Single-Cell Transcriptomic Profiling and Characterization of Endothelial Progenitor Cells: New Approach for Finding Novel Markers

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Abstract

Background: Endothelial progenitor cells (EPCs) are promising candidates for the cellular therapy of peripheral arterial & cardiovascular diseases. However, hitherto there is no specific marker(s) defining precisely EPCs. Herein, we are proposing a new in-silico approach for finding novel EPCs markers.

Methods: we assembled five groups of chosen EPCs-related genes/factors using Pubmed literature & gene ontology databases. This shortened database of EPCs factors was fed into publically-published transcriptome matrix to compare their expression between endothelial colony-forming cells (ECFCs), HUVECs and two adult endothelial cell types (ECs) from skin and adipose tissue. Further, the database was used for functional enrichment on mouse phenotype database and protein-protein interaction network analyses. Moreover, we built a digital matrix of healthy donors' PBMCs (33 thousand single cell transcriptomes) and analyzed the expression of these EPCs factors

Results: Transcriptome analyses showed that BMP2,4 & ephrinB2 were exclusively highly expressed in EPCs; the expression of neuropilin-1 & VEGF-C were significantly higher in EPCs & HUVECs compared with other ECs; Notch 1 was highly expressed in EPCs & skin-ECs; MIR21 was highly expressed in skin-ECs; PECAM-1 were significantly higher in EPCs & adipose ECs. Moreover, functional enrichment of EPCs-related genes on mouse phenotype and STRING protein database has revealed significant relations between chosen EPCs factors and endothelial & vascular functions, development and morphogenesis, where ephrinB2, BMP2 and BMP4 were highly expressed in EPCs and were connected to abnormal vascular functions. Single cell RNA-sequencing analyses has revealed that among the EPCs regulated markers in transcriptome analyses: i-ICAM1 & Endoglin were weekly expressed in the monocyte compartment of peripheral blood; ii-CD163 & CD36 were highly expressed in CD14+ monocyte compartment whereas CSF1R was highly expressed in CD16+ monocyte compartment; iii-L-selectin & IL6R were globally expressed in the lymphoid/myeloid compartments; iv-interestingly, PLAUR/UPAR & NOTCH2 were highly expressed in both CD14+ & CD16+ monocytic compartments.

Conclusions: The current study has identified novel EPCs markers that could be used for better characterization of EPCs sub-population in adult peripheral blood and subsequent usage of EPCs for various cell therapy and regenerative medicine applications.

Background

Endothelial progenitor cells (EPCs) are heterogeneous population of mononuclear cells (MNCs) that originate and reside in the bone marrow (BM), they are circulating in (mobilized to) adult peripheral (PB) or umbilical cord blood (UCB) [1]. EPCs have been discovered by Asahara and his coworkers in 1997 [2]. They express endothelial antigens like CD31, von Willebrand factor (vWF), endothelial nitric oxide synthase (eNOS), VE-cadherin and VEGFR2 [3, 4]. EPCs constitute 1-5% of the total BM cells and >0.0001−0.01% of PB circulating MNCs [5]. They are implicated in homeostasis, neovascularization, vascular repair, endothelial regeneration, and in angiogenesis processes [6]. There are two distinct

subpopulations of EPCs. Early EPCs which give rise to heterogeneous colonies that appear in culture after 3-5 days, they are obtained by negative selection on fibronectin, they are round cells surrounded by spindle-shaped cells in morphology, they have a slow-proliferation and their in vitro growth peak is reached after 2-3 weeks [7-10]. Moreover, early EPCs do not form vascular tubes in vitro but they have a strong paracrine activity (secrete a plethora of angiogenic factors) that contributes effectively to neovascularization [11,12], they have high expression of both hematopoietic and endothelial markers (VEGFR-2, CD31, vWf, able to uptake acLDL and bind UEA-1) [13,14], they are most likely derived from hematopoietic stem cells and had resemblance to myeloid progenitors [15], and hence they are also named "Hematopoietic EPCs" [16]. Early EPCs generate the endothelial cell colony forming units (CFU-ECs) in vitro [8, 17]. Interestingly, early EPCs [18] are also termed circulating angiogenic cells (CACs) [19]. On the other hand, the other subtype of EPCs is termed "late EPCs" [18], they are more homogenous colonies that appear after 2-4 weeks in culture, they are isolated by positive selection on collagen I, they are elongated cells that form a cobblestone-morphology monolayer in vitro which is characteristic of endothelial cells, they could be maintained in culture for ~12 weeks (up to 15 passages) and they have higher proliferative & clonogenic potential compared with early EPCs [12, 17, 20]. Moreover, late EPCs could easily form tubular/capillary-like structures in vitro, they possess high vasculogenic & angiogenic potential and in vivo they could incorporate in the existing endothelium where they form stable vessels and continue to differentiate into mature endothelial cells [17,21,22]. Noteworthy, late EPCs are phenotypically similar to mature endothelium, they are present/circulate in both PB and UCB, importantly they are not only closer to endothelium phenotypically but also by exhibiting no hematopoietic (CD45) or monocyte markers (CD14 & CD115) expression in contrast to early EPCs, whereas they express many endothelial cells (ECs) antigens (CD31, VEGFR-2, CD105, CD144, CD146, vWf, CD34, higher eNOS, Tie-2, VE-cadherin, able to uptake acLDL and bind UEA-1) [22,23]. Collectively, late EPCs are termed "Non-hematopoietic EPCs" [16,24] and thus they are considered the "EPCs" subtype that comply the most with the original endothelial phenotype and function to be the legitimate endothelial progenitor cells bearing almost all of the endothelial cells characteristics [15]. Further, late EPCs generate in vitro "endothelial colony forming cells or ECFCs" [25] and they are also called "outgrowth endothelial cells or OECs"[20, 26].

