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Dysregulation of angiogenesis-related microRNAs in endothelial progenitor cells from patients with coronary artery disease

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ABSTRACT

Endothelial progenitor cells (EPCs) play an important role in vascular repair and maintenance of vascular homeostasis through re-endothelialization and neovascularization. Cardiovascular risk factors that contribute to coronary artery disease (CAD) have been shown to negatively impact EPCs, although the mechanisms are poorly understood. MicroRNAs (miRNAs) which negatively regulate gene expression at the post-transcriptional level have been shown to impact endothelial cell (EC) angiogenic actions, but little is known about their role in modulating EPC function. In this study we first investigated if EPCs expressed EC specific, angiogenesis-related miRNAs; then determined whether the expression of these miRNAs was altered in EPCs from CAD patients as compared with healthy controls. Furthermore, we examined if atorvastatin, known to increase circulating EPC numbers, had any effect on EPC miRNA expression. We found EPCs produced miR-126, miR-130a, miR-221, miR-222 and miR-92a which have thus far been identified as the most important angiogenic miRNAs. Dysregulation of these miRNAs was detected in EPCs from CAD patients and atorvastatin treatment selectively impacted miRNA expression in EPCs.

Our data provide evidence that angiogenic miRNAs might play an important role in the control of EPC function, and that their dysregulation might contribute to EPC dysfunction in patients suffering from coronary artery disease. These findings might lead to the development of novel therapeutic modalities for the prevention and treatment of CAD.

1. Introduction

Bone marrow derived endothelial progenitor cells (EPCs) possess the capacity to differentiate into endothelial lineage cells *in vitro* and can be directly incorporated into newly formed vessels *in vivo* and they play an important role in vascular repair and maintenance of vascular homeostasis through reendothelialization and neovascularization [1,2]. Both animal and clinical studies have indicated that risk factors for coronary artery disease such as advanced age, diabetes and hypercholesterolemia cause reduced numbers and impaired function of circulating EPCs [3–5]. However, the mechanisms governing EPC dysfunction are poorly understood.

MicroRNAs (miRNAs) are a class of short, noncoding, single-stranded RNA molecules, approximately 22 nucleotides in length, that negatively regulate gene expression at the post-transcriptional level [6–8]. They bind sequences in the target messenger RNA 3′-untranslated regions through complementarity and form RNA–RNA complex, leading eventually to mRNA degradation or translational inhibition. It's known that miRNAs control a wide range of biological functions and processes, such as development, differentiation, metabolism, growth, proliferation, and apoptosis [9–12].

The involvement of miRNAs in the regulation of mammalian vascular biology was discovered in mice through the disruption of the Dicer gene, a gene, essential for miRNA synthesis. Homozygous *dicer*ex1/2 mutant mice lacking the first two exons of dicer were not viable with abnormal vasculature observed in yolk sacs from 11.5 day embryos compared to their wild type or heterozygous littermates [13]. Furthermore, expression changes of several crucial genes involved in embryonic angiogenesis, including VEGF, Flt1 (VEGF-R1), flk1 (VEGF-R2) and Tie1 was detected [13]. Poliseno et al. performed the first large-scale analysis of miRNA expression in human umbilical vein endothelial cells (HUVECs) and identified 15 highly expressed miRNAs, i.e., miR-23b, miR-23a, miR-let-7a, miR-let-7b, miR-125b, miR-221, miR-222, miR-125a, miR-24, miR-106a, miR-20, miR-16, miR-let-7c, miR-103, and miR-133a [14]. Now, it's well established that miRNAs modulate endothelial cell (EC) angiogenesis by controlling cell growth, migration, apoptosis, capillary tube formation [15,16]. The best characterized miRNAs so far include miR-221, miR-222, miR-130a, miR-126 and miR-92a [14,17–20].

Studies of miRNAs as regulators of angiogenesis have mostly focused on endothelial cells [15,16]. Therefore, we conducted this study to investigate: (1) if EPCs express endothelial cell specific

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angiogenesis related miRNAs, especially, miR-221, miR222, miR-130a, miR-126 and miR-92a, (2) if expression of these miRNAs is altered in EPCs from CAD patients as compared with healthy controls and (3) if atorvastatin which has been shown to increase the number of cells expressing EPC markers both *in vitro* and *in vivo* [21,22], has any effect on miRNA expression.

