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Jessica Laflèche

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

In Vitro Functional Comparison of Different Circulating Progenitor Cell Populations

TITRE DE LA THÈSE / TITLE OF THESIS

M. Ruel

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

Erike Suuronen

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

R. Beanlands

Skerjanc

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

In vitro Functional Comparison of Different Circulating Progenitor Cell Populations

Jessica Laflèche

This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular & Molecular Medicine

June 2010

University of Ottawa

Your file Votre référence
ISBN: 978-0-494-69025-3
Our file Notre référence
ISBN: 978-0-494-69025-3

ABSTRACT

Cell therapy has been shown to improve the process of revascularization in ischemic myocardial tissue; however, the search for a cell population that can achieve optimal vasculogenic and/or angiogenic potential is ongoing. This research compared the *in vitro* functional properties of different circulating progenitor cell (CPC) subpopulations from the peripheral blood and determined whether interactions between subsets of CPCs are involved in mediating their function. Of all populations investigated, a novel population referred to as derived $CD133^+$ cells seemed to exhibit superior functional potential. Furthermore, the migratory ability of the different CPC populations was significantly enhanced in the presence of cytokines/growth factors secreted by cultured monocytes. Lastly, we observed that CPC tubule formation in a pro-angiogenic environment required the support of a mature endothelial cell population. Taken together, these results indicate that specific phenotype and intercellular interactions may be necessary for CPCs to achieve their optimal functional potential for new blood vessel growth.

TABLE OF CONTENTS

ABSTRACT II

TABLE OF CONTENTS III

LIST OF TABLES IV

LIST OF FIGURES V

LIST OF ABBREVIATIONS VI

ACKNOWLEDGMENTS VII

INTRODUCTION 1

MATERIALS AND METHODS 16

RESULTS 25

DISCUSSION 47

REFERENCES 61

APPENDIX A 65

APPENDIX B 66

LIST OF TABLES

Table 1: Hypothesized origin, fate and action of specific cell types attributed to the 'early' endothelial progenitor cells. 10

Table 2: Phenotypic expression of different isolated CPC populations before and after sorting by FACS. 26

Table 3: Growth factors and/or cytokines secreted by CPCs 45

Table 4: Representative comparison of the different isolated CPC populations' abilities in the *in vitro* adhesion, migration and angiogenesis assays. 55

LIST OF FIGURES

Figure 1: Origin and fate of various stem or progenitor cells 3

Figure 2: Representative flow cytometric phenotypic characterization; $CD133^+CD34^+VEGFR\text{-}2^+$ population 27

Figure 3: Representative flow cytometric phenotypic characterization; derived $CD133^+$ population 28

Figure 4: Representative flow cytometric phenotypic characterization; $CD14^+CD34^+CD45^+$ population 29

Figure 5: Microscopic view of fluorescent adherent cells 31

Figure 6: Static adhesion assay on fibronectin 32

Figure 7: Static adhesion assay on collagen type I 33

Figure 8: Static adhesion assay on fibronectin and collagen type I 34

Figure 9: Migration assay using a modified Boyden chamber 36

Figure 10: Representative microscopic view of the *in vitro* angiogenesis assay; CPC alone 39

Figure 11: Representative microscopic view of the *in vitro* angiogenesis assay; CPC in combination with HUVECs. 40

Figure 12: CPC contribution to capillary-like structures in *in vitro* angiogenesis assay. 41

Figure 13: Overall density of capillary-like structures in *in vitro* angiogenesis assay. ... 42

Figure 14: Differentiation of CPCs in the *in vitro* angiogenesis assay 44

Figure 15: Cytokine antibody array 46

Figure 16: Flowcytometric phenotypic characterization 65

Figure 17: Immunomagnetic separation efficiency 66

LIST OF ABBREVIATIONS

Acronym	Definition	Acronym	Definition
Ac-LDL	acetylated-low density lipoprotein	HGF	hepatocyte growth factor
ANOVA	analysis of variance	HIF-1	hypoxia-inducible factor-1
APC	allophycocyanine	HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
autoMACS™	automated magnetically activated cell sorter	HSC	hematopoietic stem cell
BSA	bovine serum albumin	HUVEC	human umbilical vein endothelial cell
CABG	coronary artery bypass grafting	IGF-1	insulin-like growth factor-1
CAC	circulating angiogenic cell	IgG	immunoglobulin G
CAD	coronary artery disease	IL-8	interleukin-8
CD	cluster of differentiation	KDR	kinase insert domain receptor
CMTMR	(5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine)	MCP-1	monocyte chemoattractant protein-1
CPC	circulating progenitor cell	MMP	matrix metalloproteinase
CSF-1R	colony stimulating factor 1 receptor	MOMC	monocyte-derived multipotential cell
CVD	cardiovascular disease	MSC	mesenchymal stem cell
DAPI	4',6-diamidino-2-phenylindole	NO	nitric oxide
DMSO	dimethyl sulfoxide	NRP	neuropilin
EBM	endothelial basal medium	OEC	outgrowth endothelial cell
ECD	energy coupled dye	PBMC	peripheral blood mononuclear cell
ECM	extracellular matrix	PBS	phosphate buffered saline
EDTA	ethylene-diamine-tetra-acetic acid	PC7	phycoerythrin cyanine 7
eNOS	endothelial nitric oxide synthase	PCI	percutaneous coronary interventions
EPC	endothelial progenitor cell	PE	phycoerythrin
ESC	embryonic stem cell	PECAM-1	platelet endothelial cell adhesion molecule-1
FACS	fluorescence-activated cell sorting	PFA	paraformaldehyde
FBS	fetal bovine serum	PlGF	placental growth factor
FITC	fluorescein isothiocyanate	SDF-1	stromal cell-derived factor-1
FOV	field of view	VE-cadherin	vascular endothelial cadherin
GA-1000	gentamicin, amphotericin-B	VEGF	vascular endothelial growth factor
G-CSF	granulocyte colony stimulating factor	VEGFR-2	vascular endothelial growth factor receptor-2
HBSS	Hanks' balanced salt solution	vWF	von Willebrand factor
hEGF	human epidermal growth factor		

ACKNOWLEDGMENTS

I thank my supervisor Dr. Ruel and co-supervisor Dr. Suuronen for their support throughout the years. I would also like to thank the members of my advisory committee, Drs. Skerjanc and Wang, for their guidance. Finally, a special thanks to Suzanne Crowe and Branka Vulesevic, who have provided indispensable help in this study.

This research was funded in part by grants from the Canadian Institutes of Health Research and the Heart & Stroke Foundation of Canada (to Drs Ruel and Suuronen), and by an Endowed Research Chair (to Dr. Ruel).

INTRODUCTION

According to Health Canada, cardiovascular disease (CVD), or heart disease, is the number one killer and the most costly disease in Canada.[1] CVD includes ischemic heart disease, hypertension, stroke, peripheral vascular disease, and others. Ischemic heart disease, also referred as coronary artery disease (CAD), is the most common lethal form of CVD. This condition begins when the endothelium, which is the inner layer of cells of the blood vessel or in this case the coronary artery, is damaged leading to an accumulation of plaque at the site of injury. Plaque consists of macrophages, fatty acids, cholesterol, calcium and other substances that build up between the endothelium layer and the media layer of smooth muscle cells. Over time, the vessel lumen is obstructed and the heart is deprived of oxygenated blood. Consequently, the reduction of oxygen supply and nutrients damages the heart muscle, creates arrhythmias, and/or causes it to function inefficiently. Numerous treatments exist in order to relieve symptoms and treat the cause of CAD. It is possible to palliate CAD with medications[2], such as beta blockers, nitrates, calcium channel antagonists, anti-platelet agents (aspirin and clopidogrel), and lipid lowering medications including statins. Alternatively, procedural treatments are used when medical treatment fails to relieve symptoms, when the patient remains at significant risk of events, and/or when the investigation reveals significant disease in the coronary arteries. Procedural treatments include percutaneous coronary interventions (PCI; including balloon angioplasty and coronary stenting), coronary artery bypass grafting (CABG), automated implantable defibrillators, cardiac resynchronization therapy, ventricular assist devices, heart transplantation and, potentially, cell-based therapy.