There were a number of proposed combinations of surface anntigens for identifying EPCs in human, this include (but not restricted to): CD34+, CD31+, CD133+, VEGFR2+, CD144+, CD146+, CD45-/+, CD14+, VEGFR1+, FGFR1+ [16, 24, 27].

The vast variation in the surface antigens for EPCs is possibly attributed to identifying different EPCs' subpopulations at various matuartion/differentiation phases. The term "EPCs" has been haphazardly used to refer to both circulating (late EPCs) and cultured cells (ECFCs). In addition, the accumulating literature did not provide one consolidated definition of EPCs nor a specific EPCs phenotype or a unified isolation & culture protocol of them. Accordingly, different isolation techniques and culturing methods applied resulted in EPCs with various phenotypes [28]. Therefore, we aimed herein using in silico data to reach a possible novel EPCs marker or a combination of markers that could specifically characterize EPCs.

In the current manuscript, we are adding to the already ongoing efforts for the characterization analyses of EPCs by presenting a new approach for finding novel marker(s) of EPCs in peripheral blood.

Among the up-to-date "-omics", "gene-expression profiling" or "transcriptomics", is currently the most widely used tool for the characterization and functional analysis of cells, moreover, transcriptomics has provided a better understanding for EPCs' characterization analyses in an unbiased manner [28].

Large genomic data from large tissue sample collections are difficult to analyze; however if we use the individual transcriptomic data coming from the tissue-representing or "single-cell" level, this would render mass analysis of bulk single-cell(s) data to be fast, non-tedious [29, 30] and thus would introduce new insights about the ontogeny of new and rare cell types and the relation-ships between various cell lineages [31]. Collectively, single cell transcriptomics would help herein to improve our knowledge for the identification and characterization of EPCs in peripheral blood.

Using Gene ontology and literature survey, we assembled five groups of EPCs' molecules/factors/markers that have been specifically chosen for being of special interest and importance to the EPCs biology.

The categorization and choice of various factors were based on grouping different molecules/factors into groups involved in similar EPCs and ECs functions. The first group is involved in developmental angiogenesis, tumor angiogenesis and vascular development, this group comprises neuropilins (NRP1 & NRP2), semaphorins (3A, 3B, 3D, 3E, 3F, 4A, 4D, 5A & 6A), and VEGFR1, 2 &3 [32-35]. The second group is implicated in ECs/EPCs-immune cells interaction, proliferation, migration, survival, apoptosis, angiogenesis, immunogenicity and immune-modulation. It includes TNF-α, TNFR2/P75, TNFR1/P55 and TRAIL (Tumor Necrosis Factor Related Apoptosis Inducing Ligand) [36-40]. The third group of factors is engaged in proliferation, survival, migration and differentiation of vascular stem/progenitor cells which includes closely-related cells co-enhabiting the vascular niche; namely they are EPCs, smooth muscle cells (SMCs), pericytes & mesenchymal stem cells (MSCs). The representing candidates of this group were PDGF- (A, B & C), BMP (2,4 & 9), Wnt (1, 4, 11 & 5A), VEGF (A & C), TGF β, FGF2, IFG-1 and EGF [41-42]. Group 4 comprises microRNAs which are small, non-coding, single-stranded RNAs with regulatory activities. Recent studies showed that microRNAs play an important role in regulating EPC functions which includes proliferation, senecence, apoptosis & autophagy, mobilization & migration, tube formation & angiogenic capacity and differentiation. We have chosen representative microRNAs that could be involved in one or more biological process; the chosen candidates were microRNA-221/222, 34a, 126, 16,107, 150, 22, 21 & 130 a [43-45]. The fifth group is involved in the internalization (of ligands from the extracellular matrix to be recycled back to the endosomal compartment), endocytosis, migratory and/or invasive capacity and motility. It comprises urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor(uPAR), urokinase plasminogen activator receptor associated protein(uPARAP), tissue-type plasminogen activator (tPA), Neuropilin-1 NRP1, Neuropilin-2 NRP2, VEGFR1, 2 & 3, PECAM-1, ICAM-1, VE-cadherin , Ephrin-B2, EphB4 and EGFL7 [46-57].