2. Materials and methods

2.1. Recruitment of patients

Informed consent for study participation was obtained from all patients and healthy volunteers. All protocols involving human samples were approved by the Research Ethnics Board of St. Michael's Hospital, University of Toronto, in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). To exclude interference with miRNA expression, all patients prior to recruitment had not received treatment with HMG-CoA reductase inhibitors (statins). Patients with multiple cardiac risk factors and stable coronary artery disease (CAD), scheduled to undergo percutaneous coronary intervention, were recruited.

2.2. Isolation, culture, characterization and treatment of EPCs

Peripheral blood mononuclear cells (PBMNCs) were isolated from 100 mL of peripheral blood by Ficoll gradient centrifugation. The PBMNCs were plated at a density of 0.75×10^6 cells/cm^2 in human fibronectin-coated flasks and cultured in endothelial cell basal medium supplemented with 20% human serum and various growth factors (EGM-2MV, Cambrex). After 3 days non-adherent cells were removed and fresh culture medium was supplied. Cells were maintained until day seven with change of medium every other day. Cells obtained were characterized by uptake of Dil-Ac-LDL, binding of UEA-Lectin and detection of VEGF receptor 2 (KDR) gene expression (flow cytometric analysis) as described [23]. For treatment with atorvastatin, cells were in fresh medium without growth factor supplement (VEGF, FGF and insulin like growth factor−1), and atorvastatin (kindly provided by Pfizer Canada Inc.) was added (1 μM, final concentration) and maintained for 24 h.

2.3. Total RNA extraction and reverse transcription

Total RNA was extracted and purified from EPCs by using a mirVana miRNA Isolation Kit (Applied Biosystems Inc.) according to the manufacturer's instructions. Briefly, $0.5–2 \times 10^6$ EPCs were detached in 600 μL Denaturing Lysis Buffer. Cell lysate was mixed with miRNA Homogenate Additive followed by Acid–Phenol:Chloroform extraction. The aqueous phase was transferred to a fresh tube, mixed with 1/3 volume of room temperature 100% ethanol and passed through a RNA purification column. After three washes with the Wash Buffer, RNA was eluted by the addition of 100 μL of 95 °C pre-heated Elution Solution. A TaqMan miRNA Reverse Transcription Kit (Applied Biosystems Inc.) was used for reverse transcription (RT), the reaction contained: 0.15 μL 100 mM dNTP, 1.5 μL 10× RT buffer, 3 μL individual miRNA specific primer, 1 μL MultiScribe Reverse Transcriptase, 20 ng total RNA and nuclease free H_2O which was used to adjust reaction volume (15 μL). RT was then accomplished in following steps: 16 °C for 30 min; 42 °C for 30 min; 85 °C for 5 min.

2.4. Real-time PCR to detect miRNA levels

A Real Time PCR Assay Kit (Applied Biosystems Inc.) was used to detect miRNA levels in human EPCs. Each reaction was carried out in a total volume of 20 μL containing: 2 μL RT product, 1.0 μL 20× TaqMan MicroRNA assay primer, 10 μL 2× TaqMan Universal PCR Master Mix and nuclease-free H_2O to adjust volume. Real time PCR was run on the 7900HT DNA Sequence Detection System as follows: stage 1, 95 °C for 10 min, stage 2, 95 °C for 15 s, 60 °C for 1 min. Stage 2 was repeated for 40 cycles. The relative expression level of each individual miRNA after normalization to miR-RNU-6B was calculated using the $2^{-\Delta\Delta CT}$ method.

2.5. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by using Student's *t* test; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of EC specific angiogenesis-related miRNAs in EPCs

EPCs isolated from peripheral blood of healthy donors, and characterized by the uptake of Dil-Ac-LDL, FITC-labeled UEA-lectin stain, and VEGF receptor 2 (KDR) expression (data not shown), were analyzed for levels of miR-126, miR-130a, miR-221, miR-222 and miR-92a using a miRNA real-time PCR assay kit. The level of each individual miRNA was expressed as the ratio of the target miRNA and the reference miR-RNU6B levels. As shown in Fig. 1, of the five miRNAs assayed, miR92a showed the most robust expression followed by miR-221, miR-222, miR-126 and miR-130a. These findings, to our knowledge, are the first to show that EPCs express EC specific miRNAs with known angiogenic roles. Next, we detected the expression of these miRNAs in EPCs from CAD patients and compared the results with those from healthy subjects.