Cardiac Cell-Based Therapy

The hope with cell-based therapy is that it will provide a treatment in which stem or progenitor cells are induced to differentiate into specific cell types needed to repair damaged or lost cells or tissues.[3] With respect to CAD, the use of autologous cell-based therapy can be applied to enhance and facilitate the process of revascularization in ischemic myocardial tissue.[4] Revascularization or neovascularization requires the process of angiogenesis and vasculogenesis.[5] These processes are defined as the formation of new blood vessels *de novo* (vasculogenesis) or from preexisting vessels (angiogenesis), and are the key goals of this revascularization strategy. Cell-based cardiac repair, in particular vasculogenesis, has shown promising results in animal studies but has not been therapeutically established in human patients so far, with reports of modest benefits only.[6] Nevertheless, the field has received much attention in the last decade as some data suggested that cell-based therapy could be a promising treatment and, above all, has been evaluated to be feasible and safe (for reviews, please see [7,8]).

Without a doubt, many questions remain regarding the use of cardiac cell-based therapy. For example, the ideal cell population for transplantation and the mechanisms by which these cells can achieve their optimal vasculogenic and/or angiogenic potential remain to be determined. Currently, there is no persuasive evidence reported as to which cell population, or subpopulation, may potentially be more efficacious for stimulating neovascularization of ischemic tissue.[9] So far, many cell types have been tested (but not compared) for cell-based therapies, including bone marrow-derived hematopoietic cells, endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs), resident cardiac progenitor cells, and embryonic stem cells (ESCs) **(figure 1)**.[6]

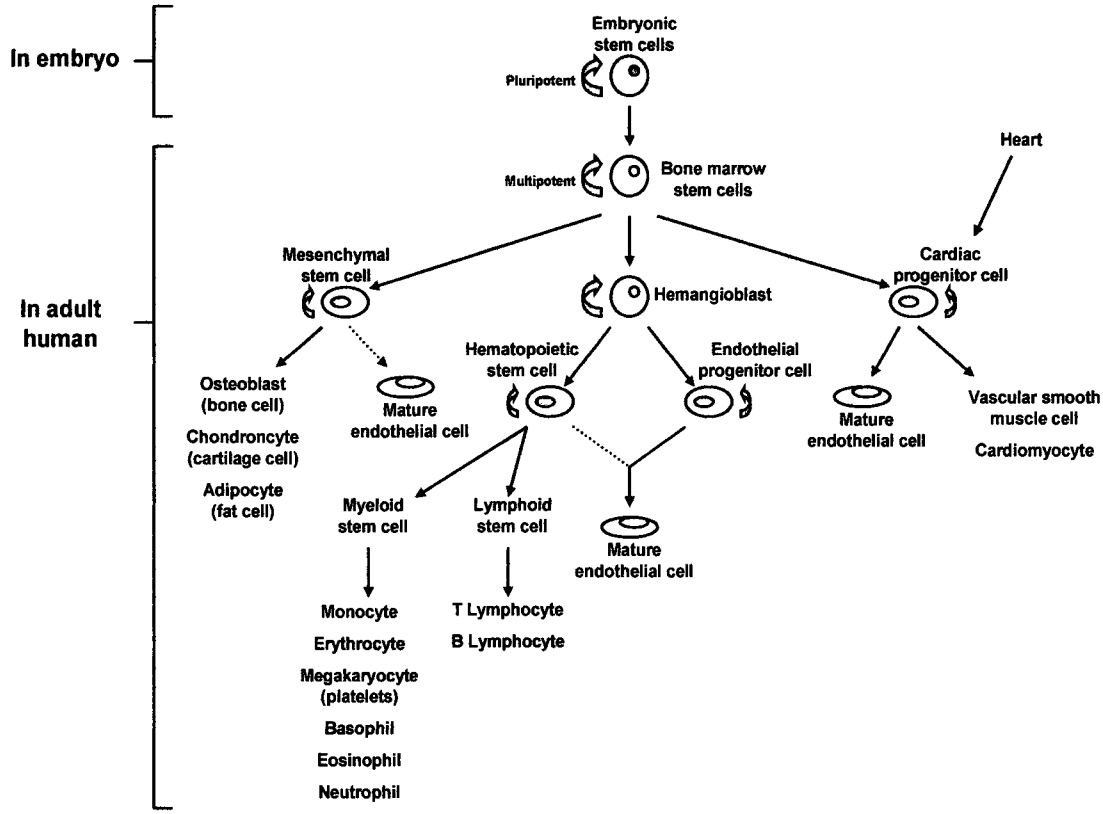


Figure 1: Origin and fate of various stem or progenitor cells. Stem or progenitor cells are theoretically committed to a specific lineage (—), but can be induced to differentiate into other cell types (·····). Various stem or progenitor cells have been found to give rise to endothelial cells.

Endothelial Progenitor Cells

The EPCs, a subset of circulating progenitor cells (CPCs), have been shown in many studies to be a promising population for vasculogenic cell-based therapy (for review, please see [10]). EPCs have the ability to improve the function of ischemic organs by vasculogenesis and/or angiogenesis, in areas where oxygen supply is reduced by stimulating the re-endothelialization of injured vessels.[11,12] EPCs are also thought to play a significant role in the homeostasis of vasculature.[13] Vascular repair is believed to occur, in part, through the migration and proliferation of neighboring mature endothelial cells. However, fully differentiated endothelial cells are recognized to have a low proliferative potential, thus have limited capacity for endothelium repair or revascularization. Notably, EPCs were found to have a better angiogenic potential in ischemic conditions compared to mature endothelial cells.[14] Many researchers have proven that measuring the level of EPCs in the peripheral blood is a useful indicator of cardiovascular outcomes in CAD patients.[15,16] However, there is a lack of consensus among scientists surrounding the definition of an EPC.[17]

The original EPC population was discovered in 1997 by Asahara and colleagues. It was described as an immature population of peripheral blood mononuclear cells (PBMCs) enriched for $CD34^+$, with the ability to differentiate into endothelial-like cells *in vitro*.[18] EPCs are predominantly located in the bone marrow in a quiescent state, prior to their activation by specific pathophysiological cues such as ischemia. Thus, in general, these cells are derived from the bone marrow, circulate within the peripheral blood, home to sites of new blood vessel formation (in ischemic tissues and tumor microenvironments), and differentiate into mature endothelial cells.[19] Over the years,

EPCs were also identified in the spleen, the intestines, the liver, the umbilical cord blood, and in adipose tissue.[20,21]

In the peripheral blood, EPCs are not abundant. It was previously thought that the number of EPCs could be increased significantly when granulocyte colony stimulating factor (G-CSF) was administrated;[15] however, recent studies have shown otherwise.[22] Experiments in mice showed that G-CSF administration resulted only in modest mobilization of EPCs within the peripheral blood, suggesting the absence of G-CSF receptors on this cell population. On the other hand, G-CSF administration increased significantly the mobilization of hematopoietic stem cells (HSCs),[22] but other studies are required in order to confirm this phenomenon.

