

EPCs in vascular repair: How can we clear the hurdles between bench and bedside?

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1. ABSTRACT

Endothelial progenitor cells (EPCs) play a fundamental role in the maintenance and repair of damaged vascular endothelium, as well as in new blood vessel formation. Based on this function of EPCs, it has been hypothesized that transfusion of these cells could be an approach to treat vascular disease. While this concept has subsequently been proven in animal models clinical trials have not been encouraging. These discrepancies have limited translation of EPCs from bench to bedside. In this review, by analyzing the reported data from the animal models and clinical trials, we describe the main factors limiting the clinical effects of EPCs infusion and the unfavorable *in vivo* reactions of the receipts. To facilitate future clinical application of EPCs, a series of strategy to overcome the obstacles have been suggested.

2. INTRODUCTION

Research into the repair mechanisms of the damaged vascular endothelial cell layer and neovascularization have identified potential therapeutic targets for a variety of vascular disease conditions. In early experiments in the 1960's, Stump *et al.* demonstrated that endothelialization could be found on a Dacron surface, which was suspended in the blood stream without direct contact to the adjacent vessel structure (1). Based on this finding, the authors hypothesized that there are cells circulating in the blood stream, with the capability of aggregating to artificial surfaces. In 1997, Asahara *et al.* purified a population of circulating cells with properties of both endothelial cell (EC) and progenitor cell, therefore termed 'endothelial

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progenitor cells' (EPCs), which were capable of promoting postnatal vasculogenesis (2).

Since then, there has been tremendous interest in the role of EPCs in the endogenous maintenance and repair of damaged endothelium, as well as for their regenerative and therapeutic potential (3-7). Experimental studies have shown that EPCs transplantation can restore both endothelial structure and function in animal models with arterial disease (6, 7). However, several clinical studies in patients with myocardial infarction showed limited efficacy following EPCs infusion (8, 9). The reasons for the discrepant results are incompletely understood but have limited the clinical application of autologous EPCs to treat vascular diseases.

In this review we will discuss the rational and methods of infusing EPCs as cell therapy for cardio-vascular as well as peripheral-vascular disease, review the molecular mechanism involving EPCs' function, and provide potential explanations for the factors limiting clinical application of EPCs. Based on this data, we finally summarize strategies to overcome these hurdles.

3. BIOLOGY OF EPCS

Based on the culture method using peripheral or umbilical cord blood, three subtypes of EPCs are defined: colony-forming-unit endothelial cells (CFU-ECs), circulating angiogenic cells (CACs), and endothelial-colony-forming cells (ECFCs). However, the cellular origin of these types of EPCs remains incompletely understood. *Gunsilius et al.* examined genetic mutation of CFU-ECs from patients with chronic myelogenous leukemia, which is characterized by the presence of a BCR/ABL fusion gene in a multipotent HSC clone and all the derived progeny. (10) Their research showed that cultured CFU-ECs, as well as some ECs in the heart displayed the translocation. *Oppliger Leibundgut et al.* found the JAK2 V617F mutation in CFU-ECs obtained from a patient with polycythemia vera, which is characterized by the JAK2 V617F mutation (11). These data provide proof of the hematopoietic origin of CFU-ECs.

However, ECFCs has been suggested to have different origin. *Yoder Mc et al.* (12) cultured ECFCs from polycythemia vera patients, but found no JAK2 V617F mutation, while CFU-ECs from same patients showed this mutation, suggesting that ECFCs are not hematopoietic in origin.

Other unclear biological aspects of EPCs include the question if these cells maintain a 'silence' status in the circulation or in blood vessel tissue, and how they can they be activated. Comparing CFU-ECs, CACs, and ECFCs, it is also incompletely understood which type of EPCs contributes to what extent in different stages of repair. Furthermore, the interaction between these cell types is unclear.

4. METHODOLOGICAL ASPECTS OF THERAPEUTIC USE OF EPCS

4.1. Isolation and Culture Methods of EPCs

Because the number/concentration of these cells is small, a critical first step is careful isolation and culture from autologous tissue. A number of methods has been described to isolate and culture EPCs from peripheral blood (PB), bone marrow (BM) or the umbilical cord (13-15), which generally can be classified into two approaches: culture methods (*in vitro* inducing and expanding specific cells, which have adhesive capacity to fibronectin, using a cocktail of cytokines) and sorting methods (using fluorescent labeled antibodies to collect cells based on cell surface marker, using fluorescence-activated cell sorting). Because of its superiority in cell expansion, culture methods are more widely used in both animal models and clinical studies.

As mentioned in last section, three subtypes of EPCs have been defined according to their culture methods: colony-forming-unit endothelial cells (CFU-ECs), circulating angiogenic cells (CACs), and endothelial-colony-forming cells (ECFCs)

Briefly, in the CFU-ECs method after 2-day incubation, there remain two types of cell with the feature of either adhering to the culture plate bottom or being suspended in the culture media. Isolation and culture of the non-adherent mononuclear cells (MNCs) give rise to the EPCs colonies (2, 16, 17). Typical colonies emerge in 5-9 days, featured by a core of round cells, with spindle-shaped cells sprouting at the periphery. Colonies of this type are commonly named as CFU-ECs or colony-forming unit-Hill (CFU-Hill) cells (16).

- CACs is a population of adherent cells emerging in a 4- to 7-day culture procedure among unfractionized MNCs (18-21). Because these cells constitute approximately 2% of the total MNCs (15), CACs can be obtained in a larger number than CFU-ECs from primary culture with the ability to promote neovascularization in animal models of critical limb ischemia or myocardial infarction (3, 22-24).

ECFCs are derived from adherent MNCs after 7-21 days culture in endothelial conditions and colonies display cobblestone morphology (15, 25). All these three subtypes are reported to have the ability of tube formation in matrigel but only ECFCs express de novo vessel-forming ability (12, 25).

