Mol Cell Biochem (2015) 400:201–206
DOI 10.1007/s11010-014-2276-z

Endothelial progenitor cells proliferated via MEK-dependent p42 MAPK signaling pathway

Ferry Sandra · Yudi Her Oktaviono · Mohammad Aris Widodo · Yanni Dirgantara · Angliana Chouw · Djanggan Sargowo

Received: 20 June 2014 / Accepted: 15 November 2014 / Published online: 25 November 2014

Abstract Endothelial progenitor cells (EPCs) clinical applications have been well reported. However, due to low number of EPCs that could be isolated, EPCs expansion study became one of the main focuses. Some optimized mediums to culture EPCs were currently available. However, the proliferation signaling pathway is not clearly disclosed yet. Peripheral blood was collected from eight healthy subjects, followed by mononuclear cells (MNCs) isolation. MNCs were then prepared and cultured for 2 days. After that, non-adherent cells were harvested and further cultured for 3 days. Resulted colony-forming unit (CFU)-Hill colonies were documented and enumerated under an inverted light microscope. To detect membrane markers, immunofluorescence was performed to detect CD34, VEGFR-2, and CD133. Cell documentation was conducted under a fluorescence microscope. To check cell proliferation, XTT Cell Proliferation Assay Kit was used according to kit insert. To detect possible activation of p44/42 MAPK, western blot was performed to detect p44/42 MAPK and phosphorylated p44/42 MAPK. All visualized bands were captured and quantified. Our results showed that EPCs markers (CD34, CD133 and VEGFR-2) were detected in 3 days culture. From XTT cell proliferation assay and CFU enumeration results, we found that EPCs proliferated significantly ($p = 0.012$) with addition of supplement. Phosphorylated-p42 MAPK expression of EPCs treated with supplement was significantly higher than the one of EPCs without treatment. Significant inhibition of p42 MAPK phosphorylation by U0126 was observed ($p = 0.012$). By pretreatment of U0126, number of viable cells and CFUs treated with supplement was significantly decreased ($p = 0.012$). Our results showed that MEK-dependent p42 MAPK pathway might play an important role in EPCs proliferation.

Keywords Endothelial progenitor cell · EPC · p42 · Erk2 · MAPK · Proliferation

F. Sandra (✉)
Prodia Clinical Laboratory, Prodia Tower, Jl. Kramat Raya No. 150, Jakarta, Indonesia
e-mail: ferrysandra@gmail.com

F. Sandra
Department of Biochemistry and Molecular Biology, Faculty of Dentistry, Trisakti University, Jl. Kyai Tapa No. 260, Jakarta, Indonesia

Y. H. Oktaviono
Department of Cardiology and Vascular Medicine, Faculty of Medicine, Dr. Soetomo Hospital, University of Airlangga, Jl. May. Jend. Prof. Dr. Moestopo No. 6-8, Surabaya, Indonesia

Y. H. Oktaviono · M. A. Widodo · D. Sargowo
Postgraduate Program in Biomedical Sciences, Faculty of Medicine, Brawijaya University, Jl. Veteran, Malang, Indonesia

M. A. Widodo
Department of Pharmacology, Faculty of Medicine, Brawijaya University, Jl. Veteran, Malang, Indonesia

Y. Dirgantara · A. Chouw
PT. Prodia Stemcell Indonesia, Jl. Kramat 7 No. 11, Jakarta, Indonesia

D. Sargowo
Department of Cardiology and Vascular Medicine, Faculty of Medicine, Dr. Saiful Anwar General Hospital, Brawijaya University, Jl. Jaksa Agung Suprapto No. 2, Klojen, Malang, Indonesia

Background

Endothelial progenitor cells (EPCs) are circulating cells believed to play an important role in tissue regeneration by promoting the repair of blood vessels and aiding in the reperfusion of ischemic areas [1, 2]. Besides tissue regeneration, EPCs are also believed to be potential for neovascularization and determination of prognosis in cardiovascular disease [3]. EPCs clinical applications have been well reported [4–6]. For coronary artery disease, EPCs improved left ventricular ejection fraction and perfusion [4, 5]. And for diabetic patients with non-healing foot ulcers, complete wound closure was observed with increased vascular perfusion [6].

