

Differential Expression of Distinct Surface Markers in Early Endothelial Progenitor Cells and Monocyte-Derived Macrophages

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Bone marrow-derived endothelial progenitor cells (EPCs) play a fundamental role in postnatal angiogenesis. Currently, EPCs are defined as early and late EPCs based on their biological properties and their time of appearance during in vitro culture. Reports have shown that early EPCs share common properties and surface markers with adherent blood cells, especially CD14⁺ monocytes. Distinguishing early EPCs from circulating monocytes or monocyte-derived macrophages (MDMs) is therefore crucial to obtaining pure endothelial populations before they can be applied as part of clinical therapies. We compared the gene expression profiles of early EPCs, blood cells (including peripheral blood mononuclear cells, monocytes, and MDMs), and various endothelial lineage cells (including mature endothelial cells, late EPCs, and CD133⁺ stem cells). We found that early EPCs expressed an mRNA profile that showed the greatest similarity to MDMs than any other cell type tested. The functional significance of this molecular profiling data was explored by Gene Ontology database search. Novel plasma membrane genes that might potentially be novel isolation biomarkers were also pinpointed. Specifically, expression of CLEC5A was high in MDMs, whereas early EPCs expressed abundant SIGLEC8 and KCNE1. These detailed mRNA expression profiles and the identified functional modules will help to develop novel cell isolation approaches that will allow EPCs to be purified; these can then be used to target cardiovascular disease, tumor angiogenesis, and various ischemia-related diseases.

Key words: Early endothelial progenitor cell (EPCs); Monocyte; Macrophage; Gene expression profile; Surface marker

INTRODUCTION

Defects in angiogenesis (blood vessel growth) or vessel repair are two of the major complications associated with cardiovascular disorders. Postnatal neoangiogenesis and vasculogenesis rely on circulating endothelial progenitor cells (EPCs), which were first identified by Asahara et al. in 1997 (1). EPCs are thought to derive from CD133⁺ stem cells that reside in bone marrow (2), and a reduced level of circulating EPCs has been found to serve as a biomarker that correlates with an increased frequency of cardiovascular events as well as more frequent death from cardiovascular causes (3–5). It has also become clear that the activation state of these cells is critical to their ability to carry out the vessel repair process.

Given their involvement in pathological and physiological angiogenesis, there has been growing interest in manipulating EPCs for therapeutic purposes. However, recently there has been much controversy in the field over the exact definition and functionality of an EPC. Current EPC definitions are based predominantly on

cell phenotype and the biological properties of the cell. Early EPCs (eEPCs; also referred to as circulating angiogenic cells) appear early (<1 week) in culture dishes, whereas late EPCs (also referred to as late outgrowth EPCs or endothelial colony-forming cells) appear late (2–4 weeks) and have a cobblestone-like morphology (1). Strikingly different angiogenic functions have been found to be associated with these two EPC subpopulations. Specifically, late outgrowth EPCs, but not eEPCs, form vascular networks de novo and can be incorporated into vascular networks; these cells can therefore be regarded as true EPCs (6). In contrast, eEPCs indirectly augment tubulogenesis even when physically separated from their target by a Transwell membrane, which implies that their effectiveness occurs via a cytokine-based paracrine mechanism (6,7). We have recently shown that eEPCs can abundantly express various inflammatory cytokines and paracrine angiogenic factors, including HGF, CCL3, CCL4, CCL18, CXCL16, and IL-10; these can promote angiogenesis in a paracrine manner (8).

Early EPCs are believed to represent a cell population that is enriched in monocytes, and this population exerts its angiogenic effects via paracrine and signaling mechanisms (9,10). It has been found that eEPCs can function as antigen-presenting cells (11). The antigen-presenting ability of eEPCs is weaker than that of monocyte-derived dendritic cells, but stronger than that of peripheral blood monocytes (11). EPCs carrying monocyte markers were recently shown to be selectively abnormal in type 1 diabetic patients with early retinopathy (12). Differences in CD14⁺ expression have been proposed as a means of distinguishing mononuclear cells (MNCs) that represent a source of either early or late EPCs (13). The majority of eEPCs have been suggested to arise from CD14⁺ subpopulations of peripheral blood mononuclear cells (PBMCs), whereas late outgrowth ones, which have the potential to differentiate toward endothelial cells, have been reported to be derived from the CD14⁻ MNC fractions (13,14). Gene expression microarray and proteomics analysis have shown that eEPCs are hematopoietic cells with a molecular phenotype linked to monocytes; this means that they should be used with caution because of their immunomodulatory nature.

As it has been shown that lineage and functional heterogeneity are present in the population of circulating cells capable of assuming an endothelial phenotype, it is critical to be able to separate out a pure population of eEPCs from immune cells such as CD14⁺ monocytes and monocyte-derived macrophages (MDMs) (14). Currently, circulating EPCs are enumerated by flow cytometry (15). Few reports exist in the literature concerning the characteristics of eEPCs, especially in relation to their surface antigen expression, other than endothelial markers. In this investigation, our aim is to better define the gene expression patterns of eEPCs and hematological cells that closely resemble them, especially monocytes and MDMs. Novel surface proteins differentially expressed between eEPCs and MDMs are identified in this study and hold the potential to be new biomarkers that will allow a more accurate isolation and/or quantification of eEPCs.