Herein, our main objective is to search for novel markers of EPCs in peripheral blood. Thus, we have created a short list divided into five groups of EPCs factors/molecules using pubmed literature, gene

ontology, and other sources. This list was used for both the transcriptomic and single cell analyses. In transcriptome analyses, the list was used to compare the relative expression of various EPCs genes (involved within this list) between ECFCs, HUVECs and two adult ECs from skin and adipose tissue. Moreover, EPCs chosen-genes were used for functional enrichment on mouse phenotype and STRING protein-protein interaction network database to decipher the involvement of these factors in endothelial and vascular development & morphogenesis. Additionally, we built a digital matrix of healthy donors' PBMCs (33 thousand transcriptomes) and analyzed the expression of the short list of EPCs factors and more specifically EPCs molecules that have shown to be significantly regulated between ECFCs and the other three adult ECs in the transcriptome analyses.

The current study has identified novel markers, which include secreted factors, miRNAs and growth factors. Among these markers we have analyzed, some of them could be used for better cytometric analyses and an optimized characterization of EPCs sub-population in peripheral blood.

Materials And Methods

Semantic search for chosen factors implicated in recent endothelial progenitor cells biology field

Using gene ontology, a vast array of EPCs' physiology/pathophysiology-related published research and literature, and Pubmed databases were used in the current work. This was followed by the selection & categorization of different factors (affecting various signaling cascades, molecular functions, and biological processes of EPCs) into five main groups of molecules/factors using a combination of keywords in the field of the EPCs-biology. The five molecular sets were described in table 1 with their related employed keywords. We have chosen sixty-one factors distributed as follows: Group 1 (purple; 14 molecules), group 2 (green; 4 molecules), group 3 (red; 19 molecules), group 4 (blue; 9 molecules) and group 5 (brown; 15 molecules) as shown in table 1.

Table 1: Table comprising semantic determination of molecule sets related to EPCs/ECs biology: Sixty-one factors distributed as follows: Group 1 (purple; 14 molecules), group 2 (green; 4 molecules), group 3 (red; 19 molecules), group 4 (blue; 9 molecules) and group 5 (brown; 15 molecules). The keywords used for each group of molecules are slightly changed between the groups depending on the biological functions that various molecules/factors are incorporated in. It has to be noted that VEGFR1, 2 and 3 were repeated in group 1 & 5 as they are differently involved in the general molecular functions of each group.

Chosen EPCs-related Factors	Group number and Keywords
Semaphorin 3A, Semaphorin 3B, Semaphorin 4A, Semaphorin 5A, Semaphorin 6A, Semaphorin 4D, Semaphorin 3D, Semaphorin 3^E, Semaphorin 3F, Neuropilin-1 (NRP1), Neuropilin-2 (NRP2), VEGFR1, VEFGR2, VEFGR3	**Group 1** Keywords: developmental angiogenesis, tumor angiogenesis and vascular development
TNF-α, TNFR2/P75, TNFR1/P55, TRAIL (Tumor Necrosis Factor Related Apoptosis Inducing Ligand)	**Group 2** Keywords: proliferation, migration, survival, apoptosis, angiogenesis, Immunogenicity, immune-modulation.
PDGF-A, PDGF-B, PDGF-C, BMP9, BMP2, BMP4, Wnt1, Wnt11, Wnt5A, Wnt4, VEGF-C, VEGF-A, TFG β, Notch , Hedghog, FGF-2, IGF-1, EGF, Alk1/TgfβR2	**Group 3** Keywords: proliferation, survival, differentiation, vascular stem/progenitor cells
miRNA-126, miRNA-34a, miRNA-16, miRNA-107, miR-150, miR-22, miR-21, miR-130α, miR-221/miR-222	**Group 4** Keywords: proliferation, senecence, apoptosis, autophagy, mobilization, migration, tube formation, angiogenic capacity, differentiation
Urokinase plasminogen activator (uPA), Urokinase plasminogen activator Receptor(uPAR), Urokinase plasminogen activator Receptor associated protein(uPARAP), Platelet endothelial cell adhesion molecule-1 (PECAM-1), Intercellular adhesion molecule-1 (ICAM-1), tissue-type plasminogen activator (tPA), Neuropilin-1 (NRP1), Neuropilin-2 (NRP2), VEGFR1, VEFGR2, VEFGR3, VE-cadherin, Ephrin-B2, EphB4, EGFL7 (Epidermal growth factor-like protein 7)	**Group 5** Keywords: internalization, endocytosis, migratoryability, invasive capacity, motility

Public datasets

ECFCs and mature ECs have been already studied by whole transcriptome analysis through Gene Omnibus Expression dataset from the series GSE55695 [58]. In these experiments, ECFCs of the peripheral blood (ECFC-PB) were compared to different kinds of endothelial cells: adipose tissue-derived endothelial cells (EC-ADIPO), dermal microvascular endothelial cells (EC-skin) and human umbilical vein endothelial cells (HUVECs). Expression matrix normalized by quantile normalization method was downloaded at the following web address: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55695. In a second step, normalized matrix was annotated with the corresponding GEO plateform GPL10558 used for microarray technology: Illumina HumanHT-12 V4.0 expression beadchip.

