약학석사 학위논문

에리스로포이에틴을 이용한 G-CSF 동원 말초혈액 자가줄기세포의 혈관 형성능 향상에 관한 연구

2014년 8월

서울대학교 대학원
분자의학 및 바이오제약 학과
분자의학 및 바이오제약 전공

윤 지 연

약학석사 학위논문

에리스로포이에틴을 이용한 G-CSF 동원 말초혈액 자가줄기세포의 혈관 형성능 향상에 관한 연구

2014년 8월

서울대학교 대학원
분자의학 및 바이오제약 학과
분자의학 및 바이오제약 전공

윤 지 연

에리스로포이에틴을 이용한 G-CSF 동원 말초혈액 자가줄기세포의 혈관 형성능 향상에 관한 연구

지도교수 김 효 수

이 논문을 약학석사 학위논문으로 제출함

2014년 6월

서울대학교 대학원

분자의학 및 바이오제약 학과

분자의학 및 바이오제약 전공

윤 지 연

윤 지 연의 석사학위논문을 인준함

2014년 6월

위 원 장 이 동 수 (인)

부 위 원 장 김 효 수 (인)

위 원 박 경 수 (인)

논 문 초 록

에리스로포이에틴을 이용한 G-CSF 동원 말초혈액 자가줄기세포의 혈관 형성능 향상에 관한 연구

Erythropoietin-Priming enhances angiogenic potential of human peripheral blood stem cells mobilized with granulocyte colony-stimulating factor

윤 지 연 (Ji-Yeon Yun)

분자의학 및 바이오제약 학과
(Molecular Medicine and Biopharmaceutical Sciences)

분자의학 및 바이오제약 전공
(Molecular Medicine and Biopharmaceutical Sciences)

The Graduate School

Seoul National University

Stem cell therapy in ischemic diseases, such as myocardial infarction (MI) has been regarded as a new method to repair ischemic tissue

and promote neovascularization. Since the first in-human clinical trial using autologous bone marrow stem cells,[1] numerous clinical trials of cell therapy have been carried out with various results. Recent several reports of meta-analysis[2-4] demonstrated the promising results that stem cell therapy improves cardiac function and reduces infarct after acute MI.[5]

Despite the proven efficacy of stem cell therapy, there is still limitation in the therapeutic efficacy of current stem cell therapy for patients with ischemic heart disease. As a source of stem cells for clinical trials, autologous bone marrow mononuclear cells have been most frequently used. Alternative to bone marrow cells that require bone marrow puncture for harvest, we have used mobilized peripheral blood stem cells (^{mob}PBSCs) that are enriched through mobilization of bone marrow cells by granulocyte colony stimulating factor (G-CSF) and apheresis process in MAGIC-CELLtrials.[6] The proportion of angiogenic progenitors in ^{mob}PBSCs was significantly increased and became comparable to bone marrow monocytes.[7] We have demonstrated that intracoronary infusion of the ^{mob}PBSCs by G-CSF was effective in improving myocardial contractility and reducing infarct volume in patients with acute MI. Furthermore it improved long-term clinical outcomes.[8]

However,the therapeutic efficacy was limited mainly due to the poor retention rate after intracoronary infusion of the cells and the insufficient regenerative potency of the adult progenitors like ^{mob}PBSCs.[9,10] As a practical breakthrough, we thought that priming of

the ^{mob}PBSCs before infusion would be a feasible and reasonable strategy and that Erythropoietin(EPO) would be a good candidate as a priming agent.

EPO, an erythropoietic growth factor that promotes survival, proliferation, and differentiation of erythroid progenitor cells,[11]has long been used for cell based therapy in ischemic diseases. The EPOreceptor (EPOR) is a 59kDa protein foundnotonly in erythroidcells, but also in endothelialcells[12] and mononuclear blood cells.[13]Along with its erythropoietic effect, EPO is known to have various ischemia-protective properties while the mechanisms were not yet fully elucidated.[14] Previous studies have shown that EPO increased endothelial cell proliferation and neovascularization,[15] induces the proliferation, differentiation and adhesion of progenitor cells,[16] and has anti-apoptotic effects.[17]

Thus in this study, we checked the presence of EPO R on ^{mob}PBSCs and evaluated the effect of EPO-priming on ^{mob}PBSCs in terms of angiogenic properties in vitro and in vivo.

Keywords :

Erythropoietin(EPO), G-CSF, Peripheral blood stem cell, Priming, Cardiovascular disease, Angiogenesis

Student Number : 2012- 24150

Contents

List of Tables

List of Figures

Introduction

Stem cell therapy in ischemic diseases, such as myocardial infarction (MI) has been regarded as a new method to repair ischemic tissue and promote neovascularization. Since the first in-human clinical trial using autologous bone marrow stem cells,[1] numerous clinical trials of cell therapy have been carried out with various results. Recent several reports of meta-analysis[2-4] demonstrated the promising results that stem cell therapy improves cardiac function and reduces infarct after acute MI.[5] Despite the proven efficacy of stem cell therapy, there is still limitation in the therapeutic efficacy of current stem cell therapy for patients with ischemic heart disease. As a source of stem cells for clinical trials, autologous bone marrow mononuclear cells have been most frequently used. Alternative to bone marrow cells that require bone marrow puncture for harvest, we have used mobilized peripheral blood stem cells(^{mob}PBSCs) that a reenriched through mobilization of bone marrow cells by granulocyte colony stimulating factor(G-CSF) and apheresis process in MAGIC-CELL trials.[6] The proportion of angiogenic progenitors in ^{mob}PBSCs was significantly increased and be came comparable to bone marrow monocytes.[7] We have demonstrated that intracoronary infusion of the ^{mob}PBSCs by G-CSF was effective in improving myocardial contractility and reducing infarct volume in patients with acute MI. Furthermore it improved long-term clinical outcomes.[8] However, the therapeutic efficacy was limited mainly due to the poor retention rate after intracoronary infusion of the cells and the insufficient

regenerative potency of the adult progenitors like ^{mob}PBSCs.[9,10] As a practical breakthrough, we thought that priming of the ^{mob}PBSCs before infusion would be a feasible and reasonable strategy and that Erythropoietin (EPO) would be a good candidate as a priming agent.

EPO, an erythropoietic growth factor that promotes survival, proliferation, and differentiation of erythroid progenitor cells,[11] has long been used for cell based therapy in ischemic diseases. The EPO receptor (EPOR) is a 59 kDa protein found not only in erythroid cells, but also in endothelial cells[12] and mononuclear blood cells.[13]Along with its erythropoietic effect, EPO is known to have various ischemia-protective properties while the mechanisms were not yet fully elucidated.[14] Previous studies have shown that EPO increased endothelial cell proliferation and neovascularization,[15] induces the proliferation, differentiation and adhesion of progenitor cells,[16] and has anti-apoptotic effects.[17]

Thus in this study, we checked the presence of EPO R on ^{mob}PBSCs and evaluated the effect of EPO-priming on ^{mob}PBSCs in terms of angiogenic properties in vitro and in vivo.

Methods & Materials

EPO priming of peripheral blood stem cells enriched by G-CSF injection

Peripheral blood (200 mL) was obtained from 4 normal volunteers with informed consent. The normal volunteers underwent daily subcutaneous injections of G-CSF (Dong-A Pharmaceutical, Seoul, Korea) at 10 μg/kg body weight for 3 days. Immediately after the blood was obtained, mononuclear cells were fractionated from other components of peripheral blood by centrifugation on Ficoll-Plaque (GE Healthcare) gradient. Mononuclear cells were immediately used for experiments with or without EPO priming for 6 hours. In experiments to test the cytokine release by EPO-primed cells, EPO-primed ^{mob}PBSCs were cultured for 36 hours and the supernatant was used to evaluate secreted cytokines.

EPO priming was consisted of a 6h incubation with human recombinant EPO (Espogen prefilled injection®, 10,000 IU/mL, LG Life Science) diluted in PBS, at a concentration of 10 IU/mL. A portion of ^{mob}PBSCs were used for analysis immediately after EPO priming and others were washed and cultured in EBM2 with 5% of FBS (Lonza).

To block the EPO pathway, EPO R blocking antibody (Santa Cruz), JAK inhibitor (AG490, Sigma) and AKT inhibitor (LY294002, Sigma) were used. Also for inhibiting integrin in the adhesion assay, integrin β1 and integrin β2 neutralizing antibodies (Santa Cruz) were used.

Cell sorting by Fluorescence-Activated Cell Sorter

^{mob}PBSCs were sorted into EPO R(+) and EPO R(-) respectively by Fluorescence-Activated Cell sorter (BD Aria). To sort the composition of ^{mob}PBSCs depending on the expression of EPOR, PE-conjugatedanti-EPO receptor antibody (R&D) was used. Isotype-matched IgG was used as a control.

qRT-PCR

To evaluate the gene expression of IL8, IL10, bFGF, MMP2, MMP9, Integrinα4, Integrinα5, IntegrinαV, Integrinβ1, Integrin β2 and Integrin β8, total RNA was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using the reverse transcription system (TAKARA), amplified with Power SYBR Green PCR master mix (Applied Biosystems), using an ABI PRISM TM 7500 Sequence Detection System (Applied Biosystems). Experiments were conducted to contrast relative levels of each transcript and endogenous control GAPDH in every sample. Information of corresponding primers is provided in Supplementary Table 3.

Acquisition of conditioned medium and measurement of cytokine concentration

After 36 hours of cell culture, the supernatant was acquired by removing cellular debris via centrifuging.The concentration of secreted cytokines/ chemokines was determined by using the Bio-PlexProt^{TM}ArraySystem(kitsandequipmentofBio-Rad,USAbasedonLu

minexxMAPtechnology)inthecaseofTNF-α,IL-8,IL-10andbasicFGF.

Matrigel tube formation assay

In vitro Matrigel tube formation assay were performed as previously described with following modifications.[2] Briefly, 80μl of growth factor-reduced(GFR) Matrigel(BDbiosciences) was coated on 35-mm confocal dish(Ibidi) and incubated 30minutes for semi-solid polymerization. $4x10^4$ HUVECs were resuspended in vehicle primed ^{mob}PBSCs culture supernatantand EPO-primed ^{mob}PBSCs culture supernatant respectively and added to each confocal dish. After 8h, the morphology of the tubes was visualized with microscope (Olympus, Tokyo, Japan).

Migration assay

For the scratch wound healing assay, HUVECs were grown on a 6-well plate to 100% confluence. The monolayer was then scratched with a constant power using a 200 μL pipette tip and washed three times with PBS. Afterwards, the media was changed to vehicle primed ^{mob}PBSCs supernatant and EPO-primed^{mob}PBSCs culture supernatant and was incubated for 36 hours. Cell migration with in the initial scratch was measured by comparing the initial photo with a photo following incubation. Scratch width was measured off line using ImageJ software.

In vitro adhesion assay

In vitro adhesion assay were performed as previously described with slight modifications.[1] Vehicle or EPO-primed^{mob}PBSCs were labeled

with 5uM carboxyfluorescein diacetate succinimidylester (CFSE;MolecularProbes), and then $2x10^5$ cells per well were incubated on Human umbilical vein endothelial cells (HUVECs) monolayer ($1x10^5$cellsperplate) and Fibronectin (50ug/ml,Sigma) coated plate respectively in EBM-2 (Lonza) with 5% FBS (Gibco) for 30 minutes, 1hour and 2 hours at 37°C. After removing non-adherent cells with PBS, the number of CFSE-labeled cells bound to the Fibronectin-coated dish was quantified under fluorescent microscopy.