3.2. Dysregulation of angiogenesis-related miRNAs in EPCs from patients with coronary artery disease

EPCs from age- and gender-matched healthy donors and CAD patients were isolated, and the angiogenic miRNA levels were detected. As shown in Fig. 2, the level of miR-130 remained the same between the two groups while the level of miR-126 was decreased, and levels of miR-221, miR-222 and miR-92a were augmented in patients suffering CAD.

3.3. Effect of atorvastatin on angiogenic miRNA expression in patients' EPCs

To determine whether atorvastatin had any effect on miRNA expression in patients' EPCs, cells were maintained for 24 h in

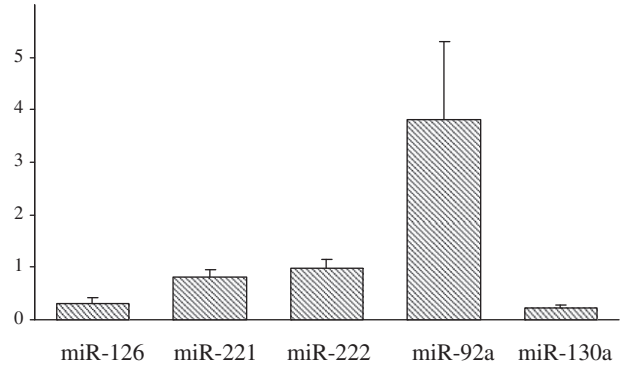

Fig. 1. Expression of EC selective angiogenesis-related miRNAs in human EPCs. EPCs were isolated from healthy donors, miR-126, miR-130a, miR-221, miR-222 and miR-92a were assessed by real time PCR. All five miRNAs were detected in EPCs with miR-92a the most robustly expressed followed by miR-222, miR-221, miR-126 and miR-130a (the level of each miRNA was expressed as the ratio of individual miRNA to miR-RNU6B levels, $n = 3$).

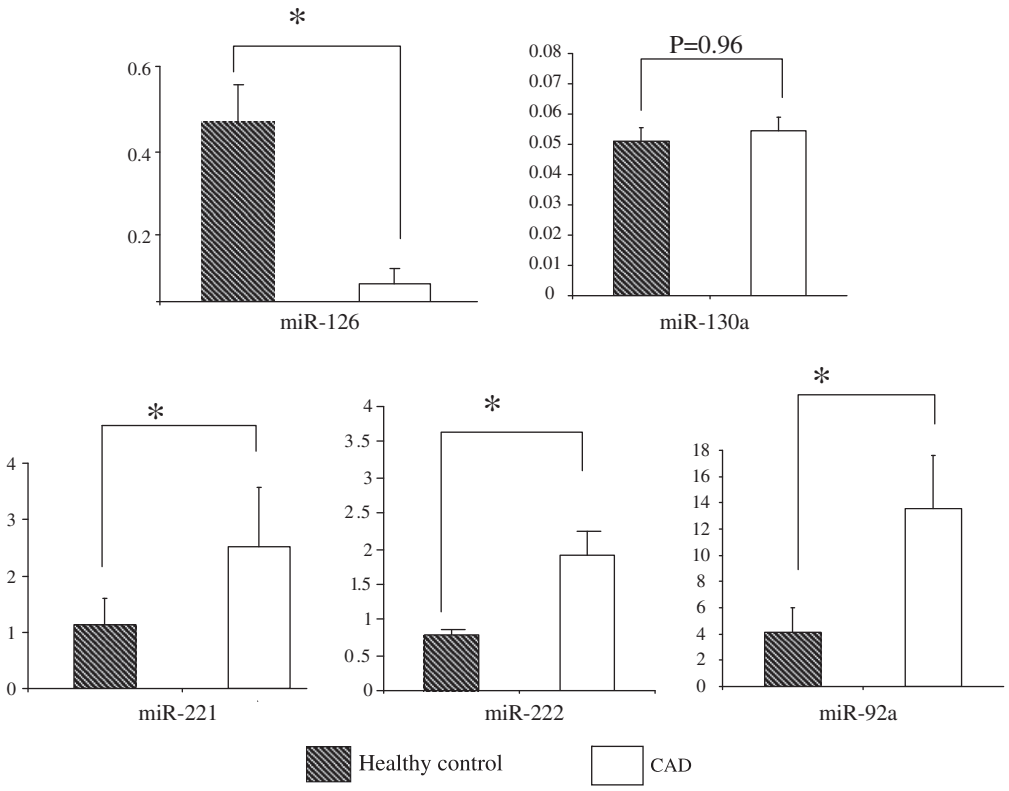

Fig. 2. Dysregulation of miRNAs in EPCs from patients with CAD. miRNA levels in EPCs were detected from age and gender matched patients and healthy subjects. The pro-angiogenic miR-126 level was reduced while anti-angiogenic miR-221, miR-222 and miR-92a levels were elevated in CAD patients compared with controls ($n = 4$, $^{*}p < 0.05$). miR-130a levels were the same between these two groups.

the presence of 1 μM atorvastatin and miRNA levels measured. As shown in Fig. 3, treatment of EPCs with atorvastatin led to enhanced production of miR-221, miR-222 and miR-92a while miR-126 and miR-130a were not affected.