All three methods can be applied to cell isolation from blood, bone marrow and umbilical cord of animals and human beings. In animal experiments, the major sources of EPCs has been results of studies. Only ECFCs have the ability of new vessel-forming as compared with CFU-ECs or CACs. Thus, the ECFCs may have greater impact on improvement of the microcirculation of target tissue. However, because of

Table 1. Summary of key animal experiments describing EPCs infusion to salvage myocardial and hindlimb ischemia

Group	Objective of EPCs therapy	EPCs subtype	Source of EPCs	Results
Asahara T. <i>et al</i> (2)	- Athymic nude mice; hindlimb ischemia through excising one femoral artery -- Rabbit model of unilateral hindlimb ischemia	Heterologous CFU-ECs	Healthy human donor	All EPCs appeared integrated into capillary vessel walls Engraftment of human EPCs in $13.4 \pm 5.7\%$ of the mouse capillaries in the injured extremity, compared with $1.6 \pm 0.8\%$ in control mice
Chade AR. <i>et al</i> (24)	- Pigs 6 weeks after induction of RAS (renal artery stenosis)	Autologous CFU-ECs and ECFCs	Pigs 3 and 5 weeks after induction of RAS	Improved renal microvascular and filtration function (ANOVA $P < 0.03$ for RBF and GFR, ANOVA $P = \text{NS}$ for perfusion) Augmented Akt ($P < 0.05$ vs RAS) and eNOS ($P < 0.05$ vs normal) expression Improved vascular volume fraction (ANOVA $P = 0.0002$) and microvascular tortuosity (ANOVA $P = 0.0007$) Attenuated renal microvascular remodeling and fibrosis ($P < 0.05$ vs either normal or RAS)
Kawamoto A. <i>et al</i> (19)	- Athymic nude rat model of MI 3 hours after induction by ligating the LAD coronary artery.	Heterologous CACs	Healthy human donor	Transplanted EPCs accumulated in the ischemic area and incorporated into foci of myocardial neovascularization Improved LV function (LVDd $P = 0.032$, LVDs $P = 0.005$, Fractional Shortening $P = 0.0004$, Regional wall motion $P = 0.0021$; all vs control) Increased capillary density ($P = 0.0009$ vs control) Less fibrosis/entire LV area fraction ($P = 0.0007$ vs control)
Kalka C. <i>et al</i> (3)	- Athymic nude mice model of hindlimb ischemia 1 day after resection of one femoral artery	Heterologous CACs	Healthy human donor	EPCs incorporated into neovascular foci Increased capillary density ($P < 0.002$ vs control media, $P = 0.0003$ vs HMVECs) Improved blood perfusion (assessed by the ratio of ischemic/normal blood flow, $P < 0.003$ vs either control media or HMVECs) Enhanced Tissue Salvage ($P = 0.003$ vs control media, $P = 0.006$ vs HMVECs)

the procedural complexity and relative lower success rate of ECFCs culture, CFU-ECs and CACs are widely used in studies, especially in clinical trials.

4.2. Experimental Concepts and Clinical Endpoints for EPCs Therapy

In contrast to the substitution of the entire hematopoietic cell population in stem cell therapy for leukemia, the concept of EPCs therapy is the local supply of EPCs, adequate to cover the affected area of target vessel or tissue. Therefore, in reported experimental and clinical studies, EPCs therapy often uses a cell infusion method. EPCs have been transfused into a target artery through a catheter with or without temporal blocking of the proximal blood flow. Less frequently, EPCs were directly injected intra-myocardial or into surrounding tissue of ischemic limb lesion.

With current technology, only complex immune-pathological methods can verify if and where labeled EPCs home and survive in the target area, and how they interact with the impaired vessel endothelium. These methods are not commonly used in clinical investigation. Typical endpoints include reendothelialization of target vessel and

microcirculation restoring of ischemic area. Functional criteria of organ, such as LVEF in coronary disease, distance of intermittent claudication in lower limb ischemia, are measured as well.

5. THERAPEUTIC EFFICACY OF EPCS IN ANIMAL MODELS AND CLINICAL STUDIES

5.1. Initial Success in Animal models

In multiple studies using animal models of ischemic disease, such as acute myocardial infarction (AMI) or hind limb ischemia, EPCs have demonstrated exciting therapeutic efficacy (2, 3, 22, 26-30). (Table 1).

In a hind limb ischemia model in nude mice, EPCs led to apparent engraftment of the human cells into the mouse capillaries of the injured extremity, remarkably improved blood flow recovery and capillary density in the ischemic and contralateral non-ischemic hind limb, and significantly reduced the rate of limb loss (2,3). In a pig model of chronic experimental renovascular disease, intrarenal artery infusion of autologous EPCs restored renal function, with ameliorated renal microvascular remodeling and fibrosis (26). In a nude rat AMI model, *ex vivo*

Table 2. Summary of key clinical studies using EPC infusion to salvage myocardial and hindlimb ischemia

