

RESEARCH ARTICLE

Proliferation of Peripheral Blood-derived Endothelial Progenitor Cells from Stable Angina SubjectsYudi Her Oktaviono^{1,2}, Djanggan Sargowo^{2,3}, Mohammad Aris Widodo^{2,4},
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Abstract

BACKGROUND: A population of circulating Endothelial Progenitor Cells (EPCs) has been reported to play important role in maintaining endothelial function and integrity. Since EPCs culture is crucial and an optimized medium is currently available. Therefore we conducted a study to investigate whether stable angina subjects peripheral blood-derived EPCs could be cultured in this medium. Here, we performed study to detect EPCs characteristics and extracellular signal-regulated kinase (Erk)1/2 Mitogen-Activated Protein Kinase (MAPK) pathway as possible underlying pathway for EPCs proliferation.

METHODS: Peripheral blood EPCs from 8 stable angina subjects were cultured in an optimized medium with/without addition of supplement for 1 or 3 days. Then, the membrane of cultured EPCs were detected with immunofluorescence method for CD34, Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2) and CD133. Colony forming unit (CFU) enumeration was performed. XTT Cell proliferation assay was performed to assess EPCs growth after 1 and 3-days culture. The western blot analysis was performed to detect possible activation of Erk1/2 MAPK.

RESULTS: Number of EPCs and CFU cultured for 3 days were significantly higher than the ones cultured for 1 day

Abstrak

LATAR BELAKANG: Populasi Sel Progenitor Endotel / *Endothelial Progenitor Cells* (EPCs) yang bersirkulasi telah dilaporkan berperan penting dalam memelihara fungsi dan integritas endotel. Oleh karena kultur EPCs sangat penting dan medium yang teroptimasi telah tersedia, maka kami melakukan suatu penelitian untuk mengetahui apakah medium tersebut dapat digunakan sebagai medium kultur EPCs yang berasal dari darah tepi subyek angina pektoris. Kami melakukan penelitian untuk menguji karakteristik EPCs dan jalur *extracellular signal-regulated kinase* (Erk)1/2 *Mitogen-Activated Protein Kinase* (MAPK) yang kemungkinan merupakan jalur mekanisme proliferasi EPCs.

METODE: EPCs darah tepi dari subyek angina pektoris dikultur dalam medium teroptimasi dengan diberikan atau tidak diberikan tambahan suplemen, selama 1 atau 3 hari. Kemudian membran EPCs hasil kultur dideteksi dengan metode imunofluoresens untuk mendeteksi CD34, *Vascular Endothelial Growth Factor Receptor 2* (VEGFR-2) dan CD133. Enumerasi *colony forming unit* (CFU) dilakukan. Uji proliferasi sel XTT dilakukan untuk mengukur pertumbuhan EPCs pada kultur 1 dan 3 hari. Analisa *western blot* dilakukan untuk mendeteksi kemungkinan adanya aktivasi Erk1/2 MAPK.

($p=0.012$). EPCs membrane markers from stable angina subjects were detected as well as CFUs were formed. There were significant increase of EPCs number, CFUs number and phosphorylated-Erk2 amount when the groups with and without supplement were compared ($p<0.05$). Meanwhile U0126, a MAPK Erk1/2 (MEK1/2) inhibitor, significantly inhibited the supplement-induced EPCs number, CFUs number and phosphorylated-Erk2 amount ($p<0.05$).

CONCLUSION: Our results showed that ERK2 MAPK signaling pathway might play an important role in supplement-induced peripheral blood EPCs proliferation in subjects with stable angina.

KEYWORDS: endothelial progenitor cell, EPC, p42, Erk2, proliferation

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HASIL: Jumlah EPCs dan CFU meningkat secara bermakna setelah dikultur selama 3 hari dibanding dengan yang dikultur 1 hari ($p=0,012$). Marker membran EPCs dari subyek angina stabil terdeteksi dan CFUs terbentuk. Terdapat peningkatan bermakna dari jumlah EPCs, jumlah CFUs dan jumlah Erk2 yang terfosforilasi ketika grup yang diberikan dan tidak diberikan suplemen dibandingkan ($p<0,05$). Sedangkan ketika diberikan U0126, inhibitor MAPK Erk1/2 (MEK1/2), jumlah EPCs, jumlah CFUs dan jumlah Erk2 yang terfosforilasi secara bermakna berkurang ($p<0,05$).