MATERIALS AND METHODS

Isolation and Cultivation of EPCs From Cord Blood

All patients gave informed consent, and the study was approved by the local research ethics committee. The protocols of this study were consistent with ethical guidelines provided in the 1975 Helsinki Declaration. EPC isolation and characterization were done as described previously with minor modifications (16,17). In brief, cord blood MNCs were isolated by Histopaque-1077 (1.077 g/ml; Sigma, St. Louis, MO, USA) density-gradient centrifugation. MNCs (1×10^7) were plated in 2 ml of endothelial growth medium-2 (Lonza Ltd., Basel, Switzerland) with supplementation (hydrocortisone, IGF-1, human EGF, human VEGF, human FGF-B, ascorbic acid, GA-1000, heparin, and 2%

fetal bovine serum) on fibronectin-coated six-well plates at 37°C in a 5% CO₂ incubator. After 3 days of culturing, attached eEPCs appeared, and medium and nonadherent cells were removed. Thereafter, the medium was replaced every 2 days, and a certain number of eEPCs continued to grow into the late EPC colonies, which emerged 2–4 weeks after the start of MNC culture. The late EPCs exhibited “cobblestone” morphology and a monolayer growth pattern that is typical of mature endothelial cells at confluence.

Characterization of Early and Late EPCs

The early and late EPCs were also assessed for endothelial and progenitor markers by indirect immunostaining with 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (DiI-acLDL; Molecular Probes, Invitrogen, Carlsbad, CA, USA) and costaining with *Bandeiraea simplicifolia* lectin I (Sigma). Briefly, the adherent cells were first incubated with DiI-acLDL (2.4 µg/ml) for 1 h and then fixed in 4% paraformaldehyde and counterstained with FITC-labeled lectin (10 µg/ml) from *Ulex europaeus* (UEA-1; Sigma). The fluorescent images were recorded under a laser scanning confocal microscope.

The antibodies used in FACS to characterize the adherent cell population were kinase insert domain receptor (KDR)/VEGF receptor 2 (R&D Systems, Minneapolis, MN, USA), AC133 (CD133), platelet endothelial cell adhesion molecule-1 (CD31; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and CD45 (Biolegend, San Diego, CA, USA). Flow cytometry was performed using a FACSCanto flow cytometer (BD Pharmingen, Franklin Lakes, NJ, USA).

Culture of CD14⁺ MDMs

Macrophage preparation was done as described (18). In brief, peripheral MNCs were isolated from the blood of healthy donors by standard density gradient centrifugation with Ficoll-Paque (Amersham Biosciences, Piscataway, NJ, USA). CD14⁺ cells were subsequently purified from peripheral MNCs by high-gradient magnetic sorting using the VARIOMACS technique with anti-CD14 microbeads (Miltenyi Biotec). CD14⁺ monocytes were cultured in complete RPMI-1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with hM-CSF (10 ng/ml) for 6 days to obtain MDMs. Fresh medium supplemented with hM-CSF (10 ng/ml) was added on day 3.

Gene Expression Microarray

Array data of CD133⁺ stem cells, CD34⁺ precursors, endothelial cells, PBMC, eEPCs, and late EPCs were from our previous publication (GSE39763 and GSE10856) (8,19,20). Public GEO microarray data sets (<http://www.ncbi.nlm.nih.gov/geo/>) included in this study were also

GSE12891 (another batch of early and late EPCs) (20) and GSE11430 (monocyte).

The array data for primary MDMs were obtained by our group using Affymetrix™ HG-U133 Plus 2.0 whole genome chips. RMA log expression units were calculated from Affymetrix GeneChip array data using the “affy” package of the Bioconductor (<http://www.bioconductor.org>) suite of software for the R statistical programming language (<http://www.r-project.org>). The default RMA settings were used to background correct, normalize, and summarize all expression values. Significant differences between the sample groups was identified using the “limma” (Linear Models for Microarray Analysis) package of the Bioconductor suite, and an empirical Bayesian moderated *t*-statistic hypothesis test between the two specified phenotypic groups was performed (21). To control for multiple testing errors, we then applied a false discovery

rate algorithm to these *p* values in order to calculate a set of *q* values, thresholds of the expected proportion of false positives, or false rejections of the null hypothesis (22).

Heat maps were created by the dChip software (<http://www.dchip.org/>). Principal components analysis (PCA) was performed by the Partek Genomics Suite (<http://www.partek.com/>) to provide a visual impression of how the various sample groups are related. The Euclidean distance between two groups of samples is calculated by the average linkage measure [the mean of all pairwise distances (linkages) between members of the two groups concerned] (23). The standard error of the average linkage distance between two groups (the standard deviation of pairwise linkages divided by the square root of the number of linkages) is quoted when intergroup distances are compared in the text. Gene enrichment analysis was performed by the Gene Ontology (GO) database