Group or Trial	Objective of EPCs therapy	EPCs type	Source of EPCs	Results								
TOPCARE-AMI Trial (29)	Human patients with AMI	Autologous CACs or selected CD34/CD45-positive BMC	Same patient who received EPCs infusion	<ol style="list-style-type: none"> Primary Endpoint: Safety and Efficacy Secondary Endpoint, but no control group: <ul style="list-style-type: none"> - At 4 month: LVEF significantly increase (50±10% to 58±10%; $P < 0.001$) and LV end-systolic volumes significantly decreased (54±19 ml to 44±20 ml; $p < 0.001$) with no variation between CACs and BMC group. - At 12 mon: increased LVEF ($P < 0.001$) and reduced infarct size ($P < 0.001$) 								
Assmus B. et al (8)	Human patients with stable ischemic heart disease and a MI at least 3 months before	Autologous CACs or Ficoll density-gradient centrifugation-isolated BMC	Same patient who received EPCs infusion	<p>At 3 mon: No significant variation of LV function between BMC, CAC and control group.</p> <table> <tr> <td>1. Global LVEF, $P=0.31$</td> <td>4. End-diastolic volume, $P=0.26$</td> </tr> <tr> <td>2. Regional contractility in central target area, $P=0.03$</td> <td>5. End-systolic volume, $P=0.26$</td> </tr> <tr> <td>3. Extent of regional left ventricular dysfunction, $P=0.50$</td> <td>6. Stroke volume, $P=0.78$</td> </tr> <tr> <td></td> <td>7. Left ventricular end-diastolic pressure, $P=0.61$</td> </tr> </table>	1. Global LVEF, $P=0.31$	4. End-diastolic volume, $P=0.26$	2. Regional contractility in central target area, $P=0.03$	5. End-systolic volume, $P=0.26$	3. Extent of regional left ventricular dysfunction, $P=0.50$	6. Stroke volume, $P=0.78$		7. Left ventricular end-diastolic pressure, $P=0.61$
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				<p>?control group?</p> <p>At 6 month: change in LVEF</p> <ol style="list-style-type: none"> an increase of 0.6% (95%CI, -3.4-4.6; $P=0.77$) on SPECT a decrease of 3.0% (95%CI, -6.1-0.1; $P=0.054$) on MRI 								
Janssens S. et al (31)	AMI Patients with successful reperfusion after percutaneous coronary intervention	Autologous MNCs from BM	Same patient who received EPCs infusion	<p>? control group?</p> <p>At 4 mon:</p> <ol style="list-style-type: none"> no significant difference in overall LVEF (95% CI 0.961-1.118, $P=0.36$) no variance in LVDD ($P=0.95$) and LVDs ($P=0.76$) decreased infarct size ($P=0.036$) and better regional function in BMC group 								

expanded EPCs accumulated in the ischemic area, incorporated into foci of myocardial neovascularization, and had beneficial effects on the preservation of left ventricular (LV) function, characterized by significantly smaller ventricular dimension, better preserved local wall motion, significantly greater capillary density and the significantly less extension of left ventricular scarring (22).

These and other findings in animal models (Table 1), have significantly raised expectations for subsequent clinical trials, with the anticipation of rapid translation into clinical therapeutic applications.

5.2. Subsequent Disappointing Results in Clinical studies

However, the results of EPCs infusion in several human clinical trials have not shown the efficacy anticipated based on pre-clinical animal studies (Table 2) (8, 31-33).

The TOPCARE-AMI Trial evaluated intracoronary infusion of either CACs or BM-derived endothelial progenitor cells (BM-EPCs) in patients with AMI, treated with coronary stenting (31). Primary endpoints were feasibility and safety, including procedural complications, in hospital course, ventricular

Table 3. Impact of pre-existing atherosclerotic disease and cardiovascular risk factors on EPCs number and function impairment

		Diabetes mellitus (33,34)	Hypercholesterolemia (35)	CAD (36,37)	Smoking (38,39)	Ageing (40)
EPCs number		dec. by 44%* (P<0.05)	dec. by 36%* (P<0.05)	dec. by 40%* (P=0.04) Inversed correlation to the number of CAD-risk factors ($r=-0.349$, $P=0.002$)	dec. vs light smoker and heavy smoker (P=0.037, 0.038). inc. rapidly after cessation (P<0.0001) and dec. again after resumption (P=0.0031).	dec. in old ApoE ^{-/-} mice vs control younger
EPCs Function	Adhesion	dec. (P<0.05)	dec. (P<0.05)	dec. by 42%* (P<0.05)	/	/
	Proliferation	dec. by 48%* (P<0.01)	dec. by 48%* (P<0.01)	dec. by 25%* (P<0.05)	/	/
	Migration	/	/	dec. by 58%* (P<0.05) Inversed correlation to the number of CAD-risk factors ($r=-0.484$, $P=0.002$)	dec. by 48-57%* (P<0.05)	/
	Tube structure formation	dec. by 60%* (P<0.05)	dec. by 60%* (P<0.05)	dec. by 45%* (P<0.05)	/	/

* Percentage is approximate value, being directly cited or calculated from the data of literature. **dec.**-decrease, **inc.**-increase

arrhythmias, infarct vessel stent thrombosis, repeated MI, cerebral infarction, and death of any cause. In addition, effects on parameters of myocardial function were recorded. The results demonstrated successful infusion without side effect. Four month after treatment, left ventricular ejection fraction (LVEF) significantly increased and end-systolic volumes significantly decreased, without differences between the two treatment groups. Contrast-enhanced magnetic resonance imaging was performed immediately after treatment and after four month and one year revealed an increased LVEF, reduced infarct size, and absence of reactive hypertrophy, suggesting functional regeneration of the infarcted ventricles. However, this feasibility trial did not include a control group without cell infusion.

Therefore, it remained unclear if the LV functional improvement was secondary to standard treatment including acute PCI and aggressive medical management, alone or if EPCs infusion contributed to the beneficial outcome.

A subsequent trial by Lunde *et al.* randomized patients with acute anterior wall STEMI treated with PCI to coronary infusion of autologous BM-EPCs versus placebo (32). The data showed no impact of BM-EPCs infusion on global left ventricular function, with a non-significant 0.6% increase by both SPECT and echocardiography and a non-

significant 3.0% decrease by MRI in the treatment group at the 6-month follow up.

In a review article, Rosenzweig *et al.* reviewed several other clinical studies employing BM- or PB- derived EPCs for cellular therapy of cardiac disease (34). Overall, the results are inconclusive.