Transcriptome analyses

Bioinformatics analyses were performed in R software environment version 3.4.1. Unsupervised principal component analysis was performed with FactoMineR R-package [59]. Molecules names from previously described semantic research in topics of endothelial cells/EPCs (see table 1) were converted in official human gene symbol with HUGO database from HUGO Gene Nomenclature Committee (HGNC consortium) [60]. Expression heatmap was performed with R-package made4 by using unsupervised classification with Euclidean distances [61]. Most variable genes between the transcriptome of the four experimental groups (ECFC-PB, EC-ADIPO, EC-skin and HUVECs) were defined by performing Fisher one way ANOVA (Analysis of Variance) with implementation of 500 permutations in order to perform multi-testing corrections on p-values with False Discovery Rate method in genomic suite Mev version 4.9.0 [62]. Functional enrichment on Mouse Phenotype database was performed with ToppGene software

suite [63]. Functional enrichment network was performed with Cytoscape standalone software version 3.6.0 [64].

Single cell RNA-sequencing analyses

Transcriptome of 33000 healthy donors' peripheral blood mononuclear cells (PBMCs) which were found publically available (10X genomics, https://www.10xgenomics.com/solutions/single-cell/), were analyzed to assess the expression of the chosen EPCs-related markers in peripheral blood as shown in Table 2. Sequencing reads were analyzed with demultiplexing solution: Cell Ranger version 1.1.0. Seurat algorithm version 2.3.0 [65] was used in R software environment version 3.4.3 to build a digital matrix of the transcriptomes and subsequent clustering by combining principal component analysis and tSNE (t-distribution stochastic neighbor embedding) mathematical reductions in order to project the quantification of the studied endothelial markers.

Table 2

Most significant EPCs related genes found by ANOVA between ECFCs and other three types of endothelial cells: most variable EPCs related genes found to be significant by ANOVA between ECFCs (in peripheral blood) and three distinct groups of endothelial cells: HUVECs, adipose and skin from transcriptome dataset GSE55695. The table shows gene symbol with their relative Illumina identifier, also ratio obtained from the Fisher statistics and their corresponding corrected p-value was adjusted for the multi-testing errors.

Gene symbol	Description	ID_illumina_DNA_beads	Fisher_F_ratio_ANOVA	Adj. p value
VEGFC	vascular endothelial growth factor C	ILMN_1701204	19.39477	0.001
BMP4	bone morphogenetic protein 4	ILMN_1693749	14.54601	0.018
BMP4	bone morphogenetic protein 4	ILMN_1709734	10.868284	0.004
NOTCH2	notch 2	ILMN_2405297	10.400303	0.018
BMP4	bone morphogenetic protein 4	ILMN_1740900	9.894238	0.008
SEMA3F	semaphorin 3F	ILMN_1761540	8.470199	0.01
PLAUR	plasminogen activator, urokinase receptor	ILMN_2408543	8.119817	0.006
PDGFA	platelet derived growth factor subunit A	ILMN_2342695	7.7437563	0.018
BMP2	bone morphogenetic protein 2	ILMN_1722718	7.219204	0.016
PDGFC	platelet derived growth factor C	ILMN_1683023	7.012548	0.028
SEMA6A	semaphorin 6A	ILMN_1713529	6.958105	0.016
NOTCH4	notch 4	ILMN_1711157	6.7292013	0.014
SEMA3A	semaphorin 3A	ILMN_1765641	6.5665183	0.008
PECAM1	platelet and endothelial cell adhesion molecule 1	ILMN_1689518	6.2686167	0.032
TNF	tumor necrosis factor	ILMN_1728106	5.749965	0.024
NOTCH1	notch 1	ILMN_1729161	5.5108757	0.032
PLAUR	plasminogen activator, urokinase receptor	ILMN_2374340	5.3354907	0.008
MIR21	microRNA 21	ILMN_3310840	5.189098	0.036
MIR34A	microRNA 34a	ILMN_3308455	5.005866	0.016

Gene symbol	Description	ID_illumina_DNA_beads	Fisher_F_ratio_ANOVA	Adj. p value
NRP1	neuropilin 1	ILMN_1742547	4.502312	0.038
EFNB2	ephrin B2	ILMN_1703852	4.091059	0.046
SEMA5A	semaphorin 5A	ILMN_1880012	3.2281258	0.026

Protein-protein interaction network

Molecular identifiers of EPCs selected markers were used to build a protein-protein interaction network with STRING proteomic database [66]. High confident interaction score over 800 was set to select interactions which were validated experimentally. Network Analyst web tool [67] was used to perform functional inference with biological process gene ontology database.

Statistical Analysis

Statistical analysis was performed in R software environment version 3.4.1. Statistical hypothesis between groups were verified by performing Fisher one way Analysis of variance with Tukey post Hoc test. A significance threshold on alpha error $p<0.05$ was defined during these analyses.