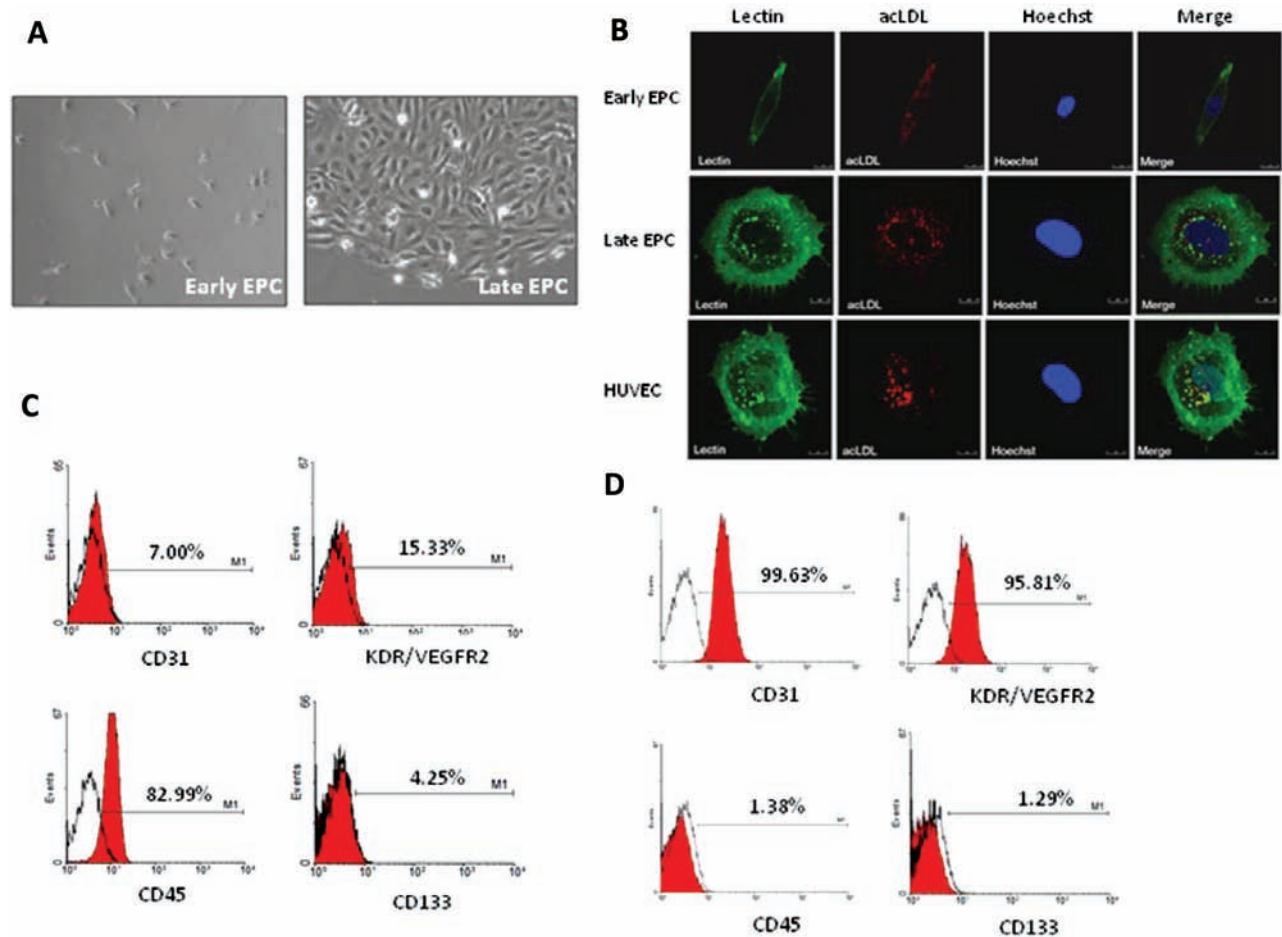


Figure 1. Cultivation and characterization of early and late EPCs. (A) Cord blood MNCs were isolated and plated on fibronectin-coated culture dishes for 4 days. Adherent eEPCs are shown in the left panel. Twenty-one days after plating, late EPCs with a cobblestone-like morphology were selected, reseeded, and grown to confluence (right). (B) Both early and late EPCs endocytose DiI-acLDL (acetylated low density lipoprotein; red) and bind fluorescein isothiocyanate UEA-1 (lectin) (green). Cells were counterstained with Hoechst 33258 to show the nucleus (blue). (C, D) Expression of indicated progenitor, endothelial, and hematopoietic markers in early (C) and late (D) EPCs by flow cytometric analysis.

(<http://www.geneontology.org/>) using the DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resources v6.7 at the National Institute of Allergy and Infectious Diseases (NIAID), NIH (<http://david.abcc.ncifcrf.gov/>).

RESULTS

Isolation and Characterization of Human Endothelial Precursor Cells

EPCs were obtained from the cord blood of healthy subjects as described (17). The peripheral blood MNCs that were initially seeded on fibronectin-coated wells were round. After the medium was changed on day 4, attached eEPCs with an elongated morphology appeared; then, late EPCs with a cobblestone-like morphology similar to mature endothelial cells grew to confluence at days 14–21 (Fig. 1A). Both EPCs were confirmed as having an endothelial lineage because both precursors were double positive for acLDL uptake and lectin (UEA-1) binding affinity (Fig. 1B).

The expression of cell lineage markers on the two EPCs was further validated and quantified by flow cytometry analysis. The majority of eEPCs expressed the hematological marker CD45, whereas the endothelial markers CD31 and KDR were present on only part of the isolated eEPC population (Fig. 1C). In contrast, the majority of late EPCs expressed the endothelial markers CD31 and

KDR (Fig. 1D). Both early and late EPC populations expressed the stem cell marker AC133/CD133 to only a limited extent (Fig. 1C, D).

Microarray Analysis Reveals Close Relationships Between eEPCs and Blood Cells, Especially MDMs

Early EPCs are believed to show greater similarity to hematopoietic cells with a molecular phenotype linked to monocytes (9,10). To evaluate the relationships between eEPCs and blood cells, especially monocytes and MDMs, we analyzed the transcriptomes of eEPC and of blood cell using whole-genome chips and then compared these to those of other endothelial lineage cells obtained by our group (19). A PCA plot using genes differentially expressed between CD133⁺ ancestor stem cells and mature endothelial progeny cells [$n=8,880$, positive false discovery rate (pFDR) $q < 10^{-4}$] represents the differentiation hierarchical relationship (Fig. 2A). Instead of being close to endothelial lineage cells, eEPCs were found to cluster with PBMCs, monocytes, and MDMs (Fig. 2A).