Initially, EPCs were classified as a subtype of $CD34^+$ hematopoietic stem cells and characterized by co-expression of CD133, also called prominin 1 [7]. EPCs were also characterized by co-expression of kinase insert domain receptor (KDR)-1, also known as vascular endothelial growth factor receptor (VEGFR) 2 [7]. For EPCs enumeration purpose, the standardized International Society for Hematotherapy and Graft Engineering (ISHAGE) protocol for enumeration of hematopoietic stem cells was adapted [3, 7]. $CD45^{dim}$ $CD34^+$ cells were quantified for KDR, in order to have higher EPCs count accuracy [3]. Meanwhile, colony-forming unit (CFU) enumeration is frequently assessed as a part of EPCs functional confirmation, and correlated with degeneration and various diseases [8–10].

EPCs generally account for only $\sim$0.01 % of circulating cells [11], meanwhile, particular EPCs number should be reached for therapeutic purposes. For example, in a clinical study, diabetic patients with non-healing foot ulcers were effectively treated with 2×10^7 peripheral blood EPCs with high number of CFUs and CD34/KDR double positivity [6]. Therefore, intensive investigations were pursued in collecting EPCs with induction of granulocyte colony-stimulating factor (G-CSF) [12], and in vitro EPCs expansion with induction of epidermal growth factor (EGF) [13], fibroblast growth factor (FGF)1 [14], granulocyte-macrophage colony-stimulating factor (GM-CSF) [15], or interleukin-1 beta (Il-1β) [16]. These factors could enhance EPCs proliferation through mitogen-activated protein kinase (MAPK) signaling pathways [13–15] and phosphatidyl-inositol-3-kinase (PI3K) [15, 16]. Along with EPCs research development, some optimized mediums were currently available. These mediums could be potential to accelerate EPCs proliferation. However, EPCs proliferation signaling pathway induced in these mediums is not clearly disclosed yet. Therefore, we conducted an investigation to find out the underlying signaling pathway.

Methods

Peripheral blood collection and mononuclear cells (MNCs) isolation

Peripheral blood was collected from eight healthy subjects. All subjects were males, aged 50–55 years old. This protocol was approved by The Ethical Committee of Medical Faculty Brawijaya University. MNCs isolation was performed 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 $800 \times g$ for 20 minutes, MNCs layer was formed and collected.

EPCs culture

EPCs culture was performed using CFU-Hill Liquid Medium Kit (Stemcell Technologies). Briefly, collected MNCs were rinsed in PBS plus 2 % FBS and re-suspended in CFU-Hill Liquid Medium to achieve concentration of 2.5×10^6 cells/ml. In each well of 6-well plate, 2 ml of MNCs suspension was plated and incubated for 2 days in humidified 37 °C 5 % CO_2 incubator. After 2 days, non-adherent cells were harvested and further cultured for 3 days to allow formation of CFU-Hill colonies with/without addition of supplement as a part of CFU-Hill Liquid Medium Kit. Supplement was 1/5 of CFU-Hill Basal Medium volume. U0126, a MEK inhibitor (Cell Signaling Technology, Inc., Danvers, MA) was applied in the concentration of 10 μM for 2 hours. CFU-Hill colonies were enumerated using an inverted light microscope (Axio Vert, Carl Zeiss, Jena, Germany).

Immunofluorescence

Cells were rinsed with PBS, fixed with 4 % paraformaldehyde, and rinsed again with PBS. Fixed cells were treated with 100 mM glycine and 0.2 % Triton X-100. Samples were incubated in 0.1 % bovine serum albumin in PBS to block nonspecific binding. A combination of 1:400 diluted mouse monoclonal Alexa Fluor 488 anti-human CD34 (BioLegend, San Diego, CA), 1:200 diluted mouse monoclonal R-phycoerythrin-conjugated anti-CD309 (VEGFR-2/KDR) (Miltenyi GmbH, Bergisch, Germany), and 1:180 diluted rabbit polyclonal anti-CD133 (Biorbyt, San Fransisco, CA) antibodies was applied. Then 1:1000 diluted Alexa Fluor 350 F(ab″)2 fragment of goat anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR) was applied as a secondary antibody for the anti-CD133 antibody. To have better cell determination, 4′,6-diamidino-2-phenylindole (DAPI) (Wako, Osaka, Japan) staining was performed. Cell

observation and documentation was conducted under an inverted fluorescence microscope (Axio Observer, Carl Zeiss). In each step, samples were rinsed with PBS.

