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Endothelial progenitor cell miR-126 promotes homing of endothelial progenitor cells within arterial thrombus in patients with cerebral infarction and its molecular mechanism

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Abstract. – OBJECTIVE: The present study was designed to investigate the effect of microRNA-126 (miR-126) on the migration and homing of endothelial progenitor cells (EPCs) within arterial thrombus of cerebral infarction patients.

MATERIALS AND METHODS: EPCs from rat bone marrow were isolated, and miR-126 overexpressed EPCs were constructed by lentiviral transfection. Then, the middle cerebral artery occlusion (MCAO) model was established by the method of thread ligation. Successfully established model rats were randomly divided into miR-126 overexpression EPC group, miR-126 wild type EPC group, and normal saline group. One day after the infarction, the miR-126 overexpression EPCs, miR-126 wild type EPCs, and normal saline, were injected into the lateral ventricle of the corresponding groups. Also, the transplanted cells were tracked by cell dye SP-DiIC18. The expression of tight junction proteins ZO-1 and Claudin-5 in brain tissue was detected by Western blotting.

RESULTS: Transplanted cells were detected in the cerebral infarction area 3 days after transplantation by cell dye SP-DiIC18. The number of homing EPCs in miR-126 overexpression group was significantly higher than that of miR-126 wild type EPC group ($p < 0.05$). Also, the protein expression of ZO-1 and Claudin-5 in the miR-126 overexpression EPC group was significantly higher compared with that of the miR-126 wild type EPC group and the normal saline group.

CONCLUSIONS: miR-126 overexpression EPCs, which were transplanted in the lateral ventricle, can home to the cerebral infarction areas via increasing increase the expression of ZO-1 and Claudin-5.

Key Words:
Endothelial progenitor cell, miR-126, Cerebral infarction.

Introduction

Cerebral infarction is a serious threat to the human health and life, with high mortality and morbidity. In addition to the primary injury caused by nerve tissue necrosis due to cerebral infarction, the secondary injury further exacerbates the central nervous system structural damage and dysfunction. Therefore, seeking treatment strategy for secondary brain injury after the occurrence of cerebral infarction is a concerning topic. Endothelial progenitor cells (EPCs) are derived from precursor bone marrow cells[1]. Meng et al[2] first isolated and confirmed them. EPCs have both the characteristics of stem cells and vascular endothelial cells, with the ability of self-renewal and self-differentiation. In response to trauma, ischemia, and hypoxia, EPCs are mobilized from the bone marrow into peripheral blood to form circulating EPCs, homing to the site of injury, and differentiating into endothelial cells[3]. Over the past decade, EPCs have become a hot research topic of heart disease, cerebral ischemia, organ transplantation, cancer, and many other diseases, that are closely related to ischemia, angiogenesis, and regeneration[4,5]. Previous animal experiments[6] have confirmed that EPCs transplantation can promote the functional recovery from limb ischemia, myocardial ischemia, cerebral infarction, and other ischemic diseases. MicroRNAs (miRs) are short oligonucleotides (about 22-25 bases), which regulate gene expression by inhibiting target gene translation. Growing evidence showed that miRNAs can regulate angiogenesis and vascular remodeling through regulation of

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cell proliferation, adhesion, migration, differentiation, and signaling pathways during angiogenesis process[7,8]. Although an increasing amount of evidence that miR-126 plays an important role in angiogenesis and vascular remodeling, the role of miR-126 in EPCs migration and homing has not been reported yet. This study aimed to investigate the effect of miR-126 on migration and homing of EPCs in arterial thrombosis in patients with cerebral infarction. This may provide a new treatment strategy for the secondary brain injury caused by cerebral infarction.

Materials and Methods

Experimental Animals

A total of 30 male Sprague Dawley (SD) rats (aged 4 weeks and weighed 75-110 g) were purchased from Experimental Animal Center of West China Medical Center, Sichuan University (Chengdu, Sichuan, China). All experimental animals were operated in accordance with the relevant provisions of Sichuan University Laboratory Animal Ethics Committee.

Main Reagents and Instruments

Rat lymphocyte separation fluid and MMT reagents were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). EGM-2MV medium was purchased from Lonza Inc. (Allendale, NJ, USA). GTVisionTM III anti-mouse immunohistochemistry kit was purchased from Shanghai Gene Technology Co., Ltd (Shanghai, China). miR-126 mimics, inhibitors and negative control were purchased from Rui Bo Biotechnology Co., Ltd (Guangzhou, Guangdong, China).

