

AMNIOTIC FETAL STEM CELLS – DERIVED ENDOTHELIAL PROGENITORS, A SUPPORT FOR PERSONALIZED CARDIOVASCULAR REGENERATIVE MEDICINE

Florin IORDACHE^{1,2,3}, Cosmin A. BUZILA^{3,4}✉

¹ University of Agronomic Science and Veterinary Medicine Bucharest, Romania

² Oncological Institute “Alexandru Trestioreanu” Bucharest, Romania

³ Academy of Romanian Scientists, Bucharest, Romania

⁴ Central Military Emergency University Hospital “Dr. Carol Davila”, Bucharest, Romania

Received 12 Dec 2021, Accepted 19 Jan 2022

<https://doi.org/10.31688/ABMU.2022.57.1.01>

ABSTRACT

Introduction. Worldwide, cardiovascular diseases represent the first cause of morbidity and mortality. The genetic contribution in this pathology is heterogenous, and the microenvironment-genes interactions (mediated by epigenetic mechanisms) influence the prevalence of the diseases.

The objective of the study was to find the micro ribonucleic acids (miRNAs) that alter gene expression in endothelial progenitor cells, to reveal the regulation mechanisms involved and to clarify the function of miRNAs in endothelial cell differentiation, that may facilitate the improvement of angiogenesis and stem cell-based therapies in cardiovascular disease.

Material and methods. The differentiation of endothelial progenitor cells from amniotic fluid stem cells was done by using endothelial specific media, followed by characterization with Real-time PCR, flow-cytometry, LDL-uptake assay, and Matrigel network formation. PCR array was used to analyse the expression of 84 miRNAs to identify molecular markers

RÉSUMÉ

Les progéniteurs endothéliaux dérivés des cellules souches fœtales amniotiques, un support pour la médecine régénérative cardiovasculaire personnalisée

Introduction. À l'échelle mondiale, les maladies cardiovasculaires (maladie coronarienne, insuffisance cardiaque et maladie artérielle périphérique) représentent la première cause de morbidité et de mortalité. La contribution génétique dans cette pathologie est hétérogène, et les interactions micro-environnement-gènes (médiées par des mécanismes épigénétiques) influencent la prévalence des maladies.

L'objectif de l'étude était de trouver les miARN qui modifient l'expression des gènes dans les cellules progénitrices endothéliales, de révéler les mécanismes de régulation impliqués et de clarifier la fonction des miARN dans la différenciation des cellules endothéliales, ce qui pourrait faciliter l'amélioration de l'angiogenèse et des thérapies à base de cellules souches dans les maladies cardiovasculaires.

✉ Address for correspondence:

Cosmin A. BUZILA
Central Military Emergency University Hospital “Dr. Carol Davila”, Bucharest,
Romania
Address: Calea Plevnei Str. no. 134, District 1, Bucharest, Romania
Email: buzilacosmin@yahoo.com

for certain stem cells and/or specific stem cell differentiation processes.

Results. The results obtained using PCR array technique showed a significantly increased expression for miRNA-106b-5p, miRNA-155-5p, miRNA-126-3p, miRNA-22-3p and a decrease in miRNA-let7e-5p expression, miRNA125a -5p, miRNA-125b-5p, miRNA-92a-3p.

Conclusions. Upregulation or antagonization of certain circulating miRNAs may represent promising therapeutic strategies in the cardiovascular diseases management. Furthermore, establishing the role of circulating miRNAs as biomarkers and independent predictors of cardiovascular disease outcome, correlated with the medical and surgical treatment, could improve the patients' lifespan.

Keywords: cardiovascular disease, epigenetics, microRNAs, biomarkers, cell therapies.

List of abbreviations:

DNA - deoxyribonucleic acid
 RNA - ribonucleic acid
 AFSCs - amniotic fluid-derived stem cells
 EC - endothelial cells
 EPC - endothelial progenitor cells.
 FBS - fetal bovine serum
 VEGF - vascular endothelial growth factor
 bFGF - basic fibroblast growth factor
 PBS - phosphate-buffered saline solution
 PFA - paraformaldehyde
 UEA - Ulex europaeus agglutinin

INTRODUCTION

Worldwide, cardiovascular diseases (coronary artery disease, heart failure, and peripheral artery disease) represent the first cause of morbidity and mortality¹. Despite of the best medical therapy, the atherosclerotic cardiovascular disease is often progressing, and patients are referred to endovascular or open surgical treatment. However, the outcome may not be always optimal².

The genetic contribution in this pathology is heterogeneous, and the microenvironment-genes interactions (mediated by epigenetic mechanisms) influence the prevalence of the diseases. Epigenetics has an important role in vascular biology³. Deoxyribonucleic acid (DNA) methylation, histone modifications, or non-coding ribonucleic acid (RNA) may influence gene expression and generate different cardiovascular disease patterns with a significant variability of clinical outcomes³.

Literature data⁴ show that DNA methylation plays an important role in cardiovascular diseases.