An overview of the experimental workflow undertaken in the current work is depicted in figure 5

Results

Specific transcriptome analyses of endothelial colony forming cells (ECFCs) compared with other adult endothelial cells revealed a distinct expression profile implicated in abnormal vascular development

In peripheral blood, ECs are derived from endothelial precursors, which are population of cells called endothelial colony forming cells (ECFCs). In order to investigate the importance of EPCs-affecting molecules/factors in endothelial cells & vascular biology; a semantic research of important chosen molecules/factors was investigated through querying Gene Ontology and Pubmed databases with different keywords (table 1). Merging this database of EPCs chosen molecules with annotated transcriptome normalized matrix allowed reducing dimensions of the matrix to 72 Illumina identifiers (data not shown). On this reduced/minimized expression matrix, a Fisher one way Analysis of Variance (ANOVA) was performed to compare experimental conditions comprising ECFCs from peripheral blood (ECFC-PB) and three adult types of endothelial cells from different tissues: skin (EC-skin), adipose tissue (EC-ADIPO) and HUVECs. This statistical test performed (with 500 hundred permutations and with corrected p-value adjusted for the multi-testing errors, threshold adjust p-value < 0.01) with multi-testing correction identified 19 EPCs-related genes which correspond to 22 unique Illumina identifiers (table 2).

Unsupervised principal component analysis performed with expression of these EPCs-related genes significantly discriminate samples through the different experimental conditions (group discrimination based on the principal component map, p-value = 0.000107, figure 1-A).

Unsupervised classification (clusters of samples with Euclidean distances and complete method, figure 1-B) was performed with these significant EPCs-related genes confirming the stratification of the samples by their experimental conditions.

Significant high levels of expression of BMP2, BMP4 and EFNB2 were found for ECFC-PB compared with the other three ECs (figure 1-B). Moreover, significant high levels of expression of MIR34A, NOTCH4 and SEMA3F were found for EC-ADIPO compared with other groups (figure 1-B). Further, significant high levels of expression of PDGFA and SEMA3A were found for EC-skin compared with other groups (figure 1-B). The most significant gene found between the four types of cells was VEGF-C ((vascular endothelial growth factor C; p=0.001, table 2) and VEGF-C was found to have a high level of expression specifically in HUVECs (figure 1-B).

 Functional enrichment of EPCs-related genes on Mouse Phenotype database allowed finding significant relations between these EPCs-related genes and endothelial functions (table 3). These relations were used to build a functional enrichment network (figure 1-C): EFNB2, BMP2 and BMP4 molecules were found to have a significant high level of expression exclusively in ECFCs (figure 1-B) and after functional enrichment were also found to be connected to several enriched endothelial phenotypes, which includes abnormal arterial morphology, abnormal angiogenesis and also abnormal vascular development (figure 1-C and table 3).

Table 3
Functional enrichment table performed with EPCs related genes on database of mouse phenotype.
Columns respectively describe database employed during the functional enrichment, mouse phenotype identifier with their description, number of genes found to be implicated in the enriched phenotype with respective p-values for each phenotype (p-values of enrichment were obtained with Toppgene application).

Database	Mouse phenotype identifiers	Mouse phenotype description	Number of EPCs-related genes implicated	p-values of enrichment
Mouse Phenotype	MP:0000260	abnormal angiogenesis	8	2.254E-7
Mouse Phenotype	MP:0002191	abnormal artery morphology	8	1.953E-6
Mouse Phenotype	MP:0000259	abnormal vascular development	8	2.402E-6
Mouse Phenotype	MP:0001614	abnormal blood vessel morphology	10	1.440E-5
Mouse Phenotype	MP:0005602	decreased angiogenesis	4	2.439E-5
Mouse Phenotype	MP:0005592	abnormal vascular smooth muscle morphology	4	3.091E-5
Mouse Phenotype	MP:0003227	abnormal vascular branching morphogenesis	3	5.270E-5

Some EPCs-related genes were also found to have a high level of expression shared between ECFCs and other types of endothelial cells. NRP1 (neuropilin 1) was found to share a high level of expression between ECFCs and HUVECs compared with other groups (ANOVA; p-value=0.0125, figure 2-A) and especially compared with EC-skin (ANOVA; p-value = 0.0104, figure 2-A). Moreover, VEGF-C was found to share a high level of expression between ECFCs and HUVECs compared with other groups (ANOVA; p-value=0.00364, figure 2-A) and especially compared with EC-skin. Further, some EPCs-related genes also shared a high level of expression between ECFCs and EC-skin (figure 2-B) which may contribute to cluster ECFCs and EC-skin as near neighbors on the expression heat map (figure 1-B). NOTCH1 shared a significant high level of expression in ECFCs and EC-skin (p-value=0.0073, figure 2-B), more particularly compared with EC-ADIPO (p-value=0.0103, figure 2-B) and also compared with HUVECs (p-value=0.0347, figure 2-B).