In Vivo Vasculogenesis of mixed cells in Ischemic Limb of Nude Mouse

All procedures were approved by the Experimental Animal Committee of Clinical Research Institute, Seoul National University Hospital, Seoul, Korea. Male athymic nude mice 7 to 9 weeks old, and 17 to 20 g in weight, were anesthetized with 50 mg/kg intraperitoneal pentobarbital for Matrigel plug injection or unilateral femoral artery ligation. For in vivo 3D Matrigel angiogenesis assay, four Matrigel (BD) plugs which contained four groups of $2x10^5$ cells in 350uL Matrigel were subcutaneously injected into each quadrant of the dorsum of the mice. For evaluation of the therapeutic neovasculogenesis using EPO-primed^{mob}PBSCs, surgery to induce hindlimb is chemia was performed as described. Three to six hours afte r surgery, $2x10^5$ cells from the respective groups in 50μL serum-free EBM medium were injected into the hindlimb intramuscularly.Laser Dopplerper fusion image analyzer (MoorInstrument, Wilmington,DE) was used to record serial blood

flow measurements over the course of 3 weeks after operation. Mice were sacrificed on day 21 for histologic evaluation and immunofluorescent staining. For the 3D Matrigel angiogenesis assay, Matrigel (350mcl) plugs containing $3x10^5$ cells, basicFGF (1μg/ml) and VEGF (1μg/ml) was injected subcutaneously in each quadrant of the dorsal region of 6-9 week old mice. Plugs were recovered after 3 weeks.

For histologic evaluation of arterioles, deparaffinized 4 mm-thick sections of adductor muscles were stained with anti-a-smooth muscle actin (a-SMA, Sigma) antibody. Five fields were randomly selected by a blinded observer and the area and count of arterioles were assessed using ImageJ software in five different randomized fields per slide (original magnification, *40). Also to evaluate the incorporated human cells in the ischemic muscles, the samples were costained with anti-human CD34 antibody (DAKO) followed by incubation with FITC conjugated secondary antibodies with anti-mouse SMA antibodies.

Myocardial infarction models of Nude Mouse

The mice were anesthetized with 50 mg/kg intraperitoneal pentobarbital and then fixed in the supine position. Then, the skin was dissected by a lateral 1.0 cm cut along the left side of the sternum, and the main trunk of the left coronary artery was ligated. Immediately after ligation, $2x10^5$ cells from the respective groups in 30μL serum-free EBM medium, or PBS were injected into the a pex of the mouse. Harvest was done 2 weeks after surgery.

Image Acquisition and Analysis

Images were obtained by an Olympus IX2 inverted fluorescence microscope (Olympus) equipped with an Olympus DP50 CF CCD camera and analySIS 5.0 software and Confocal images were obtained by Zeiss LSM-710 META confocal microscope (Olympus) and ZEN 2009 analysis software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, Calif). Results are expressed as the mean±standard error of the mean. Comparisons between groups were performed using analysis of variance, with post hoc testing performed with Bonferroni analysis or unpaired t-tests, as appropriate. Values of $P<0.05$ were considered statistically significant.

Results

EPO-priming induced the angiogenic factors and integrins on EPO R (+) ^{mob}PBSCs

The volunteer demographics and the complete blood count before and after G-CSF injection are shown in Table 1. The effect of G-CSF mobilization was proven by the Complete Blood Count, which was done immediately before peripheral blood collection. G-CSF injection significantly increased the number of total leukocytes, segmented neutrophils and monocytes (Table 2). We analyzed the effect of EPO-priming on ^{mob}PBSCs in two experimental steps; with or without FACS sorting by EPOR (Figure1).

EPO R was expressed on 36.8±4.16% of ^{mob}PBSCs. We sorted the cells by the presence of EPORs ,into EPOR(+) ^{mob}PBSCs and EPOR(-)^{mob}PBSCs. The purity of the sorted cells was confirmed by FACS (Figure2A) and immunofluorescence staining (Figure2B). Before and after EPO-priming of the FACS sorted cells, we screened them RNA level of several cytokines. The expression of genes before EPO-priming, varied according to the presence of EPOR.The expression of several genes such as IL8, TNFα and MMP9 was higher in EPOR(+) cells than in EPOR(-) one seven before priming. However, after EPO-priming, the gene expression difference was significantly augmented. EPO-priming induced specifically EPOR(+) cells to markedly express IL8, IL10, bFGF, MMP2, integrinαV, β1, β2 and β8, which is assumed as the effect of EPO-priming (Figure1C).

Considering that IL-8, IL-10, bFGF, and TNFα are well known cytokines having angiogenic potentials and that MMP9 and integrinsareessentialinangiogeniccellrecruitment,wefocusedontheangiogen iceffectof ex vivo EPO-priming.

The effect of EPO-priming in the whole ^{mob}PBSCs and its signaling pathway

In the future clinical application, FACS sorting of ^{mob}PBSCs by EPOR would be a certain burden in terms of practic ability of the protocol.Moreover ^{mob}PBSCs are currently used cells for stem cell therapy and have proven to be effective in our prior trials.[8-10] Thus, using the whole cell group (without EPO R sorting), we assessed the effect of EPO-priming. EPO-priming of whole ^{mob}PBSCs also significantly induced the expression of IL8, IL10, basicFGF, MMP9, IntegrinαV, and β8 compared to veh-priming (Figure3). Although the significant increase in some molecules disappeared in the whole cell group (i.e. integrinβ1 and β2), EPO-priming was still proved to increase the expression of angiogenic molecules in whole ^{mob}PBSCs that are used in MAGIC-CELL clinical trial.[8]

To confirm the signaling pathway down to EPO R, we performed Western blot analysis for total and phosphorylated JAK2 and Akt protein which are known as downstream of EPO/EPO R axis. After 5 minutes of EPO-priming, these signaling molecules are phosphorylated and activated, which was blocked by the EPO R antibody (Figure 4A). Next, we examined the effect of JAK or Akt inhibitor on the

release of pro-angiogenic growth factors from these cells. EPO-priming significantly increased mRNA expression of IL8, IL10, and bFGF, which was effectively prevented by EPO R blocking antibody, JAK inhibitor, or Akt inhibitor (Figure 4B). ELISA of supernatant from ^{mob}PBSCs demonstrated that secretion of these factors was remarkably induced by EPO-priming, which was obliterated by EPOR blocking antibody, JAK inhibitor, or Akt inhibitor (Figure4C).

Two faceted effects of EPO-priming on ^{mob}PBSCs: paracrine action and adhesion on endothelial cells

^{mob}PBSCs can directly take part in angiogenesis, whereas the paracrine effect may also be important. Especially, monocytes are known to differentiate into various cells and secrete cytokines which are pro-angiogenic.[18] A Matrigel tube formation assay was used to check whether the culture supernatant of EPO-primed ^{mob}PBSCs can induce angiogenesis. Formation of capillary-like structures was significantly enhanced when human umbilical vein endothelial cells (HUVECs) were cultured in the supernatant of EPO-primed^{mob}PBSCs rather than in the supernatant from veh-primed^{mob}PBSCs (Figure5A). Also in a random migration assay, the supernatant of EPO-primed ^{mob}PBSCs induced a significantly large amount of migration of HUVECs (Figure5B). Both tube formation and migration of endothelial cells were significantly disturbed by pretreatment with EPO R blocking antibody, JAK inhibitor, or Akt inhibitor (Figure5A,B).

Also, adhesion of ^{mob}PBSCs to either endothelial cells or extracellular matrix is known to be the initial step during formation of new blood vessels.[19] Therefore, we assessed the consequence of the induced integrins on the surface of EPO-primed ^{mob}PBSCs through in vitro adhesion assay on fibronectin(50μg/ml) or on HUVECs(1X10^{5}cellsperplate). EPO-priming increased ^{mob}PBSCs adhesion to both fibronectin and HUVECs, which were reduced by EPO R blocking antibodies, integrinβ1 and β2 neutralizing antibodies (Figure6A-C).

EPO-primed ^{mob}PBSCs enhance neovascularization in ischemic limb and repair myocardium after infarction through cellular and humoral mechanisms

Based on the prior results of increased angiogenic potential by EPO-priming, we investigated the angiogenic effect of EPO-primed ^{mob}PBSCsusingischemichindlimbmodelofathymicnudemice.Also,weinjected PBS,veh-primed^{mob}PBSCs,orEPO-primed^{mob}PBSCsintotheischemichindlimbofathymicnudemice. By serial measurement of the blood flow by the LDPI analyzer on day 0, 3, 7, 14 and 21, the blood flow recovery to the ischemic hindlimb was significantly facilitated by EPO-primed ^{mob}PBSCs, compared to PBS or veh-primed^{mob}PBSCs(Figure7A). For quantification of angiogenesis, we counted the number and area of newly formed mature vessels in the ischemic limb. EPO-primed^{mob}PBSCs significantly increased the number and area of SMA(+) vessels(Figure7B). Furthermore, we confirmed the direct

incorporation of human ^{mob}PBSCs into mouse vessels through staining with human specific endothelial marker anti-CD34 antibody(Figure7C).

Another important indication of EPO-primed cell is the myocardial repair after infarction. Thus we made myocardial infarction in nude mice and compared therapeutic efficacy of EPO-primed ^{mob}PBSCs versus veh-primed^{mob}PBSCs versus PBS control. EPO-primed^{mob}PBSC showed a significantly better therapeutic efficacy than veh-primed cell or PBS control in terms of infarct size and infarct wall thickness. Veh-primed cell was better than PBS control(Figure8A). Furthermore, injection of EPO-primed^{mob}PBSC resulted in the least a mount of cardiac fibrosis compared with the other two treatments(Figure 8B).

Next, to show the paracrine angiogenic properties of ^{mob}PBSCs in vivo study, we created mouse ischemic hind limb models and injected cell supernatant from primed^{mob}PBSCs(Figure 9 A-B). As a result, injection of supernatant from EPO-primed ^{mob}PBSC showed a significantly better efficacy than the supernatant from veh-primed^{mob}PBSC or PBS control in terms of blood flow recovery to ischemic limb(Figure9A), as well as neovascularization with mature vessels(Figure9A-B).

Discussion

Through this study we have shown the beneficial effect of *ex vivo* EPO-priming to increase the angiogenic potential of human mobilized peripheral blood stem cells after G-CSF administration. By EPO-priming, ^{mob}PBSCs increased the synthesis of cytokines and integrins that involve angiogenesis. In vivo models also demonstrated that injection of EPO-primed ^{mob}PBSCs achieved a greater vasculogenesis in the ischemic tissue than that of veh-primed^{mob}PBSCs did.

These findings strongly suggest that ex vivo EPO-priming of ^{mob}PBSCs can be a feasible and effective method to augment the efficacy of cytokine-base peripheral blood stem cell therapy for patients with MI(Figure10).