KESIMPULAN: Hasil kami memperlihatkan jalur pensinyalan ERK2 MAPK berperan penting pada proliferasi EPCs yang berasal dari darah tepi subyek angina stabil.

KATA KUNCI: sel progenitor endotel, EPC, p42, Erk2, proliferasi

Introduction

Cardiovascular disease (CVD) is the main cause of mortality in developed and developing countries.(1) Multiple cardiovascular risk factors cause endothelial injury and endothelial dysfunction leading to vasoconstriction, thrombosis, infiltration of monocytic cells, smooth muscle cell proliferation, and formation of atherosclerosis.(2,3) Maintenance of endothelial integrity and function are important to preserve a healthy vasculature.(4) Indeed, the balance between endothelial injury and endothelial recovery is paramount to reduce cardiovascular events.(5)

Many studies have identified a population of circulating Endothelial Progenitor Cells (EPCs) integrating into sites of neovascularization and endothelial impairment. (2) Circulating EPCs are mobilized immature cells from bone marrow into the bloodstream, in response to particular growth factors and cytokines. EPCs may contribute in vascular repair after differentiate into endothelial cell.(6)

Clinical studies showed that risk factors of atherosclerosis are related to the reduced levels of circulating EPCs.(2,7) The functional integrity of the endothelium is also comparable to the activities of EPCs.(2) The amount of circulating EPCs correlates negatively with the established risk.(8) Decreased level of circulating EPCs has become an independent predictors of atherosclerotic disease progression and measurement of EPCs has become a predictive value for cardiovascular outcomes in stable coronary artery disease patients.(4,9) The reduced circulating EPCs can reflect the

potential cardiovascular morbidity and mortality.(10-12) A long-term study shows that assessment of subpopulations of circulating EPCs in patients with stable angina treated with percutaneous coronary intervention can improve characterization of long-term prognosis, suggesting the possibility of using EPCs as biomarkers for the prediction of cardiovascular outcome.(13)

In regards to mechanisms and underlying signaling pathways, spleen-derived EPCs proliferation was shown to be regulated by the phosphatidylinositol-3-kinase (PI3K)/Akt/nuclear factor kappa B (NFκB)/survivin signaling pathway.(14) Meanwhile, in liver regeneration, Notch signaling differentially regulated bone marrow-derived two types of EPCs, early EPCs and endothelial outgrowth cells. (15) Transforming Growth Factor beta 1 (TGF-β1) was reported to play a role in the growth and differentiation of EPCs. Involvement of extracellular signal-regulated kinase (Erk)1/2 Mitogen-Activated Protein Kinase (MAPK) signaling pathway was shown in Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)-induced peripheral blood-derived EPCs culture.(16) Since EPCs culture is crucial, and an optimized mediums is currently available. This medium could be potential to accelerate EPCs proliferation. However, by using this medium, characteristics, proliferation and signaling pathway of cultured-EPCs derived from different sources, are not disclosed yet. In our current study, stable angina subjects peripheral blood-derived EPCs were cultured in an established culture medium, and then the EPCs characteristics and Erk1/2 MAPK activation were investigated.

Methods

Sample Collection and Mononuclear cells (MNCs)

Isolation

Upon signing informed consent, peripheral blood was collected from 8 volunteers with inclusion criteria are as follows: stable angina pectoris, males, 50-55 years old, coronary angiogram showing >50% stenotic lesions. Subjects with history of coronary stent, acute myocardial infarction, diabetes mellitus, smoking, critical limb ischemia, or coronary artery bypass graft surgery were excluded from this study. This protocol was approved by The Ethical Committee Medical Research Medical Faculty Brawijaya University (No.273/EC/KEPK-S3/06/2013) and The Ethical Committee / Institutional Review Board of National Cardiac Center Harapan Kita Hospital (No. LB.05.01.1.4/55/2013). MNCs was isolated using Lymphoprep (Stemcell Technologies, Vancouver, Canada). Briefly, collected peripheral blood was immediately diluted with equal amount of phosphate buffer saline (PBS) plus 2% fetal bovine serum (FBS), then layered on top of Lymphoprep. With centrifugation at 800x g for 20 minutes, MNCs layer was formed and collected.

