

## ORIGINAL ARTICLE

# Functional characterization of late outgrowth endothelial progenitor cells in patients with end-stage renal failure

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## Keywords

chronic rejection, end-stage renal failure, endothelial progenitor cells, integrin, mesenchymal stem cells, renal transplantation.

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## Conflicts of interest

The authors have declared no conflict of interests.

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## Introduction

Kidney transplantation is the preferred treatment for many patients with end-stage renal failure, but its long-term success is limited by chronic rejection [1]. Although the exact mechanism of chronic rejection remains unclear, a contributory factor is endothelial damage and dysfunction resulting from ischemia/reperfusion injury. This is further exacerbated by immunologic stimuli. There is no effective treatment, but attempting to preserve the integrity of endothelial cells and repair the damaged endothelium is a logical therapeutic option.

## Summary

Renal transplantation is potentially curative in renal failure, but long-term efficacy is limited by untreatable chronic rejection. Endothelial damage contributes to chronic rejection and is potentially repairable by circulating endothelial progenitor cells (EPC). The frequency and function of EPC are variably influenced by end-stage renal failure (ESRF). Here, we isolated and functionally characterized the late outgrowth EPC (LO-EPC) from ESRF patients to investigate their potential for endothelial repair. Patients with ESRF generated more LO-EPC colonies than healthy controls and had higher plasma levels of IL-1 $\alpha$ , IL-16, IL-6, MIF, VEGF, Prolactin, and PLGF. Patients' LO-EPC displayed normal endothelial cell morphology, increased secretion of PLGF, MCP-1, and IL-1 $\beta$ , and normal network formation *in vitro* and *in vivo*. They demonstrated decreased adhesion to extracellular matrix. Integrin gene profiles and protein expression were comparable in patients and healthy volunteers. In some patients, mesenchymal stem cells (MSC) were co-isolated and could be differentiated into adipocytes and osteocytes *in vitro*. This is the first study to characterize LO-EPC from patients with ESRF. Their behavior *in vitro* reflects the presence of elevated trophic factors; their ability to proliferate *in vitro* and angiogenic function makes them candidates for prevention of chronic rejection. Their impaired adhesion and the presence of MSC are areas for potential therapeutic intervention.

The cell type which is key to this aim is the recently identified endothelial progenitor cell (EPC) population. Mobilizing and/or delivering *ex vivo* expanded autologous EPC could be a novel approach to repairing damaged endothelium and could lead to the regeneration of vascular networks in an allograft.

Previous studies have shown that progressive or chronic renal failure is associated with a decreased number of circulating EPC [2,3] and impaired angiogenic function [3], while long-term hemodialysis is associated with either reduced numbers of EPC [4] or an increased number of EPC with impaired function [5]. EPC function in patients

with end-stage renal failure (ESRF) improves after transplantation [6], while EPC numbers in renal transplant recipients depend on kidney graft function [7].

EPC are a heterogeneous population; two subtypes have recently been identified—early outgrowth endothelial progenitor cells (EO-EPC) and late outgrowth endothelial progenitor cells (LO-EPC) [8–11]. Published studies have not hitherto distinguished between these cell types but probably mostly refer to EO-EPC [12]. Both subtypes express endothelial cell (EC) surface markers and can restore EC function and enhance angiogenesis. EO-EPC, however, do not differentiate to EC but act via a paracrine effect [13]. Although previously used for vascular repair in an ischemia model [14–16] and to restore renal function in chronic renovascular disease [17], EO-EPC are unsuitable in organ transplantation because they include monocyte lineage cells with immune functions [13,18]; delivery of an *ex vivo* expanded autologous population risks exacerbating immune rejection. By contrast, LO-EPC are homogeneous, highly proliferative, possess *de novo* vessel-forming ability, and directly contribute to endothelialization and angiogenesis [8,11,19–21].

There are no published data on LO-EPC in patients with ESRF. Therefore, we evaluated the feasibility of isolating and functionally characterizing LO-EPC from ESRF patients, to assess their suitability for autologous endothelialization therapy to prevent chronic rejection.

## Methods and materials

### Study subjects

Fifteen prerenal transplant patients with ESRF, most of whom were on maintenance hemodialysis (mean age 43.8 years, 40% female), and 15 healthy volunteers (mean age 41.4 years, 33% female) without a history of kidney disease were selected for this study (Table 1). The study had full ethical approval, and written informed consent was obtained from all patients and volunteers.

### Isolation and culture of late outgrowth endothelial progenitor cells

Mononuclear cells (MNC) were isolated from 40 ml venous peripheral blood by density gradient centrifugation (Ficoll-paque 1.077; GE Healthcare, Hatfield, UK). Plasma was separated and stored at  $-80^{\circ}\text{C}$  for cytokine analysis. MNCs were plated on a type I collagen (BD, Oxford, UK)-coated T115 flask and maintained in endothelial basal medium (EBM) supplemented with SingleQuots (Lonza, Slough, UK) and 20% Hyclone fetal calf serum (Fisher Scientific, Loughborough, UK). Nonadherent cells were removed after 3 days in culture, and the medium changed on alternate days. Attached EO-EPC appeared after

5–7 days in culture, displaying a typical spindle shape, and detaching from the culture flask after 2 weeks. Colonies of LO-EPC appeared after 2–3 weeks in culture and exhibited cobblestone morphology. Once individual colony size reached 500–1000, the cells were passaged into a new collagen-coated flask. Subsequently, cells were passaged at 1:3 ratios into noncoated flasks. LO-EPC from passage 2–6 were used. Cell phenotype and function were studied before and after freezing/thawing, or at different passages, and did not differ significantly.

### Uptake of Dil-acetylated low-density lipoprotein (Dil-Ac-LDL)

Cells were incubated with 10  $\mu\text{g/ml}$  DiI-Ac-LDL (Molecular Probes and Invitrogen, Paisley, UK) at  $37^{\circ}\text{C}$  for 1 h, washed twice with PBS, and viewed by fluorescence microscopy.

### Flow cytometric analysis of cell surface markers

LO-EPC and MSC phenotypes were determined by flow cytometry using conjugated antibodies: Alexa Fluor 488 anti-CD31 (BD Pharmingen, Oxford, UK), PE-anti-VEGFR-2 (BD Pharmingen), APC-anti-CD14 (BD Pharmingen), APC-anti-CD34 (BD Pharmingen), FITC-anti-CD90 (AbD Serotec, Kidlington, UK), and PE-anti-CD29 (Invitrogen). Isotype control antibodies were used, and at least 10 000 cells were analyzed for each marker.

### Vascular network formation in Matrigel

*In vitro assay:* 50  $\mu\text{l}$  Matrigel (BD Biosciences) was added to a precooled 96-well plate and allowed to solidify for 1 h at  $37^{\circ}\text{C}$ .  $1 \times 10^4$  cells in 150  $\mu\text{l}$  complete growth medium were added to each well. Network formation was observed by microscopy after overnight incubation.

*In vivo assay:* NOD-SCID-IL2R $\gamma^{-/-}$  mice were injected subcutaneously with 500  $\mu\text{l}$  of Matrigel (BD Biosciences) alone or including  $2 \times 10^5$  LO-EPC from patients or controls. Matrigel plugs were excised after 1 week. After being photographed, Matrigel plugs were embedded in OCT compound (Bright Instrument Company Ltd, Huntingdon, UK). Ten-micrometer cryostat sections were stained by hematoxylin–eosin or by immunohistochemistry with anti-human CD31 antibodies.

### LO-EPC proliferation

Proliferation was determined by BrdU incorporation during DNA synthesis using the Cell Proliferation ELISA kit (Roche, Burgess, UK) according to the manufacturer's protocol. Results were expressed as absorbance at wavelength 450 nm compared with a 690 nm reference.