Methods

Isolation of Rat Bone Marrow-Derived EPCs

Rat bone marrow mononuclear cells (BMMNCs) were isolated as previously reported. BMMNCs were inoculated into 25 cm^2 FN-coated plastic culture flasks at a density of 1×10^6/ml and cultured in Microvascular Endothelial Cell Growth Medium-2 BulletKit™ (EGM-2MV). After incubation for 72 h at 37°C in a 5% CO_2 incubator, the medium was removed and non-adhered cells were aspirated. The medium was changed every 2-4 days. After 10-14 days, as the primary cells confluence up to 80%, the 2^{nd}-3^{rd} passage of cells was collected for identification and for following experiments.

Identification of Rat Bone Marrow-Derived EPCs

The surface antigens vWF and VEGFR-2 of the 2^{nd}-3^{rd} passage of adherent cells were detected by immunohistochemistry by following the manufacture's instruction of GTVisionTM III (Assington Green, UK) anti-mouse immunohistochemistry kit.

Transfection of miR-126 Mimics and Inhibitors

The EPCs were transfected with miR-126 mimics and inhibitors by electroporation. Briefly, after the EPCs were digested by trypsin, EPCs were re-suspended in serum-free EGM-2 medium and cell concentration was adjusted to 5×10^7 cells/mL. Next, 100 µl of EPCs and 6 µl of miR-126 mimics, corresponding mimic negative control, miR-126 inhibitor, or corresponding inhibitor negative control, were added to the Eppendorf (EP) tubes (Hamburg, Germany). The electroporation conditions were voltage 180 V and 25 ms.

The Construction of Rat Cerebral Infarction Model

Middle cerebral artery occlusion (MCAO) model was established by suture ligation method. The 4% sodium pentobarbital solution was injected intraperitoneally for anesthesia. Rats were fixed in the supine position. The incision was made in the middle of the neck, and each layer of tissue was dissected until the bifurcation of internal and external carotid arteries. The common carotid and internal carotid arteries were clamped with the arterial clamp. After the external carotid artery (ECA) was found, the proximal end was ligated with suture 0, and a small opening was cut out with the ophthalmic scissors. The suture was slowly started, and the external carotid artery was ligated. After the arterial clamp was released, the suture was continued to the anterior cerebral artery. The tissues were sutured layer by layer.

Intraventricular Transplantation of EPCs

EPCs were digested and re-suspended in EGM-2MV medium. The cell density was adjusted to 2×10^7cells/200 µL. Anesthesia was achieved with intraperitoneal injection of 4% pentobarbital so-

dium solution. The rat was fixed in the supine position and the head was fixed on the head holder of the stereotaxic apparatus to expose the left skull. A micro-hole was made 0.5 mm behind the crevice suture and 1.5 mm left to the sagittal suture. The Hamilton microinjection device containing 3 μL of cell suspension was immobilized on a stereotactic injection rack. The position of the microinjection device was adjusted to the micro-hole, and the needle was slowly lowered until it touched the brain surface. Then, it was plunged 2.5 mm into the lateral ventricle. The cell suspension was injected slowly at a rate of 1 μL/min. After the injection, the micro-hole was filled with a small piece of bone wax. The tissues were sutured layer by layer.

Western Blot Analysis

Western blot analysis was performed as previously described[18]. Briefly, cell lysates containing equal amounts of protein (50 μg) were separated in 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF) at 100 V for 40-100 min (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline/0.5% Tween-20 (TBST) at room temperature for 3-6 h, and then incubated at 4°C overnight with anti- ZO-1 (Rabbit, 1:1000; Abcam, Cambridge, MA, USA) and anti-Claudin-5 (Rabbit, 1:3000; Bosterbio, Pleasanton, CA, USA). The membranes were washed four times with TBST and incubated at 37°C for 1 h with the corresponding horseradish peroxidase (HRP) conjugated secondary antibodies (1:3000; Boster Biological Technology, Pleasanton, CA, USA). Next, the membranes were washed four times again with TBST and immunoreactive traces were detected by using an enhanced chemiluminescence (ECL) Kit (Millipore, Billerica, MA, USA). The intensity of protein expression on the membranes was analyzed by ImageJ software.