To provide more quantitative evidence, we calculated the average linkage distances between eEPCs and various cell types in order to assess the similarity between pairs of gene expression profiles, as described previously (19). As shown in Figure 2B, the distance between eEPCs and MDMs was smaller than the distances between eEPC and

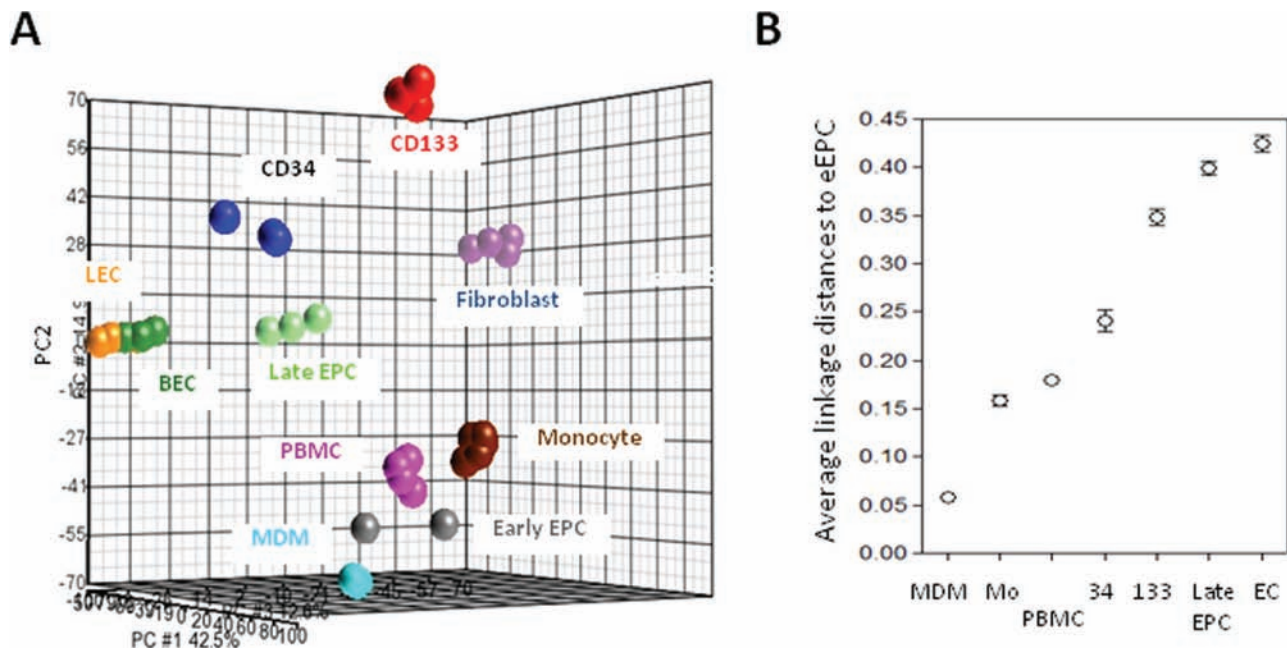


Figure 2. Similar yet distinct gene expression patterns between eEPCs and MDMs. (A) A PCA plot using genes differentially expressed between CD133⁺ stem cells and mature endothelial cells (8,880 probe sets, $q < 10^{-4}$). LEC, lymphatic endothelial cell; BEC, blood vessel endothelial cell; CD133, CD133⁺ stem cells; CD34, CD34⁺ progenitor cells. (B) Transcriptome distance analysis for eEPCs and various blood cell or endothelial cell types. Average linkage distances between transcriptomes were calculated as described using the aforementioned 8,880 probe sets. Mo, monocyte.

Table 1. Top 50 Known Genes in Early EPCs (Compared With MDMs)