**Table 1.** Clinical characteristic of patients with end-stage renal failure.

Characteristic	All subjects with ESRF	Outgrowth MSC clone obtained		P-value
		No	Yes	
Subjects, <i>n</i>	15	12	3	
Male, <i>n</i>	9 (60%)	7 (58%)	2 (67%)	
Female, <i>n</i>	6 (40%)	5 (42%)	1 (33%)	
Age	43.8 ± 4.0	38.6 ± 3.7	64.7 ± 1.45	<0.01
Body weight	77.8 ± 4.4	78.3 ± 4.8	75.5 ± 12.4	0.85
Systolic blood pressure (mmHg)	142.4 ± 6.0	137.5 ± 7.1	158.7 ± 2.0	0.02
Diastolic blood pressure (mmHg)	77.6 ± 3.6	78.4 ± 4.6	75.0 ± 3.5	0.59
Hemoglobin (mm)	11.3 ± 0.5	11.2 ± 0.5	11.8 ± 0.8	0.58
Creatinine (μM)	685.4 ± 74.7	709.3 ± 91.4	589.7 ± 76.6	0.34
Urea (mm)	16.4 ± 1.7	16.5 ± 2.1	15.6 ± 0.9	0.71
Total cholesterol (mm)	4.4 ± 0.2	4.0 ± 0.3	4.0 ± 0.6	0.96
eGFR (ml/min/1.73 cm <sup>2</sup> )	9.7 ± 2.7	10.1 ± 3.4	8.3 ± 0.7	0.63
Hemodialysis, <i>n</i> (%)	13 (87%)	10 (83%)	3 (100%)	1.00
Use of Erythropoietin, <i>n</i> (%)	11 (73%)	8 (67%)	3 (100%)	0.52
Use of statin, <i>n</i> (%)	7 (47%)	4 (33%)	3 (100%)	0.08

Data are mean ± SE. P-values are derived from comparing results from patients with and without outgrowth of MSC colonies. The causes for ESRF included autosomal dominant polycystic kidney disease (*n* = 3), immunoglobulin A nephropathy (*n* = 1), Alport's syndrome (*n* = 1), type II diabetes mellitus nephropathy (*n* = 1), focal segmental glomerulosclerosis (*n* = 2), type II membranoproliferative glomerulonephritis (*n* = 1), congenital hydro-nephrosis/vesicoureteric reflux (*n* = 1), chronic pyelonephritis (*n* = 2), multiple renal infarction (*n* = 1), and unknown etiology (*n* = 2).

### Cytokine and angiogenesis analyses

The levels of multiple cytokines/chemokines and angiogenic factors in plasma were measured using a commercial Cytokine Antibody Array kit (R&D Systems, Abingdon, UK) or Angiogenesis Array kit (R&D Systems) according to the manufacturer's instructions. The array data were analyzed by Image J software (freeware, <http://rsbweb.nih.gov/ij/>) and expressed as pixel density. To evaluate the secretion of angiogenic factors in LO-EPC *in vitro*, cells were cultured in growth-factor-free basal medium EBM with 10% FBS for 24 h. The supernatant was then collected, and the levels of multiple angiogenic factors measured.

### Migration assay

LO-EPC migration was evaluated using the ORIS™ Cell Migration Assembly kit (AMS Biotechnology (Europe) Ltd, Abingdon, UK) as described previously [22].

### LO-EPC adhesion to matrix molecules

24-well plates were coated with 100 μg/ml collagen type I (BD), 100 μg/ml fibronectin (Sigma, Gillingham, UK), 10 μg/ml vitronectin (Sigma) or 2 μg/ml laminin (Sigma) for 2 h at 37 °C.  $1 \times 10^5$  LO-EPC were added to each well, and nonadherent cells removed after 1 h. Adherent cells were stained with Dil-Ac-LDL and fixed with 2% paraformaldehyde. Cells were then counted in five fields at low magnification. Controls were uncoated plates.

### Integrin gene expression and cell surface expression analyses

The integrin gene profile of LO-EPC was analyzed using a custom-made PCR Array (RT<sup>2</sup> Profiler™ PCR Array System; Qiagen, Manchester, UK). Optimized primers for 18 integrin α and 8 integrin β subunits were used, with GAPDH as a reference gene to calculate delta CT values. Integrin gene expression was expressed as  $2^{-\Delta C_t}$ .

A panel of conjugated antibodies was used to detect cell surface integrin expression: PE-anti-integrin α5 (eBioscience, Hatfield, UK), FITC-anti-αv (Southern Biotech, Cambridge, UK), PE-Cy7-anti-αE (BioLegend, London, UK), PerCp-anti-α6 (eBioscience), Pacific blue-anti-β1 (EXBIO, Upper Heyford, UK), and APC-anti-β3 (Invitrogen), together with isotype control antibodies. To compensate for background fluorescence, geometric mean fluorescence intensity of the relevant isotype control was subtracted from that of the experimental sample.

### Culture and differentiation of mesenchymal stem cells from peripheral blood

Colonies of MSC were identified in some LO-EPC preparations after 2 weeks in culture. Spindle-shaped MSC colonies were visually distinct from LO-EPC and were isolated using cloning cylinders (Sigma) and cultured separately in MSC culture medium, consisting of Dulbecco's modified Eagle's medium (DMEM) with 10% Hyclone fetal calf serum.

MSC differentiation and functional characterization were performed using a Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems) according to the manufacturer's instructions.

Oil Red O staining was performed by fixing cells in 10% formalin for 10 min at room temperature. After washing three times with distilled water, the cells were incubated with 60% isopropanol for 2 min and left to dry at room temperature. Cells were incubated in 60% Oil Red O solution for 5 min at room temperature. Excess stain was removed by washing with distilled water.

Alizarin red staining was performed by fixing cells with 70% ethanol for 1 h on ice. After washing with distilled water, cells were incubated in 2% alizarin red S solution for 5 min, followed by multiple washes with distilled water.

### Statistical analysis

All values are expressed as mean  $\pm$  SE unless otherwise stated. Within each independent experiment, at least duplicate measurements were performed. The difference between two groups was compared using Student's unpaired two-tailed and unequal-variance t-test (GRAPHPAD PRISM software, La Jolla, CA, USA). Comparison of continuous variables was performed using one-way ANOVA analysis. Categorical variables were evaluated by Fisher's exact test. Univariate correlations were made with Spearman's two-tailed and unequal-variance correlation coefficient. A probability value of  $P < 0.05$  was considered statistically significant and indicated by \*;  $P < 0.01$  indicated by \*\*. Trends in cell adherence between patients' cells and those of healthy volunteers were compared using the Fisher's combined probability test.

## Results

### Culture characteristics of patients' LO-EPC

Culture of patients' PBMC for 2–3 weeks resulted in emergence of LO-EPC colonies displaying characteristic cobblestone morphology homogeneously in most cases (12 of 15 patients; Fig. 1a). LO-EPC took up acetylated LDL (Fig. 1b) and formed networks in Matrigel (Fig. 1c) identically to LO-EPC cultured from healthy volunteers (Fig. 1d–f). Immunophenotyping revealed the expression of mature EC surface markers CD31 and VEGFR-2, but an absence of the monocyte cell surface marker CD14, indicating their endothelial lineage. CD34 positivity confirmed their progenitor nature (Fig. 1g and h).

### LO-EPC growth kinetics in patients with ESRF

Although the total mononuclear cell number isolated from 40 ml blood was significantly lower in patients with ESRF compared with healthy volunteers ( $68.45 \pm 2.42$

vs.  $121.14 \pm 3.41$  respectively,  $P = 0.0008$ ), there was no significant difference in time of appearance of LO-EPC colonies between the two groups ( $11.77 \pm 0.98$  vs.  $14.50 \pm 1.33$  days for patients and controls respectively,  $P = 0.07$ ). However, the number of LO-EPC colonies was significantly elevated in patients compared with controls ( $P < 0.05$ , Fig. 2a) and could be accounted for by their increased capacity for LO-EPC proliferation (Fig. 2b).