Statistical Analysis

SPSS17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Measurement data are expressed as the mean ± standard deviation, and a two-independent-sample *t*-test was performed for group comparisons. One-way analysis of variance (ANOVA) with repeated measures was performed to analyze changes in continuous variables from baseline between the two groups. To validate ANOVA, the Bonferroni method was used as the post-hoc test. $p < 0.05$ was considered statistically significant.

Results

Identification of Rat Bone Marrow Endothelial Progenitor Cells

The morphological and quantitative changes of adherent bone marrow mononuclear cells (BMMNCs) were observed under inverted phase contrast microscope. On day one, the round-shaped, small-sized cells were isolated and suspended in the culture medium. On day two, a small proportion of cells began to adhere. On day three and four, most of cells were adhered. The shape of cells began to change, appearing tail or polygonal. On day five and six, some cells gathered to form a cell population. The size increased, and the shape gradually elongated. On day seven, most of the cells gradually expanded from round or fusiform-shaped to irregular shaped. On day ten to fifteen, the cells gradually fused, and there were many pavement-like cells with vigorous growth and remarkable proliferation (Figure 1). Immunohistochemistry showed that the 2nd and 3rd passage of cells were VEGFR-2 (Figure 2) and vWF positive (Figure 3).

EPCs Homing After Transplantation

Three days after transplantation, SP-DiICl8 (3) positive cells, namely transplanted EPCs, were detected in the cerebral infarction. The number of homing EPCs in the miR-126 overexpression

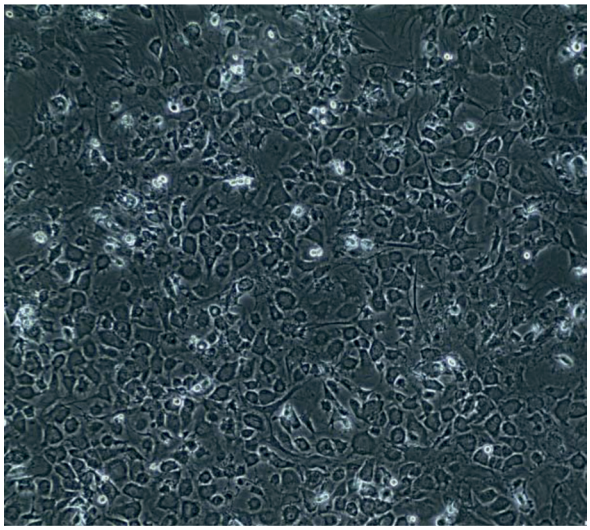

Figure 1. On the 15th day, the cells achieved confluence and appeared pavement-like (× 200).

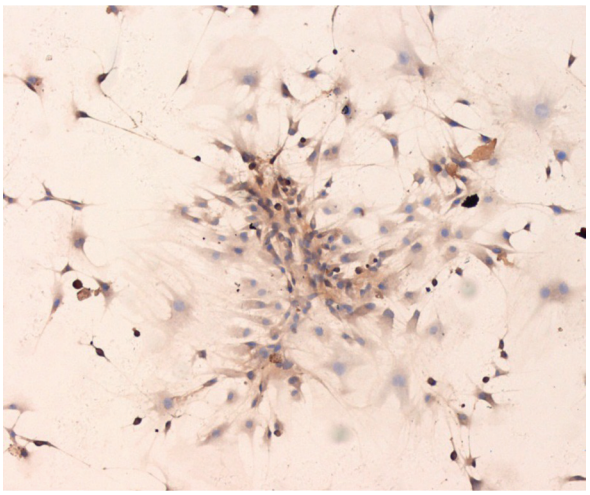

Figure 2. VEGF-2 immunohistochemical staining positive (× 200).

EPC group was significantly higher than that in the miR-126 wild type EPC group (40.57 ± 7.55/field *vs.* 10.15 ± 3.15/field, $p < 0.05$ (Figures 4-5). In addition, the number of homing EPCs in the saline group was zero.

The impact of EPCs Transplantation on the Expression of Tight Junction Proteins ZO-1 and Claudin-5

The expression of ZO-1 and Claudin-5 protein in the cerebral hemisphere of the rats on the third day after transplantation (the fourth day after cerebral infarction) were detected by Western blotting. The results showed that the ZO-1 and Claudin-5 protein expression in the miR-126 overexpression group were significantly

Figure 3. vWF immunohistochemical staining positive (× 200).