Probe Set ID	UniGene ID	Gene Title	Gene Symbol	Location
206171_at	Hs.281342	Adenosine A3 receptor	ADORA3	chr1p13.2
222416_at	Hs.500645	Aldehyde dehydrogenase 18 family, member A1	ALDH18A1	chr10q24.3
1555416_a_at	Hs.111256	Arachidonate 15-lipoxygenase, type B	ALOX15B*	chr17p13.1
203381_s_at	Hs.654439	Apolipoprotein E	APOE	chr19q13.2
202686_s_at	Hs.590970	AXL receptor tyrosine kinase	AXL	chr19q13.1
202953_at	Hs.8986	Complement component 1, q subcomponent, B chain	C1QB	chr1p36.12
229070_at	Hs.126409	Chromosome 6 open reading frame 105	C6orf105	chr6p24.1
214038_at	Hs.271387	Chemokine (C-C motif) ligand 8	CCL8*	chr17q11.2
221019_s_at	Hs.464422	Collectin subfamily member 12	COLEC12	chr18pter-p11.3
232136_s_at	Hs.592285	Ccortactin binding protein 2	CTTNBP2	chr7q31
209687_at	Hs.522891	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12*	chr10q11.1
202887_s_at	Hs.523012	DNA-damage-inducible transcript 4	DDIT4	chr10pter-q26.12
228057_at	Hs.480378	DNA-damage-inducible transcript 4-like	DDIT4L	chr4q23
219424_at	Hs.501452	Epstein-Barr virus induced gene 3	EBI3	chr19p13.3
241981_at	Hs.268874	Family with sequence similarity 20, member A	FAM20A	chr17q24.2
224840_at	Hs.407190	FK506 binding protein 5	FKBP5	chr6p21.3-p21.2
206674_at	Hs.507590	Fms-related tyrosine kinase 3	FLT3	chr13q12
218706_s_at	Hs.363558	GRAM domain containing 3	GRAMD3	chr5q23.2
204018_x_at	Hs.449630	Hemoglobin, alpha 1 /// hemoglobin, alpha 2	HBA1 /// HBA2	chr16p13.3
204419_x_at	Hs.712539	Hemoglobin, gamma A /// hemoglobin, gamma G	HBG1 /// HBG2	chr11p15.5
203819_s_at	Hs.700696	Insulin-like growth factor 2 mRNA binding protein 3	IGF2BP3	chr7p11
227297_at	Hs.113157	Integrin, alpha 9	ITGA9*	chr3p21.3
236407_at	Hs.121495	Potassium voltage-gated channel, Isk-related family, member 1	KCNE1*	chr21q22.1-q22.2/21q22.12
221583_s_at	Hs.144795	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	KCNMA1	chr10q22.3
228977_at	Hs.130652	Hypothetical protein LOC729680	LOC729680	chr13q12.11
203414_at	Hs.463483	Monocyte to macrophage differentiation-associated	MMD	chr17q
204475_at	Hs.83169	Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	chr11q22.3
225520_at	Hs.591343	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	MTHFD1L	chr6q25.1
228056_s_at	Hs.636624	Napsin B aspartic peptidase pseudogene	NAPSB	chr19q13.33
33767_at	Hs.198760	Neurofilament, heavy polypeptide 200 kDa	NEFH	chr22q12.2
203708_at	Hs.198072	Phosphodiesterase 4B, cAMP-specific	PDE4B	chr1p31
225207_at	Hs.8364	Pyruvate dehydrogenase kinase, isozyme 4	PDK4	chr7q21.3
220952_s_at	Hs.188614	Pleckstrin homology domain containing, family A member 5	PLEKHA5	chr12p12
204285_s_at	Hs.96	Phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	chr18q21.32
201876_at	Hs.530077	Paraoxonase 2	PON2	chr7q21.3
204044_at	Hs.513484	Quinolate phosphoribosyltransferase (nicotinate-nucleotide pyrophosphorylase)	QPRT	chr16p11.2
212651_at	Hs.148670	Rho-related BTB domain containing 1	RHOBTB1	chr10q21.2
225202_at	Hs.445030	Rho-related BTB domain containing 3	RHOBTB3	chr5q15
205578_at	Hs.98255	Receptor tyrosine kinase-like orphan receptor 2	ROR2	chr9q22
204900_x_at	Hs.591715	Sin3A-associated protein, 30 kDa	SAP30	chr4q34.1
208253_at	Hs.447899	Sialic acid binding Ig-like lectin 8	SIGLEC8*	chr19q13.33-q13.41
223044_at	Hs.643005	Solute carrier family 40 (iron-regulated transporter), member 1	SLC40A1	chr2q32
204955_at	Hs.15154	Sushi-repeat-containing protein, X-linked	SRPX	chrXp21.1
223939_at	Hs.279575	Succinate receptor 1	SUCNR1	chr3q24-q25.1
222116_s_at	Hs.369819	TBC1 domain family, member 16	TBC1D16	chr17q25.3
204043_at	Hs.417948	Transcobalamin II; macrocytic anemia	TCN2	chr22q12.2
209676_at	Hs.516578	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	TFPI	chr2q32
223594_at	Hs.444668	Transmembrane protein 117	TMEM117	chr12q12
219410_at	Hs.658956	Transmembrane protein 45A	TMEM45A	chr3q12.2
205194_at	Hs.512656	Phosphoserine phosphatase	PSPH	chr7p15.2-p15.1

*Verified by RT-qPCR or discussed in the text.

any other cell type examined, indicating that the genetic profile of eEPCs is closer to that of MDMs.

We compared gene expression profiles between eEPCs and MDMs, the two most similar cell types. A total of 290 signature probe sets were found to be differentially expressed between MDMs and eEPCs, with 199 probe sets being unique to eEPCs (fold change > 2, top 50 eEPC genes in Table 1) (full gene lists can be viewed in Supplementary Table 1, available at <https://docs.google.com/file/d/0B3m2MizcxKoDcEsxaVIOsZlGc1k/edit?usp=sharing>). A gene expression heat map for these genes presents the unique expression patterns of each cell type (Fig. 3A). The differential expression pattern of CD68, ALOX15B (arachidonate 15-lipoxygenase, type B), and Lyz (lysozyme) by eEPCs was verified by qPCR (Fig. 3B). Among the eEPC-enriched genes, CXCL12 [chemokine (C-X-C motif) ligand 12, also known as stromal cell-derived factor 1 (SDF-1)] (Table 1, indicated by an asterisk) plays an important role in angiogenesis by recruiting EPCs from the bone marrow through a CXCR4-dependent mechanism (24). The abundant expression of CXCL12 by eEPCs, but not MDMs, reflects the concept that eEPCs exert their angiogenic effects via a paracrine mechanism rather than by being incorporated into blood vessels directly (9,10). Consistently, CCL8, CCL18, and MMP1 (matrix metalloproteinase 1/interstitial collagenase) were

also found to be abundantly expressed in eEPCs (Table 1) (also Supplementary Table 1 available at <https://docs.google.com/file/d/0B3m2MizcxKoDcEsxaVIOsZlGc1k/edit?usp=sharing>).