Cell culture methods have also been developed in an attempt to increase the number of EPCs for therapeutic use.[14,18,23] The common method is to plate the low-density mononuclear cells from the peripheral blood on fibronectin-coated tissue culture dishes in endothelial basal medium (EBM). The EBM includes endothelial growth supplements, vascular endothelial growth factor and fetal calf serum.[16] Before culturing, the EPC population can also be enriched by direct sorting, either by immunofluorescence or immunomagnetic techniques, using a single or a combination of antibodies directed against specific cell surface markers.[4] The characterization of the cell population can be performed by using flow cytometry, based on the presence of specific markers classified for the most part according to the cluster of differentiation (CD) nomenclature.[24]

EPC Subpopulations

A general property of EPCs is their uptake of acetylated-low density lipoprotein (Ac-LDL) and their lectin *Ulex europaeus* binding.[17] More primitive sub-populations of EPCs are characterized by the concomitant cell-surface expression of antigens CD34, CD133, and vascular endothelial growth factor receptor-2 (VEGFR-2). They are found to be extremely rare (about 0.002% of cells) in the peripheral blood.[25] CD34 is a 110 kD (approximately) cell surface glycophosphoprotein that was initially found to be selectively expressed on HSCs. It was later discovered to be expressed on EPCs and, in a lower intensity, on mature endothelial cells.[18,26] As of today, CD34 has been one of the markers of preference in isolating EPCs from the peripheral blood (for review, please see [8]). CD133, which may also be referred as AC133 or prominin-1, is a five-transmembrane domain cell surface glycoprotein with a molecular weight of 120 kD. Here again, it was originally identified on HSCs and its expression was found to be restricted to the $CD34^+$ population.[27] Nowadays, CD133 is considered a more novel endothelial progenitor cell surface marker; given that its expression is depleted as EPCs differentiate to mature endothelial cells.[28] Both CD34 and CD133 may have biological functions that are still unknown. Lastly, VEGFR-2, which is also known as kinase insert domain receptor (KDR), is a type III receptor tyrosine kinase that is predominantly expressed on endothelial lineage cells.[29] It is a 230 kD glycoprotein that binds to four of the seven members of the vascular endothelial growth factor (VEGF) family, which are VEGF-A, -B, -C and –E.[30] Gene knock-out experiments of flk-1 (homolog gene in mice) have resulted in embryonic lethality, interfering with the HSCs and EPCs precursor lineage.[31]

Hence, VEGFR-2 appears to be an important signaling protein involved in both vasculogenesis and angiogenesis process.

The EPC population is defined to be heterogeneous and, therefore, may show variance in phenotype, potency, and plasticity. Consequently, many studies have agreed that at least two types of endothelial-lineage cells can be obtained by the *in vitro* culture of PBMCs. These are commonly described as the early endothelial progenitor cells (early EPCs) and the late outgrowth endothelial cells (OECs) based on their cultured period; 4 to 7 days for the early EPCs and 7 to 21 days for the OEC.[32,33,34,35] The population of cells referred as early EPCs are hematopoietic-derived cells which express both endothelial and hematopoietic cell surface antigens.[36] Among those, early EPCs co-express CD34, CD133, VEGFR-2, CD31 (also referred to as PECAM-1, platelet endothelial cell adhesion molecule-1), CD115 also known as colony stimulating factor 1 receptor (CSF-1R), CD14 which is preferably expressed by monocytes and macrophages, and CD45 which is expressed in hematopoietic cells. Early EPCs are spindle-shaped cells that secrete angiogenic growth factors to facilitate angiogenesis.[32,33,36] On the other hand, late OECs are thought to be non-hematopoietic-derived EPCs, which co-express VEGFR-2, CD34 (in a lower intensity), CD31, as well as specific endothelial antigen like CD105, CD146, von Willebrand factor (vWF), vascular endothelial (VE) cadherin, E-selectin and endothelial nitric oxide synthase (eNOS).[32,36] However, at this stage they are losing their expression of CD14[34], CD45[35] and CD133, as they are a step closer to becoming mature endothelial cells.[36] The late OECs, which are morphologically identified as having a cobblestone appearance,[33] have a robust proliferative potential and

in vivo vessel-forming capacity.[32,36] In the end, both populations may play a synergistic role in cell-based therapy.[35]

As briefly mentioned above, one of the main debates regarding EPCs surrounds their phenotypic definition. The absence of specific, distinct markers to characterize the EPC has caused issues within the scientific community. The principal reason is that EPCs and HSCs share many cell surface markers, which include among others: CD34, VEGFR-2 and CD133. This observation is not surprising as the two progenitor populations are derived from a common precursor: the hemangioblast **(see figure 1)**.[37] This multipotent precursor, which might be present in rare instances in the peripheral blood, develops from the mesodermal layer of the inner cell mass. At this time, the mechanisms regulating the differentiation of one type of progenitor over the other remain unknown.[38] However, both progenitors might be essential in angiogenic conditions, by synergistically interacting with one another.[39]

Functional Implications of the EPC Phenotype

Multiple phenotypes have been associated with the controversial EPC population, with the intention of characterizing it better. As EPCs are considered a heterogeneous cell population, the multiple phenotypes described may consist of a range of cell types from hemangioblasts to fully differentiated endothelial cells.[40] For that reason, some groups found it more suitable to refer to the early EPCs as circulating angiogenic cells (CACs).[41,42] A few of the many phenotypes attributed to the EPC population are enumerated in **table 1**. The EPCs with greatest angiogenic potential are believed to be the $CD133^{+}CD34^{+}VEGFR\text{-}2^{+}$ cell population.[19,25] The latter described as a more primitive EPC population because of its CD133 expression which is lost during the

differentiation process.[28] The $CD34^+CD45^-$ cell population was described as an endothelial precursor that originates from a non-hematopoietic lineage.[43] Based on its morphology and abilities during *in vitro* functional assays, the $CD34^+CD45^-$ population was specifically identified to be a precursor of late OECs.[43,44] Alternatively, Zhao and his team identified a sub-population of CPCs able to differentiate into multiple lineages.[45] This cell population, characterized as $CD14^+CD34^+CD45^+$, was also referred to as monocyte-derived multipotential cells (MOMCs) on account of its diverse plasticity.[46] In the presence of specific growth factors, notably VEGF, $CD14^+CD34^+CD45^+$ precursors were found to acquire specific endothelial markers and to differentiate toward the endothelial lineage.[45,46] More recently, EPCs were generated from the $CD133^-$ population of the PBMCs.[47] This isolated population referred to as derived $CD133^+$ cells co-expresses CD34 and VEGFR-2, which characterizes it as EPCs. This particular population of cells showed greater functional activity compared to the freshly isolated $CD133^+$ cells. More studies are required on these progenitor cell populations; however, their potentially increased activity makes them a promising option for cell-based therapy.

Throughout the years, efforts have been made to study the mechanisms for enhancement or impairment of EPCs' number and biological function (mobilization, migration, proliferation, survival). Pathological conditions such as cardiovascular diseases, diabetes mellitus, rheumatoid arthritis, hypercholesterolaemia, dyslipidaemia, hypertension, chronic renal failure and others, have all been associated with a decrease in number and function of EPCs (for reviews[7,48,49]). Contrastingly, ischemia[50] and acute myocardial infarction[51] were found to increase EPCs' mobilization. Furthermore, the effects of different drugs and hormones, like statins (HMG-CoA reductase inhibitors),

	TYPE OF CELL	CELL ORIGIN	FATE	ACTION
EARLY EPCs	CD133+CD34+VEGFR-2+	Hemangioblast HSC?	Endothelial cells Vascular smooth muscle cells All hematopoietic cell lineage	Under specific (endothelial) conditions Early EPCs were found to: ***in vitro;*** contribute to angiogenesis and differentiate into mature endothelial cell and/or realease pro-angiogenic factors ***in vivo;*** improve vascular density and perfusion; contribute to angiogenesis; act in a paracrine manner by releasing angiogenic factors; incorporate the endothelium by differentiating into endothelial cells
	CD45-CD34+	non-hematopoietic Hemangioblast Dedifferentiation of mature endothelial-lineage cell Neural stem cell? Adipose-derived stem cell?	Endothelial cells Vascular smooth muscle cells Neuronal cell lineage? Adipocytes, Osteoblasts, Chondrocytes?	
	CD34+CD45+CD14+ Monocyte-derived multipotential cell (MOMC)	Unknown subset of human peripheral blood monocyte	Endothelial cells Vascular smooth muscle cells Cardiomyocytes Neuronal cell lineage Mesenchymal cell lineage Hematopoietic cell lineage Epithelial cells Hepatocytes	
	Derived CD133+	Cultured population: generated from CD133- population	Endothelial cells ???	
	CD133-	Peripheral blood mononuclear cells deprived from a more primitive EPC population (or hemangioblast)	All lineages	