Both LO-EPC from patients and healthy controls secreted endoglin-1, IL-1 $\beta$ , IL-8, MCP-1, pentraxin 3, PLGF, PAI-1, and TIMP-1. However, LO-EPC from patients secreted significantly higher levels of PLGF, MCP-1, and IL-1 $\beta$  (Fig. 2c). Other angiogenic factors including VEGF, HGF, and GM-CSF were not secreted by LO-EPC from patients or healthy controls. This paracrine secretion pattern is distinctly different from that reported previously for EO-EPC [13,23].

### Influence of inflammatory cytokines and angiogenesis cytokines on LO-EPC growth characteristics

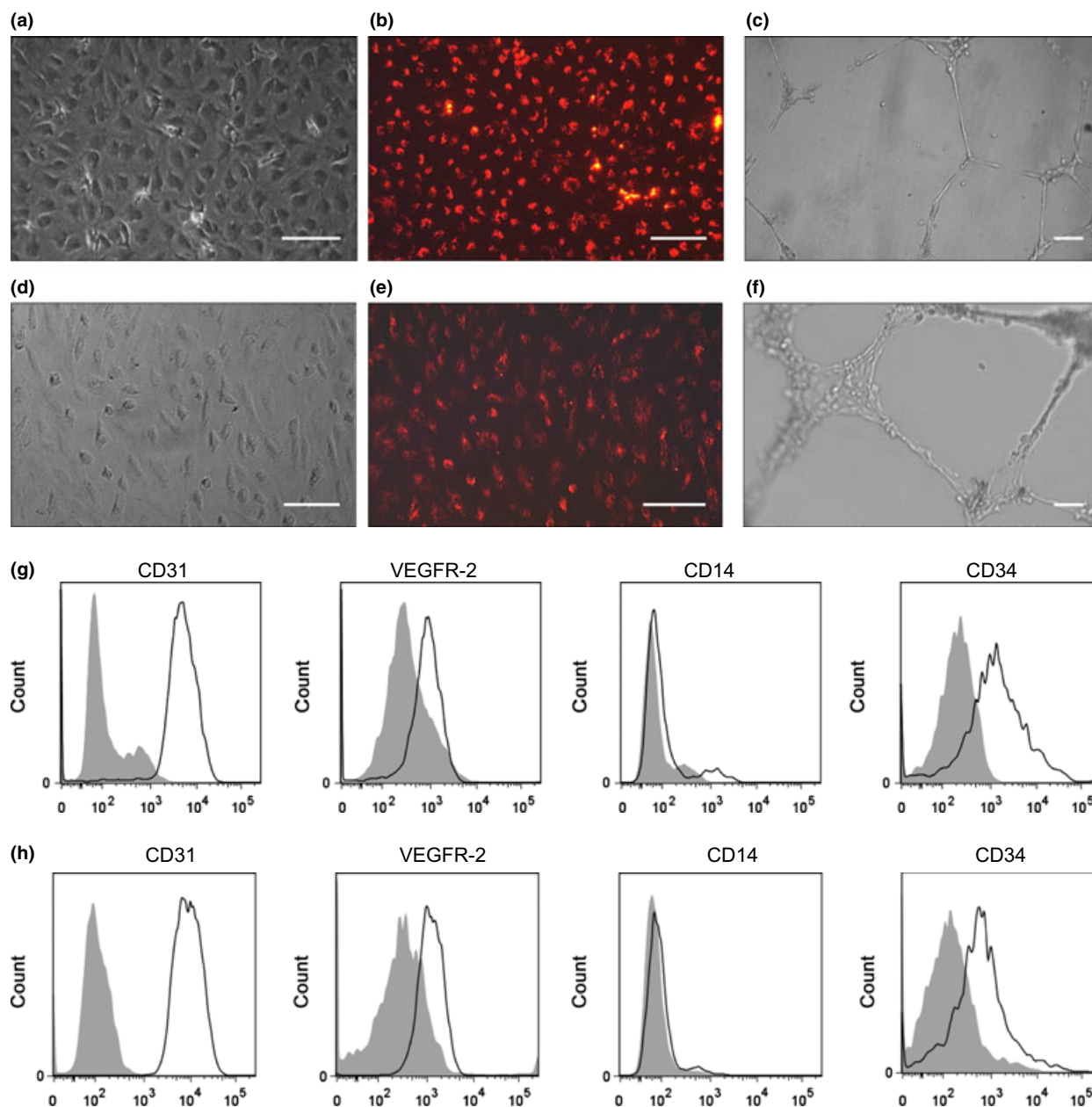
Patients with ESRF displayed significantly elevated plasma levels of IL-1 $\alpha$ , IL-16, IL-6, and MIF compared with controls (Fig. 3a), with a positive correlation between plasma levels of IL-16 and the number of LO-EPC colonies (Fig. 3c,  $P = 0.006$ ). SDF-1, a key cytokine for mobilization of progenitor cells from bone marrow, did not differ between patients and healthy controls (Fig. 3a), contrary to a previous report [6].

Angiogenesis factors PLGF, prolactin, and VEGF were significantly elevated in patients' plasma compared with controls (Fig. 3b); plasma VEGF levels correlated with the number of patients' LO-EPC colonies (Fig. 3d,  $P = 0.0008$ ). Study of the effect of supplementary IL-16 and VEGF showed that IL-16 did not affect proliferation of LO-EPC from healthy controls; however, 1  $\mu$ g/ml of IL-16 significantly increased the proliferation of LO-EPC from patients (Fig. 3e). 200 ng/ml of VEGF significantly increased proliferation of LO-EPC from both groups (Fig. 3f).

### Migration and adhesion characteristics of LO-EPC from patients with ESRF

Migration and adhesion are essential for angiogenesis. LO-EPC from patients with ESRF and controls had a similar migratory ability (Fig. 4a), although the range was wider in patients with ESRF.

LO-EPC from patients with ESRF showed a strong trend of reduced adhesion to extracellular matrix proteins collagen type I, fibronectin, vitronectin, and laminin, with  $P$ -values (equal-variance unpaired one-tailed t-test) of 0.150 (collagen), 0.153 (fibronectin), 0.072 (vitronectin), and 0.095 (laminin). The ( $\chi^2$ )  $P$ -value calculated using Fisher's combined probability test is 17.5342. The  $\alpha$  level of significance for



