Design and validation of an endothelial progenitor cell capture chip and its application in patients with pulmonary arterial hypertension


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Abstract The number of circulating endothelial progenitor cells (EPCs) inversely correlates with cardiovascular risk and clinical outcome, and thus has been proposed as a valuable biomarker for risk assessment, disease progression, and response to therapy. However, current strategies for isolation of these rare cells are limited to complex, laborious approaches. The goal of this study was the design and validation of a disposable microfluidic platform capable of selectively capturing and enumerating EPCs directly from human whole blood in healthy and diseased subjects, eliminating sample preprocessing. We then applied the “EPC capture chip” clinically and determined EPC numbers in blood from patients with pulmonary arterial hypertension (PAH). Blood was collected in tubes and injected into polymeric microfluidic chips containing microcolumns pre-coated with anti-CD34 antibody. Captured cells were immunofluorescently stained for the expression of stem and endothelial antigens, identified and counted. The EPC capture chip was validated with conventional flow cytometry counts \((r=0.83)\). The inter- and intra-day reliability of the microfluidic devices was confirmed at different time points in triplicates over 1–5 months. In a cohort of 43 patients with three forms of PAH (idiopathic/heritable, drug-induced, and connective tissue disease), EPC numbers are \(\approx50\%\) lower in PAH subjects vs. matched controls and inversely related to two potential disease modifiers: body mass index and post-menopausal status. The EPC capture chip (5 \(\times\) 30 \(\times\) 0.05 mm\(^3\)) requires only 200 \(\mu\)L of human blood and has the strong potential to serve as a rapid bedside test for the screening and monitoring of patients with PAH and other proliferative cardiovascular, pulmonary, malignant, and neurodegenerative diseases.

Keywords Bioengineering · Vascular disease · Pulmonary · Endothelium · Bone marrow · Progenitor cells · Microfluidic device · Pulmonary hypertension · Biomarker · Bedside test · Diagnostics · Biomedical engineering

Introduction

Peripheral blood contains a subtype of circulating, bone marrow-derived cells, called endothelial progenitor cells
(EPCs) [1, 2]. EPC number inversely correlates with endothelial dysfunction and impairment of angiogenesis [1, 3] and has been suggested as a biomarker for cardiovascular disease [4–6]. Flow cytometry has been the method of choice for EPC analysis but is limited in routine use due to the need for laborious, non-automated preprocessing and the size and costs of equipment and reagents [5]. There is a need for a rapid bedside test that quantifies these rare progenitor cells as a means of assessing patient risk, response to therapy, and prognosis in conjunction with traditional clinical diagnostic methodologies. Here, we describe the design, validation, and clinical utilization of a disposable, polymer-based microfluidic platform (“EPC capture chip”) which enables the isolation and detection of EPCs directly from human whole blood using surfaces coated with anti-CD34, followed by counterstaining with antibodies against characteristic EPC surface antigens, kinase insert domain (KDR), and CD31.

Based on our previous research with ovine EPCs [7], an advanced antibody-mediated microfluidic capture device was developed. While similar to previous designs for metastatic cells [8, 9], the device is distinct in it is much smaller in size (<7.5 μL) and thus designed for the capture of target cells from a single pass of a small volume of whole blood (200 μL)—important in clinical pediatrics and small (transgenic) animal research. Moreover, the device allows parallel analysis of multiple cell types (besides EPCs) within a single blood sample.

Here, we report the design, validation, and clinical application of a disposable microfluidic platform capable of selectively capturing and enumerating EPCs (CD34+/KDR+ and CD34+/KDR+/CD31+/CD45−, so-called late EPCs) directly from whole blood in healthy and diseased subjects, i.e., patients with pulmonary arterial hypertension (PAH), thereby eliminating sample preprocessing. However, this study was not designed to comprehensively investigate the role of EPCs and EPC function in PAH.

Numerous markers of EPC lineage have been proposed in the literature, subcategorized into stem cell markers (such as CD34, CD133, CD45, and c-kit) and endothelial-like markers (such as KDR, CD31, CD146, and von Willebrand factor) [2, 10]. However, the precise definition of what constitutes an EPC is the subject of an extensive debate [11, 12]. At present, the only EPC phenotype based on surface antigenic markers that provides strong and reproducible correlations across multiple studies on vascular damage and cardiovascular risk is CD34+/KDR+ [13]. An additional phenotype that has recently been utilized in the literature is the inclusion of CD133 as a secondary stem cell marker [14]; however, Timmermans et al. have recently questioned its utility as an EPC marker [10, 15]. Notably, the intersection of the CD34+/CD133+ and CD34+/KDR+ cell phenotypes (i.e., CD34+/CD133+/KDR+ cells) is known to be extremely rare [13] and, within the blood volumes used in the current investigation, no reliable enumeration of this rare cell type could be made (see Supplemental Results and Discussion).