6. DOSE EPCS DYSFUNCTION CONTRIBUTE TO THE DISCREPANCIES BETWEEN EXPERIMENTAL AND CLINIC RESULTS?

Exploring the experimental details of the animal models and clinical trials, an important difference lies in the EPCs donor population: The origin of the (homo- and heterologous) EPCs in animal experiments were healthy human donors or animals without vascular disease or cardiovascular risk factor. In contrast the autologous EPCs in clinical studies were derived from the patient subsequently treated and therefore a donor with pre-existing cardiovascular disease and cardiovascular risk factors. We hypothesize that this difference in EPCs origin has been associated with infusion of dysfunctional EPCs in the human, clinical trials, which in turn is important reason for differences in outcomes.

In fact prior data had demonstrated that many pathological conditions have adverse effects on EPCs number and function (35-42). (Table 3) The molecular mechanism responsible for the EPCs dysfunction are incompletely understood, but cellular signaling processes appear to play a

central role (Figure 1). The most important of these cellular signaling processes and their coordination are discussed below

6.1. Extracellular Signal-Regulated Kinase-2 (ERK-2)

ERK-2 belongs to the subgroup of mitogen-activated protein kinases (MAPKs), a family of conserved serine/threonine kinases that regulate cellular proliferation, differentiation, survival, and motility in response to diverse extracellular stimuli including mitogens, growth factors, and cytokines (43). ERK-2 can be activated by the key chemokine regulating EPCs homing and mobilization, stromal cell-derived factor-1 (SDF-1), through its G protein-coupled transmembrane spanning receptor CXCR4 (44, 45). In EPCs, CXCR4 blockade reduces migratory and angiogenic capacities (46), while the CXCR4 sensitizer shingosine-1-phosphate (S1P) stimulates functional capacity (47). Friedrich *et al.* recently published their research on ERK-2 for EPCs dysfunction in coronary artery disease (CAD). Their study shows an ERK-2 signaling defect in CAD-EPCs. Interestingly, their data also shows that SDF-1 can specifically, and in a dose-dependent pattern activate ERK-2, which improves CAD-EPCs adhesion *ex vivo* (48). This may identify SDF-1 as a potential target to overcome EPCs dysfunction.

6.2. Integrin-Linked Kinase (ILK)

ILK is a highly conserved 59-kD multi-domain protein with serine/threonine kinase activity that interacts with $\beta 1$ - and $\beta 3$ -integrins, signaling molecules including lipid second messengers, protein kinase B (Akt), and glycogen synthase kinase-3 (GSK-3) (49,50). Thereby, ILK bridges extracellular matrix signals and inside-out signals with receptor tyrosine kinases and the actin cytoskeleton resulting in the regulation of diverse cellular functions including proliferation, survival, differentiation, adhesion, and migration (49, 51-54). In EPCs, over-expression of ILK has been proven to provide protective effects against anchorage or nutrient deprivation *in vitro* and enhances neovascularization in the hind limb ischemic model (55). ILK is also up-regulated in endothelial cells associated with increased expression of intercellular adhesion molecule-1 (ICAM-1) and SDF-1, as well as improved induction of EPCs recruitment to ischemic areas (56). Werner *et al.* examined the role of ILK on EPCs function in patients with stable CAD (57). EPCs were isolated from blood and cultured. Their migration and dynamic adhesion function was studies *ex-vivo* using modified Boyden chambers and the laminar flow chamber respectively. The authors demonstrate that protein expression of ILK is significantly down-regulated in EPCs isolated from patients with CAD compared to healthy controls. However, if the EPCs from CAD patients were transfected with wild-type (WT)-ILK, subsequent up-regulation of ILK expression was associated with potent improvement in EPCs proliferation, migration, and adhesion ability.

6.3. Kinin B2 Receptor (B2R)

Kinins, generated through kininogen cleavage by kallikreins, are present in various tissues. The kallikrein-kinin system (KKS) contributes to the

revascularization of ischemic tissues (58). The G protein-coupled receptor B2R mediates the pro-angiogenic effects of kinins through promoting vascular cell proliferation and survival (59). B2R is also involved in the recruitment of EPCs to sites of ischemia and in their proangiogenic action. In a series of experiments, Kränkel *et al.* demonstrated that bradykinin (BK) exerts a potent chemo-attractant activity on human EPCs via a B2R/phosphoinositide 3-kinase (PI3K)/eNOS-mediated mechanism *ex-vivo* (60). BK-induced EPCs polarization, formation of filopodia, and migration could be inhibited by the B2R antagonist icatibant, PI3K inhibitor LY294002 or eNOS inhibitor L-iminoethyl-Lornithine (L-NIO). Increased phosphorylation of eNOS after stimulation of EPCs with BK and a concordant phosphorylation of Akt and β -catenin translocation to nuclear/perinuclear regions in BK-treated EPCs were all suspended in the presence of icatibant. Meanwhile, expression of B2R on human EPCs from patients with cardiovascular disease was remarkably reduced comparing with the healthy controls. Additional animal experiment verified the importance of B2R in EPCs recruitment. In a mouse model of hind-leg ischemia EPCs homing was studied after injection of EPCs from wild-type and B2R-/- mutant mice. As compared with controls given wild-type mice-EPCs in the absence of icatibant, EPCs homing was significantly reduced in ischemic adductors of icatibant treated mice injected with EPCs of wild-type mice, and in mice not treated with icatibant after injection of EPCs from B2R-/- mice.

6.4. Akt (protein kinase B)

Akt, also PKB(protein kinase B), belonging to Serine / Threonine Protein Kinase, is an important regulator of various cellular processes as the downstream effector of PI3K, which can be phosphorylated (activated) by chemokine SDF-1. Fulton *et al.* (61) have found that Akt can directly phosphorylate eNOS on serine 1179 and activate the enzyme, leading to NO production, whereas mutant eNOS (S1179A) is resistant to phosphorylation and activation by Akt. Moreover, using adenovirus-mediated gene transfer activated Akt increases basal NO release from endothelial cells, whereas activation-deficient Akt attenuates NO production stimulated by VEGF. As eNOS exerts a critical influence on EPCs functions (62, 63), these results indicate that Akt regulates EPCs' biological behavior through eNOS modulation.