New strategy to overcome the limited efficacy of adult stem cell therapy

During the past decade, a vast body of researches has been done on stem cell therapy for patients with MI and reported significant efficacy in improving contractility and reducing infarct scar.[2-4] But the problem was the limited efficacy; improvement of left ventricular ejection fraction just by 3% and reduction of infarct size just by 5 ml. Several methods to overcome such a limitation have been proposed, such as, increasing cell numbers, genetic manipulation of

the adult progenitor cells, or adoption of potent stem cells rather than adult progenitor cells. Increasing cell numbers may not be feasible because of the technical limitation of intracoronary or trans-catheter endomyocardial injection. Genetic manipulation or adoption of more potent cells derived from embryonic stem cells may encounter the safety issue before clinical application. Under these situations, priming of the adult progenitor cells before implantation would be a very feasible and safe way to augment the therapeutic efficacy, which has been well demonstrated by our previous report.[20] In this previous paper, we found that most of ^{mob}PBSCs expressed Tie-2 receptor and that priming of^{mob}PBSCs with angiopoietin-1 significantly enhanced the transdifferentiation of^{mob}PBSCs to endothelial lineage by turning on Ets-1 factor and the expression of integrins on the surface of ^{mob}PBSCs, leading to significant improvement of retention efficiency and therapeutic efficacy of ^{mob}PBSCs after intra-arterial delivery.[20] But we encountered the unexpected difficulty to realize this concept in clinics because it is very difficult to make the potent angiopietin-1 as human- or clinical-grade to meet the economic criteria during mass production. This is the reason why we searched for another cell-primer that is feasible in clinical application and paid attention to EPO because it is currently used in clinics and has several beneficial effects on cardiovascular system in the situation of MI.

EPO as a priming agent

EPO, being a well-known cytokine controlling erythropoiesis, has been tried to patients with MI with disappointing results, probably

because of the insufficient local concentration at the target infarcted myocardium or because of the unwanted systemic effects.[21, 22] With adoption of *ex vivo* cell priming strategy, we expect several benefits; to maximize the effects of EPO on target cells, i.e. enhanced proliferative, angiogenic and anti-apoptotic properties, while to avoid the systemic side effects of EPO. With this *ex vivo* cell-specific priming strategy, we could use the highest dose of 10 IU/ml, which is known to have maximal proliferative effect in prior studies.[16, 23]

In a previous study,[24] EPO was once used as a priming agent for late endothelial progenitor cells (EPCs) that already have good angiogenic potentials with high expression of VEGFR-2, CD31 and Tie2.[25] However late EPCs are not actively used in the current clinical cell therapy, due to the burden of ex vivo cell culture (requires at least 10 days for culture), limited replicative capacity and the possibility of contamination in the process of ex vivo manipulation.[26] In contrast, ^{mob}PBSCs have been used for 10 years' MAGIC-CELL program[6, 9, 10] and our strategy to prime these cells ex vivo requires just several hours' incubation without ex vivo cell culture.

Specific effects of EPO priming through EPO-EPOR pathway

About 36 % of ^{mob}PBSCs were positive for EPOR and these cells were used to evaluate the specific effects of EPO-priming through its receptor. EPO-primed^{mob}PBSCs showed dramatic increases of various angiogenic molecules. Especially IL8 increased in a 38.8-fold, IL10 in a 33.3-fold and bFGF in a 96.8-fold .EPO-priming also induced

integrins which are essential for adhesion on the vascular lumen, trans-endothelial migration, further extra-vascular migration of the circulating or intra-arterially delivered ^{mob}PBSCs. These effects of EPO-priming were all reversed by EPOR blocking antibodies. This proved the rationale of using EPO to augment the angiogenic potential of ^{mob}PBSCs. Previous studies have shown that EPO can affect endothelial cells, vascular smooth muscle cells, neuronal cells, myeloid cells, etc. and can provide cytoprotectionto ischemia through the JAK2, AKT, and ERK pathway in a parallel order. This pathway can maintain the mitochondrial membrane potential, prevent the cellular release of cytochrome C, and modulate caspase activity.[27]

Furthermore, we also checked the effect of EPO-priming on whole population of ^{mob}PBSCs without EPOR sorting. In the practical point of view, ex vivo cell sorting or purification process by FACS would be a limiting step that may be associated with damage to cell viability, risk of contamination, deprivation of possible cell-to-cell synergistic interactions, or economic burden. That is the rationale for us to use whole ^{mob}PBSCs without sorting in MAGIC-CELLtrials.[6, 8, 10, 25] We were able to reproduce the beneficial effect of EPO-priming also in the whole ^{mob}PBSCs population in terms of in vitro angiogenic gene expression as well as *in vivo* vasculogenic effect in the two different models, proving this strategy readily applicable in the clinic. The effect of EPO-priming could be classified into two levels; the paracrine effect to form a vasculogenic niche and the direct cell-modulating effect.

Paracrine action of EPO-primed ^{mob}PBSCs : Formation of an Angiogenic niche

Secretion of cytokines such as IL8 and IL10 from EPO-primed ^{mob}PBSCs may be helpful to make the angiogenic niche in the target ischemic tissue when these cells delivered. IL8 is a well-known cytokine that enhance proliferation and survival of endothelial cells by activation of MMP2 and MMP9.[28, 29] IL10 is also a well-known angiogenic cytokine that induces neovascularization and induce pro-vasculogenic phenotype of macrophages.[30] This paracrine effect was proven by functional analysis where culture supernatant of EPO-primed ^{mob}PBSCs exerted benefits to induce tube formation and cell migration of HUVECs. Also, in vivo models showed that cell supernatant of EPO-primed ^{mob}PBSCs improved perfusion in ischemia, which can support the paracrine action. Interestingly, there have been several papers concerning exosome identified as the cardioprotective component in MSC paracrine secretion.[32,33] Exosomes as small lipid vesicles, represent an ideal vehicle to effect an immediate physiological response to repair and recover from injury by delivering functional proteins rapidly into the cells. Recently, it was demonstrated not only to proteins but also microvesicles have the potential to mediate intercellular transfer of genetic material such as miRNA.[34]Although we confirmed the active components in conditioned media of EPO-primed^{mob}PBSCs limited to cytokines and growth factor-mediated extracellular signaling, there was high probability of exosome secreted by EPO-primed^{mob}PBSCs and it might have a cardioprotective effect. The need to identify the other active

surrogates remains to be done.

Direct cell-modulation by EPO-priming

EPO-priming upregulated integrin expression that is known to have an important role in vasculogenesis, including facilitation of wound healing.[5] Various combinations of integrins are known to enhance cell homing and angiogenesis.[5] Specifically, integrins αV, β1, β2 and β8 increased in gene expression by EPO-priming. The implication of integrins' induction was well demonstrated by the functional analysis where EPO-primed ^{mob}PBSCs showed stronger adhesion to HUVEC and fibronectin than the veh-primed^{mob}PBSCs.

Taken all together, EPO-priming could induce vasculogenesis by providing vasculogenic niche to target ischemic tissue through paracrine action of ^{mob}PBSCs, by directly upregulating the expression of integrins, and by inducing polarization of monocyte phenotype to vasculogenic monocytes.

Conclusion

Ex vivo EPO-priming augmented the angiogenic potential of human ^{mob}PBSCs. EPO-primed^{mob}PBSCs provide vasculogenic niche to the target ischemic tissue by expression of angiogenic cytokines and more easily incorporate into the target ischemic tissue through upregulated integrins as well as they differentiate into angiogenic monocytes. Such mechanisms are well supported by the enhanced angiogenesis, limb and myocardial salvage, and evidence of incorporation of human cell in mice limb after transplantation of EPO-primed^{mob}PBSCs in nude mice. EPO-priming can be a promising and practical method to augment the therapeutic efficacy of ^{mob}PBSCs in the cytokine-based cell therapy for patients with MI.

REFERENCES

1. Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*. 2002;106:1913-1918
2. Lipinski MJ, Biondi-Zoccai GG, Abbate A, Khianey R, Sheiban I, Bartunek J, et al. Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: A collaborative systematic review and meta-analysis of controlled clinical trials. *Journal of the American College of Cardiology*. 2007;50:1761-1767
3. Delewi R, Andriessen A, Tijssen JG, Zijlstra F, Piek JJ, Hirsch A. Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: A meta-analysis of randomised controlled clinical trials. *Heart*. 2013;99:225-232
4. Jeevanantham V, Butler M, Saad A, Abdel-Latif A, Zuba-Surma EK, Dawn B. Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: A systematic review and meta-analysis. *Circulation*. 2012;126:551-568
5. Avraamides CJ, Garmy-Susini B, Varner JA. Integrins in angiogenesis and lymphangiogenesis. *Nat Rev Cancer*. 2008;8:604-617
6. Kang HJ, Kim HS, Zhang SY, Park KW, Cho HJ, Koo BK, et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: The magic cell randomised clinical trial. *Lancet*. 2004;363:751-756
7. Kang HJ, Yoon EJ, Lee EJ, Kim MK, Suh JW, Park KW, et al. Cotreatment with darbepoetin and granulocyte colony-stimulating factor is efficient to recruit proangiogenic cell populations in patients with acute myocardial infarction. *Cell transplantation*. 2012;21:1055-1061
8. Kang HJ, Kim MK, Lee HY, Park KW, Lee W, Cho YS, et al. Five-year results of intracoronary infusion of the mobilized peripheral blood stem cells by granulocyte colony-stimulating factor in patients with myocardial infarction. *European heart journal*. 2012;33:3062-3069
9. Kang HJ, Kim HS, Koo BK, Kim YJ, Lee D, Sohn DW, et al. Intracoronary infusion of the mobilized peripheral blood stem cell by g-csf is better than

mobilization alone by g-csf for improvement of cardiac function and remodeling: 2-year follow-up results of the myocardial regeneration and angiogenesis in myocardial infarction with g-csf and intra-coronary stem cell infusion (magic cell) 1 trial. *American heart journal*. 2007;153:237 e231-238

10. Kang HJ, Lee HY, Na SH, Chang SA, Park KW, Kim HK, et al. Differential effect of intracoronary infusion of mobilized peripheral blood stem cells by granulocyte colony-stimulating factor on left ventricular function and remodeling in patients with acute myocardial infarction versus old myocardial infarction: The magic cell-3-des randomized, controlled trial. *Circulation*. 2006;114:I145-151
11. Sato T, Maekawa T, Watanabe S, Tsuji K, Nakahata T. Erythroid progenitors differentiate and mature in response to endogenous erythropoietin. *J Clin Invest*. 2000;106:263-270
12. Anagnostou A, Liu Z, Steiner M, Chin K, Lee ES, Kessimian N, et al. Erythropoietin receptor mrna expression in human endothelial cells. *Proc Natl Acad Sci U S A*. 1994;91:3974-3978
13. Lisowska KA, Debska-Slizien A, Bryl E, Rutkowski B, Witkowski JM. Erythropoietin receptor is expressed on human peripheral blood t and b lymphocytes and monocytes and is modulated by recombinant human erythropoietin treatment. *Artif Organs*. 2010;34:654-662
14. Joshi D, Tsui J, Yu R, Shiwen X, Selvakumar S, Abraham DJ, et al. Potential of novel epo derivatives in limb ischemia. *Cardiol Res Pract*. 2012;2012:213785
15. Westenbrink BD, Lipsic E, van der Meer P, van der Harst P, Oeseburg H, Du Marchie Sarvaas GJ, et al. Erythropoietin improves cardiac function through endothelial progenitor cell and vascular endothelial growth factor mediated neovascularization. *European heart journal*. 2007;28:2018-2027
16. George J, Goldstein E, Abashidze A, Wexler D, Hamed S, Shmilovich H, et al. Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a pi 3-kinase-dependent manner. *Cardiovasc Res*. 2005;68:299-306
17. Burger D, Xenocostas A, Feng QP. Molecular basis of cardioprotection by erythropoietin. *Curr Mol Pharmacol*. 2009;2:56-69
18. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer*. 2008;8:618-631
19. Bischoff J. Cell adhesion and angiogenesis. *J Clin Invest*. 1997;100:S37-39