4. Discussion

Although increasing evidence suggests that miRNAs are important regulators of the angiogenic potential of endothelial cells, little

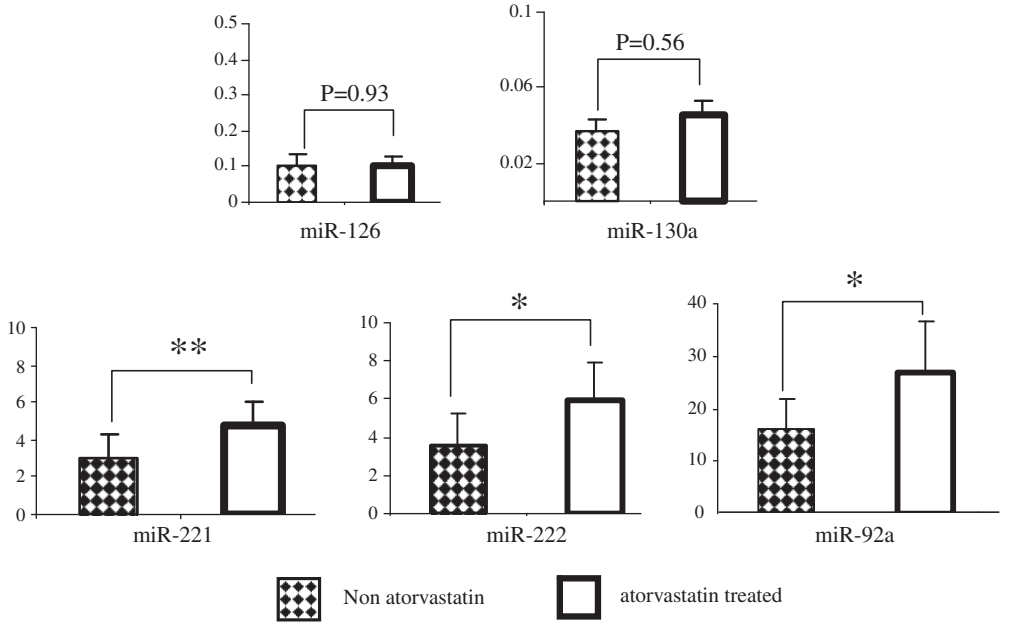

Fig. 3. Effect of atorvastatin treatment on miRNA expression in EPCs from patients suffering CAD. EPCs were maintained in the presence of atorvastatin (final concentration: 1 μM) for 24 h as detailed in section 2 and miRNA levels were measured. Atorvastatin treatment enhanced production of miR-221, miR-222 and miR-92a ($n = 4$–6, $^{*}p < 0.05$, $^{**}p < 0.01$) while had no effect on expression of miR-130a and miR-126.

is known about their involvement in EPC function. We first addressed the question if EPCs express EC specific, angiogenesis-related miRNAs. We chose to examine miR-126, miR-130a, miR-221, miR-222 and miR-92a as they have been consistently reported to be highly expressed in ECs and, more importantly, their angiogenic roles have been defined and their targets identified [14,17–20]. We detected expression of all these five miRNAs in EPCs, suggesting they might play a role in regulating EPC function as well. EPCs have been shown to display a variety of EC activities such as Dil-Ac-LDL uptake, UEA-lectin binding and VEGF receptor 2 (KDR) expression [23,24]. Our finding that EPCs express EC selective miRNAs adds a new layer of evidence that EPCs could be "coaxed" to differentiate into EC-like lineage.

