Reduced number and activity of circulating endothelial progenitor cells in patients with idiopathic pulmonary arterial hypertension

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KEYWORDS
Idiopathic pulmonary arterial hypertension; Stem cells; Endothelial progenitor cells; Endothelium; Angiogenesis

Summary
Background: Endothelial dysfunction plays a central and critical role in the initiation and development of idiopathic pulmonary arterial hypertension (IPAH), and a variety of evidence suggests that endothelial progenitor cells (EPCs) constitute one aspect of endothelium repair. In addition, transplantation of EPCs could attenuate pulmonary hypertension induced by monocrotaline in rats. However, it has not been examined and reported whether circulating EPCs from patients with IPAH are damaged.

Methods: EPCs were isolated and cultured from patients with IPAH (n = 20) and matched healthy volunteers (n = 20). Circulating EPC numbers (enumerated as AC133+KDR+ cells) as well as migratory and adhesive activity were assessed. Blood levels of vascular endothelial growth factor (VEGF), homocysteine (Hcy), B-type natriuretic peptide (BNP), von Willebrand Factor (vWF) and interleukin-6 (IL-6) were also measured.

Results: A significant decrease was observed in circulating EPC (AC133+KDR+ cells, 86.6 ± 20.7 cells/ml blood vs. 119.6 ± 25.4 cells/ml blood, P < 0.001) numbers and the cell numbers expanded in vitro (47.2 ± 14.5 vs. 70.7 ± 15.2 EPCs/200 field; P < 0.001) in patients with IPAH. EPCs from patients with IPAH were significantly impaired in their migratory capacity and ability to adhere to fibronectin. Blood levels of VEGF, Hcy, BNP, vWF and IL-6 were elevated in patients with IPAH. EPC numbers and activity were inversely related to Hcy, IL-6, BNP and vWF.

Conclusions: Our observations indicated that EPC numbers and functional capacity were impaired in patients with IPAH, which might not only give potential insight into the
Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a rare disorder of unknown etiology characterized by raised pulmonary artery pressures with pathological changes in precapillary pulmonary arteries. The disease was rapidly progressive, leading to right heart failure and death in a median of 2.8 years from diagnosis before the availability of disease-specific (targeted) therapy through the mid-1980s. Modern treatment has markedly improved physical function and has extended survival, and the 5-year mortality rate is 50%. However, we still do not understand what initiates the disease or what allows it to progress.

Although the specific mechanisms responsible for the development of IPAH remain unknown, a number of mechanisms have been proposed. The histopathologic features of IPAH suggest that endothelial injury and proliferative stimuli are fundamental processes. Abnormal bone morphogenetic protein receptor-type II (BMPR2) may play an important role in the pathogenesis of IPAH, with over 25% of patients with IPAH having abnormal BMPR2 structure or function. The BMPR2 pathway induces apoptosis in some types of cells, and the abnormal pathway activity also permits excessive endothelial cell growth and proliferation in response to a variety of injuries. Additional studies have demonstrated abnormalities in other pathways that may contribute to the pathogenesis of IPAH. These include enhanced expression of the serotonin transporter, diminished expression of the enzymes responsible for synthesis of nitric oxide (NO) and prostacyclin, altered potassium channels, and increased production of several growth factors, including endothelin, vascular endothelial growth factor, and platelet-derived growth factor.

In particular, dysfunction of the endothelium was thought to play a central and critical role in the initiation and development of IPAH. Endothelial dysfunction ultimately represents a balance between the magnitude of injury and the capacity for repair. A variety of evidence suggests that endothelial progenitor cells (EPCs) constitute one aspect of this repair process. EPCs are a cell population that have the capacity to circulate, proliferate, and differentiate into mature endothelial cells, but have neither acquired characteristic mature endothelial markers nor formed a lumen. These cells co-express hematopoietic stem/progenitor cell markers (CD34 or AC133) as well as endothelial markers (VE-Cadherin or VEGFR-2). Laboratory evidence suggests that these precursors participate in postnatal neovascularization and re-endothelialization.

Recently, experimental data suggested that transplantation of EPCs attenuated monocrotaline-induced pulmonary hypertension in the rat model. Moreover, Takahashi et al. reported that transplantation of autologous EPCs gave significant improvements in mean pulmonary artery pressure, cardiac output, and pulmonary vascular resistance in the dog dehydromonocrotaline model.

On the basis of these considerations, we hypothesized that circulating EPCs from patients with IPAH might be damaged, which is associated with endothelial dysfunction and could promote the disease progression. To test this hypothesis, the numbers and activity of EPCs from peripheral blood of patients with IPAH were determined in the present study, which has not been previously examined and reported.