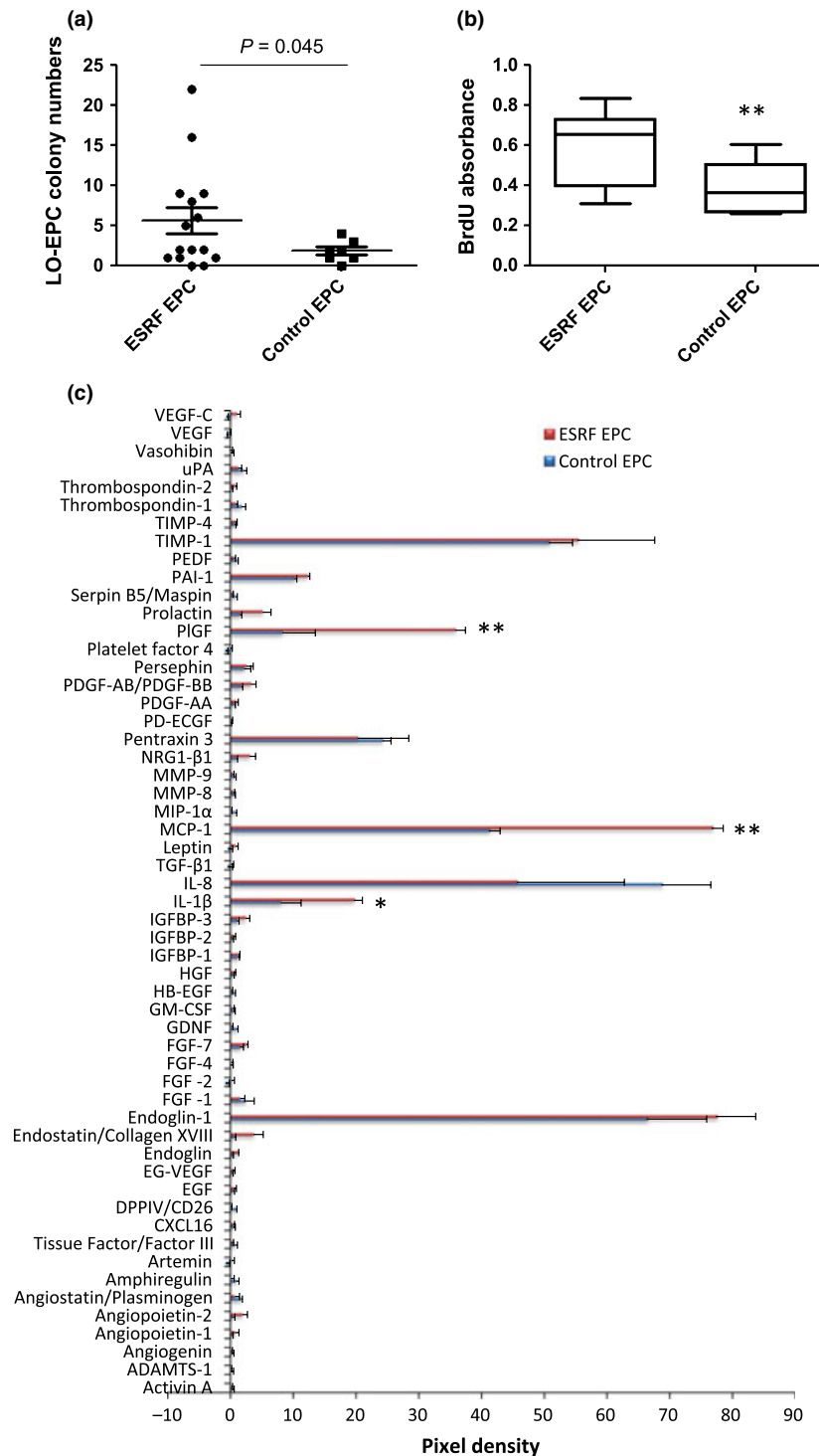
**Figure 1** Characteristics of representative LO-EPC from patients with ESRF (a–c and g) and healthy volunteers (d–e and h). Cells from both groups displayed cobblestone morphology (a and d), took up Dil-acetylated LDL (b and e) and formed networks in Matrigel (c and f). Scale bar 70  $\mu$ m. Representative flow cytometric histograms illustrating cell surface phenotype of LO-EPC from patients (g) and healthy volunteers (h). The filled histogram represents antibody isotype control, and the solid line represents LO-EPC stained with antibody.

8 d.f. (2 m) at 0.05% confidence from the chi-square table is 15.51, indicating a significant difference in adhesion capability between patients and control LO-EPC (Fig. 4b).

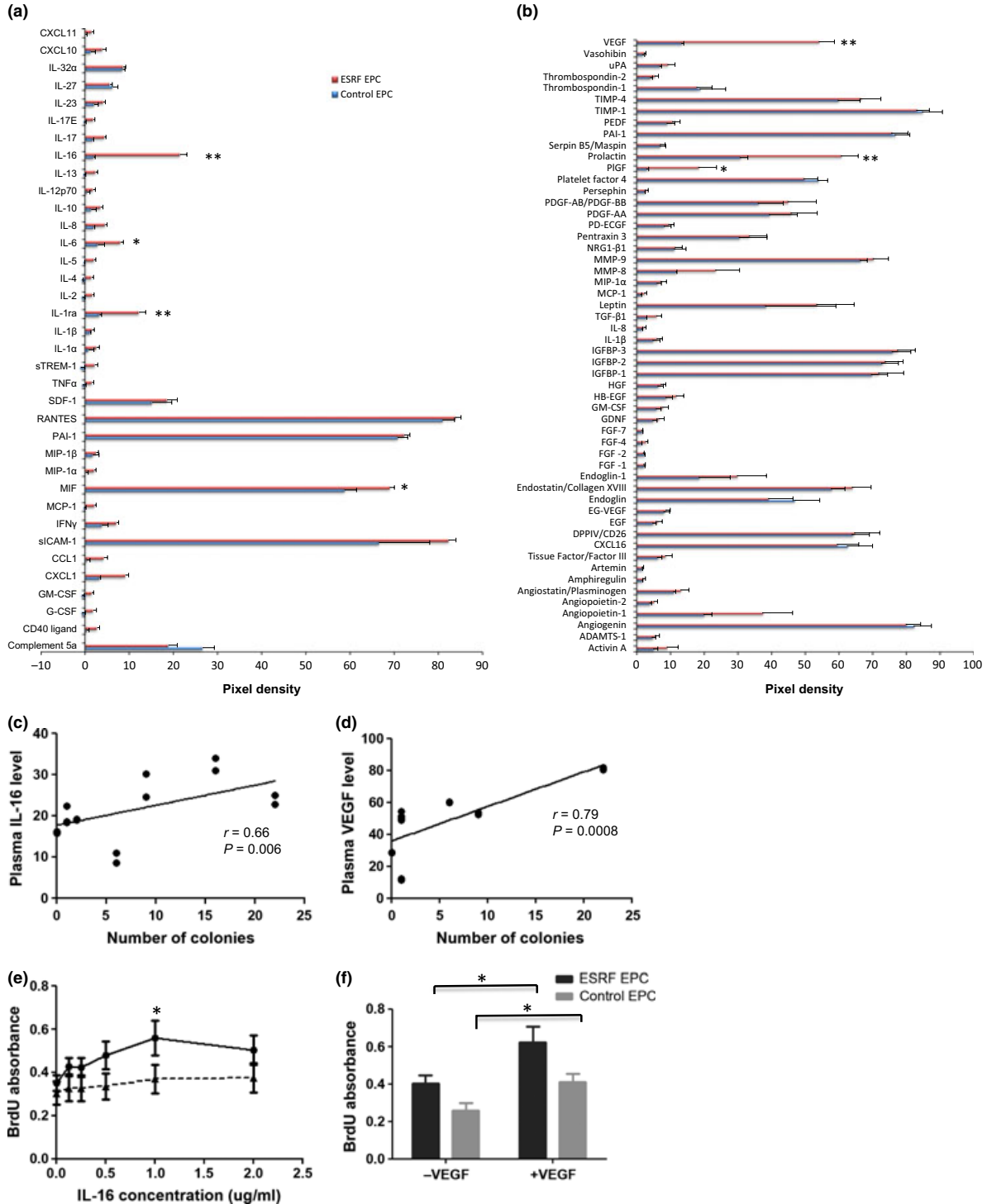
#### Integrin expression in LO-EPC from patients with ESRF

Decreased adhesion of patients' LO-EPC could not be explained by differential expression of integrins on