Matériel et méthodes. La différenciation des cellules progénitrices endothéliales dérivées des cellules souches du liquide amniotique a été effectuée à l'aide d'un milieu spécifique endothélial, suivie d'une caractérisation à l'aide de la PCR en temps réel, de la cytométrie en flux, du test d'absorption des LDL et de la formation de réseau Matrigel. Une puce PCR a été utilisée pour analyser l'expression de 84 miARN afin d'identifier des marqueurs moléculaires pour certaines cellules souches et/ou des processus de différenciation de cellules souches spécifiques.

Résultats. Les résultats obtenus à l'aide de la technique PCR array ont montré une expression significativement augmentée pour miRNA-106b-5p, miRNA-155-5p, miRNA-126-3p, miRNA-22-3p et une diminution de l'expression miRNA-let7e-5p, miRNA125a -5p, miARN-125b-5p, miARN-92a-3p.

Conclusions. La régulation positive ou l'antagonisme de certains miARN circulants peuvent représenter des stratégies thérapeutiques prometteuses dans la gestion des maladies cardiovasculaires. De plus, établir le rôle des miARN circulants en tant que biomarqueurs et prédicteurs indépendants de l'évolution des maladies cardiovasculaires, en corrélation avec le traitement médical et chirurgical, pourrait améliorer la durée de vie des patients.

Mots-clés: maladies cardiovasculaires, épigénétique, miARN, biomarqueurs, thérapies cellulaires

Moreover, the DNA methylation analysis in the peripheral blood samples may be a valuable tool in the evaluation of patients with heart failure. Local inflammation and fibrotic processes caused by myocardial ischemia and infarction trigger genomic regulation processes and are also interfering with epigenetic factors such as DNA methylation⁵. The methylation patterns were studied and further correlated with aortic plaques development, myocardial ischemia, coronary events, or dilated cardiomyopathy⁵. Bain et al.⁶ suggest the utility of methyl-binding domain capture sequencing for the assessment of peripheral blood DNA methylation profiles of patients diagnosed with heart failure, with indication for open surgical myocardial revascularization. A connection between the oxidative stress (condition associated with cardiac ischemia) and epigenetic modifications was also suggested⁷. The expression of serum miRNAs, as miR-196-5p, miR-3163-3p, miR-145-3p, and miR-190a-5p was investigated by Ying et al.⁸ in patients with incipient coronary artery disease. These authors propose that the evaluation of these

miRNAs' expression levels might help in diagnosing these patients, at a very early onset of the disease⁸.

The classical risk factors involved in cardiovascular diseases' pathogenesis, like smoking and advanced age, were described as promoters of epigenetic modifications. Gene therapies, growth factors infusion and stem/progenitor cells transplantation have been extensively studied as an alternative for patients unresponsive to medical or surgical treatment⁹.

A more recently investigated resource for regenerative medicine is represented by amniotic fluid-derived pluripotent stem cells (AFSCs). These cells possess a strong renewal capacity, express embryonic cell markers and can differentiate into all the embryonic layers – derived tissue types. The use of AFSCs has many advantages: they are low antigenicity cells, with a higher differentiation potential (they are an intermediate cell type between adult and embryonic stem cells)¹⁰, do not form tumours after *in vivo* transplantation, and may be used without ethical problems¹¹.

THE OBJECTIVE OF THE STUDY was to find the miRNAs that alter gene expression in vascular progenitor to reveal the mechanisms of regulation and clarifying the function of miRNAs in endothelial cells (EC) differentiation, that may facilitate the improvement of angiogenesis and stem cell-based therapies in cardiovascular disease.

MATERIALS AND METHODS

The study was carried out with the approval of the "Academy of Romanian Scientists" Ethics Committee, with the approval registration number 778/13.12.2021.

Amniotic fluid stem cells differentiation and characterization

AFSCs were kindly provided by Genetic Lab S.R.L. diagnoses laboratory, upon written informed consent of the patients, in agreement with national and European Union law. The primary cultures were obtained by centrifugation of amniotic fluid at 1050 rpm for 10 min. The cells were then cultured for 10 days without passages in AmnioMax medium, with medium change every two days (ThermoFischer Scientific, Waltham, Massachusetts, USA). After 10 days, the primary culture was passage and cultured in differentiation specific media supplemented with growth factors. Endothelial differentiation of AFSC was done by culture in M200 medium supplemented with 10% fetal bovine serum (FBS), 40 ng/mL vascular endothelial growth factor (VEGF), 20 ng/mL insulin growth factor (IGF-1), 10 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast

growth factor (bFGF), 100 µg/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL neomycin (all purchased from Thermo Fischer Scientific, Waltham, Massachusetts, USA).

Flow cytometry assay

Flow cytometry was used for assessing the expression of cell specific surface markers (Gallios, Beckman-Coulter, California, SUA). AFSCs (1x10⁵ cells/marker) were stained with fluorochrome-conjugated (FITC – Fluorescein-isothiocyanate and PE – Phycoerythrin) primary antibodies against CD29 (integrin β1), CD31 (PECAM-1), CD49e (integrin α5), CD54 (ICAM-1), CD56 (NCAM), CD73, CD90 (Thy-1), CD105 (endoglin), CD146 (MCAM), and VEGFR2 (Beckman-Coulter). AFSCs were detached using accutase (Sigma-Aldrich, St. Louis, MO, USA) and washed in phosphate-buffered saline solution (PBS). The cells were then incubated with the primary antibodies at room temperature in the darkness for 30 min. Further, the cells were washed and centrifuged at 400g, 10 min, in PBS (Phosphate Buffer Solution) with 1% BSA. For negative controls, AFSCs were stained with the corresponding isotype-matched IgG antibodies (Beckman-Coulter, California, SUA). Flow cytometry data were analysed using the Gallios software 1.0 (Beckman-Coulter, California, SUA).