6.5. Endothelial Nitric Oxide Synthase (eNOS) and Superoxide-Producing Enzyme Systems

eNOS is of key importance for the regulation of mobilization and function of EPCs. Mice lacking eNOS had defective EPCs mobilization, resulting in reduced VEGF-induced mobilization of EPCs (62). The provision of exogenous nitric oxide (NO), the product of eNOS, can promote microtubule formation of EPCs within the matrigel assay, however, when NO production was inhibited via the addition of L-NAME, a known inhibitor of eNOS, microtubule formation was significantly impaired (63). In addition, NO-mediated signaling pathways have been previously proposed to be essential for EPCs mobilization. Matrix

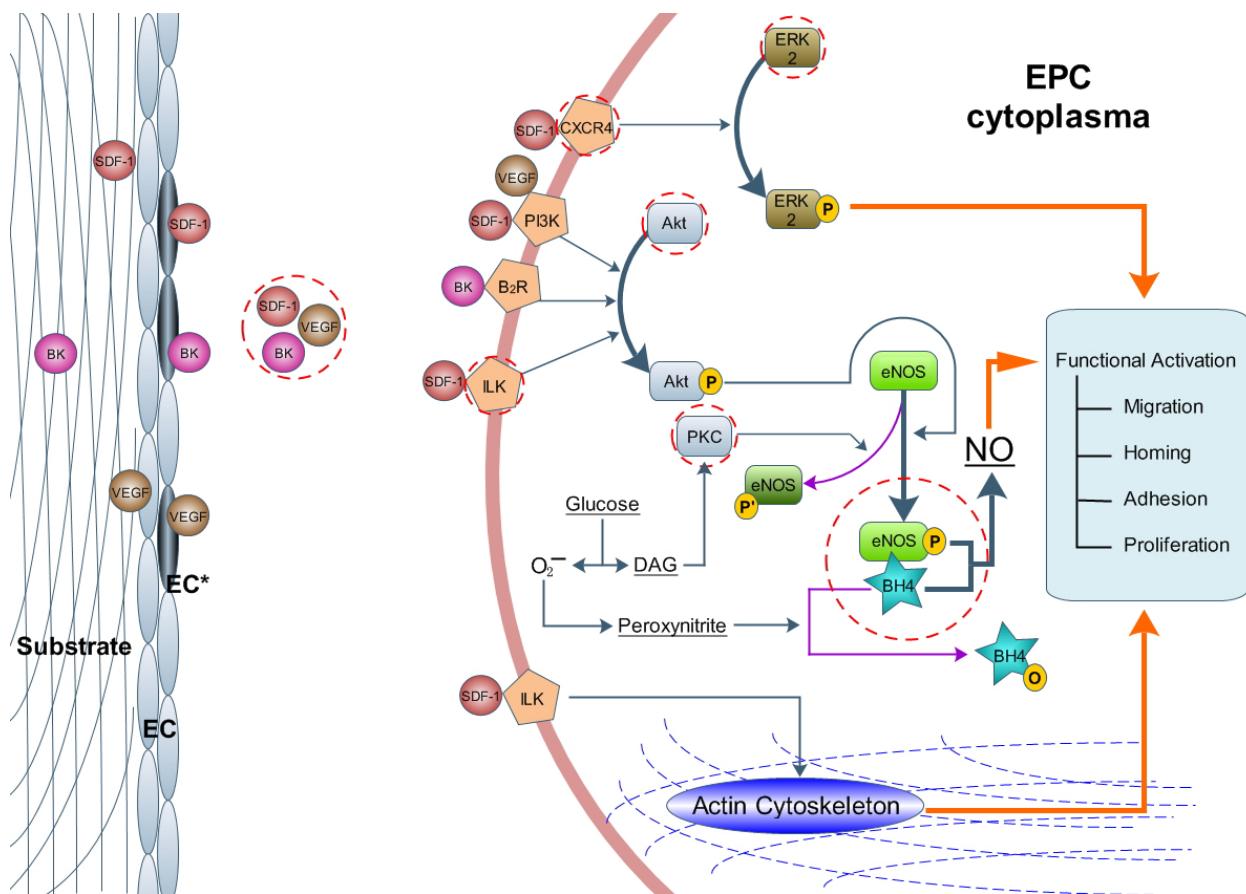


Figure 1. Cell signaling and regulation mechanism involved in EPC dysfunction. High glucose level will induces the phosphorylation of eNOS through DAG/PKC pathway, however the product of the reaction is an isotype of phosphorylated eNOS with attenuated function, hence leading to reduced NO synthesize. Meanwhile, increased O₂- induced by high glucose can oxidize BH4, the co-enzyme of eNOS, causing reduced NO synthesize and even more O₂- production, which leads to the uncoupling of eNOS. The red dashed-line circle presents some of the better documented regulating mechanisms ameliorating impaired EPCs function. Cytokines SDF-1, VEGF, BK are released by the impaired endothelial cell (EC) and substrate. SDF-1/CXCR4/ERK2 axis: SDF-1 induces ERK2 via its trans-membrane receptor CXCR4 and activate EPCs function. Akt/eNOS axis: Akt phosphorylates eNOS in response to cytokines, and ultimately produces NO and lead to EPCs activation. SDF-1/ILK axis: ILK transfers the signal of SDF-1 and finally activates EPCs function through various pathways, including activating of Akt/eNOS axis and modulating actin cytoskeleton.

metalloproteinases-9 (MMP-9), which is essential for VEGF-induced progenitor cell mobilization, is activated by NO. The number of CFU-EC was significantly greater in MMP-9^{+/+} than MMP-9^{-/-} mice following the kinetics of SDF-1 or VEGF (64). Pro-MMP-9 was significantly reduced in bone marrow plasma from eNOS deficient mice, and incubation of the NO donor S-nitrosopenicillamine increased expression of pro-MMP-9 in cultivated bone marrow (62).