Table 1: Hypothesized origin, fate and action of specific cell types attributed to 'early' endothelial progenitor cells. The five predominant phenotypes listed here are the main focus of this study, where they were tested and compared experimentally.

erythropoietin, leptin and estrogen, were found to positively influence EPCs' potential activity.[52] Other factors, like smoking[53] and aging[54] were observed to negatively affect the function and number of EPCs, while physical training[55] resulted in positive effects. Lastly, numerous cytokines and growth factors were found to influence the EPCs through various mechanisms, which, for the most part, are still unclear and require further elucidation. Some of the more studied stimulating factors include G-CSF, stromal cell-derived factor-1 (SDF-1), nitric oxide (NO), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and VEGF (for reviews[56,57]).

In the past twenty years, VEGF has been intensively studied and was found to play a critical role in vascular physiology and pathophysiology.[30,58] The VEGF family includes seven members; VEGF-A (referred to as VEGF), -B, -C, -D, -E (viral homolog), -F (snake venom) and placental growth factor (PlGF). Each member specifically binds to at least one of the three VEGF-receptors, and some to the neuropilin (NRP) receptors. VEGF-A was the first of the VEGFs to be studied and is mostly recognized under its VEGF-A_{165} isoform (composed of 165 amino acids). VEGF-A_{165} binds to receptors VEGFR-1 and R-2, as well as NRP-1 and -2. Known for its strong angiogenic potential, VEGF-A was identified as a crucial modulator of angiogenesis and vasculogenesis. Moreover, it was described as a regulator of vascular permeability and homeostasis. In relation with EPCs and the process of angiogenesis, VEGF was discovered to be involved in the proliferation, adhesion, migration, differentiation and survival of EPCs (for reviews, please see [30,59]). Finally, VEGF was found to act on progenitor cells in both paracrine and autocrine manners.[59]

The Process of Endogenous Angiogenesis

The attenuation of symptoms or cure for a specific disease relies on a great amount of research, pre-clinically (*in vitro*) and clinically (*in vivo*). In the case of ischemic disease, all pathophysiological aspects need to be addressed; thus, the processes and mechanisms behind it must be discovered and understood. To date, it is acknowledged that angiogenesis does physiologically occur to some limited extend in an attempt to relieve ischemia, restore perfusion in tissue, and regain endothelium integrity.[20] However, the exact mechanisms regulating (neo)vascularization in ischemic condition remain uncertain.[60] The process of angiogenesis is initially driven as a result of conditioned hypoxia. The oxygen deficiency causes the up-regulation of the gene hypoxia-inducible factor-1 (HIF-1), which in return is a strong mediator of multiple cytokines; VEGF, SDF-1, erythropoietic, angiopoietin, PlGF, and many others.[60,61] The high levels of cytokines produces by ischemic tissue mobilize progenitor cells from their niche (bone marrow) upon activation and, through the peripheral circulation, CPCs migrate to the site of ischemia.[10,62] These CPCs may act in a paracrine and autocrine fashion by releasing angiogenic growth factors or cytokines themselves.[35,56,60] Meanwhile, vascular permeability is increased through the activation of NO by the intermediate of VEGF.[30] This incomplete cascade of angiogenic growth factors and cytokines releases promotes the mobilization, homing, adhesion, transmigration, proliferation and perhaps differentiation of CPCs or neighboring cells, and finally confers some vascular survival protection.[63,64]

Homing of CPCs depends on the expression of growth factors (mostly chemokines) and adhesion molecules. VEGF, SDF-1 and interleukin-8 (IL-8) are

thought to be decisive chemokines in the recruitment and homing of CPCs (for review[64]). On the other hand, the adhesion process relies on the cell surface marker express on progenitor cells; E-selectin, CD-31, VE-cadherin, and most importantly integrins.[31,62] Integrins are heterodimers described as α and β subunit proteins with an affinity to extracellular matrix (ECM) and other cells' proteins. In other words, integrins are the key mediators of cell-matrix and cell-cell adhesion.[65,66] The interaction between the cells and the ECM is critical for key processes within the angiogenesis cascade, namely proliferation, transmigration, differentiation and survival. Signals exchanged through the cell-matrix complex also mediate the upcoming fate of the neo-vasculature.[66,67] Among others, the β-2 integrin was found to be abundantly express on EPCs and plays a significant role in their homing to sites of ischemia and ability for neovascularization.[68] The degradation of the ECM through matrix metalloproteinases (MMPs) was also found to influence the homing of CPCs in a paracrine fashion. MMPs are actively involved in multiple steps of angiogenesis and, more specifically, the activity of MMP-2 and MMP-9 appears to be particularly critical. These two MMPs are members of the gelatinase family which can degrade the collagen-rich ECM.[69] MMP-9 and MMP-2 were both discovered to be crucial initial stimulants for angiogenesis[70,71] and their secretion may be synergistically influenced by the presence of early EPCs and late OECs.[35]

The transmigration and proliferation of CPCs or the surrounding endothelial cells rely on the mechanisms and processes mentioned above, and constitute important targets for restoring tissue perfusion.[20,72] The angiogenic growth factors (particularly chemokines) and cytokines, the cell-ECM interactions through integrins, as well as the MMPs all likely contribute to both processes.[66,72] As stated, the mechanical signals

exchanged through the cell-ECM complex may be the key regulators of the (progenitor) endothelial cells transmigration, proliferation, differentiation and survival.[67,73] Growth factors and cytokines also induce progenitor endothelial cell differentiation (through VEGF, SDF-1 and others), and contributes to their survival.[20,60] At last, the angiogenesis process is completed by lumen formation via sprouting of the differentiated endothelial cells.[31] As discussed, the above constitutes a broad overview of the mechanisms and processes involved in angiogenesis that are more or less related to this study. The fully acknowledged process is much more complex and still to this date remains incompletely understood.[60,74]

EPCs are now well recognized to act partly in a paracrine fashion during angiogenesis.[35,63,75] It is thought that the release of cytokines and growth factors by the EPCs promote endogenous cells *in vivo* to differentiate into endothelial cells.[56] Understanding the mechanisms by which these cells contribute to angiogenesis is important. Multiple experimental assays (ELISA[63], DNA microarray[76], real-time reverse transcription polymerase chain reaction[76] and others) have been used to identify and quantify levels of specific proteins that might be released or expressed by the EPCs. More recently, a cytokine protein array was developed to easily and simultaneously observe the increase level of multiple specific cytokines and growth factors released under specific conditions.[47,75] Furthermore, the engineering of new experimental assays might bring us a step closer to fully understanding the role of EPCs in (neo)vascularization.

As formerly described, EPCs represent a heterogeneous cell population of multiple origins with distinct phenotypes and different degrees of plasticity.[77] Recent

studies have doubted the endothelial potential of the most primitive endothelial progenitor (refer in this text as early EPCs) which is defined to co-express CD34, CD133 and VEGFR-2, thus challenging the current knowledge of progenitor cell potential. It has been argued that the human population of cells co-expressing CD34, CD133 and VEGFR-2 are not EPCs but distinct, primitive hematopoietic progenitors.[36,43] Moreover, it has been suggested that the biological mechanism relating these putative cells with cardiovascular disease requires reconsideration.[43] These studies do raise questions regarding the endothelial potential of circulating EPCs; however, these have involved isolated populations only. Others have demonstrated that interactions between mononuclear cells from the peripheral blood in heterogeneous mixtures may have a significant impact on the yield and functional activity of the cultivated cells.[35,39,78] Consequently, a more precise characterization and functional understanding of CPC populations is essential.