Gene expression and functional characterization of endothelial progenitor cells (EPC)

Gene expression levels in AFSC and EPC were assessed by qRT-PCR. Total cellular RNA was isolated from cultured cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcription reaction was performed using M-MLV polymerase, High-Capacity cDNA Reverse Transcription kit (Thermo Fischer Scientific, USA). MiRNA levels of endothelial associated genes (PECAM-1, ICAM-1, VE-Cadherin, eNOS and vWF) were quantified using TaqMan hydrolysis probes (ThermoFischer Scientific, Waltham, Massachusetts, USA). Quantitative Real Time-PCR reactions were carried out in a real-time thermocycler (ViiA7, Applied Biosystems, USA), following manufacturer's guidelines. The results were expressed using relative quantitation ($2^{-\Delta C_T}$), where ΔC_T represents C_T difference between values for AFSC and EPC.

Dil-Ac-LDL uptake assay. AFSCs-derived EPC were incubated with 6 µg/mL Dil-AcLDL-PE (acetylated low density lipoprotein conjugated PE, ThermoFischer Scientific, USA). The cells were incubated for 2 hours at 37°C with 5% CO₂ and 21% O₂, washed with PBS and fixed with 1% paraformaldehyde (PFA) for 10 minutes at room temperature.

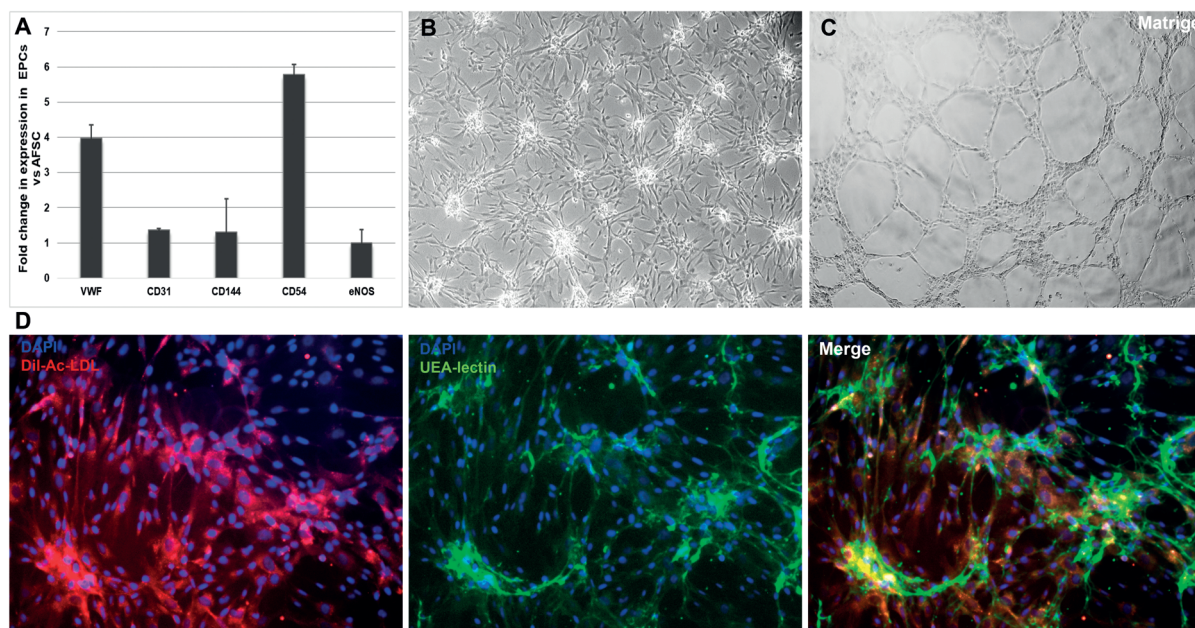


Figure 1. A. mRNA expression for vwf, CD31, CD144, CD54, eNOS in EPC by Real-time PCR technique. B. Morphology of EPC after differentiation from AFSC. C. Matrigel vascular tube test. D. Dil-Ac-LDL uptake (red) and UEA lectin binding (green) to EPC.

Ulex europaeus lectin (UEA) binding capacity to EPC. For the assessment of UEA binding capacity to EPC, the cells were incubated with 0.01 mg/mL FITC - Ulex europaeus lectin (Sigma-Aldrich, St. Louis, MO, USA) for two hours, followed by a wash with PBS. The nuclei were counterstained with DAPI (1 mg/mL). The photomicrographs were taken with a digital camera Digital Net Camera DN100 using an Eclipse TE300 microscope (Nikon, Tokyo, Japan).

