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

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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

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 (médiiées 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’angiogénèse et des thérapies à base de cellules souches dans les maladies cardiovasculaires.
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-223p 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, micro-RNAs, 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 mortality. 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 optimal.

The genetic contribution in this pathology is heterogeneous, and the microenvironment-genomes interactions (mediated by epigenetic mechanisms) influence the prevalence of the diseases. Epigenetics has an important role in vascular biology. 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 outcomes.

Literature data show that DNA methylation plays an important role in cardiovascular diseases. 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 methylation. The methylation patterns were studied and further correlated with aortic plaques development, myocardial ischemia, coronary events, or dilated cardiomyopathy. Bain et al. 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 suggested. 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. 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 disease.

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 treatment.

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), do not form tumours after in vivo transplantation, and may be used without ethical problems.

**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, 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-I), 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 (1x10^5 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^(-ΔΔCt)), where ΔΔCt 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.
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 μL 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-I, 10 ng/mL EGF and 10 ng/mL bFGF. 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 (Gallicos, Beckman-Coulter) was used
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-let-7e-5p expression, miRNA-125a-5p, miRNA-125b-5p, miRNA-92a-3p (Fig. 3, 4).

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).

Figure 2. Immunophenotypic profile of AFSC and EPC.
Figure 3. Functional cluster representation of miRNAs presents in undifferentiated AFSC and 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 vivo13.

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-κB 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 a 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 failure\(^1\). Gu et al. identified the interaction between miRNA-22 and VE-cadherin mRNA\(^16\). 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 cell function.\(^16\)

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 angiogenesis\(^17\).

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 pathway\(^18\). 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.

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