Besides the controversy on the most accurate definition of EPCs (see above), there is currently an extensive debate on the role of EPCs in PAH; in particular, it is discussed whether the number of circulating “EPCs” is actually decreased (CD34+/KDR+/CD31+, so-called late EPCs) or increased (CD34+/CD133+, so-called early EPCs) in PAH vs. healthy controls [11, 12]. Several groups have demonstrated lower number of circulating CD34+ and CD34+/KDR+ cells vs. controls [16–18] while other groups have reported an increase of CD34+/CD133+ and CD34+/CD133+/KDR+ cells [19, 20], or no change in CD34+/CD133+ cells [16, 21], in PAH patients compared to controls. Some of these apparently controversial findings may simply be explained by the different cell surface antigens targeted for EPC characterization (see Discussion). Diller et al. [16] have characterized EPCs as CD34+/KDR+ cells and demonstrated that adult pulmonary arterial hypertension (IPAH) patients have reduced numbers of such circulating EPCs when compared with healthy controls. From a clinical perspective, it is important to note that a decreased number of EPCs was associated with worse hemodynamics [16], and that treatment with the phosphodiesterase type 5 (PDE5)-inhibitor sildenafil led to a dose-dependent rise in EPC numbers [16].

Hence, we used the aforementioned most reproducible CD34+/KDR+ EPC phenotype [13] as the basis for our clinical study thereby aiming to establish a novel EPC capture chip as a new “bedside test.” Besides the technical advances (Table 1), we demonstrate that EPC numbers (CD34+/KDR+, CD34+/KDR+/CD31+/CD45−) were ≈50% lower in patients with idiopathic/heritable PAH, but also in those with PAH associated with appetite suppressant use or connective tissue disease, when compared with matched control subjects. The resulting EPC numbers were also shown to be inversely associated with two potential disease modifiers: body mass index and postmenopausal status.

### Methods

#### Blood collection

Whole blood was drawn from 14 healthy volunteers and 43 patients with pulmonary arterial hypertension (including idiopathic and heritable PAH, drug-induced PAH, and PAH associated with connective tissue disease) and collected in EDTA-coated Vacutainer® tubes (Becton Dickinson, Franklin Lakes, USA). Subjects were recruited from the "research..."
room” at the Pulmonary Hypertension Association’s Ninth International Pulmonary Hypertension Conference and Scientific Sessions, Garden Grove, CA, USA, in June 2010 (Table 2). Approvals from the Stanford University School of Medicine and Northeastern University Institutional Review Boards was obtained and all study subjects provided written informed consent.

Microfluidic device design and fabrication

The design and fabrication of the micropost array microfluidic devices followed previously described soft lithography techniques [22]. First, a negative master was fabricated and assembled at the George J. Kostas Nanoscale Technology and Manufacturing Research Center at Northeastern University using conventional photolithography techniques. Briefly, a silicon wafer was coated with SU 8–50 photore sist to a thickness of approximately 43 μm. With the transparency overlaid, the wafer was exposed to 365 nm, 11 mW/cm² UV light from a Q2001 mask aligner (Quintel Co, San Jose, CA). The unexposed photore sist was then removed using SU 8 developer. Feature height was verified using a Dektak surface profiler (Veeco Instruments, Santa Barbara, CA).

Briefly, post-array (Fig. 1) devices consisting of 100-μm diameter post with a gap, edge-to-edge distance of 50 μm were fabricated. The posts were arranged in a hexagonal pattern, where three adjacent posts form an equilateral triangle pattern. The overall dimension of the device was $5 \times 30 \times 0.05$ mm³, which results in a total volume of the channel of $7.5 \times 0.05$ mL. To form the polymeric chambers, poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning, Midland, MI) elastomer was mixed (10:1 ratio) and poured onto a negative master, degassed, and allowed to cure overnight. PDMS replicas were then removed; inlet and outlet holes were punched with a 19-G blunt-nosed needle. Prior to bonding, the PDMS replicates were extracted as described by Vickers et al. [23]. Replicas and glass microscope slides ($25 \times 75 \times 1$ mm²) were then exposed to oxygen plasma and placed in contact to bond irreversibly.

Surface modification

As described previously [22], the main steps in the surface modification protocol were (a) surface treatment with 4% (v/v) 3-mercaptopropyl trimethoxysilane (Gelest, San Francisco, CA) solution, (b) attachment of a coupling
agent, 0.28% (v/v) N-[γ-maleimidobutyryloxy] succinimide ester (GMBS; Pierce Biotechnology, Rockford, IL), to the silane, and (c) attachment of mouse anti-human CD34 protein (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 0.01 mg/mL.

Microfluidic device flow experiments

Whole EDTA blood was directly flowed through the microfluidic device with functionalized posts at 10 μL/min to a total volume of 200 μL (20 min) using a Harvard Apparatus PHD2000 syringe pump (Harvard Apparatus, Holliston, MA). The blood was then rinsed from the device using phosphate-buffered saline (PBS) at a flow rate of 10 μL/min to a total volume of 100 μL (10 min) followed by a cell fixation using 4% (v/v) formaldehyde solution in PBS, again at 10 μL/min to a total volume of 100 μL (10 min). Formaldehyde solution was flushed out with PBS prior to staining for EPC-specific surface markers.