20. Kim MS, Lee CS, Hur J, Cho HJ, Jun SI, Kim TY, et al. Priming with angiopoietin-1 augments the vasculogenic potential of the peripheral blood stem cells mobilized with granulocyte colony-stimulating factor through a novel tie2/ets-1 pathway. *Circulation*. 2009;120:2240-2250
21. Taniguchi N, Nakamura T, Sawada T, Matsubara K, Furukawa K, Hadase M, Nakahara Y, Matsubara H. Erythropoietin prevention trial of coronary restenosis and cardiac remodeling after st-elevated acute myocardial infarction (epoc-ami): A pilot, randomized, placebo-controlled study. *Circ J*. 2010;74:2365-2371
22. Kagaya Y, Asaumi Y, Wang W, Takeda M, Nakano M, Satoh K, et al. Current perspectives on protective roles of erythropoietin in cardiovascular system: Erythropoietin receptor as a novel therapeutic target. *Tohoku J Exp Med*. 2012;227:83-91
23. Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, et al. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Blood*. 1999;93:2627-2636
24. Bennis Y, Sarlon-Bartoli G, Guillet B, Lucas L, Pellegrini L, Velly L, et al. Priming of late endothelial progenitor cells with erythropoietin before transplantation requires the cd131 receptor subunit and enhances their angiogenic potential. *J Thromb Haemost*. 2012;10:1914-1928
25. Yoon CH, Hur J, Park KW, Kim JH, Lee CS, Oh IY, et al. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: The role of angiogenic cytokines and matrix metalloproteinases. *Circulation*. 2005;112:1618-1627
26. Leeper NJ, Hunter AL, Cooke JP. Stem cell therapy for vascular regeneration: Adult, embryonic, and induced pluripotent stem cells. *Circulation*. 2010;122:517-526
27. Maiese K, Li F, Chong ZZ. New avenues of exploration for erythropoietin. *JAMA : the journal of the American Medical Association*. 2005;293:90-95
28. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. Il-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol*. 2003;170:3369-3376
29. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler osis, thrombosis, and vascular biology*. 2004;24:288-293

30. Dace DS, Khan AA, Kelly J, Apte RS. Interleukin-10 promotes pathological angiogenesis by regulating macrophage response to hypoxia during development. *PLoS One*. 2008;3:e3381

31. Ho VW, Sly LM. Derivation and characterization of murine alternatively activated (m2) macrophages. *Methods Mol Biol*. 2009;531:173-185

32. Ruenn Chai Lai, Fatih Arslan et al , Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem cell resarch*, 2009;170-171

33. Ruenn Chai Lai et al, Mesenchymal stem cell exosome : a novel stem cell-based therapy for cardiovascular disease, *Regenrative medicine*, 2011;481-492

34. P.J. Quesenberry et al, The paradoxical dynamism of marrow stem cells: considerations of stem cells, niches, and microvesicles, *Stem cell Rev*, 2008;137-147.

Tables

Table 1. Baseline characteristics and CBC results of the healthy volunteers

	Volunteer				Mean±SEM
	#1	#2	#3	#4	
Gender	M	M	M	M	NA
Age (years old)	22	22	24	28	24.0±1.4
Weight (kg)	78	72	70	78	74. 5±2.0
Height (cm)	178	174	172	180	176±1.9
BMI (kg/m^2)	24.6	23.8	23.7	24.1	24.1±0.2
Comorbidity	none	none	none	none	NA
Baseline lab					
Leukocytes, $10^3/mL$	5.36	5.43	4.30	5.38	5.12±0.27
Hemoglobin, g/dL	16.1	16.5	15.8	15.8	16.1±0.2
Platelet, $10^3/mL$	213	221	205	195	209±6
Segmented neutrophil (%)	62.1	50.5	48.9	36.5	49.5±5.3
Lymphocyte (%)	32.5	38.9	40.9	54.3	41.7±4.6
Monocyte (%)	4.3	8.3	8.1	7.1	7.0±0.9
CBC after completion of G-CSF injection					
Leukocytes, $10^3/mL$	29.00	43.90	32.10	35.7	35.0±3.0
Hemoglobin, g/dL	16.2	16.1	14.2	14.2	15.2±0.6
Platelet, $10^3/mL$	189	243	185	173	198±16
Segmented neutrophil (%)	82.7	88.4	87.2	87.0	86.3±1.3
Lymphocyte (%)	10.3	5.6	7.9	8.0	8.0±1.0
Monocyte (%)	4.5	3.5	2.9	2.0	3.2±0.5

Data are presented as mean±standard error of means (SEM), n=4

Abbreviations: BMI = body mass index, CBC = Complete blood count, G-CSF = Granulocyte colony stimulating factor

Table 2. Comparison of Complete blood count results before and after G-CSF injection*

	Before G-CSF injection	After G-CSF injection	P value†
Total Leukocytes, 10^3/mL	5.12±0.27	35.2±3.2	0.002
Hemoglobin, g/dL	16.1±0.2	15.2±0.56	0.135
Platelet, 10^3/mL	209±6	198±16	0.392
Segmented neutrophil, 10^3/mL	2.53±0.31	30.46±3.14	0.003
Lymphocyte, 10^3/mL	2.13±0.28	2.71±0.13	0.134
Monocyte, 10^3/mL	0.35±0.05	1.12±0.18	0.026

Data are presented as mean±standard error of means (SEM), n=4

* G-CSF at 10 μg/kg body weight (maximum: 600μg) was injected subcutaneously for 3 days

† P value was estimated by the paired T test method

Abbreviations: G-CSF = Granulocyte colony stimulating factor

Table 3. Primer sequences and the sizes of real time PCR products.

primer	Sequence		Tm(℃)	bp
IL8	FW	5′-GTGCAGTTTTGCCAAGGAGT-3′	60	135
	RV	5′-AATTTCTGTGTTGGCGCAGT-3′		
IL10	FW	5′- GCCTAACATGCTTCGAGATC-3′	58	206
	RV	5′- TGATGTCTGGGTCTTGGTTC-3′		
bFGF	FW	5′- GGCTATGAAGGAAGATGGAAGATT-3′	60	130
	RV	5′- TGCCACATACCAACTGGTGTATTT-3′		
MMP2	FW	5′- ACAGGAGGAGAAGGCTGTGTTCTT-3′	64	148
	RV	5′- TTTGCTCCAGTTAAAGGCGGCATC-3′		
MMP9	FW	5′-GGGCTTAGATCATTCCTCAGTG -3′	58	94
	RV	5′-GCCATTCACGTCGTCCTTAT-3′		
Integrin a4	FW	5'- GCTTCTCAGATCTGCTCGTG-3'	60	131
	RV	5'- GTCACTTCCAACGAGGTTTG -3'		
Integrin a5	FW	5′- TGCAGTGTGAGGCTGTGTACA -3′	60	88
	RV	5′- GTGGCCACCTGACGCTCT -3′		
Integrin aV	FW	5'- AATCTTCCAATTGAGGATATCAC -3'	58	140
	RV	5'- AAAACAGCCAGTAGCAACAAT -3'		
Integrin b1	FW	5'- GAAGGGTTGCCCTCCAGA -3'	58	107
	RV	5'- GCTTGAGCTTCTCTGCTGTT -3'		
Integrin b2	FW	5′- CCACCTGGAAGACAACCTGT -3′	60	252
	RV	5′- TCCAGGAAGACTCTGGAGGA -3′		
Integrin b8	FW	5′- AATTTGGTAGTGGAAGCCTATC-3′	60	146
	RV	5′- GTCACGTTTCTGCATCCTTC-3′		
GAPDH	FW	5'-GAGTCAACGGATTTGGTCGT-3'	60	185
	RV	5'-GACAAGCTTCCCGTTCTCAG-3'		