Matrigel vascular tubes assay. To evaluate the formation of blood vessel networks in Matrigel, the cells were seeded into 96-well plates at a density of 3,000 cells per well. Briefly, 50 μ L of Matrigel (Sigma-Aldrich, St. Louis, MO, USA) were added in each well of a 96-well plates, and left to solidify for 30 minutes at 37°C. After Matrigel polymerization, cell suspension was added and incubated for 4 hours. Tubes formation was observed using an Eclipse TE300 microscope (Nikon, Tokyo, Japan) equipped with a digital camera (Digital Net Camera DN100).

Microarray screening of miRNAs involved in endothelial cell differentiation

Using PCR array, we analysed the expression of 84 miRNAs expressed in cell differentiation and body development. This panel provides a convenient way to make the most relevant miRNAs for cell differentiation. miRNAs were carefully selected in correlation with different stages of development, from stem cells to terminal differentiation. The results from this matrix can be used to identify molecular markers for

certain stem cells and / or specific stem cell differentiation processes. The results can also help identify miRNAs associated with the function of certain cells or tissue types. The miRNeasy Kit (Qiagen, Hilden, Germany) was used for AFSC and EPC RNA extraction, followed by reverse-transcription using miScript II RT Kit (Qiagen, Hilden, Germany). PCR array implies the miScript® kit miRNA PCR Array Human Cell Differentiation & Development kit and data were analysed using bioinformatics software (Qiagen, Hilden, Germany).

RESULTS

Our results showed that EPC differentiation from AFSCs occurred after 10 days of culture in specific endothelial medium supplemented with 40 ng/mL VEGF, 20 ng/mL IGF-1, 10 ng/mL EGF and 10 ng/mLbFGF. After 10 days, cells started to express specific endothelial markers, such as vWF, CD31, CD144, CD54, and eNOS (Fig. 1A). The cells also changed their morphology, moving from a fibroblast-like phenotype to an epithelial-like phenotype (Fig. 1B).

Differentiated AFSCs form vascular networks when were grown on Matrigel scaffold (Fig. 1C). Moreover, these cells incorporate Dil-Ac-LDL and bind to UEA lectin (Fig. 1D), suggesting an endothelial phenotype.

Flow cytometry using specific fluorescently labeled antibodies (Gallios, Beckman-Coulter) was used