MIR21 was also found to have a significant high level of expression in EC-skin and ECFCs compared with other groups (p-value=0.0302, figure 2-B) and more particularly compared with EC-ADIPO (p-value=0.0254, figure 2-B). One molecule PECAM1, platelet and endothelial cell adhesion molecule 1, was found to share a significant high level of expression between ECFCs and EC-ADIPO (p-value=0.00454,

figure 2-C) and more particularly compared with HUVECs (p-value=0.00345, figure 2-C). These results suggest that the EPCs chosen molecules that we highlighted during the transcriptomic analyses between different types of endothelial cells are implicated in vascular development and could have an impact on human endothelial phenotype because they are upregulated in these cells

Peripheral blood mononuclear cells from healthy donors expressed EPCs markers in different sub-compartments characterized by single cell RNA-sequencing

One of the actual challenges to improve the isolation protocols and the yield of isolated EPCs from peripheral blood (PB) is upgrading the characterization of EPCs using specific new markers. In this regard, in order to improve the choice of markers for EPCs sub-population, using publicly available single cell RNA-sequencing experiments, we built a digital matrix of healthy donors PBMCs (33000 single cell transcriptomes) and analyzed the expression of EPCs markers curated from the literature (table1) and more particularly EPCs markers/genes shown to be highly regulated between EPCs/ECFCs and other ECs from different tissues (table 2). Seurat algorithm allowed identifying five major cell populations after tSNE mathematical reduction (figure 3A): CD19+ cells (B lymphocytes), CD3E+ cells (general T lymphoid marker), Granzyme B cells (Natural killer cells and cytotoxic T lymphocytes), CD16+ monocytes and CD14+ monocytes. In peripheral blood, we assessed the molecular expression of endothelial markers like ICAM1 and ENG, which were at low levels in monocyte compartment and more particularly in CD14+ compartment for the ICAM1 expression (figure 3B). Other less endothelial specific markers curated from the literature, confirmed the involvement of PB monocyte compartment as source of ECs/EPCs, principally by the expression of CD163 and CD36 in CD14+ monocyte compartment and also the expression of CSF1R (CD115) in CD16+ monocyte compartment (figure 3C). These results suggest potential implication of EPCs subpopulation in monocytes sub-compartment; thus with the help of the assessed markers, a better understanding of EPCs heterogeneities could be achieved. Some EPCs-genes curated from literature harbored a mixed lympho/myeloid expression in PBMCs, this is the case for SELL (CD62L, selectin L) and IL6R which have a high expression in the lympho/myeloid compartment (figure 3D). The latter two markers with elevated expression in the lympho/myeloid compartment, especially CD62L could be interesting to be used for better EPCs characterization, where they could be used as pre-gating endothelial markers on total population of PBMCs.

Interestingly, among EPCs markers appeared in the transcriptomic analyses (figure 1 and table 2), two of them were found to have a positive expression in PBMCs: PLAUR and NOTCH2 in monocyte compartment (figure 3E) either in CD14+ or in CD16+ compartments, with a higher expression of PLAUR. Thus PLAUR could be also used as EPCs marker.

All these results of single cell RNA-sequencing obtained for EPCs-related markers expressed in PBMCs would be useful to design multi-parametric flow cytometric analyses for optimal & better characterization of EPCs sub-population in peripheral blood.

EPCs markers inferred a molecular network which is implicated in morphogenesis and vascular development

Among the sixty-one EPCs markers selected for the study (table 1), forty two of them were retained as seeds of the network (red nodes on network, figure 4) by STRING protein database with stringent parameters (interaction score over 800 and interaction validated experimentally). Building protein-protein interaction network around these 42 seeds revealed a network comprising a total of 550 nodes with 1086 edges (figure 4). Functional inference on this interaction network with Biological Process (Gene Ontology) database revealed an important involvement of these molecules partners in morphogenesis (figure Network, barplot) and also their implication in vascular development (blue nodes on network and blue bar in the barplot, figure 4 network). These results confirmed that the EPCs-related markers that we have selected for this study could influence morphogenesis and vascular development processes.

Discussion

Since the discovery of endothelial progenitor cells (EPCs) three decades ago; yet there is/are no definitive/globally-agreed upon marker or group of markers for the specific molecular characterization of EPCs. Thus, in the current work, we propose a novel in silico approach for finding novel markers of EPCs. We investigated the importance of sixty-one EPCs-affecting molecules/factors in EPCs & vascular biology; we conducted semantic research of the chosen molecules/factors curated from the literature via querying Gene Ontology and Pubmed databases with different keywords. Merging these database of EPCs markers into publically available annotated transcriptome normalized matrix to compare the expression of these chosen EPCs genes between ECFCs, HUVECs and two adult ECs from skin and adipose tissue, has revealed that BMB2, BMP4 and EFNB2 (Ephrin B2) have significant higher expression compared with other groups. Erythropoietin-producing human hepatocellular carcinoma (ephrin) receptors like Ephrin B2 are expressed by ECs [68] and EPCs [69], and they are important for embryonic angiogenesis, cellular adhesion, and migration [70]. Moreover, preconditioning EPCs with Ephin B2 increases their angionenic capacity in hind limb model [71] and in wound healing [72].

Our transcriptomic analysis has showed that both BMB2 and BMP4 are also upregulated in ECFCs. It has been demonstrated that both BMP2 and BMP4 were exclusively expressed by late EPCs (ECFCs) and they are essential for the angiogenic potential of ECFCs [73].Moreover, BMP4 is implicated in endothelial lineage differentiation of embryonic pluripotent cells [74, 75].