Figures

Figure 1

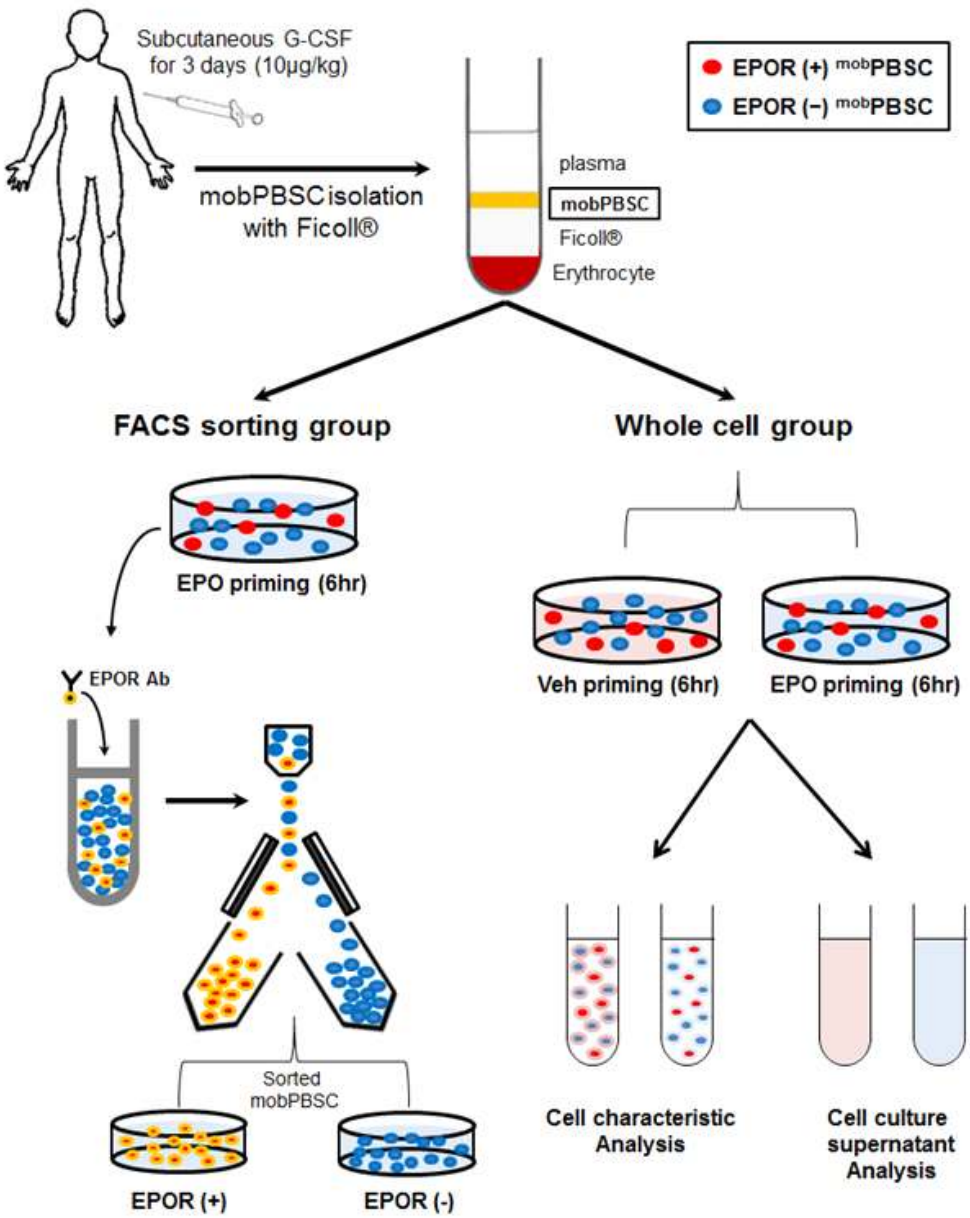


Figure 1. Detailed description of experimental scheme

Mononuclear cells were mobilized from bone marrow to peripheral blood by G-SCF injection for 3 days in healthy volunteers. After 3 days of subcutaneous G-CSF injection (10ug/kg), peripheral blood 200ml was obtained and the Complete Blood Count was checked. Immediately, we separated mononuclear cells using Ficoll-Plaque gradient. The ^{mob}PBSCs were divided into two groups; the FACS sorting group and the whole cell group. In the FACS sorting group, EPO-primed^{mob}PBSCs were sorted by FACS using PE-conjugated anti-EPOR and divided into EPOR positive and negative cells. In the whole cell group, cells were primed with PBS(vehicle) or EPO 10IU/ml for 6hours and analyzed. The culture supernatant was analyzed after an additional 36 hour cell culture.

Figure 2

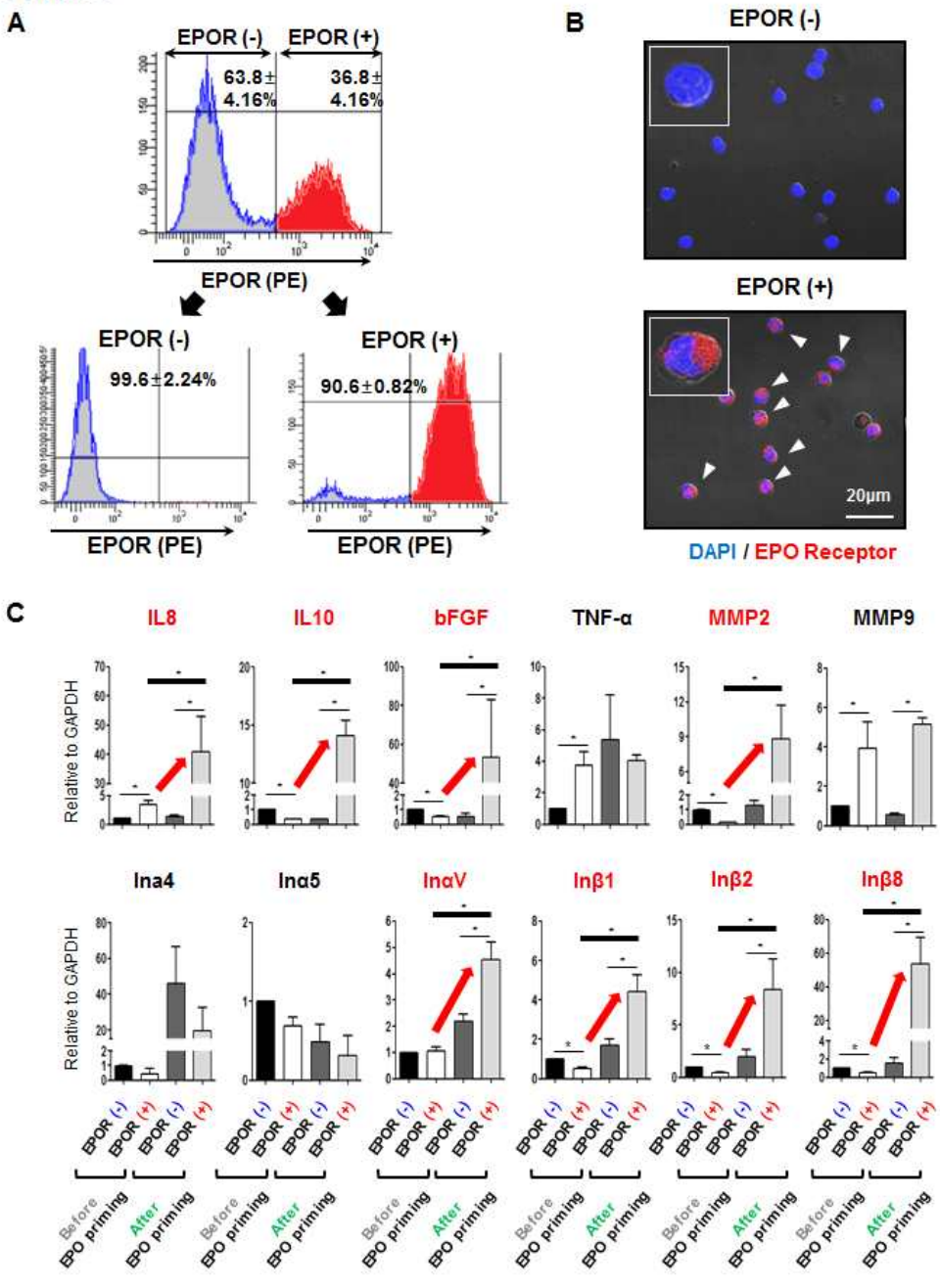

A
EPOR (-)
EPOR (+)
63.8±
4.16%
36.8±
4.16%
EPOR (PE)
EPOR (-)
99.6±2.24%
EPOR (+)
90.6±0.82%
EPOR (PE)
EPOR (PE)
B
EPOR (-)
EPOR (+)
20μm
DAPI / EPO Receptor
C
IL8
IL10
bFGF
TNF-α
MMP2
MMP9
Relative to GAPDH
Inα4
Inα5
InαV
Inβ1
Inβ2
Inβ8
Relative to GAPDH
EPOR (-)
EPOR (+)
EPOR (-)
EPOR (+)
Before EPO priming
After EPO priming

Figure 2. Expression of EPOR on ^{mob}PBSCs and Effect of EPO-priming

(A) ^{mob}PBSCs were sorted by the presence of EPOR. By FACS analysis, the purity of EPOR-sorted ^{mob}PBSCs was proven.

(B) Immunofluorescence showed again the purity of sorting that anti-EPOR antibody-stained cells were only present in the EPOR (+)^{mob}PBSCs group.

(C) Gene expression of EPOR(+) and (-) cells before EPO-priming and in response to EPO-priming. Results are shown as the relative amount of mRNA expression compared to GAPDH, and fold-expression compared to EPOR(-)^{mob}PBSCs (Thick black bars indicate difference between EPOR(+)^{mob}PBSCs, before and after EPO-priming (n=4 for all results).

Figure 3
A

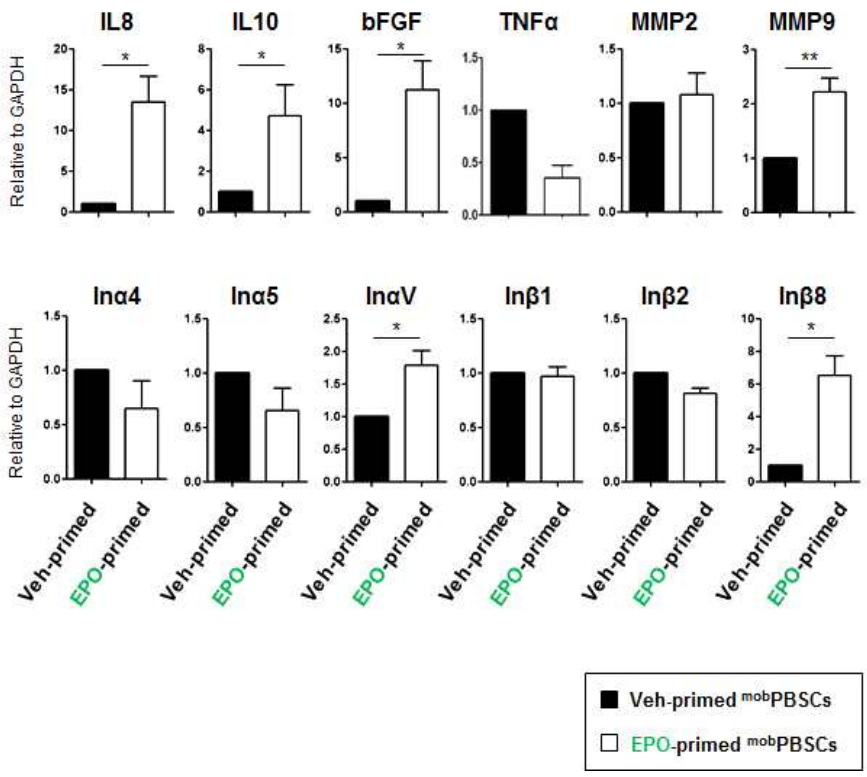
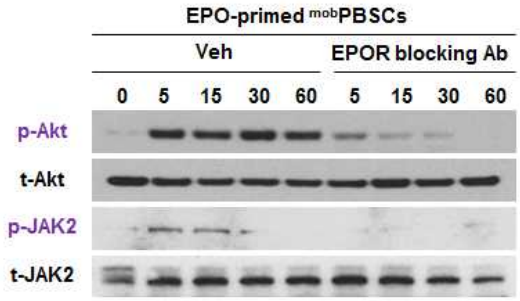


Figure 3. Effect of EPO-priming on the total ^{mob}PBSCs

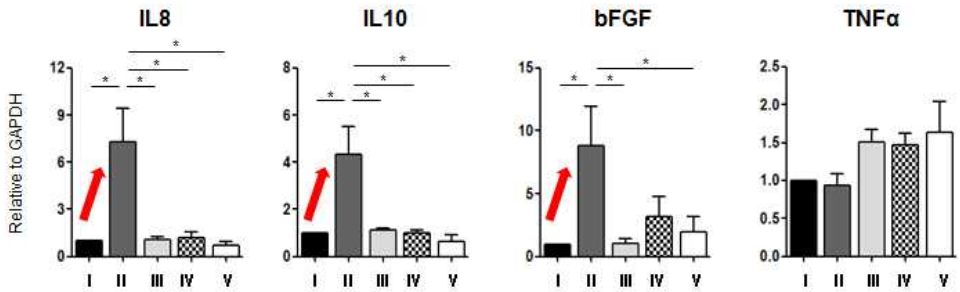
(A) Gene expression of veh-primed ^{mob}PBSCs and EPO-primed^{mob}PBSCs. EPO-priming significantly increased IL8, IL10, bFGF, MMP9, IntegrinαV and Integrinβ8. Results are shown in fold expression compared to veh-primed^{mob}PBSC. (n=4 for all results).