To this end, the objectives of the present study were to compare functional properties which include adhesion, migration and angiogenesis of different CPC populations of the PBMCs *in vitro* and to determine whether interactions between subsets of CPCs are involved in mediating their function. We hypothesized that the derived $CD133^+$ cell population possesses a greater overall functional activity compared to other circulating cell populations. As well, interactions between peripheral blood cell populations and/or their cytokines would be involved in regulating progenitor cells function. This information could improve our understanding of cell-based therapy using autologous EPCs or other CPC subpopulations, towards developing a future therapeutic option for patients with CAD.

MATERIALS AND METHODS

Outline

Different populations of cells were obtained from the peripheral circulation of healthy blood donors (n=6). In total, five populations were investigated **(see table 1)**:

1- $CD133^+CD34^+VEGFR\text{-}2^+$ cells

2- $CD34^+CD45^-$ cells (as described in [43])

3- $CD14^+CD34^+CD45^+$ cells (as described in [45])

4- Derived $CD133^+$ cells (as described in [47])

5- $CD133^-$ cells

In vitro adhesion, migration and angiogenesis assays were performed for each population with and without donor specific PBMC supernatant, in order to identify whether paracrine and/or cytokine effects may be involved in regulating the functional activity of the different progenitor populations. The data analysis of the assays was open-labeled with respect to the allocation.

Cell Isolation

The study was approved by the Human Research Ethics Board of the University of Ottawa, and informed consent was obtained from all volunteers. The total PBMC population was freshly isolated from healthy human blood donors by Histopaque 1077 (Sigma-Aldrich, Oakville ON, Canada) density-gradient centrifugation of buffy coats.[79] Approximately 30 cc of blood was gently laid onto 20 ml of Histopaque 1077 and then centrifuged at 2160 rpm for 30 minutes at room temperature (~22°C). Afterward, the isolated PBMCs found between the plasma and Histopaque 1077 fractions was removed and transferred to another tube by using a sterile plastic pipette. The transferred PBMCs

were washed and centrifuged twice at 1400 rpm for 10 minutes with a wash buffer solution; 500 µl of fetal bovine serum (FBS; Invitrogen, Burlington ON, Canada) and 833 µl of 6.5% ethylene-diamine-tetra-acetic acid (EDTA; Sigma-Aldrich, Oakville ON, Canada) in approximately 50 ml of phosphate buffered saline (PBS; Sigma-Aldrich, Oakville ON, Canada). Total PBMCs were then used in the experiments described below.

Supernatant from Unsorted PBMC Culture

Total PBMCs of approximately 30 cc of blood were cultured on fibronectin (Sigma-Aldrich, Oakville ON, Canada) coated plates in endothelial basal medium-2 (EBM-2; Lonza, Shawinigan, QC, Canada) supplemented with EGM-2-MV-SingleQuots containing 5% FBS and predetermined amounts of human VEGF, human insulin-like growth factor-1 (IGF-1), human epidermal growth factor (hEGF) and gentamicin, amphotericin-B (GA-1000). After an overnight incubation at 37°C, non-adherent cells and media were removed and centrifuged at 1400 rpm for 10 min.[80,81] The supernatant was removed and stored in a -80°C freezer until needed.

Flow Cytometry Characterization

Each isolated CPC population was labeled in a refrigerator for 30 minutes with mouse anti-human antibodies at manufacturer-recommended concentrations against the following antigens: fluorescein isothiocyanate (FITC) conjugated CD34 (Beckman Coulter, Mississauga, Canada), phycoerythrin (PE) conjugated VEGFR-2 (R&D Systems, Minneapolis MN, USA), energy coupled dye (ECD) conjugated CD14 (Beckman Coulter, Mississauga ON, Canada), allophycocyanine (APC) conjugated CD133

(Miltenyi, Auburn CA, USA) and phycoerythrin cyanine 7 (PC7) conjugated CD45 (BD Pharmingen, Mississauga ON, Canada). Cells were then washed with Hanks' balanced salt solution (HBSS; Sigma-Aldrich, Oakville ON, Canada) and centrifuged at 1400 rpm for 10 minutes. The pellet was resuspended with 500 µl of HBSS for analysis. Marker expression was evaluated by flow cytometry (FACSAria^{TM}; BD Biosciences, Mississauga, ON, Canada) and expressed as a percentage of total cells analyzed. The following appropriate fluorophore conjugated mouse immunoglobulin G (IgG) were used as a negative control: IgG FITC/PE, IgG ECD, IgG APC and IgG PC7 (All from Beckman Coulter, Mississauga, ON, Canada).[81,82]

Culture of Derived CD133$^+$ cells

CD133$^+$ cells were separated from PBMCs by using an indirect CD133-bound microbead kit (Miltenyi Biotec GmbH, Auburn CA, USA), which consists of applying a biotinylated CD133 antibody followed by anti-biotin-bound microbeads, and separation by a magnetically activated cell sorter (autoMACS^{TM}; Miltenyi Biotec GmbH, Auburn CA, USA) following the manufacturer's protocol. The CD133$^-$ fraction was plated on 24-well fibronectin-coated tissue culture plates at a concentration of $3x10^6$ cells/well with EBM. The supernatant (containing media and non-adherent cells) was removed every second day for a period of 8 days and fresh media was added to plates (adherent cells). Derived CD133$^+$ cells from the CD133$^-$ fraction were separated from the supernatant by autoMACS^{TM} and frozen in 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Oakville ON, Canada) at -80°C in 1mL cryovials before storing in liquid nitrogen until needed.[83] For use in experiments, cryopreserved derived CD133$^+$ cells (from days 2, 4, 6 and 8) were thawed, combined and washed with PBS before use.[81] Cell numbers and viability

of the combined fraction were determined by a programmed cell counter (Vi-Cell; Beckman Coulter, Mississauga ON, Canada).[79] The Vi-Cell determines cell viability by the trypan blue dye exclusion method. Specifically, the dead or non-viable cells become darker than the viable cells due to their intake of the trypan blue dye through their permeable membrane, are then quantified.

Enriched $CD133^+CD34^+VEGFR\text{-}2^+$ and $CD133^-$ populations

Total isolated PBMCs were labeled in a refrigerator for 30 minutes at manufacturer-recommended concentrations with a combination of the following fluorescent human antibodies: FITC conjugated CD34, VEGFR-2 conjugated PE and APC conjugated CD133. The cells were then washed with wash buffer, pelleted and resuspended in HBSS. The $CD133^+CD34^+VEGFR\text{-}2^+$ and $CD133^-$ populations were separated from PBMCs by FACS (Fluorescence-Activated Cell Sorting) for its (triple) positive or negative, respectively, cell surface antigen expression.[82] The two enriched populations were pelleted and then used in the *in vitro* assays.

Enriched $CD34^+CD45^-$ and $CD14^+CD34^+CD45^+$ populations

Positive and negative populations for cell surface antigen CD45 were separated from PBMCs by using CD45-bound microbeads (Miltenyi Biotec GmbH, Auburn CA, USA) and autoMACS™ following the manufacturer's protocol. The $CD45^+$ and $CD45^-$ fractions were separately stained for 30 minutes in a refrigerator at manufacturer-recommended concentrations by using fluorescent human antibody FITC conjugated CD34 for both fractions and by using ECD conjugated CD14 for the $CD45^+$ fraction only. Both fractions were then washed with wash buffer, pelleted and resuspended in

HBSS. The $CD34^+CD45^-$ and $CD14^+CD34^+CD45^+$ were further separately purified by FACS for positive specific cell surface antigen expression.[82] The two final enriched populations were pelleted and then used in the *in vitro* assays.