Further, BMP2 could enhance the vasculogenic differentiation of ECFCs co-encapsulated with mesenchymal stromal cells in synthetic scaffold [76]. Interestingly, the same three EPCs molecules were the highest significantly regulated genes in the mouse functional enrichment network. Collectively, this means that EFNB2, BMB2 & BMP4 are crucial for ECFCs commitment to the endothelial lineage and they are involved in the angiogenic capacity of ECFCs.

Some molecules have shown high level of expression between ECFCs and HUVECs, NRP1 shared a high level of expression between ECFCs and HUVECs compared with other groups. NRP1 was proved to orchestrate the committed differentiation of endothelial precursors for both human and murine

embryonic stem cells [77]. Moreover, it regulates the differentiation of murine pluripotent stem cells to vascular progenitor cells [78] and it is in general important for angiogenesis and homeostasis [79].

VEGF-C was also upregulated in both ECFCs and HUVECs, it is the most regulated gene with high level of expression in both HUVECs and ECFCs and it is known to promote lymphatic endothelial cells from human pluripotent stem cells [80]. Moreover, VEGF-C induced the differentiation of lymphatic endothelial progenitor cells (LEPCs) into lymphatic ECs and it also boosted their incorporation in the cardiac lymphatic system and thus VEGF-C stimulated cardiac lymphangiogenesis in a rat model of myocardial infarction [81].

Whereas, the expression of other molecules was elevated in both ECFCs and Skin endothelial cells, this includes NOTCH1 & MIR21. NOTCH1 via downstream action on HES1 influenced switch of hematopoietic versus endothelial fate specification [82]. Further, NOTCH1 regulates the differentiation of mouse embryonic stem cells into arterial ECs and increases the angiogenic potential of them [83]. MIR21 induces EPCs proliferation [84] and it also modulates their senescence [85]. Additionally, MIR21 is known to have a protective effect on vascular ECs [86].

On the other hand PECAM1 has shown shared high level of expression between ECFCs and adipose tissue endothelial cells. PECAM1 is a classical marker of adult ECs so it is not surprising to be upregulated in adipose-derived ECs and it has also been reported to be a maker of ECFCs [17, 27]. Thus, it can be concluded that there was a high level of expression of the chosen factors in ECFCs as compared to others endothelial cells

The functional enrichment of our chosen sixty-one EPCs-related factors on mouse phenotype database has shown the significant involvement of the chosen EPCs factors, specifically EFNB2, BMB2 and BMP4 which have the highest significant upregulation in ECFCs compared with other groups in the transcriptomic analyses, in mouse endothelial phenotypes like abnormal blood vessel morphology (with the highest number of EPCs-related genes involved), followed by abnormal vascular development, abnormal artery morphology and also in decreased angiogenesis (Table 3). Interestingly, the mouse functional enrichment analyses were consistent with the STRING analysis of functional protein-protein interaction networks, which revealed the involvement of 42 out of the chosen molecules as seeds of the network and they were crucial for vascular morphogenesis & vascular development (Figure 4). Collectively, these results clearly prove the prominence of our chosen EPCs-related factors and that they are crucial for endothelial and vascular physiology and pathophysiology.

There are two major types of blood for isolation of EPCs, namely umbilical cord blood (UCB) and peripheral blood (PB). Although, PB is the most available source, however, the number of EPCs and the probability of having EPCs colonies from PB is much lower compared with UCB [5,87]. Thus, herein, our single transcriptomic analyses derived from 33000 single cell transcriptomes of healthy donors PBMCs has revealed that EC markers like ICAM1/CD54 (activated EPCs marker) and ENG (Endolgin/CD105) were still expressed at low levels at the monocytic compartments of PB, although the previous markers are authentic established markers of both ECs and EPCs [17,27].

Further, other EPCs markers like CD163, CD36 and CD115 have been shown to be expressed in the monocytic compartment of PB; namely CD163 and CD36 EPCs in CD14+ monocyte compartment and CSF1R (CD115) in CD16+ monocyte compartment (figure 3C). Noteworthy is that both CD163 [27] & CD115 [17] are considered markers for early EPCs, whereas CD36 [27] is attributed as a late EPCs marker. Hence, this proves the existence/the involvement of EPCs as a subpopulation of the monocytic PB sub-compartment. Collectively the latter EPCs markers could improve the study of EPCs ontogeny and heterogeneities in PB and will also aid (when used with other conventional markers of EPCs) in better characterization, isolation and higher yield of EPCs colonies from PB.

Other less curated EPCs markers from literature have demonstrated high mixed lympho/myeloid expression in PBMCs which is the case of SELL (CD62L, selectin L), it has been demonstrated that CD62L has been expressed by EPCs and it is even used as marker for isolation and characterization of EPCs in combination with CD34 [27].

The same holds true for IL6R which has less expression in lympho/myeloid compartments of PBMC compared with CD62L. Actually, IL6R/CD126/gb80 is an indirect marker of activated ECs/EPCs, as IL6R is not expressed by ECs but it is expressed by neutrophils and monocytes. Moreover, IL6R is proteolytically cleaved forming a complex with IL6 and such complex binds with the gp130 receptor which is expressed ubiquitously on ECs to be activated and then they start expressing ICAM1, VCAM1 and IL6 [88]. We could conclude that the previous two markers with high expression in the lympho/myeloid compartment, especially CD62L could be used as EPCs markers for better characterization and isolation of EPCs from PBMCs population.