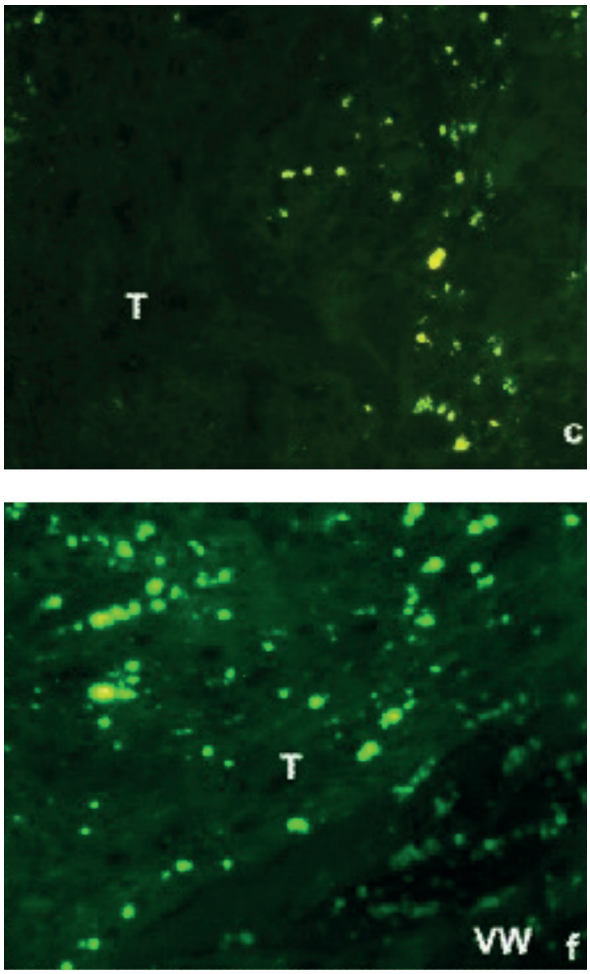

Figure 4. The EPCs from the miR-126 overexpression EPC group and the miR-126 wild type EPC group were observed in the frozen sections of infarcted area under the fluorescence microscope (× 200). Upper panel: the miR-126 overexpression EPC group; Lower panel: the miR-126 wild type EPC group.

increased compared with those of the miR-126 wild type EPC and saline groups (Figure 6).

Discussion

EPCs are precursor cells derived from bone marrow and can proliferate and differentiate into vascular endothelial cells. EPCs have the characteristics of both stem cells and vascular endothelial cells, which not only play an important role in angiogenesis of human embryos, but also participate in angiogenesis and repair of injuries after birth. Meng et al[2] first detected and isolated human EPCs from peripheral blood. Homing of EPCs to the injury areas is

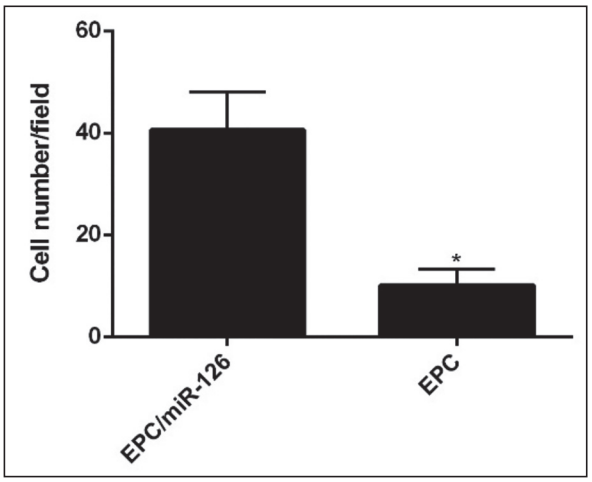

Figure 5. The number of homing EPCs in the miR-126 overexpression EPC group (EPC/miR-126 in the figure) and miR-126 wild type EPC group (EPC in the figure) in the brain infarction area; (*$p < 0.05$).

one of the prerequisite conditions for EPCs to be functional[9]. The previous study has shown that after EPCs were injected into the lateral ventricle, they demonstrated a tendency to migrate from the contralateral lateral ventricle below the corpus callosum to the ipsilateral cerebral infarction areas. Chemokines including stromal cell-derived factor 1 (SDF-1), Monocyte Chemoattractant Protein-1 (MCP-1), and hepatocyte growth factor (HGF), play an important role in the migration process (10,11). Chemokine SDF-1 and its receptor CXCR4 are involved in the activation of downstream signaling molecules such as p38MAPK, ribosomal S6 kinase, c-Jun, and paxillin. This lead to the reorganization of cytoskeletal actin, cytoskeleton phosphorylation,