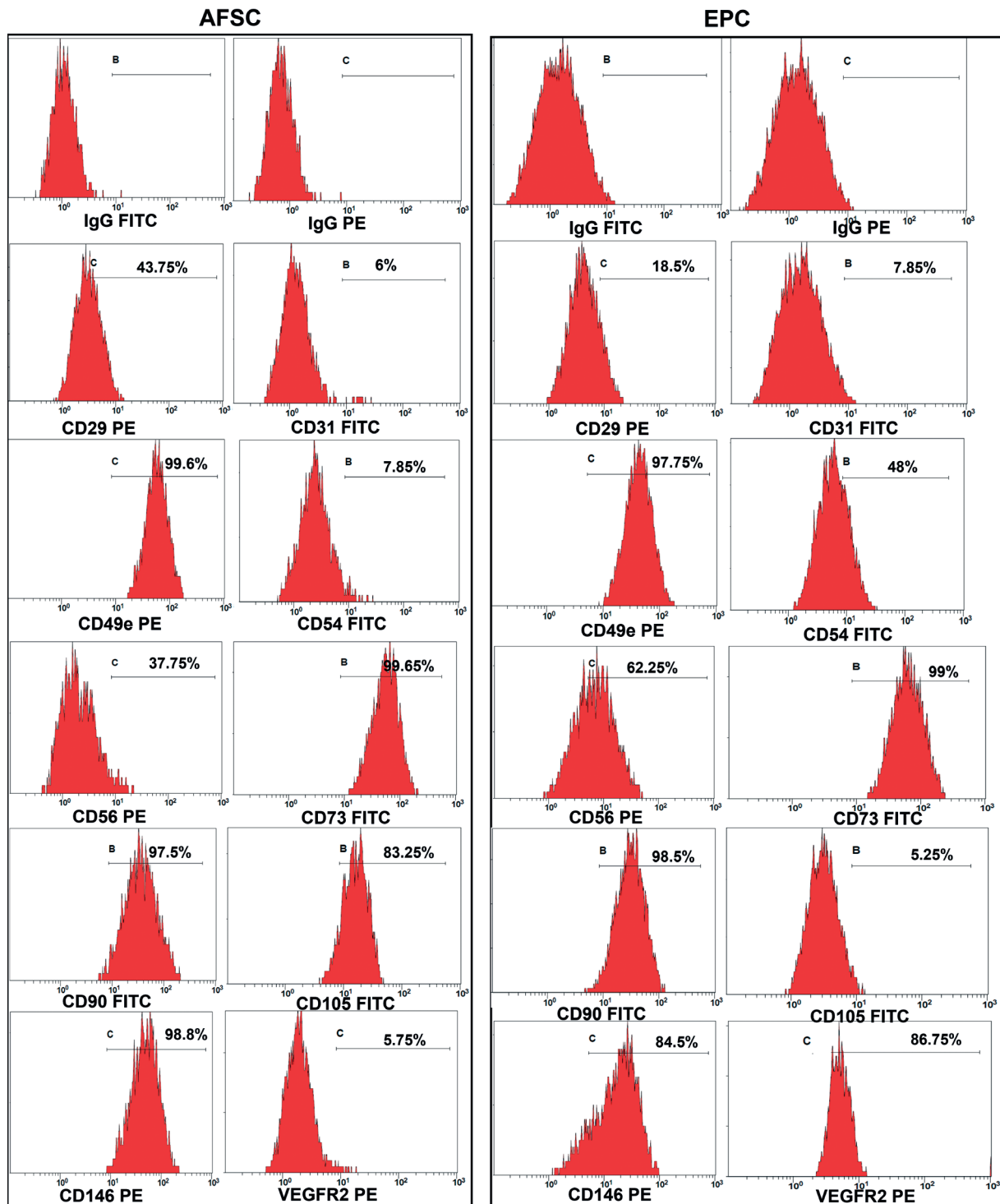


Figure 2. Immunophenotypic profile of AFSC and EPC.

to highlight AFSCs differentiation in EPC. The results showed that the differentiation to EPC was achieved, the cells expressing specific endothelial markers such as CD29 (18.5%), CD49e (97.7%), CD54 (48%), CD56 (62%), CD73 (99%), CD90 (98.5%), CD105 (5.25%), CD146 (84.5%) and VEGFR2 (86.7%) (Fig. 2).

The results obtained using PCR array technique showed a significantly increased expression for miRNA-106b-5p, miRNA-155-5p, miRNA-126-3p, miRNA-22-3p and a decrease in miRNA-let7e-5p expression, miRNA125a -5p, miRNA-125b-5p, miRNA-92a-3p (Fig. 3, 4).

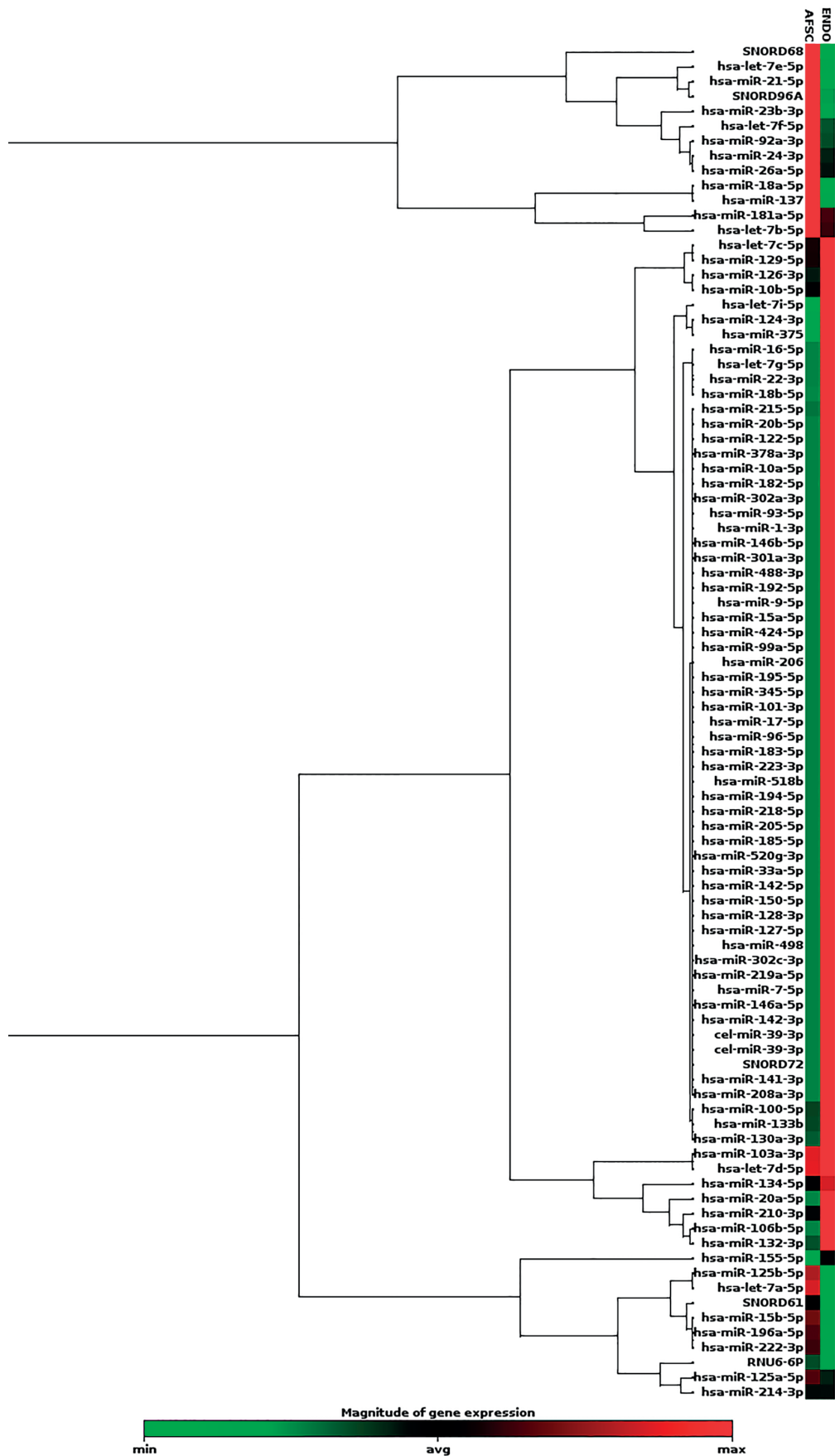


Figure 3. Functional cluster representation of miRNAs presents in undifferentiated AFSC and EPC.