Cell proliferation assay

Cell proliferation assay was performed using 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). 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 prepared and added into each well. Cells were then incubated in a 37 °C 5 % CO_2 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 then trypan blue was added to the cell suspensions. 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)

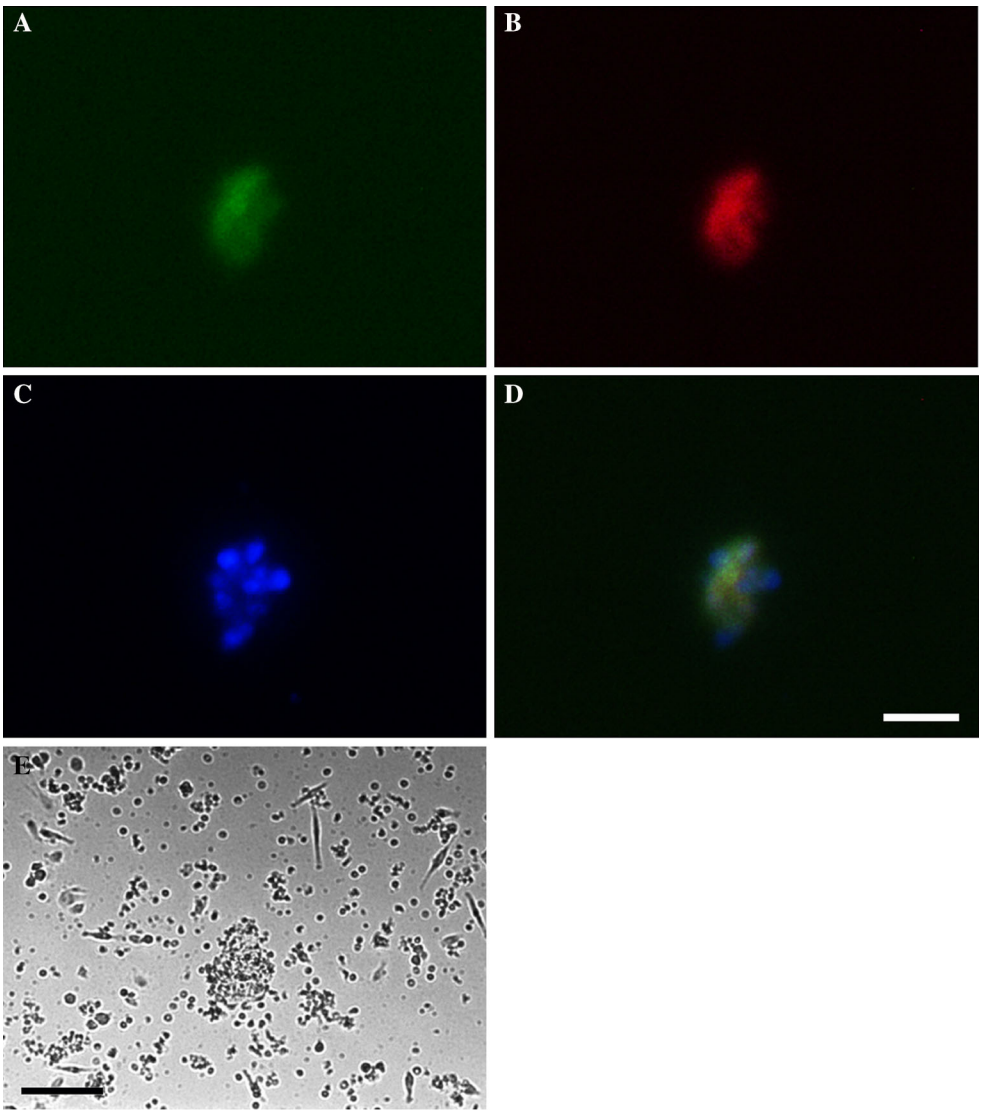

Fig. 1 EPCs Characteristics. EPCs were cultured, then characterized with immunofluorescence as described in "Methods" section. EPCs membrane markers of CD34 (**a**), VEGFR2 (**b**), and CD133 (**c**) were documented. In addition, DAPI-stained EPCs were also visualized (**c**) under an inverted fluorescence microscope. **d** Merge of a–c. **e** CFU-Hill colony was documented under an inverted light microscope. *White bar* 50 μm. *Black bar* 100 μm

benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide, leupeptin, pepstatin A) (Sigma-Aldrich, St. Louis, MO)], and 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 (pH 7.4), the membrane was probed with 1:1000 diluted rabbit polyclonal anti-phospho-p44/42 MAPK (extracellular signal-regulated kinase (Erk) 1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology, Inc.). 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-p44/42 MAPK (Erk1/2) (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

Wilcoxon Signed Rank test was performed using IBM SPSS for Windows version 19.0 (IBM Corp., Armonk, NY). A probability value <0.05 was considered to be statistically significant.