Immunomagnetic Separation Efficiency

Total PBMCs were separated into positive and negative fractions using antigen-bound microbeads, more specifically indirect CD133 or direct CD45 microbeads kit, and autoMACS™ following the manufacturer's protocol.[82] For both antigen specific separations, each fraction (positive and negative) was stained for 30 minutes in a refrigerator with appropriate fluorescent antigen, either APC conjugated CD133 or PC7 conjugated CD45. Additionally, appropriate fluorophores conjugated mouse IgG were used as negative control: IgG APC and IgG PC7. Fractions were further washed with HBSS, pelleted and resuspended in HBSS for analysis. Separation efficiency of antigen-bound microbeads by autoMACs was evaluated by characterizing each fraction by flow cytometry.

Static Adhesion Assay

Twenty-four well culture plates coated with type I collagen (100 μg/ml) (BD Pharmingen, Mississauga, ON, Canada) or fibronectin (100 μg/ml) were used and wells were blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich, Oakville ON, Canada) for 1h.[47] Prior to use in the assay, isolated CPC populations were labeled with the fluorescent dyes CellTracker orange CMTMR (Molecular Probes, Burlington ON, Canada) and 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Oakville ON, Canada) for 30 minutes at 37°C. Cells were washed with wash buffer, pelleted and

resuspended with a small volume of EBM. Then, $2x10^4$ cells per well were added with donor specific supernatant of PBMCs or EBM for each of the five cell populations described. The cells were incubated for 1h at 37°C, after which the media and non adherent cells were removed. The adherent populations were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, Oakville ON, Canada) for 20 minutes and stored in 0.1 M PBS in refrigerator. The quantification of adherent cells was done under fluorescent microscope (Model BX60F5, Olympus Optical Co., Ltd., Tokyo, Japan) at 100X from manual counts in six random microscopic fields. Each population of cells was submitted to the two treatments (EBM and supernatant of PBMCs), on fibronectin and type I collagen, in triplicate for the exception of $CD133^+CD34^+VEGFR\text{-}2^+$ cells; as the latter is a rare population, only one assay per treatment on fibronectin only was evaluated. Fold changes were calculated as the average number of adherent cells per field of view (FOV) for the different CPC population relative to the average number of each donor's adherent $CD133^-$ cell population.

Migration Assay

The migratory potential of the five CPC populations was investigated by using a modified Boyden chamber (Fisher Scientific, Ottawa, ON, Canada) with the chemotactic angiogenic factor VEGF.[81] The modified Boyden chamber consists of an upper and lower set of wells that are separated by a cell-permeable membrane.[84] To the upper chamber, $2x10^4$ cells/well were placed for each population and supplemented with either donor specific PBMC supernatant or serum-free media (without FBS or VEGF added). The lower chamber was coated with fibronectin and contained serum-free media added with 50ng/ml VEGF (Cedarlane, Burlington ON, Canada) which served as a migratory

stimulus. After a 24h incubation at 37°C, the cells that migrated and adhered to the lower chambers were fixed with 4% PFA for 20 minutes and stored in 0.1 M PBS in a refrigerator. Cell populations were previously labeled with the fluorescent dyes CellTracker orange CMTMR and DAPI for 30 minutes at 37°C. The quantification of migrated cells was done under fluorescent microscope at 100X from manual counts in six random microscopic fields. Each population of cells was submitted to the two treatments in triplicate for the exception of $CD133^{+}CD34^{+}VEGFR\text{-}2^{+}$ cells; due to their rarity, one assay per treatment was evaluated. Fold changes were calculated as the average number of migrated cells per FOV for the different CPC population relative to the average number of each donor's migrated $CD133^{-}$ cells population.

***In vitro* Angiogenesis Assay**

ECMatrix™ mix (Chemicon (Millipore), Temecula, CA, USA) was prepared following the manufacturer's protocol and 80 µl of the mix was added to wells of a 96-well plate and incubated for 1h at 37 °C.[47] Each CPC population was seeded ($1x10^4$ cells/well) on matrix either alone, or with $1x10^4$ human umbilical vein endothelial cells (HUVECs; a mature endothelial cell population) (donated by Dr.Griffith; University of Ottawa Eye Institute, Ottawa ON, Canada), and supplemented with donor specific PBMC supernatant or EBM. The cultures were incubated overnight at 37°C. HUVECs ($2x10^4$ cells/well) seeded alone were used as a control. Prior to seeding, the CPC populations were labeled with both fluorescent dyes, CellTracker orange CMTMR and DAPI, for 30 minutes at 37°C, while the HUVECs were labeled with the fluorescent dye CellTracker orange CMTMR only. Quantification of cells contributing to tubule formation as well as the overall density of capillary-like structures was performed (manually) using three to

four random microscopic fields under bright field and fluorescence microscopy at 100X. Again, each population of cells was submitted to the two treatments in triplicate for the exception of $CD133^{+}CD34^{+}VEGFR\text{-}2^{+}$ cells. Fold changes were calculated as the average number of contributed cells to tubule formation per FOV for the different CPC population relative to the average number of each donor's contributed $CD133^{-}$ cells population. The percentages of the overall density of capillary-like structures were evaluated as the area covered by capillary-like structures relative to the complete area in FOV.

Differentiation of CPCs in the *in vitro* Angiogenesis Assay

ECMatrix™ gels were digested with cell recovery solution (BD Biosciences, Mississauga, Canada) after an overnight incubation with EBM at 37°C, following manufacturer's protocol (See previous section for details on *in vitro* angiogenesis assay). For this assay, each CPC population was labelled with DAPI while HUVECs remained unlabeled. Pelleted cells were collected from the digest and labelled with the following endothelial specific cell surface markers in a refrigerator for 30 minutes; FITC conjugated CD31 (Beckman Coulter, Mississauga ON, Canada) and PE conjugated VE-Cadherin (Beckman Coulter, Mississauga ON, Canada). Then, cells were washed, pelleted and resuspended in HBSS for analysis. Each CPC populations were characterized by flow cytometry for its $DAPI^{+}$ expression, and the mentioned endothelial specific markers. Appropriate IgGs were used as negative control (see Flow Cytometry Characterization section as reference).

Cytokine Antibody Array

Raybio® human cytokine antibody array V (Raybiotech, Norcross GA, USA) was performed to assay proteins in the supernatants of the isolated CPC populations from PBMCs (n=3).[47,80] In total, the expression of 79 different cytokines was assayed in accordance with manufacturer's protocol. The supernatant of the isolated CPC populations was collected after an overnight incubation at 37°C in basal media (EBM). Differences in the soluble factors were assayed by comparing the growth factor and cytokine levels within culture EBM (control) and the supernatant of PBMCs. Quantification of protein levels were performed against internal controls in the array and against other samples as fold increases. A chemiluminescent and fluorescent imaging system (FluorChemHD® Alpha Innotech; Fischer Scientific, Ottawa ON, Canada) was used to detect the levels of cytokines expression or intensity on each array.

Statistical Analysis

Values are presented as the mean ± standard error. A one-way analysis of variance (ANOVA) between the different CPC populations in each treatment or paired student t-tests for within donor comparisons were used to assess statistical differences. A paired student t-test was performed to analyze differences between the two treatments: control (EBM) *vs* supernatant of PBMC. Differences with p-values <0.05 were considered statistically significant.