Immunofluorescent staining

Following cell capture, rinse, and fixation, immunofluorescent staining was performed. Cells within the device were incubated for 10 min in the presence of primary antibodies against CD31 (PECAM-1; 1:100, Santa Cruz Biotechnology) conjugated to fluorescein isothiocyanate (FITC), CD45 (1:100, Santa Cruz Biotechnology) conjugated to phycoerythrin (PE), and kinase insert domain receptor (KDR/Flk-1; 1:100, Santa Cruz Biotechnology) with secondary antibody AlexaFluor350 (1:100, Invitrogen, Carlsbad, CA). All incubation steps were conducted at room temperature. Following incubation, the devices were flushed with PBS, and EPCs were counted via raster scanning with fluorescence microscopy (at ×10 magnification) using a Nikon Eclipse TE2000 inverted microscope using fluorescein (480±30 nm/535±40 nm), rhodamine (540±25 nm/605±50 nm), and DAPI (360±40 nm/460±50 nm) excitation/emission filters.

Flow cytometry

Whole blood was collected in EDTA-coated tubes and kept on ice. Flow cytometry-based EPC staining was performed within 2 h after blood collection. Peripheral blood mononuclear cells were isolated from 100 μL aliquots of blood using 8.3 g/L ammonium chloride lysis buffer. A three-colored panel of antibodies was used to enumerate the EPCs: mouse anti-CD31-FITC (1:100; Santa Cruz), mouse anti-CD34-PE (1:100; Santa Cruz), and goat anti-KDR (1:100; Santa Cruz) along with donkey anti-goat PerCP secondary stain (1:500; R&D Systems, Minneapolis, MN) incubated at room temperature. Using a Beckman Coulter

Table 2 Characteristics of control subjects and PAH patients enrolled

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Numbers are mean values or the number of subjects, as appropriate

BMI body mass index, PAH pulmonary arterial hypertension

a EPC count (CD34+/KDR+) in larger control cohort (n=14; age 23–60 years) and the total PAH cohort (n=43; age 19–77 years) showed no difference between genders

b BMP-RII gene mutation, i.e., heritable PAH (n=1)

c PAH associated with appetite suppressants (fenfluramine/phentermine; n=3); PAH associated with illegal drug use (n=1)

d Systemic sclerosis (scleroderma; n=2), mixed connective tissue disease (n=2), systemic lupus erythematosus (n=1), limited scleroderma (CREST; n=2), and scleroderma/mixed connective tissue disease (n=1)
Cell Lab Quanta™ SC flow cytometer (Brea, CA), cells were processed for electronic volume, side scatter, and the three fluorescent markers. Cell populations were quantified as an absolute number of live events that were CD31, CD34, and KDR positive (CD34+/KDR+/CD31+). Gating was conducted using the FlowJo™ gating software. A representative flow cytometry scatter plot for CD34+/CD31+/KDR+ cells is shown in Fig. S1.

Statistical analysis

Values from multiple experiments are expressed as mean±SEM unless stated otherwise. Using the Kolmogorov–Smirnov normality test, we could show that the measured values were normally distributed. Statistical significance was determined via Student’s t test for side-by-side comparison or one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test, for comparisons between multiple groups. Pearson correlations and scatter plots were used to study the association between flow cytometry and EPC capture on chip and for EPC number association with age. The number of relevant subjects in each group is found in Table 1. A p value<0.05 was considered significant.

Reproducibility was tested with a Wilcoxon matched-pairs signed rank test (intra-day comparisons) and Kruskal–Wallis test (inter-day comparisons). A p value close to 1 was considered a reproducible result.

Results

Development and validation of the EPC capture chip

Briefly, peripheral blood was collected in EDTA tubes and directly injected into the polymeric microfluidic chips at a flow rate of 0.6 mL/h (Fig. 1a, b). Following capture (CD34+ cells), cells were identified and enumerated via immunofluorescent staining (Fig. 1c–f) for the expression of CD31, KDR, and CD45 antigens. Data were tabulated for cell numbers which stained for (a) KDR only (CD34+/KDR+), (b) for cells that stained for CD31 and KDR (CD34+/CD31+/KDR+), and (c) for cells that stained for CD31 and KDR while negative for CD45 (CD34+/CD31+/KDR+/CD45−)—all frequently described EPC phenotypes [1, 24, 25]. A comprehensive diagnostic readout was attained in approximately 1 h, significantly faster than traditional techniques such as flow cytometry [5, 26], magnetic bead-based approaches [2], or colony-forming cell assays [5, 27] (>2 h up to 5 days; see Table 1). To validate the efficiency and accuracy of the EPC chip, CD34+/CD31+/KDR+ cell counts from 21 separate blood draws (seven control subjects) were directly compared to traditional three-color flow cytometry measurements of the same three markers in the first set of experiments. As
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*Clinical classification of pulmonary hypertension (Dana Point 2008) as described in ref. [53]

WHO classification (1–4)