Figure 4

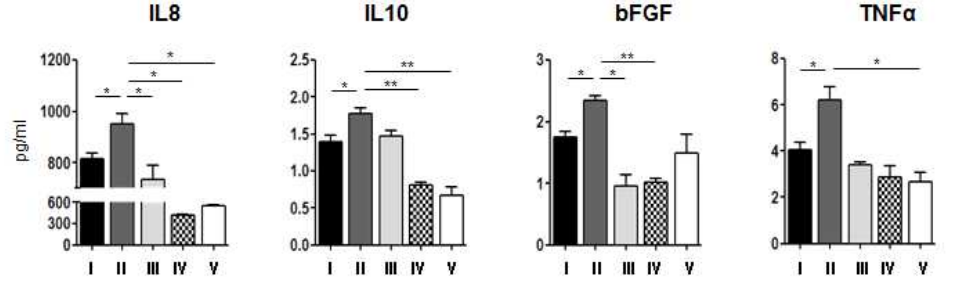
A



B



C



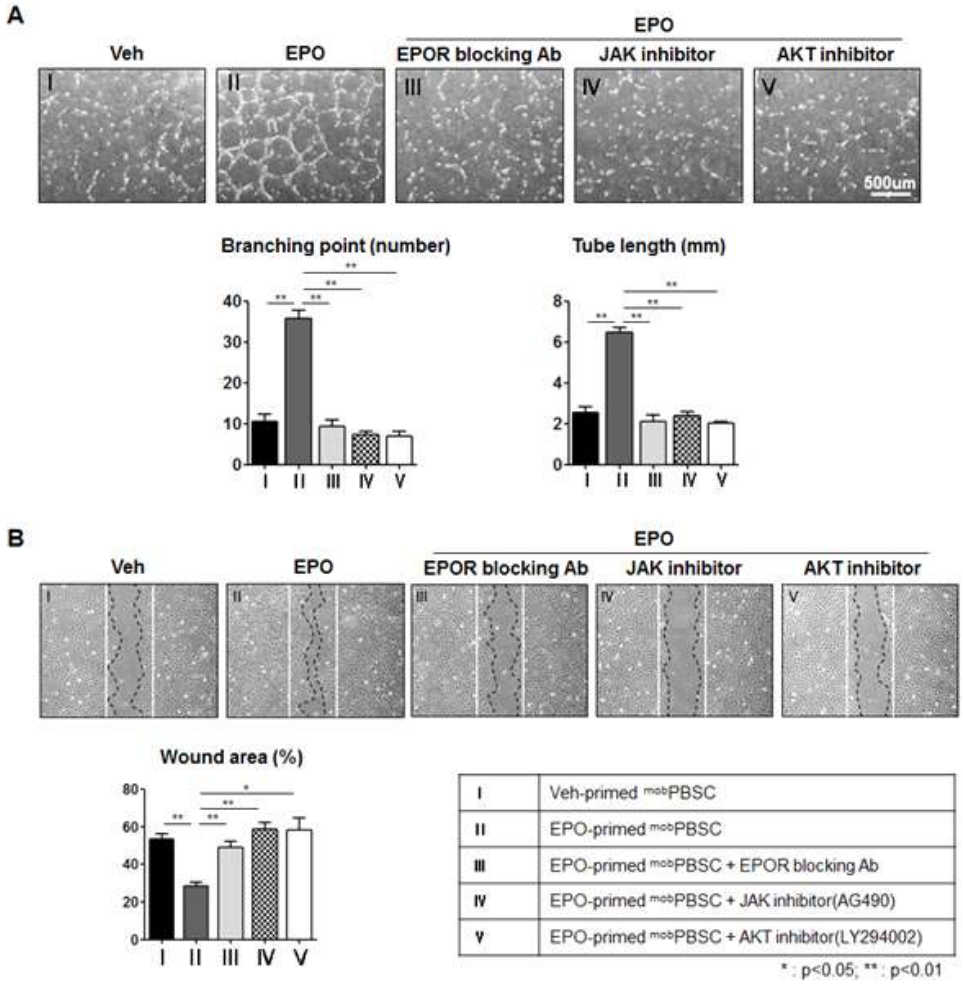
I	Veh-primed mobPBSC	III	EPO-primed mobPBSC + EPOR blocking Ab
II	EPO-primed mobPBSC	IV	EPO-primed mobPBSC + JAK inhibitor(AG490)
		V	EPO-primed mobPBSC + AKT inhibitor(LY294002)

(* : p<0.05; ** : p<0.01)

Figure 4. Signal pathway of EPO-priming

(A) Signaling pathway underlying EPO-priming of ^{mob}PBSCs. Western blot for EPO-primed^{mob}PBSCs cultured with additional veh or EPOR blocking antibodies. EPOR blocking antibodies reduced the phospho-form of Akt and JAK2, without effect on the total amount. (B,C) The anigogenic secretome primed by EPO was checked at them RNA level (B) and at the protein level (C) by qRT-PCR and ELISA respectively. IL8, IL10 and bFGF levels were augmented by EPO-priming, which were abolished by EPO R blocking antibody, JAK inhibitor, and Akt inhibitor. Results in (B) are shown as the relative amount of mRNA expression compared to GAPDH and fold-expression compared to veh-primed^{mob}PBSCs. Cytokine levels were shown in pg/ml (n=4 for all results).

Figure 5



I	Veh-primed ^{mob}PBSC
II	EPO-primed ^{mob}PBSC
III	EPO-primed ^{mob}PBSC + EPOR blocking Ab
IV	EPO-primed ^{mob}PBSC + JAK inhibitor(AG490)
V	EPO-primed ^{mob}PBSC + AKT inhibitor(LY294002)

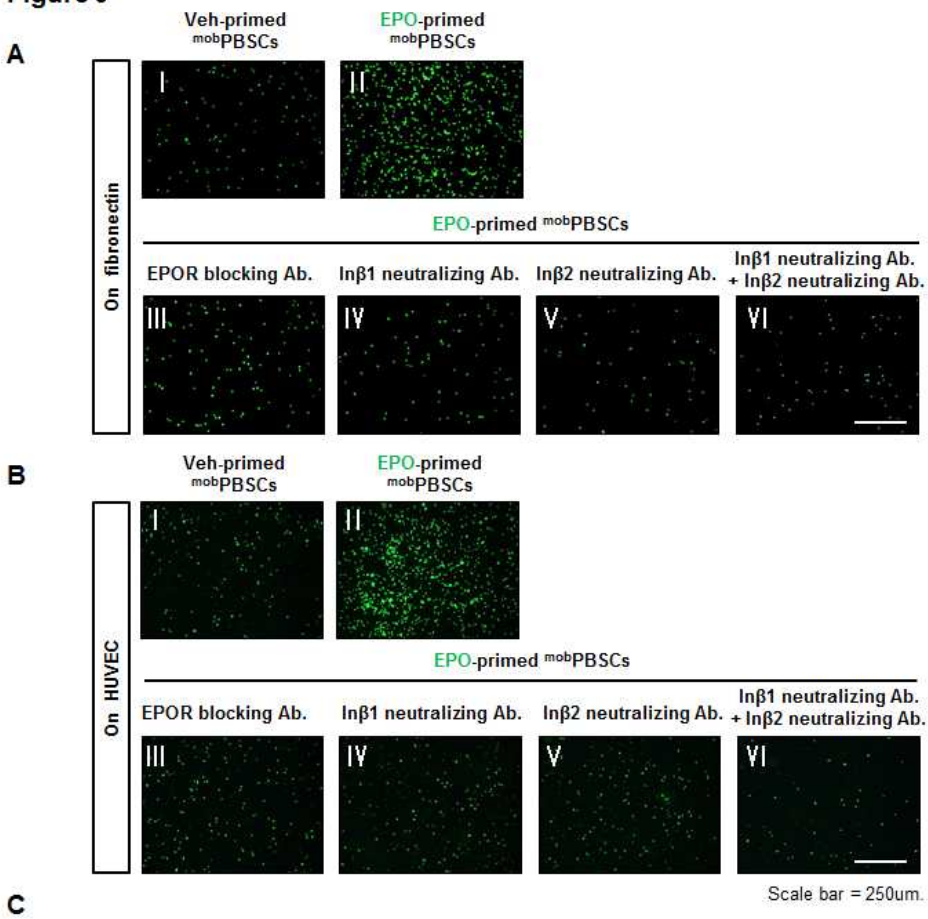
* : $p<0.05$; ** : $p<0.01$

Figure 5. Angiogenic potential of the EPO-primed ^{mob}PBSCs in vitro study

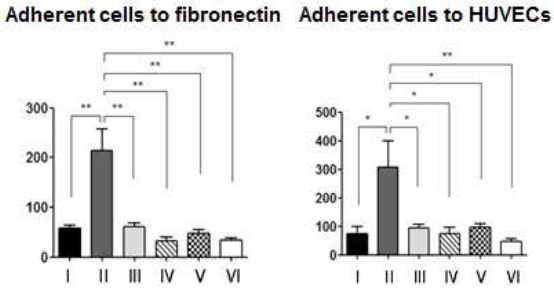
(A) HUVECs were cultured in each cell culture supernatant. Tube formation assay showed an increase in tube like structures in supernatant of EPO-primed ^{mob}PBSCs, resulting in a significant increase in branching points and tube length.

(B) Random migration assay showed a significant increase in wound healing in the HUVECs cultured in EPO-primed ^{mob}PBSCs culture supernatant. Both Tube formation and random migration activity of HUVEC were decreased by EPOR blocking antibody, JAK inhibitor, and Akt inhibitor (n=4 for all results).

Figure 6



C



I	Veh-primed
II	EPO-primed
III	EPO-primed + EPOR blocking Ab.
IV	EPO-primed + Inβ1 neutralizing Ab.
V	EPO-primed + Inβ2 neutralizing Ab.
VI	EPO-primed + Inβ1 neutralizing Ab. + Inβ2 neutralizing Ab.

* : p<0.05; ** : p<0.01

Figure 6. Adhesion of the EPO-primed ^{mob}PBSCs

(A) Adhesion assays of primed ^{mob}PBSCs were done on HUVECs and (B) Fibronectin. (C) EPO-priming increased the adhesion ability of ^{mob}PBSCs on HUVECs and fibronectin, which we reabolished by EPO R blocking antibody, integrinβ1 and β2 neutralizing antibodies. Therefore increased adhesion was an integrin mediated effect (n=4 for all results).