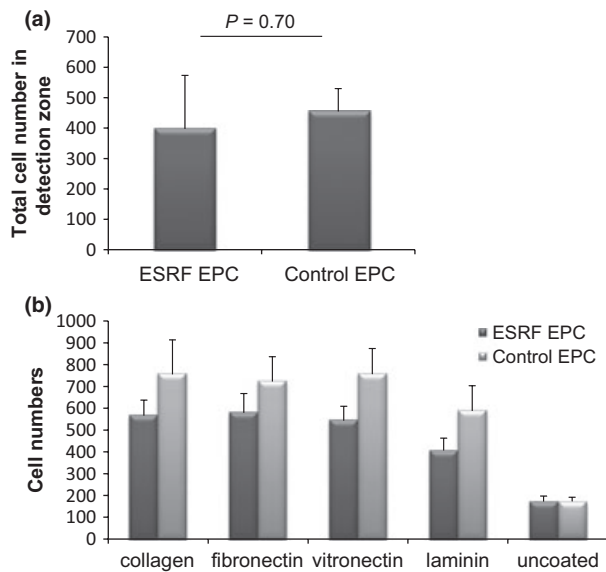
LO-EPC between patients with ESRF (Fig. 5a) and controls (Fig. 5b). Both showed high gene expression of the integrin subunits,  $\alpha$ 5,  $\alpha$ v,  $\beta$ 1, and  $\beta$ 3, moderate expression of  $\alpha$ 6 and  $\alpha$ E, and low level expression of other integrin subunits. The apparent decreased expression of integrin  $\alpha$ 5,  $\alpha$ v,  $\beta$ 1, and  $\beta$ 3 in LO-EPC from patients with ESRF compared with the healthy controls did not reach statistical significance (Fig. 5c). Consistent with integrin gene expression profiles,



**Figure 2** Characteristics of LO-EPC from patients with ESRF. Dot plot of the colony number of LO-EPC from patients and healthy volunteers (a) ( $P = 0.045$ ). Box plot of proliferation assay of LO-EPC from patients and healthy volunteers (b) ( $n = 6$ ,  $P = 0.0014$ ). Secretion of angiogenic growth factors by LO-EPC from patients (red bars) and healthy controls (blue bars) in growth-factor-free medium over a 24-h period (c). Data expressed as mean  $\pm$  SE of three experiments.



**Figure 3** Production of cytokines by LO-EPC. Comparison of plasma inflammatory cytokines (a) and plasma angiogenesis cytokines (b) from patients with ESRF (red bars) and healthy controls (blue bars;  $n = 3$ ). The level of plasma IL-16 correlated with the number of LO-EPC from patients (c) ( $r = 0.66$ ,  $P = 0.006$ ). The level of plasma VEGF correlated with the number of LO-EPC from patients with ESRF (d) ( $r = 0.79$ ,  $P = 0.0008$ ). Effect of IL-16 on proliferation of LO-EPC from patients with ESRF (solid line) and healthy controls (dashed line) (e). The effect of human recombinant IL-16 (R&D systems) on LO-EPC proliferation was determined using serial dilutions of IL-16 from 2.0 to 0.125  $\mu\text{g/ml}$  added to endothelial culture medium for 24 h. Effect of VEGF on proliferation of LO-EPC from patients with ESRF and healthy controls (f). 200 ng/ml human recombinant VEGF (Sigma) was added to LO-EPC endothelial culture medium for 24 h.



**Figure 4** Functional characterization of LO-EPC from patients with ESRF. Migration assay of LO-EPC from patients and healthy volunteers (a). Data expressed as mean  $\pm$  SE of six experiments. Adhesion assay of LO-EPC from patients and healthy volunteers binding to collagen I, fibronectin, vitronectin, and laminin (b). Black bars represent LO-EPC from patients, and gray bars represent LO-EPC from healthy volunteers. Data expressed as mean  $\pm$  SE of six experiments.

LO-EPC from patients and healthy controls expressed similar cell surface expression of integrin subunits  $\alpha 5$ ,  $\alpha v$ ,  $\alpha E$ ,  $\alpha 6$ ,  $\beta 1$ , and  $\beta 3$  (Fig. 5d and e).

#### *In vivo* angiogenesis of LO-EPC from patients with ESRF

To explore the angiogenic function of LO-EPC *in vivo*, a Matrigel plug implantation model was used. Blood vessels are clearly visible in Matrigel plugs loaded with LO-EPC from patients with ESRF (Fig. 6a) and healthy controls (Fig. 6d), but absent in Matrigel alone (Fig. 6g). Consistent with this, immunohistochemistry using anti-human CD31 antibodies showed cell populations of mixed human and mouse origin in the LO-EPC-injected Matrigel plugs (Fig. 6b and c, e and f versus h and i), suggesting that human LO-EPC could attract and recruit murine blood cells to the newly forming vasculature.

#### Characterization and differentiation of MSC from patients with ESRF

One striking difference between patients and healthy volunteers was the detection of MSC isolated from patients' PBMC. While 12 of 15 patients with ESRF produced LO-EPC colonies with typical cobblestone morphology, 3

showed a mixed population of colonies in culture. An adherent layer of elongated spindle-like cell colonies appeared at a similar time to LO-EPC colonies (Fig. 7a). When isolated and cultured separately, these cells did not take up acetylated LDL (Fig. 7b) and could not form networks *in vitro* (Fig. 7c). They were uniformly positive for MSC surface markers CD90 and CD29 and lacked the EC surface markers CD31, CD34, and CD14 (Fig. 7d).

Culture of these cells in adipogenic medium for 2 weeks induced differentiation into adipocytes confirmed by Oil Red O (Fig. 7e) and adipocyte-specific antibody FABP4 staining (Fig. 7f). Culturing in osteogenic medium for 3 weeks induced differentiation to osteocytes confirmed by Alizarin red (Fig. 7g) and osteocyte-specific antibody osteocalcin staining (Fig. 7h).

There was a trend that patients from whom outgrowth MSC clones were obtained were of higher age ( $P < 0.01$ ), had higher systolic blood pressure ( $P = 0.02$ ), and a higher statin usage ( $P = 0.08$ ; Table 1). However, the small patient number requires that further studies are performed to confirm this correlation.