Abbreviations: BMI body mass index, ID idiopathic PAH (Dana Point PH category 1.1), HPiPH heritable PAH (Dana Point PH category 1.2.1), CTD connective tissue disease (Dana Point PH category 1.4.1), IAPH PAH due to drug use (Dana Point PH category 1.3); Medications (prostanoids are in bold): ALD aldacone PO, ALL allopurinol PO, AMB ambrisentan PO, AMI amiodipine, ASA acetylsalicylic acid, ATE atenolol (beta blocker) PO, AZA azathioprine PO, BOS bosentan PO, BUM bumetanide PO, BUP bupropion PO (norepinephrine–dopamine reuptake inhibitor), CAR carisoprodol (muscle relaxant), CEL celecoxib PO, CLO clonidine PO, COL colchicin PO, DES desvenlafaxine (serotonin–norepinephrine reuptake inhibitor), DIG digoxin PO, DIL diltiazem, DIP dipyriramole, DUL duloxetine PO (serotonin–norepinephrine reuptake inhibitor), EPI epoprostenol IV, ESI escitloplam PO (SSRI), EPSC epoprostenol SC, FA folic acid PO, FEX fexofenadine PO, FFI fenofibrate PO, FLU fluoxetine PO (SSRI), FUR furosemide (Lasix) PO, GAB gabapentin PO, HCT hydrochlorothiazide PO, HYD hydrocodone, IBA ibandronate (bisphosphate) PO, INS insulin, IRO iron PO, LIS lisinopril PO, LOS losartan (AT1 receptor antagonist) PO, LOV lovastatin PO, MED medodrine, MET metoprolol PO (beta blocker), MON melotulastuk inhal., MST metformin and sitagliptin, MTX methotrexate PO, NAP naproxe PO, NIF nifedipine, O2 oxygen by nasal canula, OME omega-3-acid ethyl esters PO, PAR paroxetine PO (SSRI), PG1 prostanoid (unspec.), PPI proton pump inhibitor PO, PRA pravastatin PO, PRE pregabalin, PRED prednisone PO, RIS risedronate (bisphosphate) PO, SD PH study drug, SER sertraline PO (SSRI), SIL sildenafil PO, SIM simvastatine PO, TAD tadalafil PO, THY levothyroxine PO, TOR torsemide PO, TRA trazodone (serotonin antagonist and reuptake inhibitor, SARI), TRAM tramadolo, TREPIV trepostin IV, TREPIN trepostin inhal., TREPPPO trepostin PO, TREPSC trepostin subcutaneously, TRI triamterene PO, URS ursodiol PO, VAL valsartan PO (AT1 receptor antagonist), VEN venlaxafine PO (serotonin–norepinephrine reuptake inhibitor), WAR warfarin (Counadim) PO

illustrated in Fig. 1g, the comparison of EPC number on-chip with the flow cytometry measurements revealed a 1:2.7 ratio in EPC number (i.e., approx 37% capture rate) with excellent correlation between the two techniques ($r=0.83; p=0.0196$) for the median values of seven control subjects. In addition, we investigated the inter- and intra-day reliability of the microfluidic devices by measuring EPC number in five controls at different time points in triplicates over 1–5 months and found control EPC numbers to be consistently 24–30/200 μL of whole blood with little fluctuation (see Fig. S2 in the online data supplement).

Endothelial progenitor cell number is decreased in patients with pulmonary arterial hypertension

In the subsequent clinical study, the enrolled subjects included 43 patients diagnosed with PAH (six males, 37 females; mean age of 47.1 year) and six age- and gender-matched controls (one male, five females; mean age of 44.9 year, Table 2; see Tables 3 and 4 for individual characteristics of PAH patients and control subjects, respectively). PAH patients were further stratified into three distinct groups according to PAH subcategory [28], i.e., idiopathic and heritable PAH (IPAH/HPAH; $n=28$), drug-induced PAH ($n=4$; appetite suppressants), and PAH associated with connective tissue disease ($n=11$).

Consistent with prior reports [16, 29], we found that circulating EPC numbers, as defined by the number of CD34+/KDR+ (double-labeled) cells, were significantly decreased in PAH patients vs. controls ($p<0.001$; Fig. 2a, b) and demonstrated such a reduction also for CD34+/CD31+/KDR+/CD45− (triple-labeled) cells ($p<0.001$; Fig. 2a, b). Cells captured on-chip prior to immunolabeling were defined as CD34+ cells (see Fig. S3 in the online data supplement). The more stringent triple labeling excluded CD45+ bone marrow (BM)-derived hematopoietic stem cells, and included only EPCs that were CD31+, thereby characterizing a more differentiated EPC phenotype typical for circulatory rather than BM-stationary EPCs [2]. This particular EPC phenotype (CD34+/CD31+/KDR+/CD45−) has also been termed “late EPC” (also known as a late outgrowth endothelial cell or endothelial colony-forming cell, ECFC; for details on the significance of this subpopulation see Supplemental Results.