Functional Module Analysis as a Framework for the Interpretation of Early EPC Biology

The gene list outlined above gave us preliminary insights into the functional consequences of differential gene expression between the two types of EPCs. To understand more about how the gene expression profiles might be correlated functionally as well as to provide quantitative evidence, the signature mRNAs were subjected to a GO database search in order to pinpoint statistically overrepresented functional groups within the gene lists. The DAVID bioinformatics web tool (see Materials and Methods for details) was applied to provide the statistical analysis and to visually present the results. The GO biological process categories that were statistically overrepresented ($p < 0.05$) among genes that formed the eEPC group (compared with those from MDMs) are shown in Table 2. Genes CTTNBP2, APOE, and KCNMA1, which are involved in vasodilation, were significantly overexpressed in eEPCs ($p = 0.0197$) (Table 2, indicated by an asterisk). Another significant biological process associated with eEPCs was found to be cell proliferation (nine genes

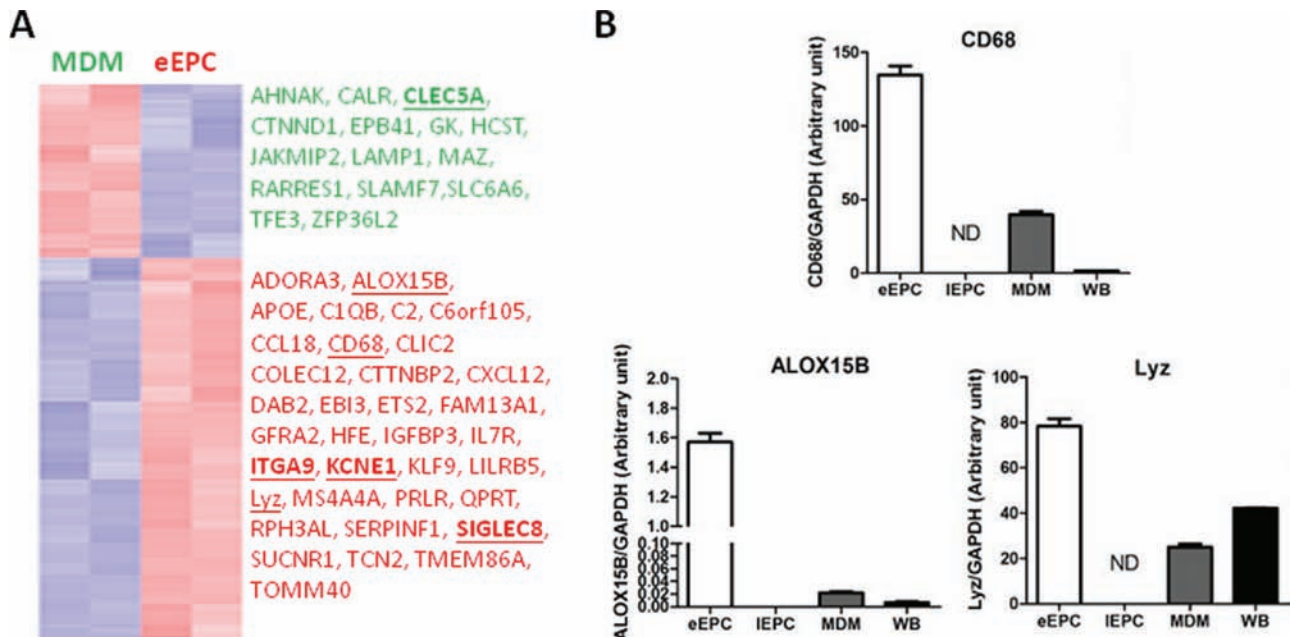


Figure 3. Genes unique in eEPCs and MDMs. (A) Heat map showing differentially expressed genes in eEPCs or MDMs. Columns represent human tissue and stem cell samples, and rows represent probe sets. Genes in red: increased expression; in blue: decreased expression. Genes picked for RT-qPCR in (B) (underlined) and Figure 4 (underlined and in bold) are also shown. (B) Validation of array data by RT-qPCR. Mean gene expression levels of eEPC proteins (compared to GAPDH control) are shown. Results are expressed as the mean \pm SD.

Table 2. Altered Functional Modules in Early EPCs (Compared With MDMs)

Biological Process	Count	%	<i>p</i>	Genes
Vasodilation*	3	1.91	0.0197	CTTNBP2, APOE, KCNMA1
T-cell activation	5	3.18	0.0212	CXCL12, CCND3, FLT3, IL7R, PRLR
Transition metal ion transport*	4	2.55	0.0215	HFE, SLC30A4, SLC40A1, TCN2
Cell proliferation*	9	5.73	0.0296	CXCL12, CSGALNACT1, CCND3, DAB2, IGFBP4, PSPH, IL7R, SERPINF1, TXN
Cation homeostasis*	7	4.46	0.0327	ATP6V0E1, CXCL12, HFE, APOE, KCNMA1, SLC30A4, SLC40A1
Oxidation reduction*	11	7.01	0.0410	BDH2, ALDH18A1, ALDH18A1, CYB5A, ALOX15B, DHRS3, ENOX2, MAOA, TXN, UQCRB
Cellular homeostasis	9	5.73	0.0413	ATP6V0E1, CXCL12, HFE, APOE, PRDX4, KCNMA1, KCNE1, SLC40A1, TXN
Lymphocyte proliferation	3	1.91	0.0479	CXCL12, CCND3, IL7R
Homeostatic process	12	7.64	0.0491	ATP6V0E1, CXCL12, HFE, APOE, IL7R, PRDX4, RPH3AL, SLC30A4, SLC40A1, TXN, KCNMA1, KCNE1

*Discussed in the text.

including CXCL12, CSGALNACT1, CCND3, DAB2, IGFBP4, PSPH, IL7R, SERPINF1, and TXN) ($p=0.0296$) (Table 2, indicated by an asterisk). Other predominant processes found to be associated with the eEPC group include genes involved in cation homeostasis and/or transportation (ATP6V0E1, CXCL12, HFE, APOE, KCNMA1, SLC30A4, SLC40A1, and HFE) and oxidation reduction (BDH2, ALDH18A1, ALDH18A1, CYB5A, ALOX15B, DHRS3, ENOX2, MAOA, TXN, and UQCRB) (Table 2). In contrast, the principal functions associated with MDMs (compared with eEPCs) included those related to cortical actin cytoskeleton organization [calreticulin (CALR), formin-like 1 (FMNL1), and erythrocyte membrane protein band 4.1 (EPB41)] ($p=0.0122$) (Table 3, indicated by an asterisk), which reflect the active cell migratory ability of macrophages.