Materials and methods

Characteristics of the patients and controls

Twenty IPAH patients were consecutively enrolled. The control group comprised 20 healthy volunteers who were matched as to gender and age with the patients, and clinical evaluation has been done to be sure about healthy status of control group. Ethics committee approval was obtained, as was informed consent from patients and controls. Table 1 shows the clinical details of the studied population. IPAH was defined as pulmonary hypertension unexplained by any secondary cause, on the basis of the criteria of the National Institutes of Health registry. The diagnosis of IPAH was based on clinical assessment, right heart catheterization, echocardiography, spiral-computed tomography of the pulmonary arteries, pulmonary angiography, ventilation/perfusion lung scan, and complete lung function testing. Pulmonary hypertension as a result of heart disease, pulmonary disease, sleep-associated disorders, chronic thromboembolic disease, autoimmune or collagen vascular disease, HIV infection, liver disease was excluded. All participants were all free of wounds, ulcers, retinopathy, recent surgery, inflammatory or malignant disease that may influence EPCs kinetics.

At the beginning of each experimental session, a clinical evaluation including 6-min walk test was performed, and 24 h after 6-min walk test venous blood was collected for measurement of laboratory variables, isolation of EPC, and flow cytometric analysis during catheterization.

The exercise capacity and hemodynamics

The 6-min walk test was performed in all subjects using a standardized protocol in accordance to the American Thoracic Society statement 2002. Patients walked along an enclosed-level corridor, and length to first turnaround point was 40 m. Technicians did not escort but encouraged patients using standard phrases such as “You are going well,” “Keep up the good work,” and were instructed not to use other encouragement. All patients were told to use their own pace, but to cover as much ground as possible in 6 min.

In IPAH patients a Swan-Ganz catheter (Arrow Deutschland GmbH, Erding, Germany) and an arterial catheter...
(Angiocath, BD) were inserted into the right internal jugular and right radial artery, respectively. Hemodynamic measurements were performed in recumbent position. Continuous monitoring included heart rate, systemic and pulmonary artery blood pressures and transcutaneous oxygen saturation. Additional parameters were pressures in wedge position and right atrium. Cardiac output was obtained, using triplicate measurements with the thermodilution method (Agilent, Boeblingen, Germany). Pulmonary vascular resistance (PVR) and systemic vascular resistance (SVR) were calculated according to the standard formulas as follows.32

\[
PVR = \frac{80}{C} \times \left( \text{mean pulmonary artery pressure} \right) - \left( \text{pulmonary capillary wedge pressure} \right) / \text{cardiac output} \text{[L/min]}
\]

and

\[
SVR = \frac{80}{C} \times \left( \text{mean systemic artery pressure} \right) - \left( \text{right atrial pressure} \right) / \text{cardiac output} \text{[L/min]} \text{ defined in dyns/cm}^5
\]

Both the walk test and right heart catheterization were administered by personnel who were unaware of the study protocol.

### Isolation, cultivation and characterization of circulating EPCs

EPCs were isolated, cultured and characterized according to previously described techniques.17,33–36 Briefly, mononuclear cells (MNCs) were isolated from peripheral blood of patients with IPAH or control subjects by Ficoll density gradient centrifugation and cultured on fibronectin (Chemicon)-coated dishes in Medium 199 (Sigma) supplemented with 10% fetal-calf serum and vascular endothelial growth factor (VEGF, 50 ng/ml, Chemicon). After 4 days in culture, adherent cells were incubated with DiLDL (Molecular Probe) and stained with FITC-labeled Ulex europaeus agglutinin (UEA-1; Sigma). After the staining, samples were viewed with an inverted fluorescent microscope (Leica) and further demonstrated by laser scanning confocal microscope (LSCM, Leica). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs.33,35,36 Two or three independent investigators evaluated the number of EPCs per well by counting 15 randomly selected high-power fields (×200) with an inverted fluorescent microscope.