### Table 4 Characteristics of control subjects

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<tr>
<th>ID</th>
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<th>Gender (M/F)</th>
<th>Weight (kg)</th>
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<th>WHO class</th>
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BMI body mass index, PMHx past medical history
and Discussion in the online data supplement). However, overall, the cell numbers for the two EPC phenotypes measured with the microfluidic chip (CD34+/CD31+; CD34+/CD31+/KDR+/CD45−) were very proportionate and in a similar range (Fig. 2). The EPC number (CD34+/KDR+) did not differ significantly between genders within a larger set of control subjects (males, 28.5±1.0; females, 27.0±1.1 EPCs/200 μL of blood; p<0.05; n=14; age range 23–60 years; data not shown) or among PAH patients (males, 16.9±1.0; females, 17.0±0.4 EPCs/200 μL blood; p<0.05; n=43; age range 19–77 years; see Table 1). A subgroup analysis by PAH subcategory revealed that not only PAH patients diagnosed with IPAH/HPAH, but also those with drug-induced PAH (due to appetite suppressants or illegal drugs), or PAH associated with connective tissue disease had comparably low EPC numbers that were approximately half the numbers in the healthy matched control subjects (Fig. 2c, d).

Association between EPC number and age in control but not PAH patients

Interestingly, while EPC number (CD34+/CD31+/KDR+/CD45−) inversely correlated with age in control subjects (r=−0.93; p=0.008), there was not such an association in the cohort of enrolled PAH patients (r=−0.28; p=0.08; Fig. 3). Thus, other modifiers must influence the EPC numbers to a greater extent than aging alone.

EPC number is inversely associated with two potential disease modifiers: postmenopausal status and body mass index

Because metabolic and hormonal factors such as insulin resistance, dyslipidemia [30], mitochondrial dysregulation [31, 32], and imbalanced sex hormone composition (ratio) [33] are increasingly recognized as influential environmental factors and potential “second hits” in PAH development, we compared EPC number in pre- vs. postmenopausal women with PAH (Fig. 4). Moreover, we measured EPCs within the IPAH/HPAH subgroup in males and females stratified by normal, mildly, or greatly elevated body mass index (BMI; Fig. 5). Interestingly, despite the lack of age dependency of the EPC number in the PAH cohort (Fig. 3b), postmenopausal women with PAH (≥50 years of age) did have lower EPC numbers (CD34+/KDR+) than younger, premenopausal-affected females (18.3±0.8 vs. 16.3±0.4 EPCs/200 μL of blood; p<0.05; Fig. 4a); this difference was seen not only in CD34+/KDR+ but also in CD34+/CD31+/KDR+/CD45− (“late EPC”) cell counts (12.4±0.9 vs. 10.7±0.4 EPCs/200 μL of blood; p<0.05) (Fig. 4b).

Stratifying the data according to BMI for both the total PAH (n=43) and IPAH/HPAH (n=28) populations illustrates that higher BMI is associated with lower numbers of circulating EPCs (Fig. 5). In the total PAH cohort, obese (16.2±0.4 EPCs/200 μL of blood; p<0.05; BMI≥30 kg/m²) and overweight (15.4±0.6 EPCs/200 μL of blood; p<0.05;
more experimental clinical therapies. (b) The number of circulating EPCs represents a promising candidate biomarker for pulmonary vascular disease severity: Adult IPAH patients have reduced numbers of circulating EPCs when compared with healthy controls; a reduced number of EPCs (CD34+/KDR+) is associated with worse hemodynamics and abnormally elevated concentrations of inflammatory markers, including tumor necrosis factor-α, interleukin-6, and C-reactive protein (CRP) [16], and asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase inhibitor [16]. Heightened plasma ADMA levels are observed in PAH patients and negatively correlate with hemodynamic performance and survival rates [34]. (c) Pilot studies have demonstrated that autologous transplantation of EPCs is safe and leads to significant improvements in pulmonary hemodynamics, exercise capacity including 6-min walk distance in children [35] and adults with PAH [36]. (d) EPC number indicates response to therapy: Treatment with the PDE5-inhibitor sildenafil, an established PAH medication, leads to a dose-dependent rise in EPC numbers in IPAH patients [16]. In separate studies, peroxisome proliferator-activated receptor-γ (PPARγ) treatment inhibits the negative effects of CRP on human EPC survival, differentiation, and function [29] and an increase in EPC number in culture [37]. Recently, we demonstrated that PPARγ agonists reverse PAH, right ventricular hypertrophy, and pulmonary vascular remodeling in rodents [38] thereby revealing their potential as a new pharmacotherapy [39–41]. We [42] and others [43] have since shown that metabolic dysregulation such as insulin resistance [42] and dyslipidemia (low HDL cholesterol [42, 43]) is more common in (female) PAH patients and associated with clinical worsening and poorer survival at six [42] and 20 [43] months follow-up. Given our current results on the lower number of circulating EPCs in obese vs. non-obese and post- vs. premenopausal PAH patients and the previously described inverse relation between EPC number and hemodynamic status of PAH patients [16], it will be important to explore the impact of metabolic regulators such as PPARs [41], mitochondrial regulatory proteins [32], micro RNAs [44, 45], and sex hormones [33, 46] on pulmonary vascular disease and associated right ventricular dysfunction.