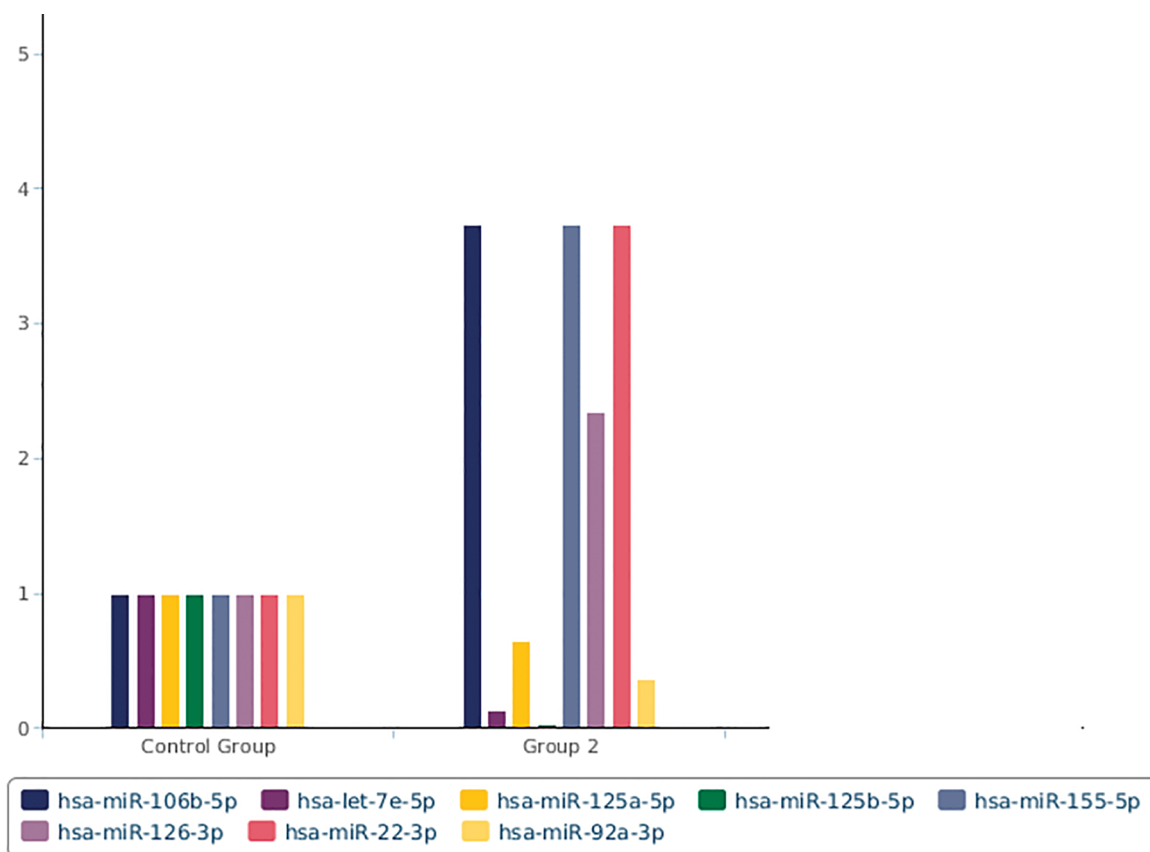


Figure 4. Significantly upregulated and downregulated miRNAs in EPC (control group – AFSC, Group 2 – EPC).

DISCUSSION

EPC contributes to reendothelialization and neovascularization and protects against vascular damage and ischemia of various organs. Leakage of miRNA-126 expression in EPC from diabetic patients contributes to EPC dysfunction, including impaired migration capacity¹². In our experiments, we found in EPC differentiated from AFSCs an increased expression of miRNA-126 (Fig. 4). This was also identified by Pan et al., who showed that miRNA-126 overexpression promoted proliferation, migration, and tube formation, decreased reactive oxygen species expression, and increased nitric oxide production in EPC by activating the PI3K/Akt/eNOS signaling pathway¹³. Also, EPCs in which miRNA-126 was overexpressed were more effective than normal EPCs in attenuating infarction volume, improving the neurological deficit score (NDS), increasing cerebral microvascular density, cerebral blood flow and angiogenesis, suggesting that miRNA-126 overexpression may improve the function of EPCs *in vitro* and *in vivo*¹³.