### Table 1 Characteristics of patients with IPAH and control subjects.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>IPAH (n = 20)</th>
<th>Control (n = 20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year</td>
<td>36.6 ± 11.4</td>
<td>37.3 ± 10.8</td>
<td>NS</td>
</tr>
<tr>
<td>Sex, no.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of symptoms, months</td>
<td>25 ± 10</td>
<td></td>
<td></td>
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<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.5 ± 0.8</td>
<td>4.7 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.2 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Medication, no.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anticoagulant agents</td>
<td>18</td>
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<td></td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrates</td>
<td>7</td>
<td></td>
<td></td>
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<tr>
<td>Digitalis</td>
<td>4</td>
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<td></td>
</tr>
<tr>
<td>Diuretics</td>
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<tr>
<td>Biomarkers</td>
<td></td>
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<tr>
<td>VEGF, pg/mL</td>
<td>603.4 ± 88.5</td>
<td>305.5 ± 75.6</td>
<td>P&lt;0.001</td>
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<tr>
<td>BNP, pg/mL</td>
<td>151.7 ± 51</td>
<td>19.2 ± 8.6</td>
<td>P&lt;0.001</td>
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<tr>
<td>IL-6, pg/mL</td>
<td>5.33 ± 1.07</td>
<td>1.75 ± 0.69</td>
<td>P&lt;0.001</td>
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<tr>
<td>vWF, %</td>
<td>215 ± 45</td>
<td>110 ± 27</td>
<td>P&lt;0.001</td>
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<tr>
<td>Hcy, mmol/L</td>
<td>19.4 ± 5.7</td>
<td>12.9 ± 3.8</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>The 6-min walk distance, m</td>
<td>257 ± 42</td>
<td>586 ± 80</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Mean pulmonary artery pressure, mmHg</td>
<td>58 ± 9</td>
<td></td>
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<tr>
<td>Pulmonary vascular resistance, dyn cm⁻¹</td>
<td>1067 ± 220</td>
<td></td>
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<tr>
<td>Cardiac output, L/min</td>
<td>3.7 ± 0.6</td>
<td></td>
<td></td>
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<tr>
<td>Pulmonary function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO₂, %</td>
<td>93 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SvO₂, %</td>
<td>61 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>85 ± 6</td>
<td></td>
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<tr>
<td>FEV₁, % predicted</td>
<td>73 ± 3</td>
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</table>

SaO₂ indicates arterial oxygen pressure; SvO₂, mixed venous oxygen saturation; FVC, forced vital capacity; and FEV₁, forced expiratory volume in 1 s. Data are mean ± SD.
Flow cytometry analysis

For flow cytometric determination of circulating EPCs (AC133+/KDR+), 100 μL of whole blood was labeled for 30 min at 4°C using manufacturer-recommended concentrations with antihuman-KDR-PE (Becton Dickinson) and antihuman-AC133-APC (Miltenyi Biotec). Fluorescent isotype-matched antibodies (Becton Dickinson) were used as controls. The suspension was then incubated with fluorescent-activated cell sorter (FACS) lysing solution (Becton-Dickinson) according to the manufacturer’s instructions for 10–15 min. After washing and fixation, samples were analyzed on a FACS—Calibur Instrument (Becton-Dickinson). Surface markers AC133 and KDR were determined on cells in the lymphocyte gate, because this is where EPCs are commonly found.33 The percentages of positive cells were converted to cells per mL of blood using the complete blood count.33,37,38

Migration assay

EPCs migration was evaluated by using a modified Boyden chamber assay (Jiangsu Qilin medical equipment factory, China). In brief, isolated EPCs were detached using 1 mmol/L EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 μL M199, and counted, then 2 × 10⁴ EPCs were placed in the upper chamber of a modified Boyden chamber. M199 and human recombinant VEGF (50 ng/mL) were placed in the lower compartment of the chamber. After 24 h incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cells were stained with Giemsa solution. Cells migrating into the lower chamber were counted manually in three random microscopic fields (×200).33,35,38

Cell adhesion assay

Human EPCs were washed with PBS and gently detached with 1 mmol/L EDTA in PBS. After centrifugation and resuspension in M199, 5% FBS, identical cell numbers were replated onto fibronectin-coated culture dishes and incubated for 30 min at 37°C. Adherent cells were counted by independent-blinded investigators.33,35,38

VEGF, homocysteine, B-type natriuretic peptide, von Willebrand factor, and interleukin-6

Blood levels of VEGF and interleukin-6 (IL-6) were measured by ELISA (R&D), according to the manufacturer’s instructions. Plasma total homocysteine (Hcy) was measured using an automated fluorescence polarization immunoassay on an IMx Analyser as described previously.38,39 Von Willebrand factor (vWF) concentrations were measured by enzyme immunoassay for the assessment of endothelial cell dysfunction. B-type natriuretic peptide (BNP) was measured by the Triage BNP test (Biosite, San Diego, CA).40

Statistical analysis

All values were expressed as mean ± SD. Student’s unpaired t test was performed for comparison of data between two groups. Categorical variables were compared by means of the χ² test. Correlations were tested by Pearson analysis. Values of P < 0.05 were considered significant. All statistical analyses were performed with SPSS 12.0.