Figure 7

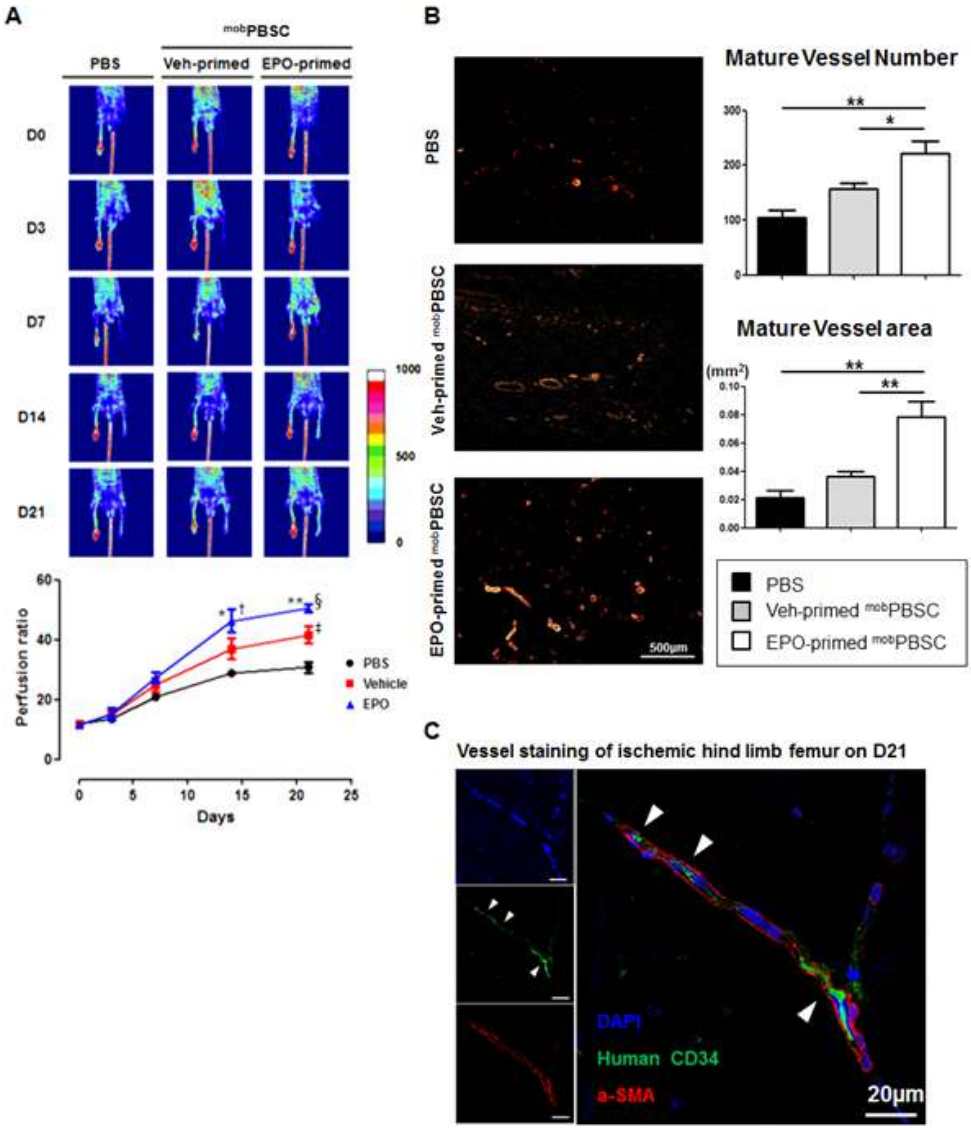

A
mobPBSC
PBS
Veh-primed
EPO-primed
D0
D3
D7
D14
D21
1000
500
0
Perfusion ratio
Days
PBS
Vehicle
EPO
B
PBS
Veh-primed mobPBSC
EPO-primed mobPBSC
500μm
Mature Vessel Number
Mature Vessel area
(mm²)
PBS
Veh-primed mobPBSC
EPO-primed mobPBSC
C
Vessel staining of ischemic hind limb femur on D21
DAPI
Human CD34
α-SMA
20μm

Figure 7. Transplantation of EPO-primed ^{mob}PBSCs improved blood flow recovery to ischemic limb.

(A) Representative figure of LDPI measurements and the perfusion ratio of the ischemic limb compared to the non-ischemic limb. After 3 weeks of femoral artery ligation and ^{mob}PBSCs injection, the perfusion ratio was 30.8±1.8% in of the PBS injected group and 41.7±2.7% in the veh-primed^{mob}PBSCs injected group, whereas it was 50.6±1.3% in the EPO-primed^{mob}PBSCs injected group.(*:P<0.01 for EPO vs. PBS, † : P of trend <0.01, ‡ :P<0.05 for veh vs. PBS, § : P<0.01 for EPO vs. PBS, ** : P of trend <0.01)

(B) The mature vessel count was analyzed by SMA(+) circular structures in low power fields (X40). EPO-primed^{mob}PBSCs induced a larger number and broader area of mature vessels than did veh-primed cells or PBS.

(C) Immunofluorescence staining showed the evidence that human specific CD34(+) cells (white arrowheads) incorporated into the mouse vascular structures.

Figure 8

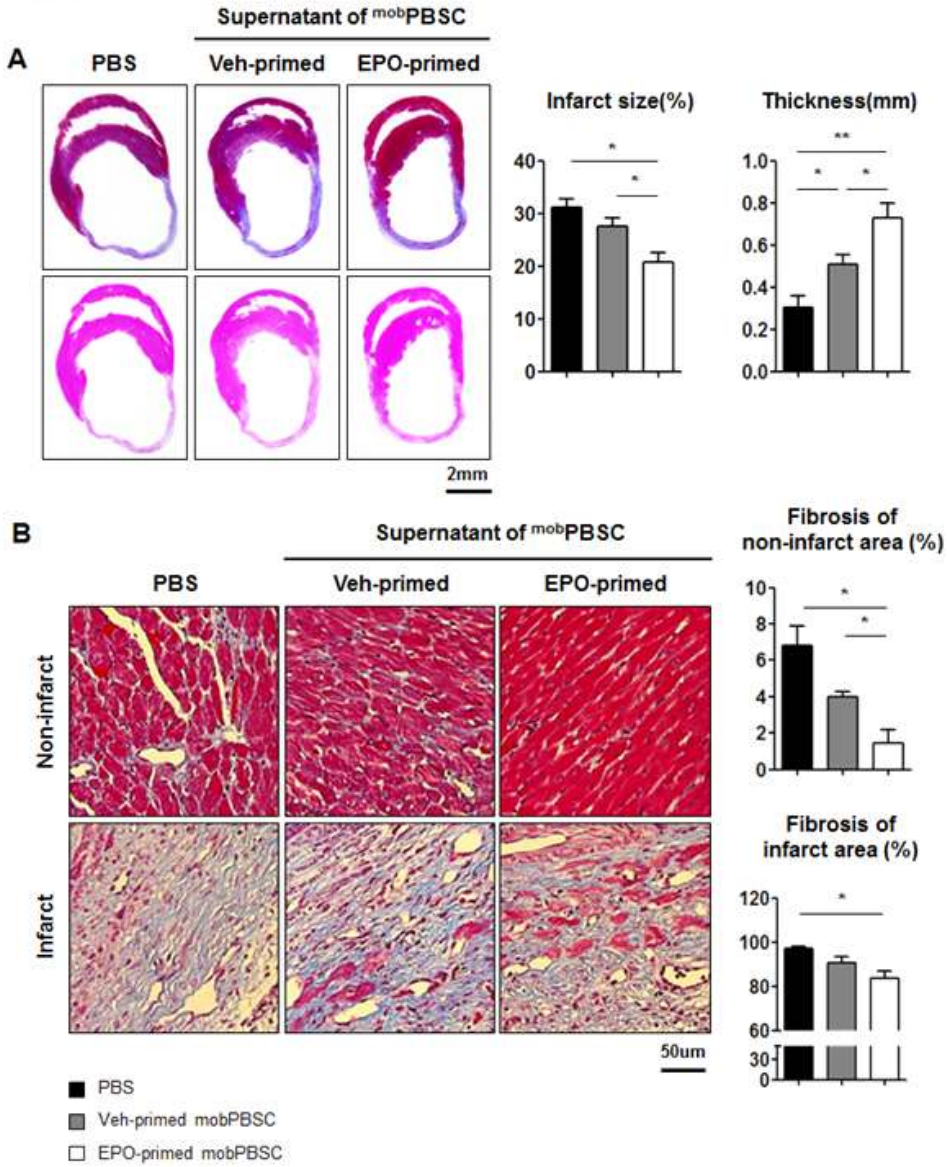

Supernatant of mobPBSC
A
PBS
Veh-primed
EPO-primed
2mm
Infarct size(%)
Thickness(mm)
B
Supernatant of mobPBSC
PBS
Veh-primed
EPO-primed
Non-infarct
Infarct
50um
Fibrosis of non-infarct area (%)
Fibrosis of infarct area (%)
PBS
Veh-primed mobPBSC
EPO-primed mobPBSC

Figure 8. Transplantation of EPO-primed ^{mob}PBSCs improved the repair of the damaged heart after myocardial infarction in nude mice.

(A) In myocardial infarction model of nude mice, transplantation of EPO-primed ^{mob}PBSCs showed the better therapeutic efficacy than veh-primed cells or PBS control in terms of infarct size and wall thickness.

(B) Cardiac ischemia models were performed and primed cells were injected for tissue regeneration. MT staining showed decreased fibrosis within the non-infarct area and infarct area, in the EPO-primed ^{mob}PBSCs injection group compared with the other two treatments.