It has been shown that EPCs have lower levels of basal and stress-induced intracellular reactive oxygen species (ROS) than primary endothelial cells, because they express higher levels of catalase, manganese superoxide dismutase (MnSOD) and glutathione peroxidase type 1 (GPx-1) (65, 66). Thus, normal EPCs are protected against oxidative stress

consistent with their progenitor cell character. Accordingly, in GPx-1 deficient mice, EPCs showed a reduced ability to neutralize oxidative stress *in vitro*, which was associated with impaired migration toward vascular endothelial growth factor (VEGF) and increased sensitivity to ROS-induced apoptosis, and an impaired capacity to promote angiogenesis in wild-type mice, whereas wild-type EPCs were effective in stimulating angiogenesis in GPx-1-deficient mice (67). Under certain pathological conditions, for example diabetes, EPCs produce excessive superoxide anion (O₂⁻) and show impaired migratory capacity compared with non-diabetic control subjects, and there is uncoupling of the eNOS (the phenomenon of a concomitant increase in eNOS expression and reduced production of NO) resulting in O₂⁻ formation instead of NO, which will subsequently cause EPCs dysfunction (68). In general,

eNOS functional impairment coupled with over-activation of superoxide-producing enzyme systems is one of essential factors for EPCs dysfunction.

6.6. Interaction between individual cellular signaling processes

The above described individual cell signaling pathways appears to be a highly interactive system, coordinating EPCs function. Although the interactions between these factors are not completely understood, recent literature describes up to 3 axes with a number of branches as illustrated in Figure 1.

7. DOES THE HOST TISSUE ENVIRONMENT CONTRIBUTE TO THE DISCREPANCIES BETWEEN EXPERIMENTAL AND CLINIC RESULTS? DOES

EPCs homing and functional integration require a complex interaction of the injected cells with the host environment. Therefore, besides the above-described dysfunction of autologous EPCs used in clinical trials, there are host-tissue related factors that might limit the efficiency of EPCs infusion in clinical settings.

7.1. Ageing

Ageing is an important factor associated with decreased number and impaired function of EPCs. Most patients enrolled in clinical trials of acute coronary ischemia have been middle-aged or older (31-34). In prior studies, EPCs from elderly individuals were significantly more frequently β -galactosidase positive compared with EPCs isolated from young subjects, demonstrating increased cellular senescence, where telomerase activity was reduced (69). Similarly, bone marrow transplantation from young, but not old non-atherosclerotic mice, prevented atherosclerosis progression in apo-lipoprotein E knock-out recipients (42). Aging also decreases hypoxia-inducible factor 1- α leading to diminished expression SDF-1 (70), resulting in impaired intracellular signaling pathways involved in inducing EPCs' function.

7.2. Pre-existing Cardiovascular Disease

Despite current incomplete understanding, it is conceivable that tissue of hosts with pre-existing, atherosclerotic disease and/or cardiovascular risk factors, would have impaired ability of homing and interacting with implanted cells. Specifically, the local composition of tissue in patients with pre-existing atherosclerotic disease and superimposed acute events, likely provides an entirely different internal environment for the transplanted cells compared to the acute injury models (previously healthy animals) typically used in the experimental studies.

As discussed above, endogenous and infused EPCs homing, adhesion and proliferation behavior is initiated and regulated by several chemokines such as SDF-1, and VEGF. These chemokines are produced and released by damaged tissue such as the stroma under the endothelial layer and endothelium itself. For example, it has been

demonstrated that SDF-1 mRNA and SDF-1 positive cells were significantly reduced in wound tissue of diabetic mice (71). These experimental results are supported by findings in diabetic patients with peripheral artery disease, where the expression of VEGF, SDF-1, and CXCR4 in human limb muscle was significantly suppressed, below the levels detected in non-diabetic controls, suggesting a lack of recruitment signals in the diabetic patients. Furthermore, the levels of these cytokines are down-regulated in chronic ischemia, as opposed to up-regulated in more acute ischemia (72).

8. STRATEGIES TO RESTORE EPCS FUNCTION AND CYTOKINE ENVIRONMENT

Summarizing the above, the senescence of autologous EPCs of elderly subjects and the changes in the cytokine environment in the tissue of subjects with pre-existing atherosclerotic disease, predispose to a decreased number and impaired function of EPCs, resulting in limited efficacy of autologous EPCs implantation in clinical studies. In order to replicate the positive results obtained in animal models, it is therefore necessary to develop strategies to improve EPCs function. Therapeutic approaches to restore normality of EPCs function and internal tissue environment are discussed below.

8.1. Heterologous EPCs Transfusion

The use of heterologous cells from healthy individual might be an option to compensate for the cell function deficiency; however, it might also exert additional risks, such as activation of the immune response on patients. In extreme condition, cell rejection might occur posing a high risk for the treated patients. Moreover, testing the compatibility between heterologous cells and the recipient will add additional cost. Therefore, the indications of heterologous cell transplantation are restricted to fields of hematopoietic system malignancy or benign diseases, immune system deficiency, and a few solid tumors.

8.2. Pretreatment of Impaired Autologous EPCs

Caballero *et al.* reported in 2007 that ischemic vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells (73). On the basis of functional impairment of EPCs in diabetic patients, this research implicated that improving and restoring the impaired cell function before transplantation would be critical in the use of 'abnormal' autologous EPCs of patients with pre-existing atherosclerotic disease and/or risk factors. According to the above-described mechanism involved in EPCs dysfunction, there have been several pertinent approaches to improve EPCs' function in or *ex vivo* (Figure 1).

8.2.1. Agents targeting eNOS and superoxide-producing enzyme systems

Transcriptionally enhancing eNOS expression in EPCs can activate the nitric oxide system.