RESULTS

Characterization of Different CPC Populations by Flow Cytometry

With the intention of better characterizing the different CPC populations used in this study, the expression of five cell surface antigens (CD14, CD34, CD133, CD45 and VEGFR-2) was evaluated for the different populations. The purity of the sorted CPC populations was not 100% **(table 2)**, but compared to the pre-sorted populations, an increase of expression was observed **(for additional information, see Appendix A)**. For $CD133^+$ cells, 40.4% of the freshly isolated $CD133^+CD34^+VEGFR\text{-}2^+$ population **(figure 2)** and 89.4% of the derived $CD133^+$ population **(figure 3)** expressed the cell surface marker CD34 and VEGFR-2. As for the $CD14^+CD34^+CD45^+$ population **(figure 4)**, 25.3% of the $CD45^+$ fraction simultaneously expressed all three markers. Therefore, this study was based on cell populations which were enriched for particular cell surface antigens. Notably, the efficiency of the immunomagnetic separation technique was evaluated; for the cell surface antigen CD45 and CD133, an average separation efficiency of 89.6±4.1% and 5.4±0.9% was obtained, respectively **(Appendix B).**

POPULATION		PHENOTYPE (%)				
		CD34	VEGFR-2	CD14	CD133	CD45
$CD133^+CD34^+$ $VEGFR\text{-}2^+$	pre-sort	8.4	16.7	-	0.2	-
	post-sort	28.3	57.1	51.8	18.4	82.4
$CD34^+CD45^-$	pre-sort	6.9	-	-	-	-
	post-sort	14.2	26.1	28.0	9.7	-
$CD14^+CD34^+CD45^+$	pre-sort	31.0	-	38.5	-	-
	post-sort	22.9	35.1	53.8	3.8	94.8
Derived $CD133^+$	n/a	0.6	0.8	0.3	3.0	17.3
$CD133^-$	n/a	1.3	9.9	7.1	-	75.9

Table 2: Phenotypic expression of different isolated CPC populations before and after sorting by FACS.

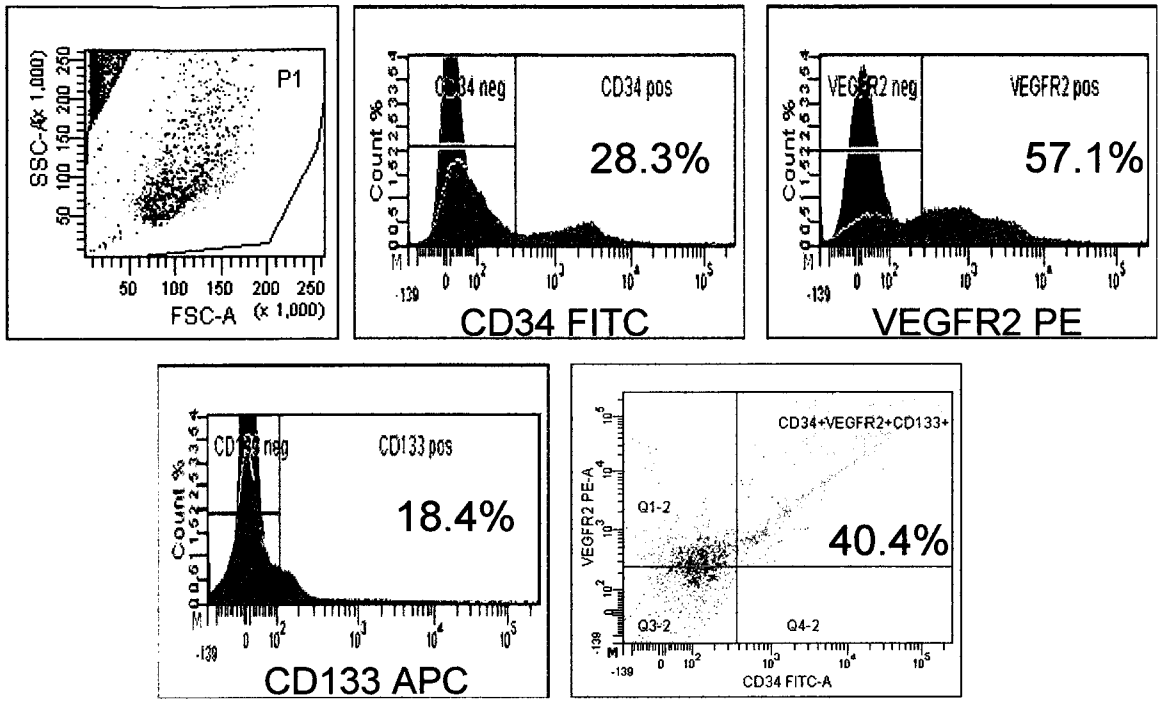


Figure 2: Representative flow cytometric phenotypic characterization. Percentage of expression for cell surface markers CD34, VEGFR-2 and CD133; approximately 40.4%, gated on CD133 expression, of the freshly isolated $CD133^{+}CD34^{+}VEGFR\text{-}2^{+}$ population simultaneously co-expressed CD34 and VEGFR-2. The expression of a specific cell surface marker for an isolated cell population is represented in green while IgG controls are in black.

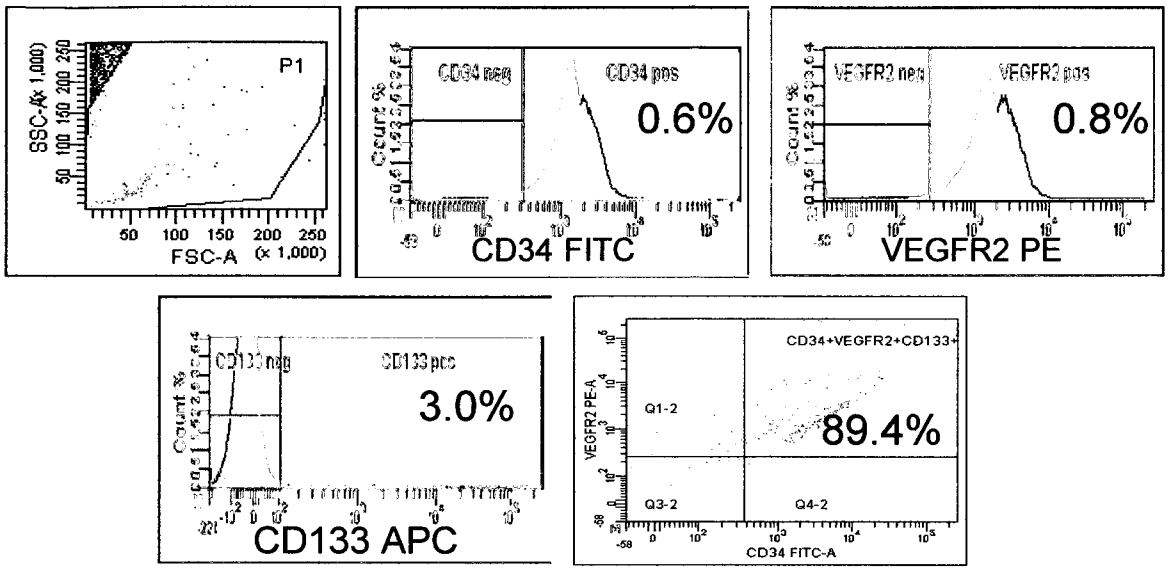


Figure 3: Representative flow cytometric phenotypic characterization. Percentage of expression for cell surface markers CD34, VEGFR-2 and CD133; an average of 89.4%, gated on CD133 expression, of the cultured derived $CD133^{+}$ population were co-expressing CD34 and VEGFR-2. The expression of a specific cell surface marker for an isolated cell population is represented in green while IgG controls are in black.