Figure 9

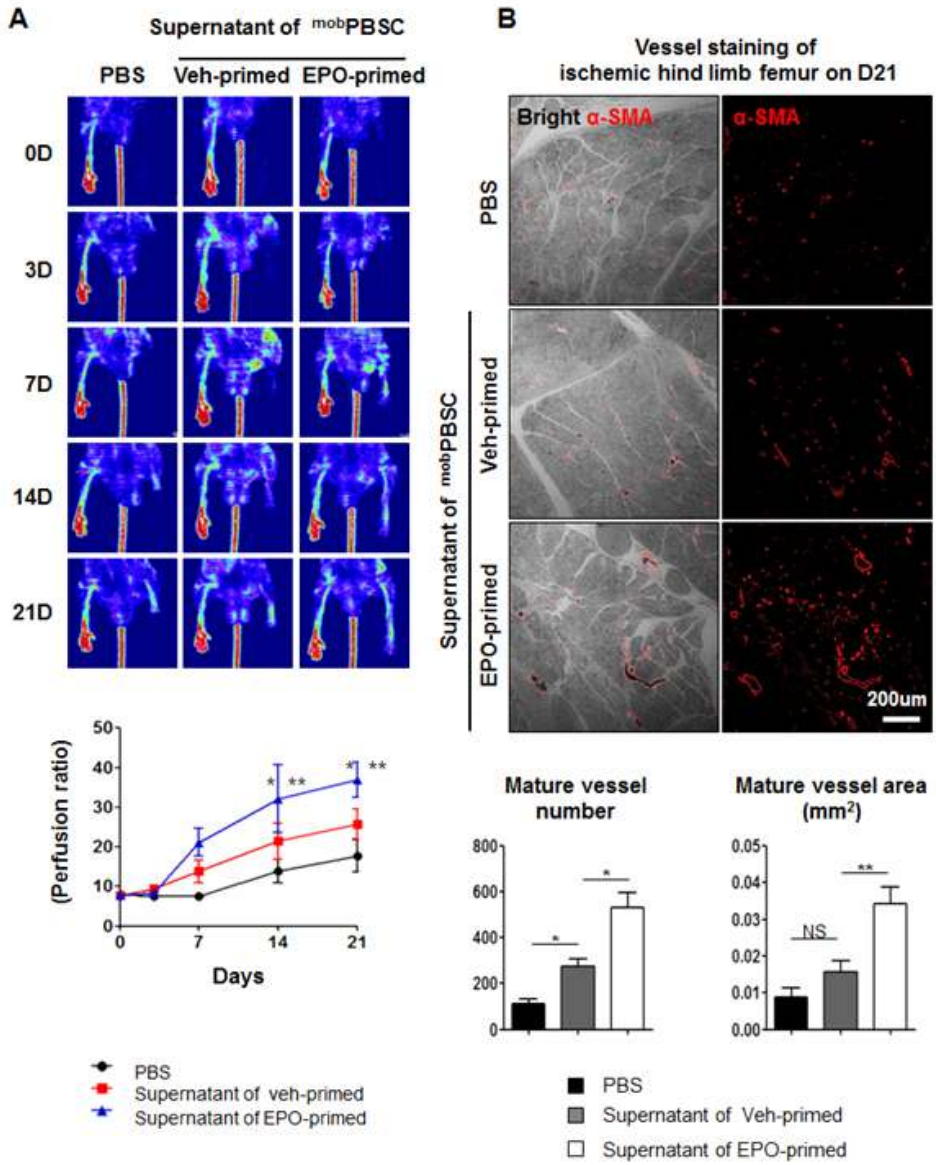

A
Supernatant of mobPBSC
PBS
Veh-primed
EPO-primed
0D
3D
7D
14D
21D
(Perfusion ratio)
Days
PBS
Supernatant of veh-primed
Supernatant of EPO-primed
B
Vessel staining of
ischemic hind limb femur on D21
Bright α-SMA
α-SMA
PBS
Supernatant of mobPBSC
Veh-primed
EPO-primed
200um
Mature vessel
number
Mature vessel area
(mm2)
NS
PBS
Supernatant of Veh-primed
Supernatant of EPO-primed

Figure 9. Supernatanof EPO-primed ^{mob}PBSCs improved blood flow recovery to ischemic limb.

(A-B) Paracrine humoral effects of EPO-primed cells were tested in the ischemic hindlimb model. Injection of supernatants from EPO-primed ^{mob}PBSCs showed the better efficacy than supernatants from veh-primed cells or PBS control in terms of blood flow recovery to ischemic limb (perfusion ratio ; 36.9±10.0% vs. 25.7±8.6% vs. 17.7±9.3%) (* : $P<0.01$ for EPO vs. PBS, ** : $P<0.05$ for EPO vs. veh, † : $P<0.01$ for EPO vs. PBS)

Figure 10

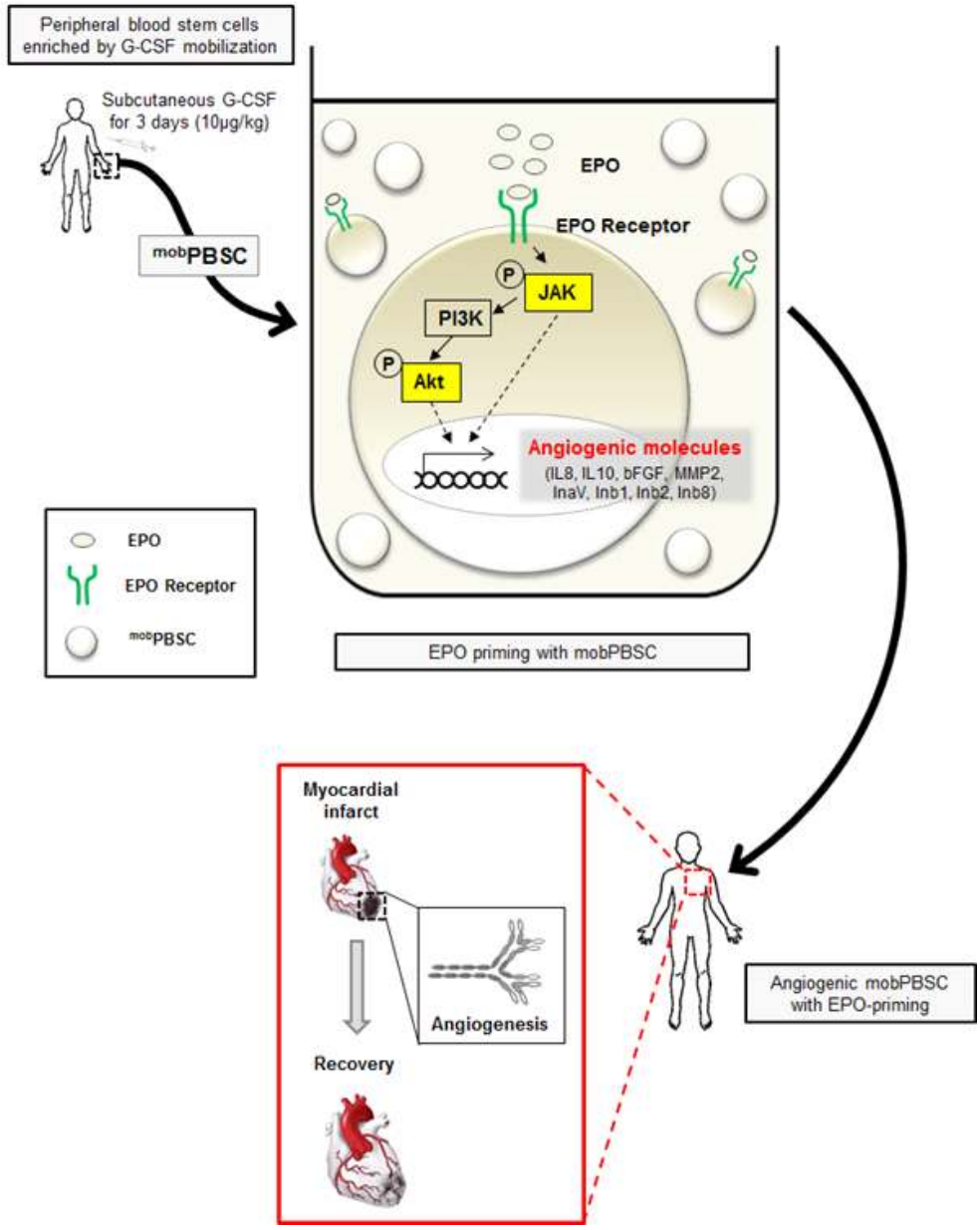

Peripheral blood stem cells enriched by G-CSF mobilization
Subcutaneous G-CSF for 3 days (10μg/kg)
mobPBSC
EPO
EPO Receptor
P
JAK
PI3K
P
Akt
Angiogenic molecules
(IL8, IL10, bFGF, MMP2, InaV, Inb1, Inb2, Inb8)
EPO
EPO Receptor
mobPBSC
EPO priming with mobPBSC
Myocardial infarct
Angiogenesis
Recovery
Angiogenic mobPBSC with EPO-priming

Figure 10. Schematic picture for therapeutic application of EPO-primied ^{mob}PBSCs

Abbreviations: ^{mob}PBSCs = mobilized peripheral blood stem cells, EPO = erythropoietin, EPOR = erythropoietin receptor, IL = interleukin, bFGF = basic fibroblast growth factor, TNFα=tumor necrosis factor alpha, VEGF = vascular endothelial growth factor, MMP = Matrix metalloproteinase, In = Integrin, FACS = Fluorescence-Activated Cell Sorter, SSC = side scatter, CD = cluster of differentiation, Veh = vehicle, ELISA = Enzyme-linked immunosorbent assay

논 문 국 문 초 록

에리스로포이에틴을 이용한 G-CSF 동원 말초혈액 자가줄기세포의 혈관 형성능 향상에 관한 연구

Erythropoietin-Priming enhances angiogenic potential of human peripheral blood stem cells mobilized with granulocyte colony-stimulating factor

윤 지 연 (Ji-Yeon Yun)

분자의학 및 바이오제약 학과 (Molecular Medicine and Biopharmaceutical Sciences)

분자의학 및 바이오제약 전공 (Molecular Medicine and Biopharmaceutical Sciences)

The Graduate School

Seoul National University

심혈관계 질환은 전 세계적으로 주요한 사망 원인이 되는 질환이며, 주요 사망 원인들 중 가장 급격하게 증가하고 있다. 특히, 심부전의 경우 아직까지 심장 이식 이외의 뾰족한 치료해법을 찾지 못하여 지속적으로

발병이 증가하고 있다. 이를 해결하기 위한 여러 새로운 치료법 개발 중 하나가 바로 말초혈액 자가 줄기세포를 이용한 심근의 재생 방법이다. G-CSF 주사를 통하여 전신순환계로 골수 유래 성체줄기세포를 동원시키고, 이를 말초 혈액을 채취 후 분리하여 이를 세포 치료원으로 사용하는 방법으로 심근경색 환자에게 관상동맥 내로 주사하는 세포치료요법을 기존 치료법과 병행하였을 경우 치료효과가 매우 호전됨이 확인되었다. 현재에는 G-CSF 동원 말초혈액 줄기세포의 치료효능을 더욱 높이기 위한 방안이 새로운 연구과제로 떠오르고 있다.

본 연구는 허혈성 심질환 치료를 위한 전도유망 세포 치료원인 G-CSF 동원 말초혈액 줄기세포의 치료효능을 증진시키기 위하여 에리스로포이에틴을 이용한 체외 priming 단계를 도입하였다. 그리고 이를 통하여 G-CSF 동원 말초혈액 줄기세포의 혈관신생능이 향상됨으로써 치료 효능이 더욱 증진됨을 확인하였다.

건강한 자원자 4명에게 3일동안 G-CSF(10ug/kg)를 피하주사 한 후 전신 순환계로 동원된 골수 성체 줄기세포를 말초혈액을 채취하여 분리하였다. 채취한 G-CSF 동원 말초혈액 줄기세포를 에리스로포이에틴 양성과 음성 세포로 분리하여 에리스로포이에틴을 이용한 프라이밍 효과를 확인한 결과 에리스로포이에틴 양성 세포에서 혈관신생관련 유전자가 음성 세포 대비 더욱 증가함을 확인하였다. 또한 에리스로포이에틴을 이용하여 프라이밍한 G-CSF 동원 말초혈액 줄기세포의 배양액이 혈관 내피세포의 혈관 형성능 및 이동능력을 대조군 대비 더욱 증진시킴을 in vitro 실험을 통하여 확인하였다. 이러한 에리스로포이에틴 프라이밍을 통한 G-CSF 동원 말초혈액 줄기세포의 혈관 형성능 증가 효과는 누드마우스의 허혈성 뒷다리 모델 및 심근경색 동물모델에서도 확인하였다.

본 연구는 체외에서 단시간 에리스로포이에틴 priming 자극은 G-CSF

동원 말초혈액 자가 줄기세포의 혈관 형성능력을 더욱 증진시킴을 증명함으로써 이를 통한 심근경색 세포치료법 개발의 더욱 효과적인 방향을 제시하였다. 더 나아가 본 연구 결과는 심근경색 세포치료법의 임상 적용을 위한 치료세포 최적화에 이바지할 수 있다.

주 요 어 :

에리스로포이에틴, G-CSF, 말초혈액 자가 줄기세포, 프라이밍, 심혈관계 질환, 혈관신생

학 번 : 2012-24150