Results

Blood levels of VEGF, Hcy, BNP, vWF and IL-6 in patients with IPAH

Blood levels of VEGF, Hcy, vWF and IL-6 were elevated in patients with IPAH (Table 1).

EPC levels in patients with IPAH

The number of EPCs in the peripheral blood was assayed via fluorescence-activated cell sorter analysis, which might more closely resemble the in vivo conditions. Circulating EPCs were enumerated as AC133’KDR’ cells.21–23,37 A significant decrease was observed in circulating EPCs in subjects with IPAH compared with matched control subjects (86.6 ± 20.7 vs. 119.6 ± 25.4 cells/mL, P < 0.001, Figure 1). AC133’KDR’ cells correlated to the 6-min walk distance (6MWD, r = 0.71, P < 0.001, n = 40), BNP (r = –0.6, P < 0.001, n = 40), vWF (r = –0.68, P < 0.001, n = 40), IL-6 (r = –0.59, P < 0.001, n = 40), Hcy (r = –0.59, P < 0.001, n = 40), mPAP (r = –0.53, P < 0.05, n = 20), and PVR (r = –0.45, P < 0.05, n = 20). In addition, EPCs were expanded from human blood in vitro and identified by DiLDL uptake and lectin staining. EPC numbers decreased in subjects with IPAH compared with matched control subjects after ex vivo cultivation (47.2 ± 14.5 vs. 70.7 ± 15.2 EPCs/× 200 field; P < 0.001, Figure 2). Differentiating EPCs also correlated to 6MWD (r = 0.65, P < 0.001, n = 40), BNP (r = –0.63, P < 0.001, n = 40), vWF (r = –0.61, P < 0.001, n = 40), IL-6 (r = –0.64, P < 0.001, n = 40), and Hcy (r = –0.52, P = 0.001, n = 40).

Migratory capacity of EPCs in patients with IPAH

To assess the functional activity of EPCs, migration of isolated EPC in response to VEGF was determined using a modified Boyden chamber. As illustrated in Figure 3, the migratory capacity of EPCs isolated from patients with IPAH was significantly impaired compared with healthy matched volunteers (12.5 ± 5.0 vs. 20.5 ± 7.4 cells/× 200 fields, P < 0.001, Figure 3). The migratory capacity of EPCs correlated to 6MWD (r = 0.63, P < 0.001, n = 40), BNP (r = –0.52, P = 0.001), vWF (r = –0.55, P < 0.001, n = 40), IL-6 (r = –0.56, P < 0.001, n = 40), and Hcy (r = –0.53, P < 0.001, n = 40).

EPCs adhesion of EPCs in patients with IPAH

We assessed the adhesion of EPCs, because adhesion to the extracellular matrix is believed to be important during new
blood vessel growth. EPCs from patients with IPAH were found to be significantly impaired in their ability to adhere to fibronectin (24.2 ± 7.2 vs. 38.9 ± 8.0, \( P < 0.001 \), Figure 4).

The ability of EPCs adhesion correlated to 6MWD (\( r = 0.69, P < 0.001, n = 40 \)), BNP (\( r = -0.59, P < 0.001, n = 40 \)), vWF (\( r = -0.63, P < 0.001, n = 40 \)), IL-6 (\( r = -0.71, P < 0.001, n = 40 \)), and Hcy (\( r = -0.43, P = 0.006, n = 40 \)).

**Discussion**

To the best of our knowledge, this is the first study to examine the number and activity of EPCs from peripheral blood of patients with IPAH. We observed that the number of differentiated EPCs and AC133-KDR-positive circulating EPCs was significantly reduced in subjects with IPAH. In addition, the functional activities of isolated EPCs such as migratory and adhesive capacity were also impaired. Therefore, an autologous application/transplantation or a pharmacological mobilization/activation of EPCs might be future possibilities for the treatment of IPAH.

Recently, Nagaya et al.\[27\] have reported transplantation of EPCs modestly attenuated monocrotaline-induced pulmonary hypertension in nude rats (16% decrease in pulmonary vascular resistance). Furthermore, very recent results from the Stewart group show near-complete prevention of pulmonary hypertension in the rat monocrotaline model using syngeneic bone marrow—derived EPCs.\[28\] In addition, in experimental dogs with dehydromonocrotaline-induced pulmonary hypertension, transplantation of autologous EPCs from peripheral blood gave significant improvements in mean pulmonary artery pressure, cardiac output, and pulmonary vascular resistance.\[29\] More recently, we have demonstrated that intravenous infusion of autologous EPCs...
appeared to be feasible and safe, and might have beneficial effects on exercise capacity and pulmonary hemodynamics in adults with IPAH.\textsuperscript{41} Thus, the results of the present study seem consistent with current efforts to ameliorate pulmonary hypertension by augmentation of circulating EPCs.