Results

Expressions of CD34, VEGFR2, CD133, and CFU

Peripheral blood MNCs were shown to differentiate into EPCs, marked by EPCs membrane markers of CD34, VEGFR2, and CD133 (Fig. 1a–c). EPCs with overlapped $CD34^+$, $VEGFR2^+$, and $CD133^+$ expressions (Fig. 1d) were formed in a colony. Under a light microscope, EPCs were clearly demonstrated as a CFU (Fig. 1e).

p42 MAPK activation in EPCs

To check the amount of protein loaded, expression of inactive (non-phosphorylated) form of p44/42 MAPK was examined. We found similar p44/42 MAPK expression for all groups treated with/without supplement and/or U0126 for 1 or 3 days (Fig. 2). Meanwhile, phosphorylated p42 expression of EPCs treated with supplement was significantly higher than the one of EPCs without supplement in 1 day culture ($p = 0.012$). As a highly selective inhibitor of MAPK/Erk Kinase (MEK), U0126 significantly inhibited phosphorylation of p42 ($p = 0.012$). With 3 days treatment of supplement, we could see a significant increase of phosphorylated p42 as well ($p = 0.012$), which could be inhibited significantly by U0126 as well ($p = 0.012$).

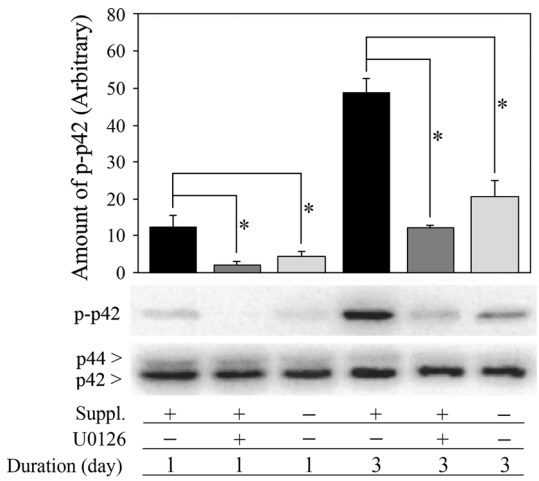

Fig. 2 Supplement induced phosphorylation of p42. After collected MNCs were incubated for 2 days as described in "Methods" section, the non-adherent cells were harvested and cultured for 1 or 3 days with/without U0126 and supplement of medium kit. Then cells were collected, lysed, and subjected to western blot as described in "Methods" section. Data were analyzed by Wilcoxon Signed Rank test, $*p < 0.05$. Each panel shows the typical results and the summary of eight independent determinations. *Suppl* supplement, *p-p42* phosphorylated p42

U0126 inhibited EPCs proliferation and CFU formation

Number of viable cells was significantly increased for the group treated with supplement than without supplement for 1 ($p = 0.012$) or 3 ($p = 0.012$) days (Fig. 3). Significant higher number of CFUs was documented as well for the group treated with supplement than without supplement for 1 ($p = 0.012$) or 3 ($p = 0.012$) days. Meanwhile, by addition of U0126, significant inhibition of EPCs proliferation for 1 ($p = 0.012$) or 3 ($p = 0.012$) days culture and CFU formation for 1 ($p = 0.012$) or 3 ($p = 0.012$) days culture was observed (Fig. 3).

Discussion

EPCs culture has been widely conducted and modified with addition of various factors [13–16], in order to accelerate