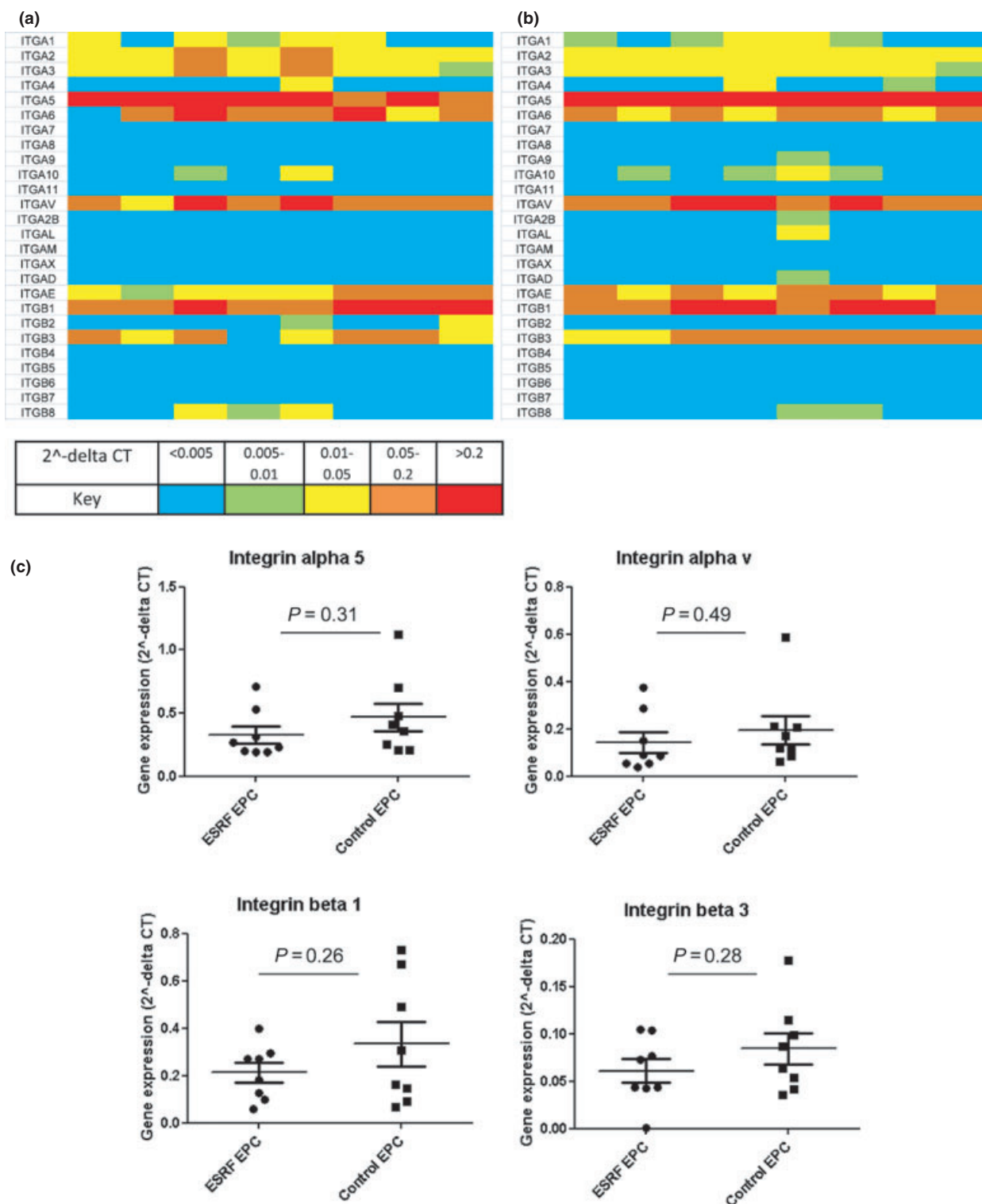
#### Discussion

This is the first study to explore the feasibility of isolating LO-EPC from patients with ESRF, to systematically characterize their angiogenic function and to investigate their phenotype, growth kinetics, paracrine activity, and the plasma environment from which they were isolated. It has implications for the utilization of LO-EPC from patients with ESRF for autologous endothelialization applications in the future.

We showed that the total PBMC number is reduced in patients with ESRF compared with healthy volunteers, possibly contributed to by changes in circulating blood volume [24] and depletion of monocytes associated with long-term dialysis [25]. However, the number of LO-EPC colonies was significantly higher in patients. This discrepancy between the number of EO-EPC and LO-EPC was also shown in patients with coronary artery disease who had reduced numbers of EO-EPC [26], but increased numbers of LO-EPC [27]. Our data suggest that the increased yield of LO-EPC colonies seen in patients was due to enhanced LO-EPC proliferation.

Release of angiogenic factors by EPC is important for their proangiogenic effect. Previous studies showed that EO-EPC could secrete VEGF, HGF, GCSF, and GM-CSF [13,23,28]. End-stage renal disease is associated with decreased angiogenic factors in EO-EPC [24]. Our study revealed that a distinctly different panel of angiogenic factors are secreted by LO-EPC compared with EO-EPC. LO-EPC did not secrete VEGF, HDG, and GM-CSF, but instead secreted high levels of endoglin-1, IL-1 $\beta$ , IL-8,





**Figure 5** Integrin gene and protein expression profile in LO-EPC from eight patients with ESRF (a) and eight healthy volunteers (b). Gene expression was expressed as 2<sup>-ΔC<sub>t</sub></sup>. Comparison of gene expression of integrin subunits α5, αv, β1, and β3 between LO-EPC from patients and healthy volunteers (c). Representative FACS analysis of surface integrin expression of LO-EPC from patients (upper panel) and healthy controls (lower panel) (d). Three separate experiments were performed. Filled histogram represents antibody isotype control, and the solid-line histogram represents LO-EPC stained antibody. Mean fluorescence intensity plot of cell surface integrin expression of LO-EPC from patients and healthy control (e).

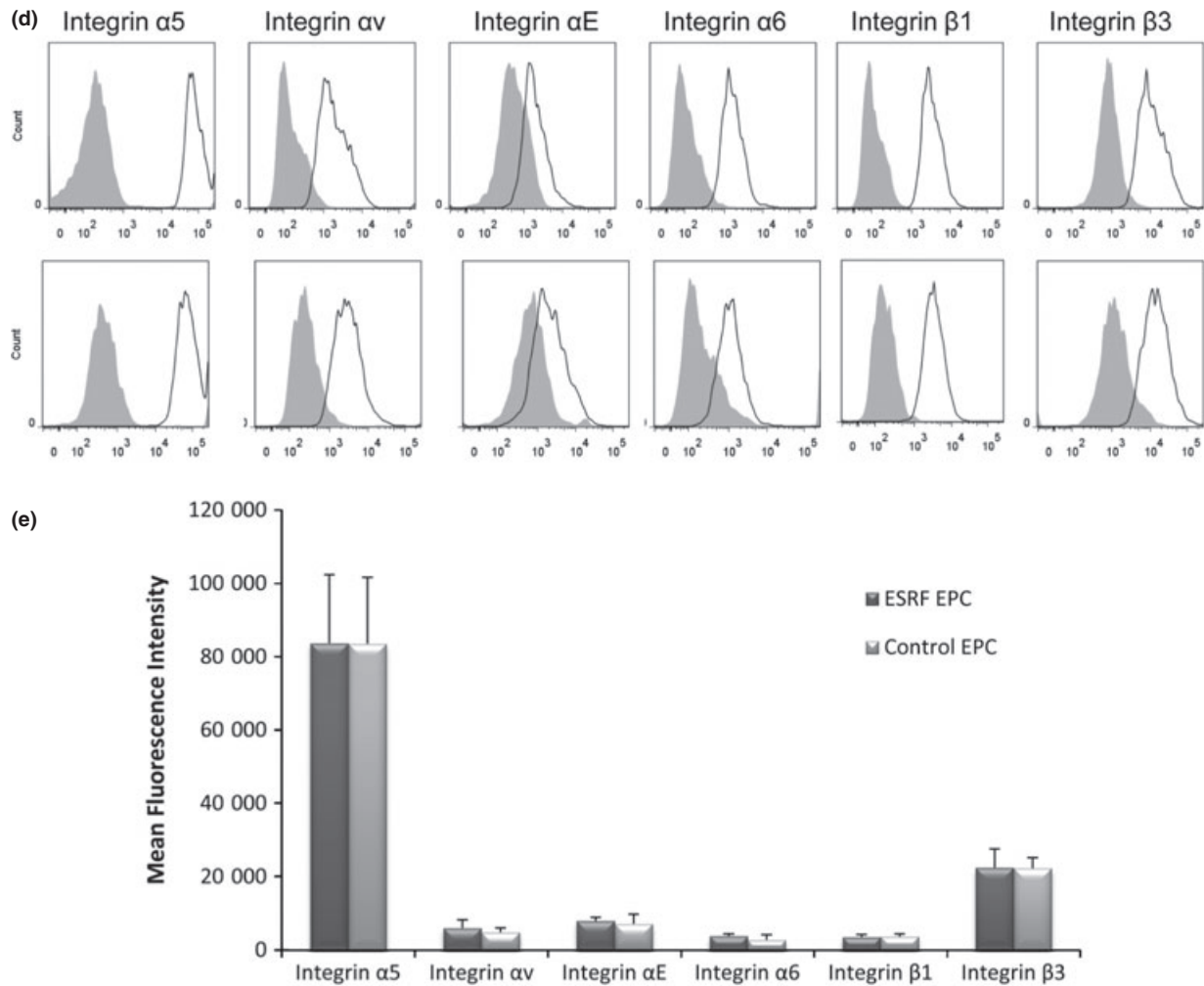
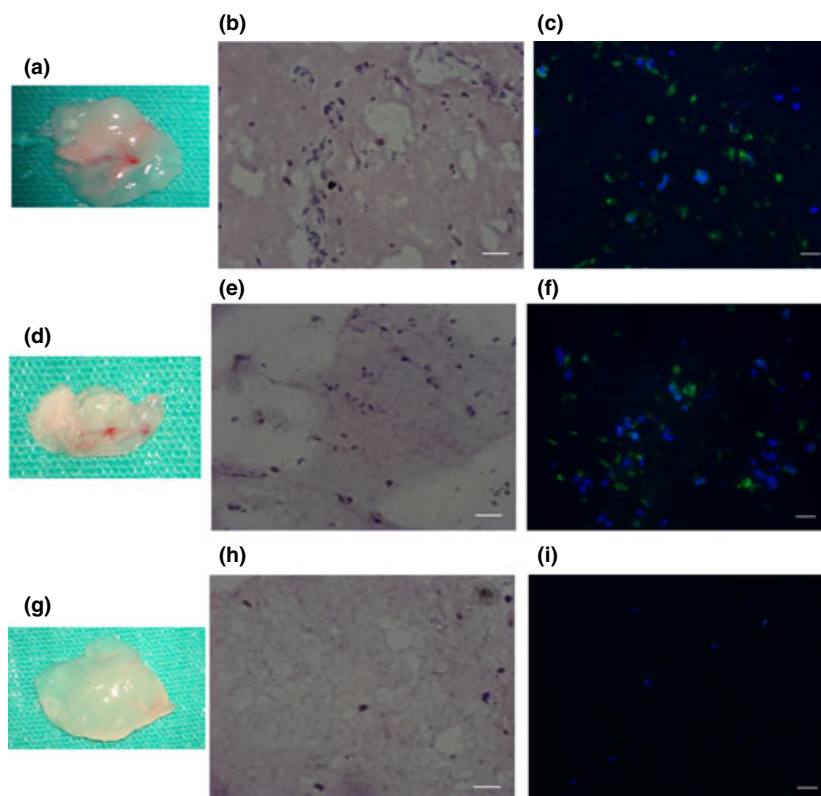