The members of the let-7 miRNA group play a key role in modulating inflammatory responses. Vascular smooth muscle cell proliferation (SMC) and endothelial cell dysfunction are critical in the

pathogenesis of atherosclerosis, including diabetes. Brennan et al. showed that let-7 levels are low in the carotid plaques in diabetes patients and in a model of diabetes-associated atherosclerosis using ApoE (-/-) diabetic mice. *In vitro*, platelet-derived growth factor and tumour necrosis factor alpha (TNF- α) induced by vascular SMC and endothelial cell dysfunction activation were associated with reduced miRNA-let-7 expression by Lin28b, a negative regulator of let-7 biogenesis. Ectopic overexpression of let-7 in SMC inhibited inflammatory responses, including proliferation, migration, monocyte adhesion, and NF- κ B activation. The therapeutic potential of restoring let-7 levels using a let-7 substitute was tested by Brennan et al. *in vitro* SMC using an endogenous anti-inflammatory lipid (lipoxin A4) and *ex vivo* in murine aorta model. They observed significant changes in secretome in response to let-7 therapy. The restoration of let-7 expression could provide a new target for an anti-inflammatory approach in diabetic vascular disease¹⁴. These data also support our results, the miRNA-let 7 expression being low in EPC differentiated from AFSCs (Fig. 4).

VE-cadherin (CD144) functions as an endothelial barrier protein that controls endothelial permeability and leukocyte transmigration. Studies show that VE-cadherin also plays a vital role in angiogenesis.

miRNA-22 plays an important role in cardiovascular disease, including heart hypertrophy and heart failure¹⁵. Gu et al. identified the interaction between miRNA-22 and VE-cadherin mRNA¹⁶. The overexpression of miRNA-22 in endothelial cells increases the synthesis of proinflammatory cytokines. The injection of miRNA-22 leads to increased myeloperoxidase activity in mouse's lungs. Furthermore, injection of miRNA-22 into fluorescently labeled zebrafish transgenic embryos (Tg(fli1:EGFP)) caused defective vascular development in the dorsal and intersegmental vessels, and vascular markers were significantly suppressed in these embryos. In conclusion, the study conducted by Gu et al. demonstrates that miR-22 conserved targeting of EV-cadherin regulates endothelial inflammation, tissue damage and angiogenesis¹⁶.

In our experiments, the expression of microRNA-125a-5p in EPC was lower compared to AFSCs (Fig. 3, 4). Using qRT-PCR, Che et al. showed that miRNA-125a-5p expression was approximately 2.9-fold higher in aged endothelial cells compared to samples collected from young animals. Western blot tests showed a lower level of expression of a miRNA-125a-5p target known as related transcription amplification factor (RTEF-1) in aged endothelial cells compared to its expression levels in young cells. Overexpression of miRNA-125a-5p in young endothelial cells using pre-miRNA-125a-5p caused inhibition of RTEF-1, endothelial nitric oxide synthase (eNOS), and vascular endothelial growth factor (VEGF), impairment of angiogenesis, as evidenced by *in vitro* spheroid and tube formation tests. In contrast, repression of miRNA-125a-5p in aged endothelial cells using anti-miRNA-125a-5p increased RTEF-1, eNOS, and VEGF expression and improved EC angiogenesis¹⁷.

Atherosclerosis is induced by multiple factors, including high blood pressure, hyperlipidemia, and smoking, and its pathogenesis has not been fully elucidated. Xu et al. have shown that miRNAs have great anti-atherosclerotic potential, but the precise function of miRNA-92a-3p in atherosclerosis and its potential molecular mechanism have not been well elucidated. Using flow cytometry and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay) test, they evaluated the effects of oxidized low-density lipoprotein (ox-LDL) on the proliferation and apoptosis of human umbilical vein endothelial cells (HUVECs). Expression levels of miRNA-92a-3p and Sirtuin 6 (SIRT6) in HUVEC exposed to ox-LDL were estimated by the RT-qPCR method. In addition, they measured the protein levels of SIRT6, c-Jun N-terminal kinase (JNK), p-JNK, mitogen-activated protein kinase p38 (p38 MAPK) and p38 MAPK (p-p38 MAPK). The relationship between miRNA-92a-3p and SIRT6 was confirmed

by the dual-luciferase reporter test. Xu et al. showed that Ox-LDL induced apoptosis and oxidative stress in HUVECs in concentration- and time-dependent ways. In contrast, miRNA-92a-3p blockade inhibited apoptosis and SIRT6 expression in HUVEC. Overexpression of miRNA-92a-3p increased apoptosis and phosphorylation levels of JNK and p38 MAPK, and inhibited proliferation in HUVECs induced by ox-LDL. In addition, SIRT6 was a target of miRNA-92a-3p. miRNA-92a-3p negatively regulated ox-LDL-induced SIRT6 expression in HUVEC to activate the MAPK signaling pathway *in vitro*. In conclusion, miRNA-92a-3p promoted HUVEC apoptosis and suppressed HUVEC proliferation induced by ox-LDL by targeting SIRT6 expression and activating the MAPK signaling pathway¹⁸. These results also correlate with our microarray experiments, in which we determined a low level of miR-92a-3p in EPC compared to AFSC (Fig. 4).

CONCLUSIONS

Upregulation or antagonization of certain circulating miRNAs may represent promising therapeutic strategies in the cardiovascular diseases management. The role of circulating miRNAs as biomarkers and independent predictors of cardiovascular disease outcome, correlated with the medical and surgical treatment should be further investigated. Future studies are needed to establish how much the peripheral blood methylome analysis may help in risk assessment and postoperative outcome for patients undergoing cardiac surgery. Although the results of clinical and preclinical studies investigating gene and stem cell therapies for cardiovascular regeneration are promising, the long-term effects of these approaches should be evaluated.