Next, we addressed the question if expression of miRNAs in EPCs from CAD patients is altered due to genetic predisposition and environment influences. With a real time PCR approach, we found that miR-126 was decreased, miR-221, miR-222 and miR-92a were increased and miR-130a remained the same level in CAD patients compared with healthy controls. Our findings that miR-221 and miR-222 were augmented in CAD patients are in accordance with a previous report by Minami and colleagues, who limited their analysis only to miR-221 and miR-222 [25]. Of the five miRNAs selected for current analysis, miR-126 promotes angiogenesis by the inhibition of suppressors of the PI-3 kinase pathway, namely Spred-1 and PI-3 kinase regulatory subunit two, resulting in VEGF and FGF induced endothelial cell proliferation and tube formation [18,19]. miR-130a targets and down-regulates the anti-angiogenic homeobox proteins GAX and HoxA5 [17]. miR-221 and miR-222 negatively regulate expression of c-kit, a tyrosine kinase receptor for stem cell factor (SCF) that is involved in EC survival, migration, and capillary tube formation [14]. Over-expression of miR-221 and miR-222 complexes in HUVECs results in reduced cell migration and tube formation in response to SCF stimulation [14]. miR-221 and miR-222 also inhibit expression of eNOS, an essential molecule in the regulation of vessel tone and angiogenesis. miR-92a targets a number of genes related to angiogenesis, including integrin subunits α5 (ITGA5) and αv (ITGAV), which mediate cell-matrix interactions and cell migration [20]. Therefore, miR-126 and miR-130a exert pro-angiogenic effects while miR-221, miR-222 and miR-92a are anti-angiogenic miRNAs. Dysregulation of these miRNAs as demonstrated in this study, i.e., down-regulation of miR-126 and up-regulation of miR-221, miR-222 and miR-92a might contribute to the dysfunction and reduced regenerative capacity of EPCs observed in CAD patients.

Minami et al. reported that after 12 months of atorvastatin therapy in CAD patients, miR-221 and miR-222 levels in EPCs were reduced [25]. However, we found that atorvastatin treatment did not affect miR-126 and miR-130a and enhanced the production of miR-221, miR-222 and miR-92a in cultured EPCs from patients with CAD. The difference between the two observations might be explained by the method of atorvastatin administration. The oral administration of atorvastatin results in multiple metabolic effects, which may indirectly influence EPC biology. In addition, metabolic products after oral administration of atorvastatin might also impact intracellular miRNA levels [26]. In our study, the application of atorvastatin directly to cultured cells provides information on the receptor dependent and independent effects of the parent compound, without confounding environmental influences.

In conclusion, we reported here that EPC expressed EC miRNAs which are involved in angiogenesis. Furthermore, we found that miRNA expression in EPCs from patients with CAD was altered and that atorvastatin affected miRNA expression in EPCs. These data suggest that miRNAs may play an important role in regulating EPC function. Modulation of miRNA activities, thereby restoring the regenerative potential of EPCs, may provide a therapeutic target for the prevention or treatment of cardiovascular disorders.