Novel Surface Biomarkers That Distinguish eEPCs From Monocytes/MDMs

One critical and practical question after gene pattern analysis is how we can make it easier to purify eEPCs without monocyte and/or MDM contamination. In order to pinpoint novel surface antigens that are able to distinguish eEPCs from monocyte/MDMs, we searched for target membrane proteins that might act as novel surface markers when separating eEPCs and immune cells during their purification and isolation. The expression of CLEC5A [C-type lectin domain family 5, member A; also known as myeloid DAP12-associating lectin (MDL-1)], which contains a C-type lectin-like fold similar to the natural killer T-cell C-type lectin domains and associates with a 12-kDa DNAX-activating protein (DAP12) on myeloid cells (25), was found to be unique to MDM

Table 3. Altered Functional Modules in MDMs (Compared With Early EPCs)

Biological Process	Count	%	<i>p</i>
Negative regulation of gene expression	8	10.96	0.0033
Response to organic substance	9	12.33	0.0066
Negative regulation of macromolecule metabolic process	9	12.33	0.0073
Negative regulation of transcription	7	9.590	0.0088
Cortical actin cytoskeleton organization*	3	3.371	0.0122
Negative regulation of nucleobase and nucleic acid metabolic process	7	9.590	0.0145
Negative regulation of nitrogen compound metabolic process	7	9.589	0.0154
Negative regulation of macromolecule biosynthetic process	7	9.589	0.0195
Negative regulation of cellular biosynthetic process	7	9.589	0.0218
Negative regulation of biosynthetic process	7	9.589	0.0239
Cell proliferation	6	8.219	0.0275
Blood circulation	4	5.479	0.0363
Steroid metabolic process	4	5.479	0.0446
Response to inorganic substance	4	5.479	0.0462
Posttranscriptional regulation of gene expression	4	5.479	0.0496
Positive regulation of TGFB receptor signaling pathway	2	2.741	0.0498

*Discussed in the text.

and whole-blood cells (Fig. 4A). On the other hand, 3 of the top 50 eEPC genes, including ITGA9 (integrin, alpha 9), KCNE1 (potassium voltage-gated channel, Isk-related family, member 1), and in particular SIGLEC8 (sialic acid binding Ig-like lectin 8), were found to be uniquely expressed in eEPCs (Fig. 4B, C).

DISCUSSION

EPCs play an important role in postnatal vascular repair and the maintenance of vascular homeostasis because they are involved in reendothelialization and neovascularization. CD34, KDR, and CD133⁺ are considered to be the critical markers associated with isolating circulating EPCs, which need to be different from those of hematopoietic progenitors or leukocytes. Combinations of markers, including CD133⁺CD34⁺KDR⁺, CD34⁺KDR⁺, or CD14⁺CD34^{low}, have been widely used to define or select cells that express the properties attributed to EPCs (26,27). These approaches, however, are unable to distinguish eEPCs from the late EPCs. The lack of known surface biomarkers for the differentiation of the two categories of EPCs and the absence of a standardized protocol in terms of reagents and gating strategy may account for

the widespread interlaboratory variations when quantifying EPCs. In our previous study, we have identified, also by mRNA profiling of various novel surface markers, how to distinguish early and late EPCs (8). Among endothelial lineage cells, CD204, CD169, GPNMB, and many other membrane proteins are uniquely expressed on eEPCs, whereas genes such as CXADR, OSAP, and CD106 are uniquely expressed on late EPCs (8). In the present study, new findings provide another group of surface genes that should help to separate circulating eEPCs from other blood cells that closely resemble them. It should now be possible to combine these studies and use these new biomarkers in parallel with CD34 and KDR for the direct isolation and counting of early and late EPCs from cord blood and peripheral blood. The latter point is of clinical importance because the number of EPCs in peripheral blood has been found to correlate with disease prognosis in a number of studies (3–5).

Nevertheless, there are still challenges to the harnessing of EPCs from blood for direct cell therapy. One of these is their rarity (0.01–0.02 per 10⁶ MNCs), which makes the isolation of EPCs challenging. Optimization of the cultivation and amplification of EPCs is therefore