There are several limitations to our study. Although most of the published competitive techniques solely use cell surface markers [13], it should be noted that the definition of a stem/progenitor cell optimally should be based on both surface markers and functional assays. Specifically, EPC characteristics including cell surface proteins have been shown to differ depending on the culture techniques and stage of differentiation in which the cells are isolated (CFU-Hill, early EPCs, or late EPCs; see Supplementary Results and Discussion for details). Among the existing EPC

**Fig. 3** Age association of EPC counts in controls subjects (a) and PAH patients (b). The number of circulating EPCs defined by the expression of CD34 and KDR, inversely correlated with age in (a) controls (n=6; Pearson correlation coefficient $r=-0.93$ (p=0.008). However, (b) PAH patients (n=43) illustrated no such age-dependent decline with age ($r=-0.28$; $p=0.08$). Results with CD34+/CD31+/KDR+/CD45− cells illustrated a similar trend, where controls (n=6) showed a clear decline in cell numbers with age, but PAH patients (n=43) had no statistically significant correlation with age ($r=-0.26$; $p=0.054$)

BMI=25–29.9 kg/m²) PAH patients had a lower number of circulating EPC vs. PAH patients with normal BMI (17.9±0.7 EPCs/200 µL of blood; BMI=18.5–24.9 kg/m²) (Fig. 5; see also Supplementary Results and Discussion).

**Discussion**

We chose PAH for the first clinical application of the EPC capture chip for several reasons: (a) PAH is a prototype of proliferative cardiovascular diseases. Its pathobiology is characterized by endothelial cell death and progressive obliteration of the peripheral pulmonary arteries and involves multiple signalling pathways which currently make tailored PAH therapy extremely difficult. Novel PAH biomarkers that indicate disease severity, progression, and prognosis of PAH and associated right ventricular dysfunction would be extremely helpful in guiding established and
assays, only the laborious colony-forming unit (CFU) assays (processing time 5 days) give information on EPC function (Table 2). Hence, as an EPC characterization tool, the described device is somewhat limited because the captured cell population is defined solely based on surface markers. However, as a practical diagnostic device, characterization of EPC function is secondary to quantifying a novel, reliable, and validated cellular PAH biomarker such as EPC number that is inversely associated with the hemodynamic status of PAH patients and increased by the phosphodiesterase inhibitor sildenafil [47], an established PAH medication. This study was not designed to com pre-

Fig. 4 Age association of EPC counts in female PAH patients. Circulating EPCs were enumerated on-chip. The number of EPCs defined by the expression of (a) CD34 and KDR was lower in women over or equal to 50 years (postmenopausal) when compared to women younger or equal to 35 years of age (premenopausal). b This pattern was also illustrated in cells expressing CD34, CD31, KDR, and exclusion of CD45 (also called “late” EPCs or ECFCs). Each point represents an individual patient’s EPC count and the bar represents the median value. A comparison between females younger than or equal to 35 years and patients over or equal to 50 years was made via a Student’s t test. *p<0.05

Fig. 5 BMI association with EPC counts in PAH patients. Circulating EPCs defined by the expression of (a, c) CD34 and KDR or defined by the expression of (b, d) CD34, CD31, and KDR while not expressing CD45 in controls and PAH. Subjects were stratified by BMI for (a, b) PAH (n=43) and (c, d) IPAH patients (includes one HPAH patient; n=28) along with the controls. Normal weights were considered as BMI=18.5–24.9, overweight as BMI=25–29.9, and obese as BMI≥30 kg/m². It was determined that EPC numbers decline with BMI in PAH patients for both (a) CD34+/KDR+ cells and (b) CD34+/CD31+/KDR+/CD45− cells. Furthermore, for both (c, d) EPC phenotypes, EPC numbers were lower in obese IPAH patients when compared to IPAH patients with normal BMI. There was no significant difference in controls for all BMI subcategories and no significant difference in overweight IPAH patients vs. either obese or non-overweight IPAH patients. Comparisons were made via one-way ANOVA analysis with Bonferroni post hoc test. *p<0.05; **p<0.001
hensively investigate the role of EPC and EPC function in PAH rather this report merely presents a novel EPC enumeration modality as a potential “bedside test” and facile alternative to the conventional laborious techniques.

Currently conflicting data exist in the literature on the relative EPC number in PAH patients [11, 12]. A number of studies have described a reduction in circulating EPCs in PAH patients when compared with healthy controls [16–18], consistent with our data presented herein. However, others have found an elevation [19, 20] or no difference [48] in (CD133+) EPC numbers in PAH patients vs. healthy controls. A possible explanation for this discord in the literature may be attributed to the various methods and cell surface protein markers used to identify, isolate, and quantify the cells, as well as possible differences in patient selection. Secondly, it is possible that “early” (immature) EPCs (CD133+) may be released from the bone marrow as an early adaptive response to pulmonary vascular injury [47]. We speculate that environmental factors such as inflammation [49], sex hormone resistance [30], and/or dyslipidemia [30, 43] subsequently inhibit the differentiation of the circulating EPCs from “early” to the “late” (mature) EPC (CD31+) phenotype. Such a biphasic EPC response may account for the different findings in patients with IPAH published to date [16–20, 48]. Nevertheless, the beneficial role of EPCs in PAH is supported by recent reports on the successful autologous transplantation of CD34+/KDR+ cells that lead to hemodynamic and clinical improvement in children [35] and adults [36] with IPAH.

