI3K/Akt/eNOS signal pathway.[22] In our study there was an increased expression of miR-126 in DM1 patients compared to healthy controls, in contrast with findings from previous studies.[22,23,31,32] All these studies were performed in type 2 diabetic patients. Furthermore, alterations in circulating miR-126 have been proposed as a marker for endothelial dysfunction in type 2 diabetes[32] and coronary artery disease (CAD).[33] In our group, recent macrovascular disease was an exclusion criteria, and only 10 patients suffered macrovascular disease before 6 months prior to entering the study. These differences among study groups could explain the conflicting results. We observed a negative association between miR-126 and diastolic blood pressure and

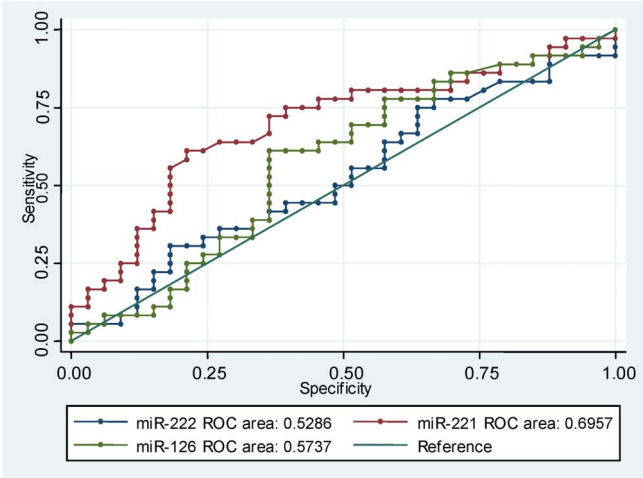

FIGURE 5. ROC curve for miR expression.

BMI. A significantly lower miR-126 expression in PBMNCs from hypertensive patients compared with healthy controls has been shown[34]; and in a model of mice with renal ischemia/reperfusion injury, animals overexpressing miR-126 reduced weight.[35]

To our knowledge this is the first report of increased expression of miR-221 in DM1 patients with DR compared to patients without DR. Overexpression of miR-221 in EPCs significantly decreases EPC proliferation through the MEK/ERK pathway by targeting PAK1.[36] Exposure to high levels of glucose induced expression of miR-221 in HUVECs, impairing c-kit expression and decreasing migration of HUVECs[21]; but there are no studies in diabetic patients reported to date. We have found an increased expression of miR-221 in DM1 patients with DR compared to patients without DR and controls, although there was no difference in miR-221 expression in DM1 patients compared to controls. Healthy controls presented a very similar distribution of miR-221 expression to DM1 patients without DR but significantly lower than in DM1 patients with DR. It is possible to hypothesize that when retinal damage is widespread with chronic hypoxia and nonperfusion, the EPCs would respond by increasing the expression of miR-221 and specific chemokines, but this process is not activated in earlier stages in noncomplicated diabetic patients.

Data obtained in this study showed that miR-221 expression was increased in patients treated with ACE inhibitors or ARB II compared with those without treatment. Since patients with DR needed ACE inhibitors/ARB II therapy significantly more often than patients without DR, further studies are needed to elucidate whether this difference is mainly due to retinal endothelial damage or influenced by an upregulation of miR-221 expression in ACE inhibitor- or ARB II-treated patients.

We performed a ROC analysis and found an AUC of 0.696 for miR-221. Considering miR-221 expression as a marker of DR, a cutoff point of 1.14 established according to our data would have a 0.72 sensitivity and a 0.60 specificity. The association between miR-221 and DR remained statistically significant after adjusting by HbA1c levels, smoking status, use of statin and ACE inhibitors/ARB treatment, and triglyceride concentration. Nevertheless, these data should be validated subsequently since our sample was limited and the cross-sectional design does not allow proposing miR-221 as a biomarker for DR.

Our data showed a relationship between miR-221 expression and triglyceride concentration. The relation between DR and triglycerides has been reported previously. Patients with combined dyslipidemia, but not familial hypercholesterolemia, have an increased incidence of retinal abnormalities. This suggests that elevated cholesterol and triglycerides may be implicated in the development of retinovascular lesions occurring in DR (e.g., hemorrhage and cotton-wool spots).[37] There is now consistent evidence from two major trials, the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study[38] and the Action to Control Cardiovascular Risk in Diabetes Eye (ACCORD-Eye) study,[39] that the triglyceride-lowering drug fenofibrate reduces the risk of development and progression of DR. A number of putative therapeutic mechanisms for fenofibrate have been proposed, including anti-inflammatory and antiapoptotic effects and modulation of angiogenesis through a mechanism dependent on VEGF.[40] We could not analyze differences between patients with and without fibrate treatment since only one of our patients followed this treatment.

In summary, this study shows a significantly reduced circulating level of late EPCs and an increased expression of miR-126 in patients with DM1 compared to healthy controls. In addition, the miR-221 expression in EPCs from DM1 patients with DR was significantly higher than in patients without DR and controls. The identification of diabetic biomarkers and pathogenic determinants of progression would not only assist detection of early complications, but would also serve to monitor markers for disease progression and potentially open new possibilities of treatment. Current therapies for DR are not fully efficacious; hence there is an imperative need for a better understanding of the molecular mechanisms underlying diabetic complications in order to identify newer therapeutic targets. The therapeutic potential of miRs as antiangiogenic agents has been shown in multiple animal models. Westenskow et al.[41] observed that intraocular injection of anti-miR-132 is a potent inhibitor of pathological neovascularization, but not the normal quiescent vasculature, in the eye in various mouse models. Eye drops containing large amounts of miR-410 and intravitreal injections of miR-126 efficiently downregulated VEGF-A expression, prevented retinal angiogenesis, and effectively treated retinal neovascularization in oxygen-induced retinopathy in mice.[42,43] Therefore, we may be just a few years away from using these molecules to target aberrant angiogenesis in humans.

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