Author Contributions:

Conceptualization, F.I and C.B.; methodology, F.I.; software, F.I.; validation, C.B. and F.I.; formal analysis, F.I.; investigation, F.I. and C.B.; resources, F.I.; data curation, C.B. and F.I.; writing-original draft preparation, F.I. and C.B.; writing-review and editing, F.I. and C.B.; visualization, F.I. and C.B.; supervision, C.B.; project administration, C.B. All the authors have read and agreed with the final version of the article.

Compliance with Ethics Requirements:

"The authors declare no conflict of interest regarding this article".

"The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as

well as the national law. Informed consent was obtained from all the patients included in the study“

Acknowledgement

This work was supported by a Postdoctoral Fellowship Project entitled “Epigenetic impact in the development of cardiovascular diseases“ funded by the Academy of Romanian Scientists.

REFERENCES

1. Mahmood SS, Levyb D, Vasan RS, Wang TJ. The Framingham Heart Study and the epidemiology of cardiovascular diseases: ahistorical perspective. *Lancet* 2014; 383(9921): 999–1008.
2. Heuslein JL, Gorick CM, Price RJ. Epigenetic regulators of the revascularization response to chronic arterial occlusion. *Cardiovascular Research* 2019; 115:701–712.
3. Bain CR, Ziemann M, Kaspi A, et al. DNA methylation patterns from peripheral blood separate coronary artery disease patients with and without heart failure. *ESC Heart Failure* 2020; 7: 2468–2478.
4. Baccarelli A, Wright R, Bollati V, et al. Ischemic heart disease and stroke in relation to blood DNA methylation. *Epidemiology* 2010; 21: 819–828.
5. Aslibekyan S, Agha G, Colicino E, et al. Association of methylation signals with incident coronary heart disease in an epigenome-wide assessment of circulating tumor necrosis factor α . *JAMA Cardiol* 2018; 3: 463–472.
6. Bain CR, Ziemann M, Kaspi A, et al. DNA methylation patterns from peripheral blood separate coronary artery disease patients with and without heart failure. *ESC Heart Failure* 2020; 7: 2468–2478.
7. Lakshmi SVV, Naushad SM, Reddy CA, et al. Oxidative stress in coronary artery disease: epigenetic perspective. *Mol Cell Biochem*, 2013;374(1-2):203-11.
8. DU Y, Yang SH, Li S, et al. Circulating microRNAs as novel diagnostic biomarkers for very early onset (≤ 40 years) coronary artery disease. *Biomed Environ Sci.* 2016;29(8):545-554.
9. Golledge J, Biros E, Bingley J, Iyer V, Krishna SM. Epigenetics and peripheral artery disease. *Curr Atheroscler Rep* 2016;18(4):15.
10. Antonucci I, Pantalone A, Tete S, et al. Amniotic fluid stem cells: a promising therapeutic resource for cell-based regenerative therapy. *Curr Pharm Des.* 2012;18(13):1846-63.
11. Cananzi M, Atala A, De Coppi P. Stem cells derived from amniotic fluid: new potentials in regenerative medicine. *Reprod Biomed Online.* 2009;18 Suppl 1:17-27.
12. Pei CZ, Liu B, Li YT, et al. MicroRNA-126 protects against vascular injury by promoting homing and maintaining stemness of late outgrowth endothelial progenitor cells. *Stem Cell Res Ther* 2020;21;11(1):28.
13. Pan Q, Zheng J, Du D, Liao X, Ma C, Yang Y, Chen Y, Zhong W, Ma X. MicroRNA-126 priming enhances functions of endothelial progenitor cells under physiological and hypoxic conditions and their therapeutic efficacy in cerebral ischemic damage. *Stem Cells International* 2018; 13:1-14.
14. Brennan E, Wang B, McClelland A, et al. Protective effect of let-7 miRNA family in regulating inflammation in diabetes-associated atherosclerosis. *Diabetes* 2017;66(8):2266-2277.
15. Diaconu C, Nastasa A, Zaki AR, Arsalan M. Type 2 diabetes: a driver for chronic heart failure. *The 2nd International Conference on Interdisciplinary Management of Diabetes Mellitus and its Complications – Diabetes mellitus as cardiovascular disease, INTERDIAB 2016 Proceedings*, pp. 201-210. Ed. Niculescu. Editors Serafinceanu C, Negoita O, Elian V.
16. Gu W, Zhan H, Zhou XY et al. MicroRNA-22 regulates inflammation and angiogenesis via targeting VE-cadherin. *FEBS Lett.* 2017;591(3):513-526.
17. Che P, Liu J, Shan Z, et al. miR-125a-5p impairs endothelial cell angiogenesis in aging mice via RTEF-1 downregulation. *Aging Cell* 2014;13(5):926-34.
18. Xu Y, Miao C, Cui J, Bian X. miR-92a-3p promotes ox-LDL induced-apoptosis in HUVECs via targeting SIRT6 and activating MAPK signaling pathway. *Braz J Med Biol Res.* 2021;54(3):e9386.