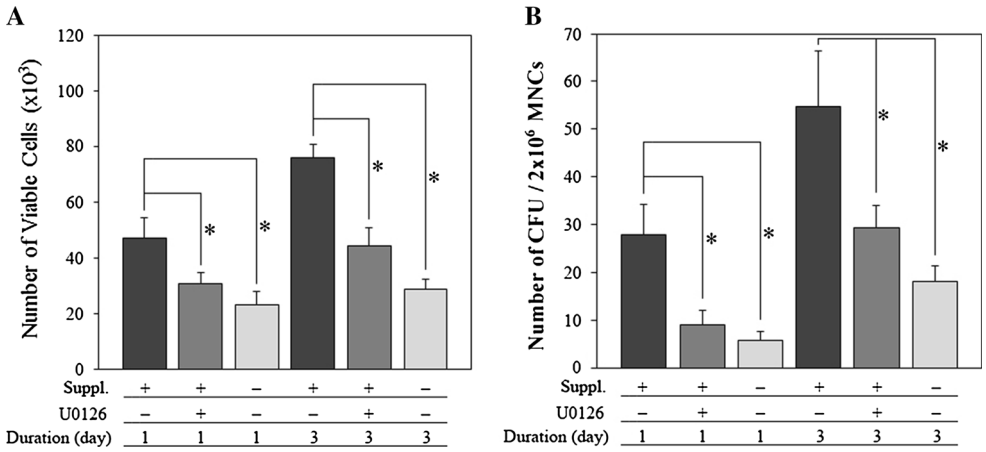

Fig. 3 U0126 inhibited EPCs proliferation and CFU formation. After collected MNCs were incubated for 2 days as described in "Methods" section, the non-adherent cells were harvested and cultured for 1 or 3 days with/without U0126 and supplement of medium kit. For EPCs proliferation, XTT assay was performed as described in "Methods" section. Meanwhile, CFU was enumerated under an inverted light microscope. Data were analyzed by Student's *t* test, $*p < 0.05$. Each result shows the typical results and the summary of eight independent determinations. *Suppl* supplement

EPCs proliferation. In our current study, we used an optimized medium. Peripheral blood-derived EPCs were generated and confirmed by immunofluorescence detection of CD34, CD133, and VEGFR2. In addition, CFUs were also resulted.

Growth factor signals regulate a variety of outputs, ranging from cell division, cell migration, regulation of apoptosis, cellular adhesion and differentiation, as well as alterations in gene expression and intracellular metabolism [17]. The p44/42 MAPK cascade was usually identified as a growth-promoting pathway [17]. Through the phosphorylation of carbamoyl phosphate synthetase II, which is involved in pyrimidine nucleotide biosynthesis, p44/42 can support DNA synthesis during the S phase [17, 18]. Initially, p44 and p42 MAPK were suggested to regulate and contribute similarly to intracellular signaling by phosphorylating a largely common subset of substrates, both in the cytosol and in the nucleus [19]. However, later on antagonizing activities between p44 and p42 were reported. The p42 was suggested to be the one that has a positive role in controlling normal and Ras-dependent cell proliferation [19]. In our current study, we noticed that under induction of supplement, p42 MAPK was highly phosphorylated. Meanwhile, only few amounts of p44 MAPK were detected and phosphorylated p44 MAPK was undetected. This result suggested that EPCs proliferation was highly induced with very less antagonizing activity of p44 MAPK. Phosphorylation of p42 MAPK was diminished by U0126, a MEK inhibitor, showing that activated pathway was MEK dependent.

Since U0126 could diminish supplement-induced phosphorylation of p42 MAPK, we conducted a study to see the influence of U0126 on the growth of supplement-induced EPCs. Our results showed that U0126 diminished supplement-induced EPCs growth and CFU. Taken together, our results showed that an important role of p42 MAPK in supplement-induced EPCs. However, PI3 K was also reported as another underlying mechanism [15, 16] and might play an important role in supplement-induced EPCs growth; therefore, PI3 K pathway should be further investigated.

Acknowledgments Authors are thankful to National Cardiovascular Center Harapan Kita for human sample collection, PT. Prodia Stemcell Indonesia for laboratory support, Dr. Budi Bakti Jasa Darmajati for invaluable support in this project, and Dr. Ika Christine for statistical analyses.

Conflict of interests The authors declare that they have no conflict of interests.

References

1. Lois N, McCarter RV, O'Neill C, Medina RJ, Stitt AW (2014) Endothelial Progenitor Cells in Diabetic Retinopathy. Front Endocrinol (Lausanne) 5:44. doi:10.3389/fendo.2014.00044
2. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967
3. Schmidt-Lucke C, Fichtlscherer S, Aicher A, Tschöpe C, Schultheiss HP, Zeiher AM, Dimmeler S (2010) Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol. PLoS One 5:e13790. doi:10.1371/journal.pone.0013790
4. Kawamoto A, Losordo DW (2008) Endothelial progenitor cells for cardiovascular regeneration. Trends Cardiovasc Med 18:33–37. doi:10.1016/j.tcm.2007.11.004
5. Britten MB, Abolmaali ND, Assmus B, Lehmann R, Honold J, Schmitt J, Vogl TJ, Martin H, Schächinger V, Dimmeler S, Zeiher AM (2003) Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging. Circulation 108:2212–2218
6. Tanaka R, Masuda H, Kato S, Imagawa K, Kanabuchi K, Nakashioya C, Yoshiba F, Fukui T, Ito R, Kobori M, Wada M, Asahara T, Miyasaka M (2014) Autologous G-CSF-mobilized