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Sasaki *et al.* discovered that ex-vivo pretreatment of EPCs with endothelial NO synthase enhancer AVE9488 enhances their functional activity for cell therapy (74). Their study showed *ex vivo* treatment for 18–24 h with AVE9488 increased eNOS mRNA expression in PB-EPCs, CD34+ cells, and BMC by 2.1-fold ($P < 0.05$) and eNOS activity by >3-fold ($P < 0.05$). The increased eNOS expression was associated with an enhanced migratory capacity *in vitro* ($P < 0.01$) and improved neo-vascularization capacity of the infused BMC in an ischemic hind limb model *in vivo* ($P < 0.001$).

Tetrahydrobiopterin (BH4) is an essential cofactor for eNOS-mediated NO formation (75). BH4 levels reduced by 59% in human EPCs after glucose-challenge, whereas oxidized biopterin levels raised by 36%. Exogenous treatment of glucose-challenged EPCs with BH4 increased their intracellular availability by fivefold compared with untreated controls. As a result, glucose-mediated exaggerated O₂- production was attenuated. Normalization of BH4 levels by exogenous BH4 addition completely rescued EPCs migratory capacity and normalized ROS levels, which indicated recoupling of uncoupled eNOS by BH4 treatment (64). Prevention of BH4 oxidation may be of additional value to rescue the decreased BH4 levels in EPCs from diabetic patients.

Protein kinase C (PKC) inhibition is another effective way to resolve the uncoupling of eNOS and ROS-mediated EPCs dysfunction in diabetic subjects. PKC is involved in vascular O₂- production in diabetic vessels (76). On a molecular level, high glucose conditions increase intracellular diacylglycerol (DAG) levels ultimately leading to PKC activation (77). Subsequently PKC mediated phosphorylation of nitric oxide synthase (NOS) III protein may reduce, but not increase, the activity of the enzyme (78, 79). On the other hand, hyperglycemia greatly enhances endothelial superoxide production, leading to increased vascular formation of peroxynitrite (80, 81). Peroxynitrite in turn oxidize avidly BH4 leading to BH4 deficiency, ultimately resulting in production of superoxide rather than NO (82, 83). Although a similar mechanism for PKC in eNOS uncoupling has not been verified in EPCs, inhibition of PKC by chelerythrine has been proved to attenuate O₂- production after treatment of EPCs with glucose (64).

8.2.2. Regulating the cell signaling pathways controlling EPCs function

As demonstrated in Figure 1, there are several key regulatory mechanisms in the signaling pathways of maintaining normal EPCs behavior. These key mechanisms could be potential targets for improving and restoring impaired EPCs functions. Indeed, beneficial results have already been described in a few studies.

As described above, the SDF-1/CXCR4/ERK2 axis plays a critical role in EPCs homing and adhesion. SDF-1 acts as chemokine, CXC4

and ERK2 are important second messengers. Walter *et al.* demonstrated that the CXCR4 sensitizer S1P stimulates the functional capacity of CAD-EPCs (47). Specifically, tyrosine phosphorylation of CXCR4 by S1P and its synthetic analog FTY720 occurred rapidly reaching a 2-fold induction after 30 minutes incubation (1.96±0.57, $P < 0.01$). Phosphorylation of JAK2, a known downstream target of the CXCR4 receptor, was also significantly increased after pre-incubation of EPCs with S1P or FTY720. As a result, CAD-EPCs' homing and recovery of blood flow in hind limb ischemia were improved. On the ERK-2 level, augmenting ERK-2 signaling was demonstrated by transfecting EPCs from patients with CAD with a commercially available activating MEK-2 (MAPKs Kinase 2) cDNA construct. This led to encoding of activated MEK-2 and robust activation of ERK-2 signaling, which in turn reversed the adhesion defect of CAD-EPCs and restored adhesion activity to control levels (47).

As described above in detail, the SDF-1/ILK axis is another regulating pathway for EPCs migration, homing and proliferation. Werner *et al.* showed that WT-ILK transfection significantly improved EPCs migration (57). The restoration of ILK protein expression in EPCs from patients with CAD reversed the migratory defect of CAD-EPCs as compared to healthy controls, and there was a significant increase in colony forming units in cells transfected with WT-ILK to 191±12.9% of control-transfected cells. Moreover, adhesion was significantly increased in WT-ILK-transfected EPCs

Akt/eNOS axis is proposed to be a reliable target to restore EPCs function, considering its great importance in regulating NO release. It has been demonstrated that Akt itself is activated through PI3K in response to various stimuli (84). Downstream, Akt directly phosphorylates eNOS on serine 1179 and activates the enzyme, leading to NO production. This activating effect of Akt on eNOS is selective and specified (68). Transfection of WT-Akt, but not the kinase-inactive variant into COS-7 cells (which do not express NOS) results in markedly increased NO accumulation, which is enhanced by co-transfection of eNOS (68).

8.2.3. Agents improving EPCs tolerance to the host tissue environment

Tripterygium wilfordii is a chemical compound extracted from the Chinese plant Tripterygium wilfordii, which has demonstrated anti-inflammatory properties in several animal models (85-89). Our group has tested the effect of tripterygium on vascular endothelial cell (EC), the progeny of EPCs (90). In inflammatory conditions, tripterygium inhibits the expression of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) in human umbilical vein endothelial cells (HUVEC) in a dose-dependent manner. Effects on endothelial CAM of other proinflammatory cytokines, such as interleukin-1 β and interferon- γ , were also inhibited significantly by tripterygium. Moreover,

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tripterine inhibited adhesion of human monocytes and T lymphocytes to TNF- α -stimulated HUVEC. Therefore, tripterine can attenuate the damage of EC in inflammatory tissue conditions. Because an inflammatory tissue environment is found in many pathologic conditions (including diabetes, atherosclerosis, and hyperlipidemia) in which EPCs dysfunction have been demonstrated, on-going research of our group examines the hypothesis that tripterine could also ameliorate the impairment of EPCs in such inflammatory circumstance (sponsored by National Natural Science Foundation of China, Grant number 30770614).