EPCs Culture

Colony Forming Unit (CFU)-Hill Liquid Medium Kit (Stemcell Technologies) was used for EPCs culture. Briefly, after rinsed in PBS plus 2% FBS, MNCs were suspended in CFU-Hill Liquid Medium. In each well of 6-well plate, 5×10^6 cells of MNCs suspension was plated and incubated for 2 days in humidified 37°C 5% CO₂ incubator. Then, non-adherent cells were harvested and further cultured for 3 days to allow formation of CFU-Hill colonies. CFU-Hill colonies were enumerated according to kit insert.

Immunofluorescence

After rinsed with PBS, cells were fixed with 4% paraformaldehyde. Fixed cells were then treated with 100 mM glycine and 0.2% Triton X-100 for permeabilization. To block nonspecific binding, 0.1% bovine serum albumin in PBS was applied. For antibodies probing, a combination of 1:400 diluted mouse monoclonal Alexa Fluor 488 anti-human CD34, 1:200 diluted mouse monoclonal R-phycoerythrin-conjugated anti-CD309 (VEGFR-2/KDR) and 1:180 diluted rabbit polyclonal anti-CD133 antibodies, was applied. Then 1:1000 diluted Alexa Fluor 350 F(ab')₂ fragment of goat anti-rabbit IgG (H+L) was applied as a secondary antibody for the anti-CD133 antibody. To have

better cell determination, 4',6-diamidino-2-phenylindole (DAPI) staining was performed. Cell expression was documented under a fluorescence microscope.

Cell Proliferation Assay

Na,3'-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) Cell Proliferation Assay Kit (Cayman Chemical Company, Ann Arbor, MI) was used for cell proliferation assay. The assay is based on the extracellular reduction of XTT by nicotinamide adenine dinucleotide (NADH) produced in the mitochondria via transplasma membrane electron transport and an electron mediator. Briefly, XTT mixture was added into each well and incubated in a 37°C 5% CO₂ incubator for 4 hours. The absorbance was measured using a microplate reader at a wavelength of 450 nm. To interpolate cell number, some wells containing cells were trypsinized and stained with trypan blue. The cell was then counted using hemocytometer under an inverted light microscope.

Western Blot

Cells were harvested and incubated with lysis buffer containing 25 mM Tris HCl buffer (pH 7.6), 150 mM NaCl, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), Protease Inhibitor Cocktail [containing 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide, leupeptin, pepstatin A] (Sigma-Aldrich, St.Louis, MO), Phosphatase Inhibitor Cocktail 3 [containing cantharidin, (-)-p-bromolevamisole oxalate, calyculin A (Sigma-Aldrich)]. Samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA). After blocking with 5% skim milk in PBS, the membrane was probed with 1:1000 diluted rabbit polyclonal anti-phospho-Erk1/2 MAPK (Thr202/Tyr204) antibody (Cell Signaling Technology, Inc., Danvers, MA). The secondary antibody was 1:2000 diluted goat anti-rabbit IgG horseradish peroxidase-linked antibody (Cell Signaling Technology, Inc.). The bound antibodies were visualized using Immun Star HRP Chemiluminescent Kit (Bio-Rad Laboratories, Inc.). Membrane was then stripped with Seppro stripping buffer (Sigma-Aldrich), blocked with 5% skim milk in PBS, probed with rabbit polyclonal anti-Erk1/2 MAPK (Cell Signaling Technology, Inc.), bound with same secondary antibody and visualized with chemiluminescent kit. All visualized bands were captured using Alliance 4.7 (UVItech, Ltd., Cambridge, UK) and quantified using UVIband software (UVItech, Ltd.).

Statistical Analysis

Analyses were performed using IBM SPSS for Windows version 19.0 (IBM Corp., Armonk, NY). Wilcoxon Signed Rank Test was used to determine the statistical differences. A probability value <0.05 was considered to be statistically significant.