peripheral blood $CD34^+$ cell therapy for diabetic patients with chronic nonhealing ulcer. Cell Transplant 23:167–179. doi:10.3727/096368912X658007
7. Grisar JC, Haddad F, Gomari FA, Wu JC (2011) Endothelial progenitor cells in cardiovascular disease and chronic inflammation: from biomarker to therapeutic agent. Biomark Med 5:731–744. doi:10.2217/bmm.11.92
8. Fabbri-Arrigoni FI, Clarke L, Wang G, Charakida M, Ellins E, Halliday N, Brogan PA, Deanfield JE, Halcox JP, Klein N (2012) Levels of circulating endothelial cells and colony-forming units are influenced by age and dyslipidemia. Pediatr Res 72:299–304. doi:10.1038/pr.2012.76
9. Campioni D, Zauli G, Gambetti S, Campo G, Cuneo A, Ferrari R, Secchiero P (2013) In vitro characterization of circulating endothelial progenitor cells isolated from patients with acute coronary syndrome. PLoS One 8:e56377. doi:10.1371/journal.pone.0056377
10. Cribbs SK, Sutcliffe DJ, Taylor WR, Rojas M, Easley KA, Tang L, Brigham KL, Martin GS (2012) Circulating endothelial progenitor cells inversely associate with organ dysfunction in sepsis. Intensive Care Med 38:429–436. doi:10.1007/s00134-012-2480-9
11. Khoo CP, Pozzilli P, Alison MR (2008) Endothelial progenitor cells and their potential therapeutic applications. Regen Med 3:863–876. doi:10.2217/17460751.3.6.863
12. Meier P, Gloekler S, Oezdemir B, Indermuehle A, Traupe T, Vogel R, de Marchi S, Seiler C (2013) G-CSF induced arteriogenesis in humans: molecular insights into a randomized controlled trial. Curr Vasc Pharmacol 11:38–46
13. Kimura H, Okubo N, Chosa N, Kyakumoto S, Kamo M, Miura H, Ishisaki A (2013) EGF positively regulates the proliferation and migration, and negatively regulates the myofibroblast differentiation of periodontal ligament-derived endothelial progenitor cells through MEK/ERK- and JNK-dependent signals. Cell Physiol Biochem 32:899–914. doi:10.1159/000354493
14. Takahashi M, Okubo N, Chosa N, Takahashi N, Ibi M, Kamo M, Mizuki H, Ishisaki A, Kyakumoto S (2012) Fibroblast growth factor-1-induced ERK1/2 signaling reciprocally regulates proliferation and smooth muscle cell differentiation of ligament-derived endothelial progenitor cell-like cells. Int J Mol Med 29:357–364. doi:10.3892/ijmm.2011.847
15. Qiu C, Xie Q, Zhang D, Chen Q, Hu J, Xu L (2014) GM-CSF Induces Cyclin D1 Expression and Proliferation of Endothelial Progenitor Cells via PI3 K and MAPK Signaling. Cell Physiol Biochem 33:784–795. doi:10.1159/000358652
16. Yang L, Guo XG, Du CQ, Yang JX, Jiang DM, Li B, Zhou WJ, Zhang FR (2012) Interleukin-1 beta increases activity of human endothelial progenitor cells: involvement of PI3K-Akt signaling pathway. Inflammation 35:1242–1250. doi:10.1007/s10753-012-9434-9
17. Katz M, Amit I, Yarden Y (2007) Regulation of MAPKs by growth factors and receptor tyrosine kinases. Biochim Biophys Acta 1773:1161–1176
18. Graves LM, Guy HI, Kozlowski P, Huang M, Lazarowski E, Pope RM, Collins MA, Dahlstrand EN, Earp HS 3rd, Evans DR (2000) Regulation of carbamoyl phosphate synthetase by MAP kinase. Nature 403:328–332
19. Vantaggiato C, Formentini I, Bondanza A, Bonini C, Naldini L, Brambilla R (2006) ERK1 and ERK2 mitogen-activated protein kinases affect Ras-dependent cell signaling differentially. J Biol 5:14