8.3. ENVIRONMENT MODULATION

8.3.1. Modification of arteriosclerosis risk factors *in vivo*

Aggressive modification of cardiovascular risk factors is generally indicated in the patient population enrolled in clinical EPCs trials. Besides its many documented clinical benefits, risk factor modification has been shown to improve EPCs function. Specifically, the effect of statins has been widely investigated and data demonstrates an increase number of EPCs, accelerated re-endothelialization and reduced neointimal formation during treatment (91). Single or combined administration of oral anti-diabetic drugs and insulin can increase number of EPCs (92). Pistrosch *et al.* demonstrated that the PPAR γ -agonist rosiglitazone could improve migratory activity of cultured EPCs and increase EPCs number (464 \pm 33, $P < 0.01$), in patients receiving a dose of 4 mg, b.i.d for 12 weeks (93).

8.3.2. Administration of cytokines *in vivo*

Since SDF-1 expression and the number of SDF-1 positive cells are significantly reduced under certain chronic disease conditions (69, 70), the administration of exogenous SDF-1 may facilitate the recruitment of EPCs. In an animal model, exogenous administration of SDF-1 α via local wound injections enhanced EPCs mobilization and homing in diabetic peripheral cutaneous wounds, as well as wound healing (69). SDF-1 can be cleaved by exopeptidases and matrix metalloproteinase-2, thereby generating an inactive protein. By mutating the SDF-1 cleavage motif, Seger *et al.* generated a stabilized SDF-1 mutant, which was cleavage resistant, and more efficiently achieved improved cell recruitment and functional recovery after AMI compared to wild type SDF-1 (94). Besides direct injecting into ischemic local tissue, there exist physical methods to improve the endogenous expression of chemokines. For example, low energy shock wave application can activate the tissue and stimulated the expression of SDF-1 and VEGF within the target tissue and promote homing of intravenously infused EPCs in uninjured and chronically ischemic rats (95).

In summary, among the above described strategies, medications used for atherosclerosis risk factor modification (environment modulation) are the most convenient methods to improve EPCs function *in*

vivo. However, the known fact that chronic atherosclerotic disease progression and acute events occur in many patient despite good risk factor control, demonstrates the need for further investigation.

Pretreatment of the impaired patient-derived autologous EPCs is currently extensively investigated as tool to reverse the molecular mechanism underlying EPCs dysfunction. These approaches can be conducted *ex vivo*, and do not disrupt the routine therapy. In the context of planned EPCs therapy, they can expand the cell quantity and function. However, the clinical efficacy has not been documented in randomized trials.

Heterologous EPCs transfusion is currently not a major clinical target, secondary to the risk of immune reject, the need to suppress the immune system, the high cost, and more complex ethical concerns.

It is important to consider that different diseases may require different therapeutic strategy consistent with different mechanism of EPCs dysfunction, for example, eNOS uncoupling in diabetes vs. reduced ERK-2, ILK and Akt/eNOS signaling in coronary artery disease or advanced age.

9. SUMMARY AND FUTURE PERSPECTIVES

In the last decade, EPCs have been extensively studied and accumulating evidence highlights their importance in vascular repair. However, the mechanisms of EPCs participation in the vasculogenesis and vascular repair are incompletely understood. There is increasing evidence that EPCs-based therapy could accelerate the process of re-endothelialization of damaged vascular endothelium in various vascular disease conditions. However, despite encouraging results in animal models, clinical trials in patient with atherosclerotic disease have so far been disappointing, emphasizing that there are multiple critical issues concerning the biology of the EPCs that are incompletely understood and need to be investigated further.

The functional impairment of EPCs in subjects with pre-existing atherosclerotic disease and/or risk factors poses a primary hurdle in using autologous cell for implantation. There is increasing data describing approaches to reverse EPCs dysfunction. Using modulating procedures on the genomic level, molecular signaling level, and related to key regulation enzymes, researchers have successfully improved or restored functions of dysfunctional EPCs.

Another important hurdle for autologous EPCs infusion is the host tissue environment in patient with preexisting atherosclerotic disease and/or risk factors. Studies on tissue environment modulation also achieved some encouraging results.

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Specifically, local or systemic administration of cytokines involved in EPCs mobilization, such tissue exhibits improved homing and adhesion ability of EPCs to impaired endothelium.

Besides EPCs function, a sufficient number of EPCs is critical in clinical study. Compared with the limited size of typical experimental animals such as mouse, rat and rabbit, human will demand a much larger number of EPCs to allow sufficient re-endothelialization and recovery of the ischemic tissue. Up to now, culture methods seem to be able to expand cell number *in vitro* to match the quantity needed in clinic applications. During the culture process of cell expansion, functional and environmental adjustment procedure can be accomplished simultaneously.

Similar to the profound impact of transluminal interventional therapy of vascular disease in last century, EPCs-based endovascular therapy may allow future advances in the treatment of atherosclerotic disease, which remains a leading cause of morbidity and mortality worldwide. However, as described above further evaluation is necessary to overcome the hurdles separating bench results from bedside application.

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Abbreviations: SDF-1: stromal derived factor 1; VEGF: vascular endothelial growth factor; BK: bradykinin; PI3K: phosphoinositide-3-kinase; B₂R: kinin B₂ receptor; ILK: integrin-linked kinase; ERK2: extracellular signal-regulated kinase 2; Akt: serine/threonine protein kinase Akt (protein kinase B); eNOS: endothelial nitric oxide synthase; BH4: tetrahydrobiopterin; PKC: protein kinase C; DAG: diacylglycerol; P and P': with yellow cycle, normal and abnormal phosphorylation; O: with yellow cycle, oxidization; EC: endothelial cell; EC: impaired endothelial cell

Key Words: Endothelial Progenitor Cells; Vascular Repair; Colony-Forming Unit-Endothelial Cells; Circulating Angiogenic Cells; Endothelial Colony-Forming Cells, Review

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