Results

Peripheral blood-derived EPCs Membrane Marker Expression

EPCs membrane markers of CD34 (Figure 1A), VEGFR2 (Figure 1B) and CD133 (Figure 1C) were expressed under a fluorescent microscope. EPCs with CD34⁺, VEGFR2⁺ and CD133⁺ expressions were merged (Figure 1D). The captured EPCs were shown as a CFU under a light microscope (Figure 1E).

Increased number of EPCs and CFU

Number of EPCs and CFU cultured for 3 days were significantly higher than the ones cultured for 1 day

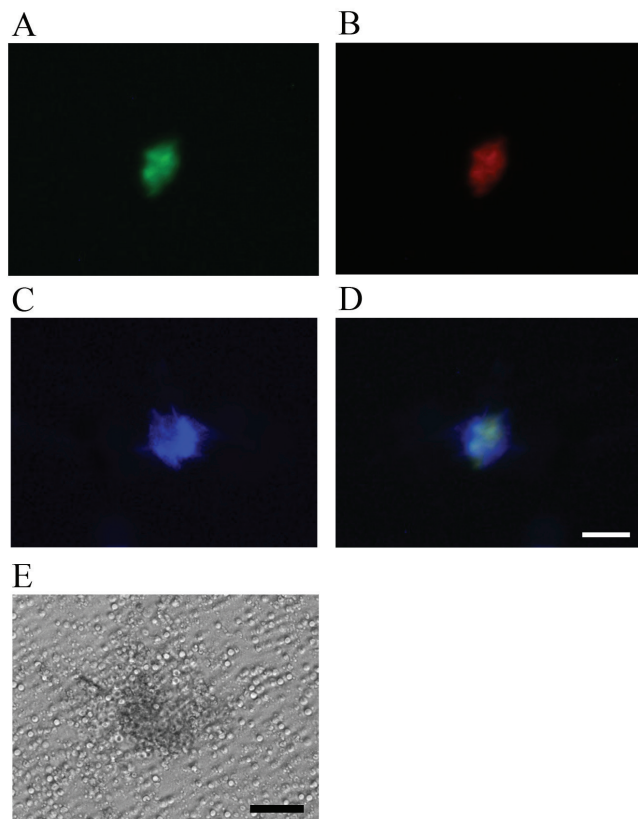


Figure 1. Expression of EPCs membrane markers and CFU. Collected MNCs were cultured and characterized with immunofluorescence as described in Methods. EPCs membrane markers of CD34 (A), VEGFR2 (B) and CD133 (C) were documented. In addition, DAPI-stained EPCs were also visualized (C) under an inverted fluorescence microscope. D: Merge of A, B & C. E: CFU-Hill colony was documented under an inverted light microscope. White bar: 50 μm . Black bar: 100 μm .

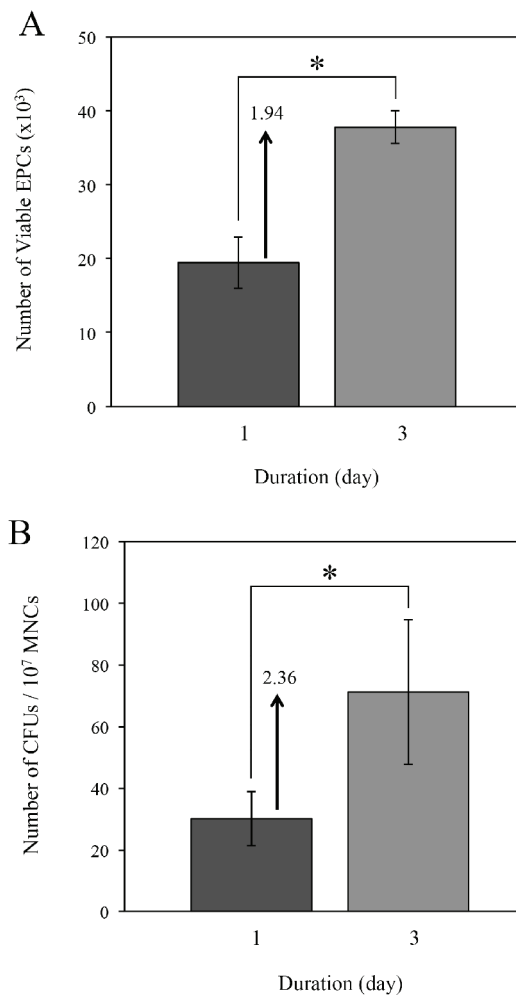


Figure 2. Supplement induced EPCs proliferation and CFU formation.