Figure 5 Continued.

MCP-1, pentraxin 3, PLGF, PAI-1, and TIMP-1. Secretion of IL-8 and MCP-1 by LO-EPC was reported previously [29,30]. Compared with healthy controls, LO-EPC from patients produced significantly more PLGF, MCP-1, and IL-1 $\beta$ . PLGF is a pleiotropic factor whose proangiogenic activity relies on enhanced proliferation, migration, and survival of EC [31–33]. Although MCP-1 and IL-1 $\beta$  are generally regarded as proinflammatory cytokines, both have been shown to play a role in the regulation of EC proliferation [34–36]. Together, increased secretion of these factors may be responsible for increased proliferation of LO-EPC from patients.

Circulating cytokine levels are important for mobilization of EPC from the bone marrow and their angiogenic functions and trafficking to sites of vascular injury [37,38]. We showed that patients with ESRF had increased blood levels of the angiogenesis-related cytokines VEGF, PIGF, and prolactin and increased pro-

inflammatory cytokines IL-1 $\alpha$ , IL-16, IL-6, and MIF. Increased serum VEGF has been reported in ESRF [5,39], diabetic nephropathy [40], and cardiac allograft arteriosclerosis [41]. Both VEGF and IL-16 elevations correlated with the number of LO-EPC colonies isolated from patients with ESRF. VEGF plays important roles in regulation of blood vessel growth, maintenance of endothelial integrity, and EPC release from bone marrow, but is also reported to contribute to ESRF progression [42,43], and to have both vasoprotective [44] and atherogenic effects [45]. The reason for increased levels of VEGF in patients with renal dysfunction is unknown and may reflect increased oxidative stress associated with chronic renal failure [39] or may be a compensatory mechanism for triggering endothelial repair. Consistent with this, kidney transplantation improves EPC function, and this is associated with a decreased level of VEGF [6]. Increased colony number, proliferation, and paracrine



**Figure 6** Representative images of *in vivo* angiogenesis assay. Macroscopic view of Matrigel plugs (a, d, and g). Blood vessels are visible in plugs loaded with LO-EPC from patients with ESRF (a) and healthy controls (d), but absent from Matrigel containing no LO-EPC (g). H&E staining of explanted Matrigel plug sections (b, e, and h), scale bar 40  $\mu\text{m}$ , and immunohistochemistry staining of explanted Matrigel plug sections with anti-human CD31 antibodies (green) and Hoechst nuclear stain (blue) (c, f, and i), scale bar 50  $\mu\text{m}$ . Infiltrating cells of mixed human and mouse origin are present in the plugs loaded with EPC from patient (b and c) and healthy control (e and f).

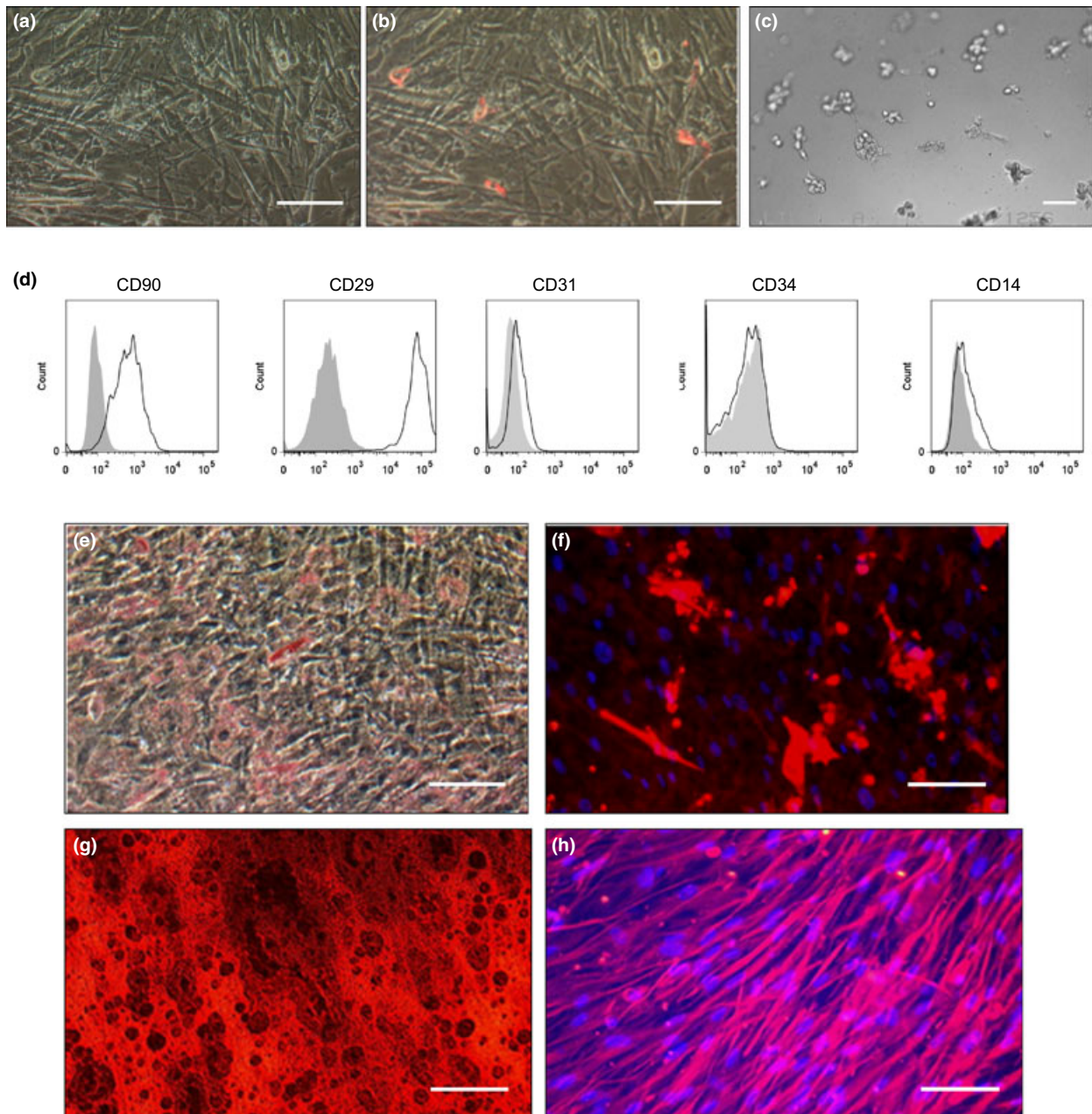
angiogenic activity in patients' LO-EPC may be part of this compensatory mechanism regulated by VEGF and is supported by our finding that VEGF had a direct effect on LO-EPC proliferation.