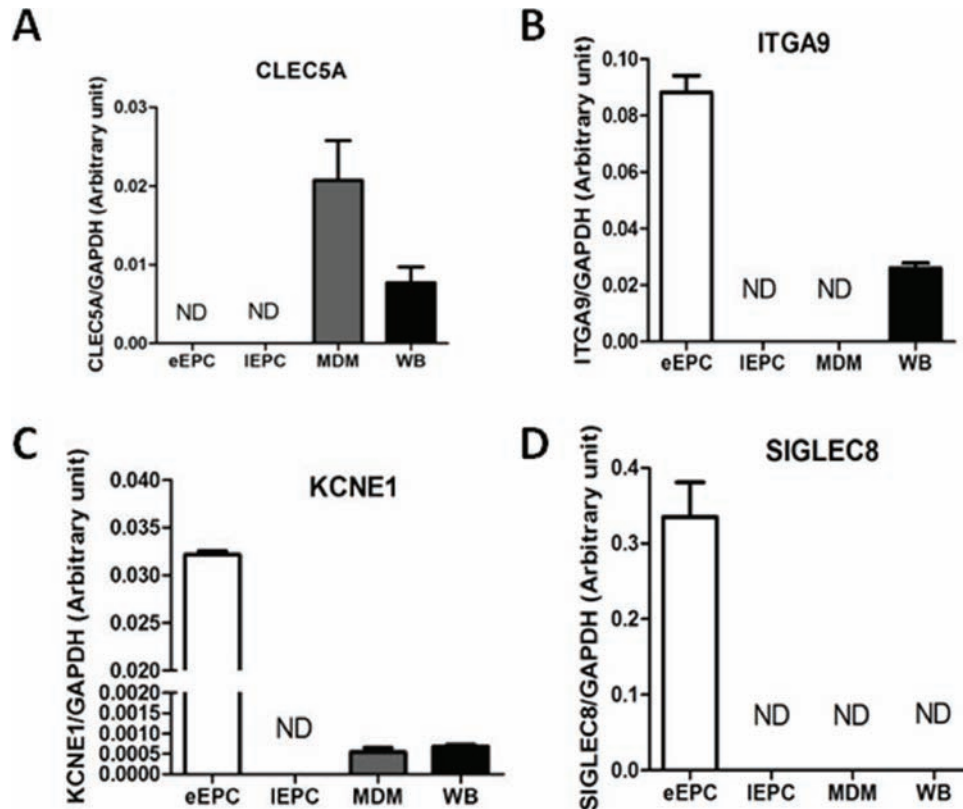


Figure 4. Cell membrane proteins specifically expressed in eEPCs or MDMs. These four genes were selected according to the “Cellular Component” ontology in the GO database. Mean gene expression levels of eEPC proteins (compared to GAPDH control) by RT-qPCR are shown. Results are expressed as the mean \pm SD.

required before these cells may be appropriately investigated to determine their use in clinical therapies. It has been shown that eEPCs do not proliferate significantly in vitro (28). The use of these cells is also handicapped by the difficulties associated with separating eEPCs, which are normally regarded as the cells that appear early (<1 week) in culture dishes, from adherent blood cells such as monocytes and macrophages. Finding surface markers distinguishing eEPCs and adherent blood cells (especially monocytes and MDMs) (Fig. 4) should help with the purification of cultured eEPC for use in cell-based therapy. Contamination due to monocytes and macrophages in transplanted eEPCs may cause graft-versus-host disease (GVHD), a common complication of allogeneic cell transplantation. The presence of these immune cells in the cell graft may recognize the recipient (the host) as “foreign” and then attack the host’s body cells.

Early EPCs have long been recognized to have molecular phenotypes and surface markers linked to hematopoietic cells (10,11,13), yet most comparative studies have focused on the relationships between eEPCs and monocytes. Specifically, proteomic analysis has revealed that 90% of spots identified by 2D gel analysis are common between OECs and endothelial cells, whereas eEPCs share 77% spot identity with monocytes (9). In this study, we show that MDMs rather than monocytes share the closest relationship to eEPCs (Fig. 2B). Owing to the inflammatory nature of macrophages, eEPCs should be used clinically with caution and care taken with respect to the disease they are used to treat. If injected into a proinflammatory tissue microenvironment such as ischemic cardiovascular disease, it is possible they might intensify the pre-existing pathology because of their inflammatory potential. Late EPCs, on the other hand, should be a better source for cell-based transplantation and therapy that involves neoangiogenesis because these cells have greater similarity to mature endothelial cells and distant in terms of gene expression from immune cells or fibroblasts (Fig. 2A).

GO analysis and qPCR validation work revealed the functional significance of our gene list analysis and in the process identified the functional variations between eEPCs and MDMs. For example, CLEC5A, which is critical to inflammation and autoimmune diseases such as arthritis as well as being involved in the activation of myeloid cells (25,29), is unique to MDM and whole-blood cells (Fig. 4A). CLEC5A/MDL-1 also serves as a receptor for several viruses that infect macrophages and induce lethal inflammation responses. Specifically, in human macrophages, CLEC5A interacts with the dengue virion (DV) directly and is responsible for dengue virus-induced lethal disease and inflammasome activation (30,31). Blockade of CLEC5A-DV’s interactions suppresses the secretion of proinflammatory cytokines. Moreover, anti-CLEC5A monoclonal antibodies inhibit

DV-induced plasma leakage as well as subcutaneous and vital organ hemorrhaging; these result in a reduction in the mortality of DV-infected mice (30). CLEC5A also regulates Japanese encephalitis virus (JEV)-induced neuroinflammation and lethality (32). The unique expression of CLEC5A in MDMs (Fig. 4A) indicates that macrophages, but not eEPCs, serve as a natural host for DV or JEV and are involved in viral pathogenesis. This emphasizes that eEPCs and MDMs are actually two distinct cell populations with different physiological and pathological roles. eEPCs seem to be able to contribute more to the area of angiogenesis because they express more genes related to vasodilation, including CTTNBP2, APOE, and KCNMA1 (Table 2). MDMs, on the other hand, are more involved in inflammatory responses. In addition, macrophages may possess better motility than eEPCs because genes involved in cortical actin cytoskeleton organization are more abundant in MDMs (Table 3); this may reflect the host defense nature of this immune cell population.

In summary, our results, which combine mRNA profiling and gene set analysis, help to decipher the RNA expression situation in eEPCs compared to various types of blood cells that closely resemble them, especially monocytes and MDMs. Although new research directions and hypotheses can be formed based on the gene expression data provided by this study, careful functional studies of the genes in context using in vitro and in vivo models of angiogenesis are still necessary in order to further support the clinical relevance of the data set. We envision that our report will serve as a resource for future studies that are aimed at improving our understanding of the various regulatory mechanisms that ultimately modulate EPC and EC activities.

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