After collected MNCs were incubated for 2 days as described in Methods, the non-adherent cells were harvested and cultured for 1 or 3 days with supplement of medium kit. For EPCs proliferation, XTT assay was performed as described in Methods. Meanwhile, CFU was enumerated under a standard light microscope. Data were analyzed with Wilcoxon Signed Rank Test, $*p < 0.05$.

($p=0.012$) (Figure 2). Number of EPCs was increased significantly at 1.94-fold ($p=0.012$). In the same comparison between culture of 3 days and 1 day, number of CFU was increased significantly at 2.36-fold ($p=0.012$).

Erk2 MAPK activation in EPCs derived from stable angina subjects

Expressions of Erk1/2 MAPK were observed in EPCs derived from stable angina subjects treated with/without supplement, a part of CFU-Hill Liquid Medium Kit, and/or U0126, a MAPK Erk1/2 (MEK1/2) inhibitor, for 1 or 3 days (Figure 3). Meanwhile phosphorylated-Erk2 expression of stable angina subjects was slightly observed only in EPCs treated with supplement for 3 days. Number of viable EPCs was significantly increased for the group treated with supplement than without supplement in both durations of 1

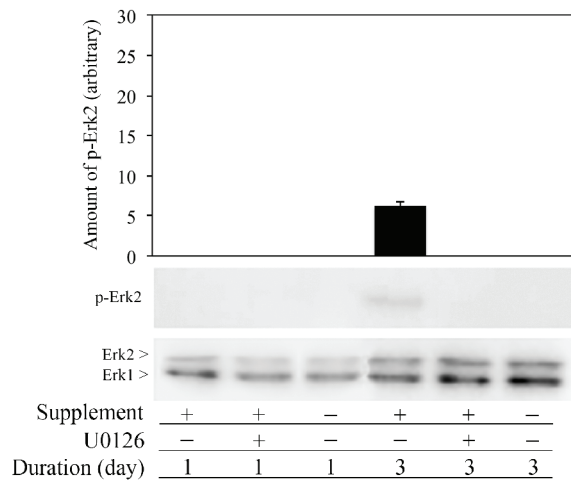


Figure 3. Supplement induced phosphorylation of Erk2.

After collected MNCs were incubated for 2 days as described in Methods, the non-adherent cells were harvested and cultured for 1 or 3 days with/without U0126 and supplement of medium kit. Then cells were collected, lysed and subjected to western blot to detect Erk1/2 and phosphorylated Erk1/2 as described in Methods. p-Erk2: phosphorylated Erk2.

day ($p=0.012$) and 3 days ($p=0.012$) (Figure 4A). Number of CFUs was also significantly increased for the group treated with supplement than without supplement in both durations of 1 day ($p=0.012$) and 3 days ($p=0.012$) (Figure 4B). Meanwhile, number of EPCs was significantly decreased for the group treated for the group treated with U0126 prior to supplement, compared to the one treated with supplement merely, in both durations of 1 day ($p=0.012$) and 3 days ($p=0.012$) (Figure 4B).

Discussion

There is an increasing interest in EPCs due to their contribution in the maintenance of endothelial integrity.(5) At first, the description of EPCs isolation from peripheral blood was introduced by Asahara et al.(17) Then later on, the evolving process of EPCs into mature and functional endothelial cells was reported.(8,18) In this current study, we aim to analyze the cultured-EPCs derived from stable angina subjects in an established commercially available medium. Peripheral blood-derived EPCs were generated and confirmed by verifying EPCs membrane marker CD34, CD133 and VEGFR2. In addition, CFUs were also resulted. We found that the number of EPCs and CFUs significantly increased upon addition of supplement provided along with medium ($p=0.012$). When we cultured EPCs in a longer duration for 3 days, higher numbers of EPCs and CFUs were observed.