In summary, the new EPC capture chip captures a significant percentage of EPCs in a single step, requiring minimal blood volume (200 μL) which is important for pediatric clinical care and small animal research. To the best of our knowledge, this is the first report on the application of a polymeric cell affinity microfluidic diagnostic platform in cardiovascular disease and the first on the validation and clinical application of such a microfluidic device for the analysis of human-circulating EPCs. In addition to patients with idiopathic PAH, those with PAH associated with either connective tissue disease or drug use (appetite suppressants) have about half the number of circulating EPCs relative to healthy, age- and gender-matched controls. Beyond the technical aspects, we found that the clinically relevant number of circulating EPCs (CD34+/KDR+, CD34+/KDR+/CD31+/CD45−) is inversely related to BMI and postmenopausal status in PAH patients. The novel EPC capture chip has the potential to become a practical diagnostic tool in the risk assessment and clinical monitoring of patients with PAH and other cardiopulmonary and neurodegenerative diseases [50] as well as cancer [51], requiring only small blood volumes and no laborious preprocessing steps.

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Potential conflict of interest None

References


Design And Validation Of An Endothelial Progenitor Cell Capture Chip And Its Application In Patients With Pulmonary Arterial Hypertension

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Online Supplemental Results and Discussion

EPC Phenotype and Limitations of the Current Study

There still remains an extensive debate on the most accurate definition of an EPC. At present, the only antigenic EPC phenotype that provides strong and reproducible correlations across multiple studies on vascular damage and cardiovascular risk is CD34+/KDR+ [1]. Hence, we used this EPC phenotype (CD34+/KDR+) as the basis for our clinical study. A more stringent triple labeling excluded CD45+ bone marrow (BM) derived hematopoetic stem cells, and included only EPCs that were CD31+, characterizing a more differentiated EPC phenotype typical for circulatory rather than BM-stationary EPCs [2]. This particular EPC phenotype (CD34+/CD31+/KDR+/CD45-) has also been termed “late-EPC” [3]. We believe that the majority of the cells captured are EPCs, consistent with our prior studies on EPCs capture under dynamic flow condition [4].

It should be noted that a common EPC marker, CD133, was intentionally excluded from this study. Several control samples were tested with microfluidic devices where CD133+/CD34+ expression was investigated. The circulating CD133+/CD34+ progenitor cell number was shown to be too low for any reliable diagnostic evaluation (n=5; < 2 EPCs/200µL whole blood). This result is in agreement with studies, which have illustrated that CD34+/CD133+ cells are rarer than CD34+/KDR+ cells in circulation [5, 6]. It has also been hypothesized that EPCs in the bone marrow are CD133+/CD34+/KDR+ whereas more mature, differentiated circulating EPCs, which lose CD133 expression, are better characterized as CD34+/KDR+/CD31+ [2]. Furthermore, in a recent study by Timmermans et al. [7] it was reported that functional EPCs are not derived from a CD133+ cell population. The particular EPC phenotype (CD34+/CD31+/KDR+/CD45-) enumerated in our study has been termed endothelial colony...
forming cell (ECFC) or “late-EPC” [7, 8]. In studies with PAH patients in which an EPC was solely defined as CD133+/CD34+ cell, it was found that the EPC number was either not significantly different [8-10] or even increased [11] when compared to matched control blood samples. On the other hand, when additional markers (including KDR, CD31 and CD45) were probed there were consistently lower EPC numbers in PAH patients versus matched controls [12], consistent with the results of the present study.

Although the EPC capture chip has several distinct advantages over current EPC isolation and counting techniques, as described in Table 1 in the main text, it must be underlined that the most comprehensive definition of a stem/progenitor cell is based on surface markers as well as functional assays. EPC characteristics have been shown to differ depending on the culture techniques used to obtain these cells and stage of differentiation in which the cells are isolated (CFU-Hill, early-EPCs, or late-EPCs). Late-EPCs have been shown to be clonogenic and to contribute more directly to neovascularization by supplying new endothelial cells and blood vessels in vivo. Hence, late-EPCs are rather true endothelial precursors whereas early-EPCs (as defined by expression of CD133) display features of angiogenic cells [3]. The CD133+ progenitor cells may play a role in vascular remodeling by releasing cytokines and growth factors [1]. Toshner et al. [8] found that early-EPCs specifically isolated from PAH patients, including those patients with BMP-RII gene mutations, have an impaired ability to form vascular networks versus early-EPCs isolated from healthy volunteers. Conversely, cord blood plated in the CFU-Hill assay gives rise to cells that do express many proteins similar to primary endothelial cells, but the cells which form the CFU-Hill also express numerous myeloid progenitor cell markers and ultimately fail to mature into endothelial cells [13]. Unlike colony forming-unit assays that take several days to be completed, our EPC capture chip defines EPCs
based on surface marker only, and requires a processing time of approximately 1 hour – a feature desirable for rapid bedside testing. Moreover, among the existing EPC assays only the laborious CFU assays (processing time 5 days) give information on EPC function (see Table 1, main text). Hence, as an EPC characterization tool, the described device is somewhat limited because the captured cell population is defined solely based on surface markers – an approach that has been used by many other research groups. However, as a practical diagnostic device, characterization of EPC function is secondary to quantifying a novel, reliable and validated cellular PAH biomarker such as EPC number (CD34+/KDR+) that is inversely associated with the hemodynamic status of PAH patients and increased by the established PAH drug, sildenafil [12]. The EPC capture chip may in essence be capturing a mixed population of the aforementioned EPCs sub-populations, but the individual function and clinical significance of these EPC-subtypes is vastly unclear at this point.