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References

[1] T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. Van der Zee, T. Li, B. Witzenbichler, G. Schatteman, J.M. Isner, Isolation of putative progenitor endothelial cells for angiogenesis, Science 275 (1997) 964–967.
[2] Q. Shi, S. Rafii, M.H.D. Wu, E.S. Wijelath, C. Yu, A. Ishida, Y. Fujita, S. Kothari, R. Mohle, L.R. Sauvage, M.A.S. Moore, R.F. Storb, W.P. Hammond, Evidence for circulating bone marrow-derived endothelial cells, Blood 92 (1998) 362–367.
[3] M. Vasa, S. Fichtlscherer, A. Aicher, K. Adler, C. Urbich, H. Martin, A.M. Zeiher, S. Dimmeler, Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease, Circ. Res. 89 (2001) E1–E7.
[4] C. Heeschen, R. Lehmann, J. Honold, B. Assmus, A. Aicher, D.H. Walter, H. Martin, A.M. Zeiher, S. Dimmeler, Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease, Circulation 109 (2004) 1615–1622.
[5] R. Tamarat, J.S. Silvestre, S. Le Ricousse-Roussanne, V. Barateau, L. Lecomte-Raclet, M. Clergue, M. Duriez, G. Tobelem, B.I. Lévy, Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor treatment, Am. J. Pathol. 164 (2004) 457–466.
[6] R.C. Lee, R.L. Feinbaum, V. Ambros, The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14, Cell 75 (1993) 843–854.
[7] B. Wightman, I. Ha, G. Ruvkun, Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. Elegans, Cell 75 (1993) 855–862.
[8] B.J. Reinhart, F.J. Slack, M. Basson, A.E. Pasquinelli, J.C. Bettinger, A.E. Rougvie, H.R. Horvitz, G. Ruvkun, The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans, Nature 403 (2000) 901–906.
[9] V. Ambros, The functions of animal microRNAs, Nature 431 (2004) 350–355.
[10] D.P. Bartel, MicroRNAs: genomics biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[11] N. Bushati, S.M. Cohen, MicroRNA functions, Annu. Rev. Cell Dev. Biol. 23 (2007) 175–205.
[12] T.C. Chang, J.T. Mendell, MicroRNAs in vertebrate physiology and human disease, Annu. Rev. Genomics Hum. Genet. 8 (2007) 215–239.
[13] W.J. Yang, D.D. Yang, S. Na, G.E. Sandusky, Q. Zhang, G. Zhao, Dicer is required for embryonic angiogenesis during mouse development, J. Biol. Chem. 280 (2005) 9330–9339.
[14] L. Poliseno, A. Tuccoli, L. Mariani, M. Evangelista, L. Citti, K. Woods, A. Mercatanti, S. Hammond, G. Rainaldi, MicroRNAs modulate the angiogenic properties of HUVECs, Blood 108 (2006) 3068–3071.
[15] Y. Suárez, W.C. Sessa, MicroRNAs as novel regulators of angiogenesis, Cir. Res. 104 (2009) 442–454.
[16] A. Bonauer, RA. Boon, S. Dimmeler, Vascular microRNAs, Curr Drug Targets 11 (2010) 934–939.
[17] Y. Chen, D.H. Gorski, Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5, Blood 111 (2008) 1217–1226.
[18] J.E. Fish, M.M. Santoro, S.U. Morton, S. Yu, R.F. Yeh, J.D. Wythe, K.N. Ivey, B.G. Bruneau, D.Y. Stainier, D. Srivastava, MiR-126 regulates angiogenic signaling and vascular integrity, Dev. Cell 15 (2008) 272–284.
[19] S. Wang, A.B. Aurora, B.A. Johnson, X. Qi, J. McAnally, J.A. Hill, J.A. Richardson, R. Bassel-Duby, E.N. Olson, The endothelial-specific microRNA MiR-126 governs vascular integrity and angiogenesis, Dev. Cell 15 (2008) 261–271.
[20] A. Bonauer, G. Carmona, M. Iwasaki, M. Mione, M. Koyanagi, A. Fischer, J. Burchfield, H. Fox, C. Doebele, K. Ohtani, E. Chavakis, M. Potente, M. Tjwa, C. Urbich, A.M. Zeiher, S. Dimmeler, MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice, Science 324 (2009) 1710–1713.
[21] S. Dimmeler, A. Aicher, M. Vasa, C. Mildner-Rihm, K. Adler, M. Tiemann, H. Rütten, S. Fichtlscherer, H. Martin, A.M. Zeiher, HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway, J. Clin. Invest. 108 (2001) 391–397.
[22] J. Llevadot, S. Murasawa, Y. Kureishi, S. Uchida, H. Masuda, A. Kawamoto, K. Walsh, J.M. Isner, T. Asahara, HMG-CoA reductase inhibitor mobilizes bone marrow–derived endothelial progenitor cells, J. Clin. Invest. 108 (2001) 399–405.
[23] S. Verma, M.A. Kuliszewski, S.H. Li, P.E. Szmitko, L. Zucco, C.H. Wang, M.V. Badiwala, D.A. Mickle, R.D. Weisel, P.W. Fedak, D.J. Stewart, M.J. Kutryk, C-reactive protein attenuates endothelial progenitor cell survival differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease, Circulation 109 (2004) 2058–2067.

[24] J. Rehman, J. Li, C.M. Orschell, K.L. March, Peripheral blood endothelial progenitor cells are derived from monocyte/macrophages and secrete angiogenic growth factors, Circulation 107 (2003) 1164–1169.
[25] Y. Minami, M. Satoh, C. Maesawa, Y. Takahashi, T. Tabuchi, T. Itoh, M. Nakamura, Effect of atorvastatin on MicroRNA 221/222 expression in endothelial progenitor cells obtained from patients with coronary artery disease, Eur. J. Clin. Invest. 39 (2009) 359–367.
[26] A.E. Black, R.N. Hayes, B.D. Roth, P. Woo, T.F. Woolf, Metabolism and excretion of atorvastatin in rats and dogs, Drug Metab. Dispos. 27 (1999) 916–923.