MAPK pathway relies, amplifies and integrates signals from any enormous array of stimuli and establish

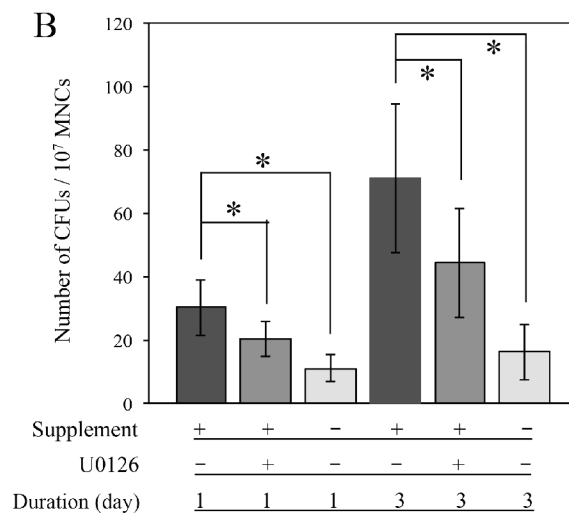
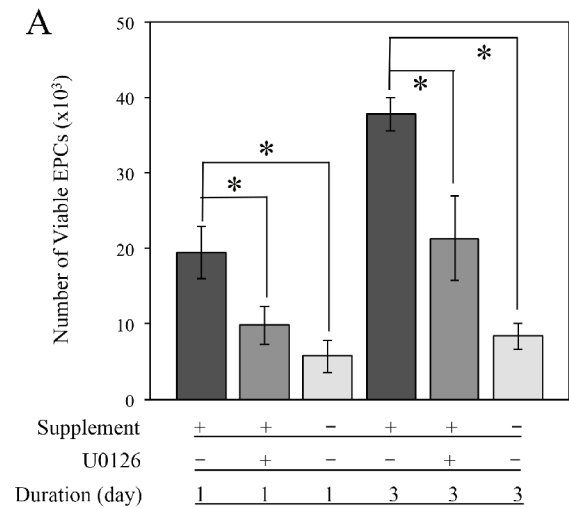


Figure 4. U0126 inhibited EPCs proliferation and CFU formation.

After collected MNCs were incubated for 2 days as described in Methods, the non-adherent cells were harvested and cultured for 1 or 3 days with/without U0126 and supplement of medium kit. For EPCs proliferation, XTT assay was performed as described in Methods. Meanwhile, CFU was enumerated under a standard light microscope. Data were analyzed with Wilcoxon Signed Rank Test, $*p<0.05$.

some physiological responses, such as: cellular proliferation, differentiation, development, transformation, inflammatory response and apoptosis.(19,20) Moreover, it regulates gene expression, metabolism, cell division, morphology, and survival as well as proteins phosphorylation.(20) There are 3 common subfamilies of MAPK: Erk, Jun kinase (Jnk) and p38 MAPK in which Erk MAPK has been the most extensively studied subfamily by far.(19,21) Mitogenic stimulation by some particular growth factors may cause activation of the classical Erk family (ERK1/p44 or ERK2/p42 MAPK).(19,21-23) It has been hypothesized that the activation of MAPK at G1 seems to be related with its ability to enter S phase.(19) Since involvement of Erk1/2 MAPK

signaling pathway was reported in peripheral blood-derived EPCs culture (16), we then pursued our study in Erk1/2 signaling pathway as potential pathway for the supplement to induce EPCs proliferation and CFUs formation.

Our current results showed that under induction of supplement, Erk2 MAPK of EPCs derived from stable angina subjects were slightly phosphorylated. In contrast, by addition of U0126, phosphorylation of Erk2 was totally diminished. A long with the Erk2 phosphorylation inhibition, EPCs growth and CFU formation were also inhibited by U0126, showing that activated pathway was ERK2 MAPK-dependent.

Conclusion

Taken together, our results showed that an important role of p42 MAPK in supplement-induced peripheral blood EPCs derived from stable angina subjects. Further investigation in component of supplement and other potential activated signaling pathway should be carried out to clarify the mechanism of EPCs proliferation induction in this medium.

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