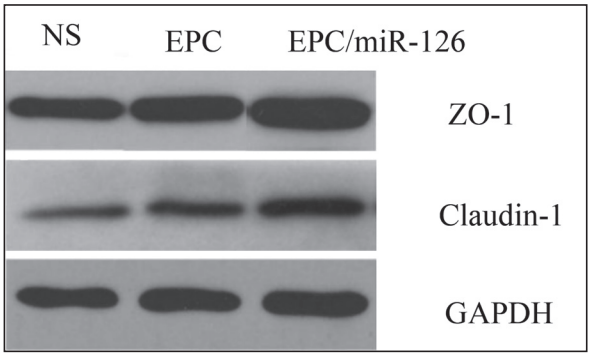

Figure 6. The comparison of protein expression of ZO-1 and Claudin-1 in the miR-126 overexpression EPC group (EPC/miR-126 in the figure), the miR-126 wild type EPC group (EPC in the figure), and the normal saline group (NS in the figure).

and local adhesion molecules phosphorylation, resulting in cell axon elongation and cell migration[12,13]. Our study showed that homing EPCs were detected in the infarcted areas three days after EPCs transplantation. Homing EPCs exerted their effects via integrating the existing blood vessels or directly generating new blood vessels. The tight connection between brain capillary endothelial cells is a major part of the blood-brain barrier (BBB), which consists of transmembrane proteins, adhesion proteins, adhesion molecules, as well as other complex protein structures. They rely on cytoskeletal contraction forces and the tight coupling forces between endothelial cells to limit the free movement of the macromolecules between the brain and the blood[14,15]. Changes of tight junction protein expression and the location in the central nervous system may disrupt the integrity of tight junctions, resulting in severe BBB dysfunction[16]. Previous animal experiments showed that tight junction protein expression was significantly decreased after craniocerebral injury, cerebral ischemia, and cerebral infarction. Also, it is known that the tight junction proteins ZO-1 and Claudin-5 play important roles in maintaining the tight junction structure and function[17]. As an adhesion protein, ZO-1 is a recognition and signal transduction protein of the tight junction, and participates in maintaining the integrity of cytoskeletal. Loss of ZO-1 lead to the destruction of the tight junction. Claudin-5 is a transmembrane protein, which is directly involved in the regulation of BBB structure and function. A previous study[18] has shown that the increase of Claudin-5 expression reduces BBB permeability. Our work has shown that after EPCs were injected into the lateral ventricle, the expression of ZO-1 and Claudin-5 were significantly increased. These data suggested that EPCs transplantation repairs the BBB via upregulating ZO-1 and Claudin-5 expression. miRs are small RNAs which function in RNA silencing and post-transcriptional regulation of gene expression. They have shown critical roles in regulating tumor development and angiogenesis[19,20]. The miR-126 is specifically expressed in endothelial cells. The previous study[9] found that endothelial cells express miR-126, which can activate the vascular endothelial growth factor (VEGF)-dependent signaling pathways promoting angiogenesis. The miR-126 antagonist (antagomiR-126) can inhibit the expression of miRNA-126 in arterial endothelial cells, thus reducing the angiogenesis in ischemic

injury region and increasing the ischemic injury of the lower extremity arteries. Also, miRNA-126 is essential for the maintenance of vascular structure, which directly affects Spred-1, Vascular Cell Adhesion Molecule 1 (VCAM1) and PIK3R2. This will enhance VEGF and fibroblast growth factor (FGF)-induced angiogenesis by inhibiting Spred-1 and PIK3R2 expression[10]. Our study showed that the number of homing EPCs in the miR-126 overexpression EPC group was significantly higher than that in miR-126 wild type EPC group.