Interestingly, the same two EPCs-related genes markers, namely PLAUR and NOTCH2 that have been shown to be highly regulated between EPCs and other ECs from different tissues (figure 1 and table 2), have also been shown in our single cell RNA-sequencing analyses to be highly expressed in PBMC monocyte sup-compartment (figure 3E) either in CD14+ or in CD16+ sup-compartments, where PLAUR has a much higher expression. UPAR/PLAUR/CD87 is the receptor of UPA and both of them in addition to uPARAP form the UPA/UPAR/uPARAP system. This system is involved in the migration, proliferation and adhesion of cells. Moreover, this system is a key orchestrator of angiogenesis besides other cellular processes that include receptor shedding and internalization, protein expression, phenotype modulation and tissue remodeling, cancer progression and metastasis [47, 51, 53-55]. In order for angiogenesis to occur EPCs have to be released from the basement membrane then they migrate to distant regions where there is injury or neovascularization. UPA binds to UPAR on ECs/EPCs surface resulting in formation of plasmin (activation or conversion of plasminogen to plasmin) which activates matrix metalloproteinases (MMPs) like MMP-3 & MMP-12 that in turn cleaves basement membrane releasing EPCs free to migrate and recruited to sites where neovascularization occurs where they differentiate progressively to mature ECs, moreover MMPs also releases growth factors like VEGF, FGF2 and HGF which activate the proliferation of EPCs [89]. Additionally, it has been shown that that EPCs showed higher uPAR levels & uPA activity compared with mature ECs [90]. Adding to this, UPAR is a crucial pro-angiogenic regulator for ECFCs and it is also inducing VEGF activity [91]. Also it has been shown that UPAR-CD36 interaction is

important for the pathogenesis of atherosclerosis [92]. Collectively, UPAR/PLAUR has been proven to be a key player in angiogenesis, vasculogenesis and EPCs function and physiology. To summarize, in the current study, we are introducing a novel set of EPCs markers (which include secreted factors, miRNAs and growth factors),where we would propose novel combination of conventional ECs/EPCs markers (like CD31, VEGFR2 (KDR) and vWF) and novel EPCs markers emerging from the current study, like UPAR/PLAUR and CD36, as plausible panel of markers to be used for EPCs pre-gating on total PBMCs population to design multi-parametric flow cytometric analyses and thus would aid in an improved characterization, isolation and higher yield of EPCs colonies from peripheral blood.

Conclusions

In conclusion, we report a new single-cell transcriptomic in silico approach for delineating a novel characterization panel of novel EPCs markers that would help to design a multi-parametric cytometric analyses for optimal & better characterization of EPCs sub-population in peripheral blood, and thus improving the isolation and yield of EPCs from peripheral blood for subsequent use of EPCs in cell therapy and regenerative medicine applications

List Of Abbreviations

EPCs: endothelial progenitor cells; MNCs: mononuclear cells; BM: bone marrow; PB: peripheral blood; UCB: umbilical cord blood; vWF: von Willebrand factor; eNOS: endothelial nitric oxide synthase; UEA-1: Ulex europaeus agglutinin I; ECFCs: endothelial colony forming cells; uPA: Urokinase plasminogen activator; uPAR: Urokinase plasminogen activator Receptor; uPARAP: Urokinase plasminogen activator Receptor associated protein; PECAM-1:Platelet endothelial cell adhesion molecule-1; ICAM-1: Intercellular adhesion molecule-1;tPA: tissue-type plasminogen activator; NRP1: Neuropilin-1; NRP2: Neuropilin-2; VEGFR1: Vascular endothelial growth factor receptor 1; VEFGR2: Vascular endothelial growth factor receptor 2; VEFGR3: Vascular endothelial growth factor receptor 3; EGFL7: Epidermal growth factor-like protein 7; TNFR1/P55: Tumor necrosis factor receptor type 1 (p55); TNFR2/P75 : Tumor necrosis factor receptor type 2 (p75); TRAIL :Tumor Necrosis Factor Related Apoptosis Inducing Ligand; LEPCs: lymphatic endothelial progenitor cells; PBMCs: peripheral blood mononuclear cells; tSNE: t-distribution stochastic neighbor embedding; HES1: hairy and enhancer of split 1; MPPs: matrix metalloproteinases; HUVEC: human umbilical vein endothelial cells

Declarations

Ethics approval and consent to participate

Not applicable, since the study is an in silico study and there are no patients or animal studies involved, and all the bioinformatics analyses were derived from publicly available data

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Authors' Contributions

MEA identified the scientific problem and conceptualization of the manuscript. MEA and CD assisted in designing the study, performed and analyzed the data, and wrote the manuscript. MEA, CD, GU and SN critically edited, drafted and revised the manuscript. All authors read and approved the final manuscript.

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