Pulmonary vascular endothelial dysfunction was thought to play a major role in the initiation and development of IPAH.\textsuperscript{15,16} This endothelial dysfunction ultimately represents an imbalance between the magnitude of injury and the capacity for repair.\textsuperscript{17} There is strong evidence that EPCs may play an important role in endothelium maintenance, being implicated in both re-endothelialization and neovascularization.\textsuperscript{17–19,24–26} In this study, we have observed that circulating VWF levels were significantly higher in patients with IPAH, which reflect the severity of endothelial injury and dysfunction, and it inversely correlated with EPC numbers and functional capacity. Therefore, the reduction in the number of EPCs and their functional impairment may contribute to an insufficient regeneration of the endothelium, which is associated with endothelial dysfunction.

Migration is essential for stem/progenitor cells to invade the sites of injured vessels or the ischemic tissue, which might reflect their homing capacity.\textsuperscript{33} In this study, we observed EPCs isolated from patients with IPAH exhibited a decreased migration in response to VEGF. The reduced response to VEGF might be due to a downregulation of the VEGF receptor KDR, or/and defects in the downstream signaling pathway.\textsuperscript{33} However, at least the expression of KDR receptor was not reduced in EPCs derived from patients with IPAH compared with those from matched volunteers (data not shown), suggesting that defects in the downstream signaling pathways might be responsible for the impairment of cell migration. Future studies will be needed to address this question. The impaired response of EPCs to VEGF in IPAH patients might be responsible for the paradoxical phenomenon between the alteration in endothelial function and the elevated level of VEGF.\textsuperscript{42} In addition, this might also explain the paradoxical phenomenon between reduced number and activity of circulating EPCs and the elevated level of VEGF, which was observed in this study, since VEGF has been shown to promote the mobilization of EPCs.

An additional interesting finding in our study is the reduced capacity of circulating EPCs from patients with IPAH to adhere to fibronectin. This observation, which may be related to a dysregulated integrin synthesis,\textsuperscript{43} can also aid in explaining the reduction in EPCs number and endothelial dysfunction evident in patients with IPAH, for the adhesive capacity of EPCs is thought to be important in EPCs mobilization and homing.

Although the mechanisms by which circulating EPC numbers and activity were impaired in IPAH patients remain to be uncertain to date, there are several possible scenarios which could account for the damaged EPCs. Firstly, our finding of a negative correlation between the IL-6 level and the number and activity of EPCs over the entire study group may support the hypothesis that the individual microinflammatory state is related to the number of circulating EPC. Furthermore, it is known from in vitro experiments that C reactive protein (CRP) attenuates EPC survival and, recently, it has been demonstrated that CRP has the potential to decrease the angiogenic function of EPC.\textsuperscript{44,45} However, hscRP was not significantly increased in the serum of IPAH patients (data not shown). Secondly, the reduced number and activity of circulating EPCs in patients with IPAH might be associated with hyperhomocysteinemia. Arroliga et al.\textsuperscript{46} have reported that IPAH patients are significantly more likely to have hyperhomocysteinemia. In this study, we also have observed that plasma total Hcy levels of IPAH patients are significantly higher than that of controls, and there is an inverse correlation between the number and activity of EPCs and Hcy levels. Moreover, we previously reported that homocysteine dose and time dependently decreased EPC numbers and impaired the cells proliferation, migration, adhesion and in vitro vasculogenesis capacity.\textsuperscript{24} Lastly, the continuous endothelial damage or dysfunction may lead to an eventual depletion or exhaustion of a presumed finite supply of EPCs.

Obviously, because of the limitations imposed by studying patients, we cannot determine the molecular pathway(s) responsible for impairment in circulating EPCs in our patients with IPAH. Future studies will be needed to address this question. The second limitation of this pilot trial is a relatively small quantity of patients with IPAH enrolled in this study. Future studies on a larger scale will be required to perform.

Taken together, the present study demonstrated that EPCs could play a role in patients with IPAH, which might not only give potential insight into the pathophysiological mechanisms but might be useful for identifying suitable therapeutic targets in these patients. However, further studies are needed to define the mechanisms that underlie the reduction in the number and activity of circulating EPCs in patients with IPAH.

Conflict of interest statement

None of the authors have a conflict of interest to declare in relation to this work under this sub-heading.

References