Conclusions

We have showed that miR-126 overexpression EPCs, which were transplanted in the lateral ventricle, can home to the cerebral infarction areas via increasing the expression of ZO-1 and Claudin-5.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Fadini GP, Losordo D, Dimmeler S. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. Circ Res 2012; 110: 624-637.
2) Meng Q, Li X, Yu X, Lei F, Jiang K, Li C. Transplantation of ex vivo expanded bone marrow-derived endothelial progenitor cells enhances chronic venous thrombus resolution and recanalization. Clin Appl Thromb Hemost 2011; 17: E196-201.
3) Meng QY, Li XQ, Yu XB, Lei FR, Jiang K, Li CY. Transplantation of VEGF165-gene-transfected endothelial progenitor cells in the treatment of chronic venous thrombosis in rats. Chin Med J (Engl) 2010; 123: 471-477.
4) Bonauer A, Boon RA, Dimmeler S. Vascular microRNAs. Curr Drug Targets 2010; 11: 943-949.
5) Nicoli S, Standley C, Walker P, Hurlstone A, Fogarty KE, Lawson ND. MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. Nature 2010; 464: 1196-1200.
6) Zhang Q, Kandic I, Kutryk MJ. Dysregulation of angiogenesis-related microRNAs in endothelial progenitor cells from patients with coronary artery disease. Biochem Biophys Res Commun 2011; 405: 42-46.
7) Zhang J, Zhang Z, Zhang DY, Zhu J, Zhang T, Wang C. microRNA 126 inhibits the transition of endothelial progenitor cells to mesenchymal cells via the PIK3R2-PI3K/Akt signalling pathway. PLoS One 2013; 8: e83294.
8) Meng S, Cao JT, Zhang B, Zhou Q, Shen CX, Wang CQ. Downregulation of microRNA-126 in endothelial progenitor cells from diabetes patients, impairs their functional properties, via target gene Spred-1. J Mol Cell Cardiol 2012; 53: 64-72.
9) Yan T, Liu Y, Cui K, Hu B, Wang F, Zou L. MicroRNA-126 regulates EPCs function: implications for a role of miR-126 in preeclampsia. J Cell Biochem 2013; 114: 2148-2159.
10) Mocharla P, Briand S, Giannotti G, Dörries C, Jakob P, Paneni F, Lüscher T, Landmesser U. AngiomiR-126 expression and secretion from circulating CD34(+) and CD14(+) PBMCs: role for proangiogenic effects and alterations in type 2 diabetics. Blood 2013; 121: 226-236.
11) Jodon de Villeroché V, Avouac J, Ponceau A, Ruiz B, Kahan A, Boileau C, Uzan G, Allanore Y. Enhanced late-outgrowth circulating endothelial progenitor cell levels in rheumatoid arthritis and correlation with disease activity. Arthritis Res Ther 2010; 12: R27.
12) Naito H, Kidoya H, Sakimoto S, Wakabayashi T, Takakura N. Identification and characterization of a resident vascular stem/progenitor cell population in preexisting blood vessels. EMBO J 2012; 31: 842-855.
13) Fadini GP, Agostini C, Avogaro A. Autologous stem cell therapy for peripheral arterial disease meta-analysis and systematic review of the literature. Atherosclerosis 2010; 209: 10-17.
14) Medina RJ, O'Neill CL, Sweeney M, Guduric-Fuchs J, Gardiner TA, Simpson DA, Stitt AW. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. BMC Med Genomics 2010; 3: 18-24.
15) Navarro-Sobrino M, Hernández-Guillamon M, Fernandez-Cadenas I, Ribó M, Romero IA, Couraud PO, Wekssler BB, Montaner J, Rosell A. The angiogenic gene profile of circulating endothelial progenitor cells from ischemic stroke patients. Vasc Cell 2013; 5: 3.
16) Tura O, Skinner EM, Barclay GR, Samuel K, Gallagher RC, Brittan M, Hadoke PW, Newby DE, Turner ML, Mills NL. Late outgrowth endothelial cells resemble mature endothelial cells and are not derived from bone marrow. Stem Cells 2013; 31: 338-348.
17) Chen J, Song M, Yu S, Gao P, Yu Y, Wang H, Huang L. Advanced glycation endproducts alter functions and promote apoptosis in endothelial progenitor cells through receptor for advanced glycation endproducts mediate overpression of cell oxidant stress. Mol Cell Biochem 2010; 335: 137-146.
18) Ii M. Bone marrow-derived endothelial progenitor cells: isolation and characterization for myocardial repair. Methods Mol Biol 2010; 660: 9-27.
19) Wu HX, Wang GM, Lu X, Zhang L. miR-542-3p targets sphingosine-1-phosphate receptor 1 and regulates cell proliferation and invasion of breast cancer cells. Eur Rev Med Pharmacol Sci 2017; 21: 108-114.
20) Liu H, Wu HY, Wang WY, Zhao ZL, Liu XY, Wang LY. Regulation of miR-92a on vascular endothelial aging via mediating Nrf2-KEAP1-ARE signal pathway. Eur Rev Med Pharmacol Sci 2017; 21: 2734-2742.