Potential Disease Modifiers: Impact of Post-Menopausal Status and Body-Mass Index in PAH

Recently, we have demonstrated that PPARγ agonists reverse PAH and pulmonary vascular remodeling in rodents [14] thereby revealing their potential as a new pharmacotherapy [15-17]. We [18] and others [19] have since shown that metabolic dysregulation such as insulin resistance (IR) [18] and dyslipidemia (low HDL-cholesterol [18, 19]) is more common in (female) PAH patients and associated with clinical worsening and poorer survival at six [18] and twenty [19] months follow up. Because metabolic and hormonal factors such insulin resistance, dyslipidemia [17], mitochondrial dysregulation [20, 21], and imbalanced sex hormone composition (ratio) [22] are increasingly recognized as influential environmental factors and potential "second hits" in PAH development (as recently discussed: “2010 Strategic Plan for Lung Vascular Research: An
NHLBI-ORDR Workshop” [23]), we compared EPC number in pre- vs. postmenopausal women with PAH and in normal weight vs. overweight and obese PAH patients (Figure 4 and Figure 5 main text, respectively). We demonstrate that both higher body-mass-index (BMI; male and female) and postmenopausal status (for literature on lower EPC number in non-PAH subjects see refs [24, 25]) are associated with lower circulating EPC number in patients with PAH. In a recent retrospective chart analysis of 541 female pulmonary hypertension patients in Colorado, 56% of all pulmonary hypertensive women were found to be postmenopausal; 39% of these postmenopausal PAH patients with “primary” PH (i.e. IPAH/HPAH) and 48% of those with secondary severe PH were found to be obese [26]. In our published Stanford cohort of 81 women with PAH, the mean age was 46.1 years (SD = 11.4 years); 45.7% of those had insulin resistance as defined by a triglyceride to HDL-cholesterol ratio > 3 [27]. Hence, future clinical sequelae of the epidemic “metabolic syndrome” (i.e. dyslipidemia, insulin resistance/glucose intolerance, obesity), may include a pronounced rise in the incidence and prevalence of progressive PAH both in children and adults. We speculate that treatment aimed at improving insulin resistance, such as simple exercise programs [28] or pharmacological PPARγ activation [14, 15, 17, 21, 27, 29, 30], may benefit a large percentage of PAH patients.

Within the IPAH/HPAH subgroup of our present study, obese patients (BMI $\geq$ 30 kg/m$^2$) had lower EPC numbers than those with normal BMI (15.6±0.4 vs. 17.8±0.8 EPCs/200 µL blood; $p<0.05$; BMI < 25 kg/m$^2$), but EPC number was not different from overweight patients with only moderately lower BMI (16.2±0.9 EPCs/200 µL blood; $p<0.05$; BMI $\geq$ 25-29.9 kg/m$^2$). The same inverse relation between EPC number and BMI was seen for triplet-stained “late” EPCs (CD34+/CD31+/KDR+/CD45−). Finally, EPC numbers were significantly lower in PAH and IPAH/HPAH patients versus controls independently of BMI ($p<0.001$). Subgroup analysis
according to BMI in the control group was underpowered and did not allow any meaningful conclusions (Figure 5 main text).

Several investigators have suggested that a decrease in EPC number and function may contribute to adiposity-related cardiovascular risk [31, 32]. EPCs from obese subjects failed to respond to pro-angiogenic factors such as vascular endothelial growth factor (VEGF). Moreover, basal p38 mitogen-activated protein kinase (MAPK) phosphorylation was elevated in EPCs from obese subjects [33]. Six-month follow-up of 26 obese subjects who achieved significant weight reduction revealed a normalization of p38 MAPK phosphorylation levels and improved EPC function [33]. In addition to dysfunctional EPC signaling, it is likely that deleterious “vasocrine signaling” from fat cells contributes further to vascular dysfunction in humans with obesity and/or abnormal insulin and lipid profiles [14, 17, 34]. Because both excess adiposity and sedentary behavior adversely affect not only insulin action but also EPC number and function, an obvious choice would be weight loss and increased physical activity. PAH patients who underwent a 15-week exercise program had a significant improvement in 6-minute walk distance (91±61 m) when compared with control PAH patients (-15±54 m), similar or even greater than improvements achieved with established oral pharmacotherapy [28].

Supplemental References


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