IL-16 is a pleiotropic cytokine that functions as a lymphocyte chemoattractant [46]; its role in chronic renal failure is unclear. Wang *et al.* showed renal expression of the IL-16 precursor, which can be cleaved to a mature form of IL-16 under stress, and indicated its involvement in renal ischemia reperfusion injury [47]. The levels of urine IL-16 are a biomarker for the severity of renal injury in both native and transplanted kidneys [47]. Our study has shown significant positive correlations between blood levels of IL-16 and firstly the number of LO-EPC, and secondly, blood VEGF levels (data not shown). We also demonstrated a correlation between IL-16 concentration *in vitro* and LO-EPC proliferation from patients with ESRF but not controls. The nature of the IL-16 receptor is unclear. There is evidence that it is CD4 [48,49], but this is not found on LO-EPC. A whole range of other cell surface molecules have been implicated to interact with IL-16 [50,51], which

may exert its effect on patients' LO-EPC through synergistic interaction with other angiogenic factors.

An important goal for using autologous LO-EPC is the repair and reformation of vascular endothelium. We show that LO-EPC from patients with ESRF and healthy controls have a comparable ability to form vascular networks *in vitro* and *in vivo* and for migration, the latter contrasting with previous studies using EO-EPC [3,5].

Repair and the regeneration of damaged vessels depend critically on the ability of EPC to migrate normally and to adhere firmly to the site of injury. Both LO-EPC from patients and healthy volunteers could attach to the extracellular matrix proteins collagen, fibronectin, vitronectin, and laminin, but there was a statistically significant trend of reduced adhesion of LO-EPC from patients to all four extracellular matrix proteins. Our findings match previous studies on EO-EPC showing impaired adhesion of EPC from patients with ESRF [3,5]. This occurred despite elevated blood levels of VEGF in patients with ESRF which would be predicted to be associated with enhanced adhesion ability. In addition, despite increased angiogenic factor



**Figure 7** Representative phenotypic and functional characteristics of mesenchymal stem cells from patients with ESRF. MSC displayed spindle-shape morphology (a), did not uptake Dil-acetylated LDL (b), and could not form vascular networks in Matrigel (c). Scale bar 70  $\mu\text{m}$ . Representative FACS analysis of phenotypic markers specific for MSC isolated from patients with ESRF (d). Cells express CD90 and CD29 and lack expression of CD31, CD34, and CD14. The filled histogram represents isotype control antibody, and the solid-line histogram represents cells stained with specific antibodies. Functional characterization of MSC from patients with ESRF is shown in e–h. Adipogenic differentiation potential of MSC from patients was determined by Oil Red O staining for lipid droplets (e) and antibody staining for the adipocyte-specific antigen FABP4 (f). Osteogenic differentiation potential of MSC from patients was determined by Alizarin red S staining for the mineralized matrix (g) and antibody staining for the osteocyte-specific antigen osteocalcin (h). Scale bar 70  $\mu\text{m}$ .

secretion in LO-EPC from patients, which enhanced LO-EPC proliferation, this failed to enhance adhesion, suggesting that proliferation and adhesion are regulated independently.

Cell adhesion is mediated by integrins [52,53], which are glycosylated heterodimeric cell surface proteins [54] comprising noncovalently linked alpha and beta subunits [54]; 18 integrin  $\alpha$  and 8 integrin  $\beta$  subunits combine to form 24

different transmembrane integrin receptors [55]. The full integrin profile of LO-EPC has not previously been determined. We quantified mRNA levels of all  $\alpha$  and  $\beta$  subunits; no difference was detected between patients and healthy controls. Both had high levels of integrins,  $\alpha 5$ ,  $\alpha v$ ,  $\beta 1$ , and  $\beta 3$ , moderate expression of  $\alpha 6$  and  $\alpha E$ , and minimal expression of the remainder. Consistent with this, there was no significant difference in cell surface integrin expression. Thus, the decreased adhesion in LO-EPC from patients could not be explained by differences in integrin expression and was unrelated to their ability to form vascular network in Matrigel.

We report here for the first time outgrowth of MSC from the peripheral blood of some patients with ESRF. These cells not only possess MSC surface markers, but also could differentiate to adipocytes and osteocytes, suggesting that they are truly functional MSC. The identification of MSC raises the intriguing possibility of their contribution to vascular disease and opens up the prospect of investigating their role in transplant vasculopathy. MSC may be a double-edged sword in transplant vasculopathy. Under appropriate conditions, MSC have the potential to differentiate into several cell types including smooth muscle cells [56], which participate in the pathological process of atherosclerosis [57–59]. Increased levels of smooth muscle progenitor cells have been observed in patients with chronic kidney disease [60] and end-stage renal disease [24]. However, MSC have been shown to play an immunosuppressive role [61] and to exert a profound inhibitory effect on proliferation of T cells, B cells, and other immune cells *in vitro* and *in vivo* [62]. More importantly, the inflammatory cytokine interferon- $\gamma$  could promote the immunosuppressive function of MSC [63]. Taken together, these studies imply that administration of MSC in transplant patients has the potential to ameliorate allograft rejection, although with the caveat that they express HLA class I and therefore have the potential also to sensitize the nonautologous recipient [64].

The subset of patients from whom MSC were derived did not show a differential expression of inflammatory/angiogenic cytokines compared with the other patients, but were characterized by a higher age, higher systolic blood pressure, and higher rate of usage of statins. However, our sample size was too small to confidently associate these factors with MSC outgrowth, or systematically assess disease or treatment-specific subsets of these patients.

In conclusion, we present the first phenotypic and functional evaluation of LO-EPC from patients with ESRF and highlight similarities and contrasts between these and LO-EPC from healthy volunteers. LO-EPC isolated from patients with ESRF display normal endothelial cell surface markers, have increased secretion of angiogenic factors, are able to attach to extracellular matrix proteins, and form

vascular networks *in vitro* and *in vivo*. Our results strongly confirm that LO-EPCs isolated from patients with ESRF are a clinically relevant candidate for applications involving autologous endothelial repair. They may have impaired ability to adhere to extracellular matrix, and this is an area of potential therapeutic intervention. Moreover, we report here for the first time an outgrowth of functional MSC from the peripheral blood of some patients with ESRF. Further work will clarify the importance of these cells in transplant vasculopathy in these patients.

### Authorship

JZ: designed research study, performed research, collected, and analyzed data, and wrote manuscript. EMB: contributed to design of the research study, data analysis, and preparation of the manuscript. LR: contributed to collection of samples and data. JAB: contributed to design of the research study and preparation of the manuscript. AML: contributed to design of the research study, data analysis, and preparation of the manuscript.

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