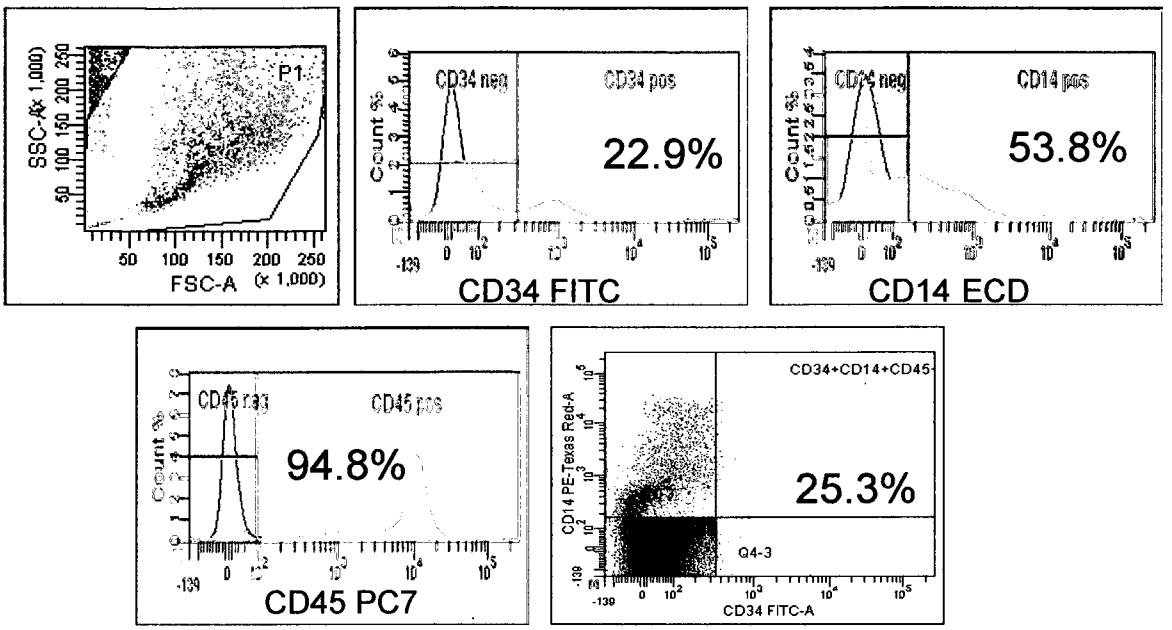


Figure 4: Representative flow cytometric phenotypic characterization. Percentage of expression for cell surface antigens CD34, CD14 and CD45; an average of 25.3%, based on the $CD45^+$ expression, of the $CD14^+CD34^+CD45^+$ population simultaneously co-expressed CD14 and CD34. The expression of a specific cell surface marker for an isolated cell population is represented in green while IgG controls are in black.

Static Adhesion Assay

The adhesiveness of the different cell populations was tested on fibronectin and collagen type I, and the effect of possible cell-cytokine interactions was investigated. The derived $CD133^+$ population showed a significant increase in its adhesive potential when cells were co-cultured with donor specific supernatant of PBMCs (**figure 5)** on both substrates (fold increases); fibronectin (1.6±0.1; p=0.032) and collagen type I (2.2±0.5; p=0.019) **(figures 6a and 7a)**. Significance between the two treatments (+/- supernatant) was also observed for the following populations: $CD14^+CD34^+CD45^+$ (1.5±0.2; p=0.037) on fibronectin **(figure 6a)** and $CD133^-$ (1.8±0.3; p=0.042) on collagen type I **(figure 7a)**. Comparison between the different populations investigated in this study indicated that some CPCs possess a better adhesive potential than others. Consequently, significant differences were observed between the different cell populations; regardless of the treatment, more derived $CD133^+$ cells adhered to both substrates than other cell types **(figures 6b-c and 7b-c)**. Compared to the other cell populations, the derived $CD133^+$ cells showed fold change increases of 4.1±1.2 (fibronectin) and 3.7±0.9 (collagen type I) when supplemented with EBM and 7.0±2.1 (fibronectin) or 7.4±1.7 (collagen type I) when supplemented with donor specific supernatant of PBMCs. No significant differences were observed between cell populations plated on fibronectin versus collagen type I **(figures 8a-b)**.

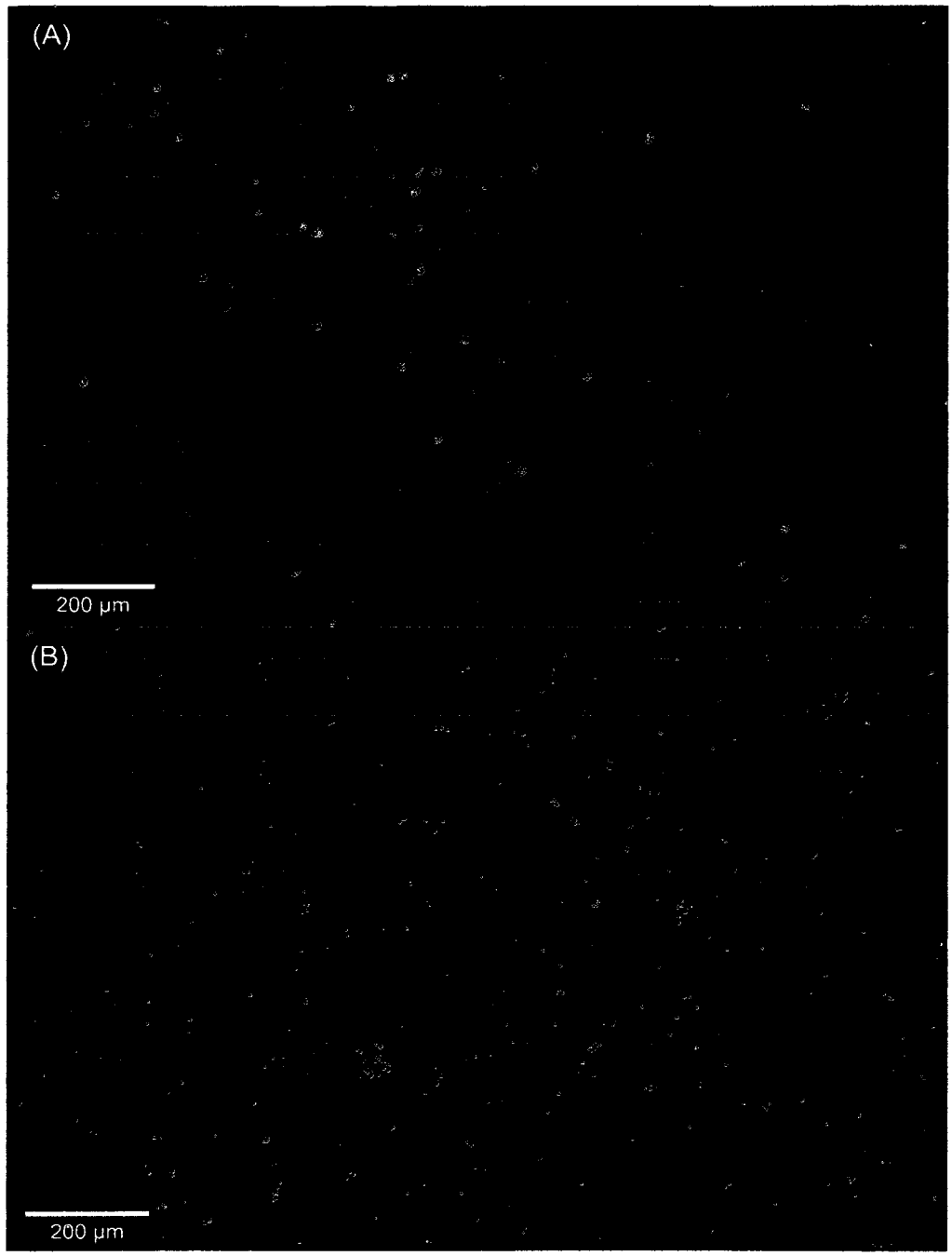


Figure 5: Microscopic view (100X) of fluorescent (DAPI-stained) adherent cells. Representative image of adherent derived $CD133^+$ cells on collagen type I supplemented with EBM medium **(A)** and donor specific supernatant of PBMCs **(B)**. The same observation was made when cells were cultured on fibronectin.

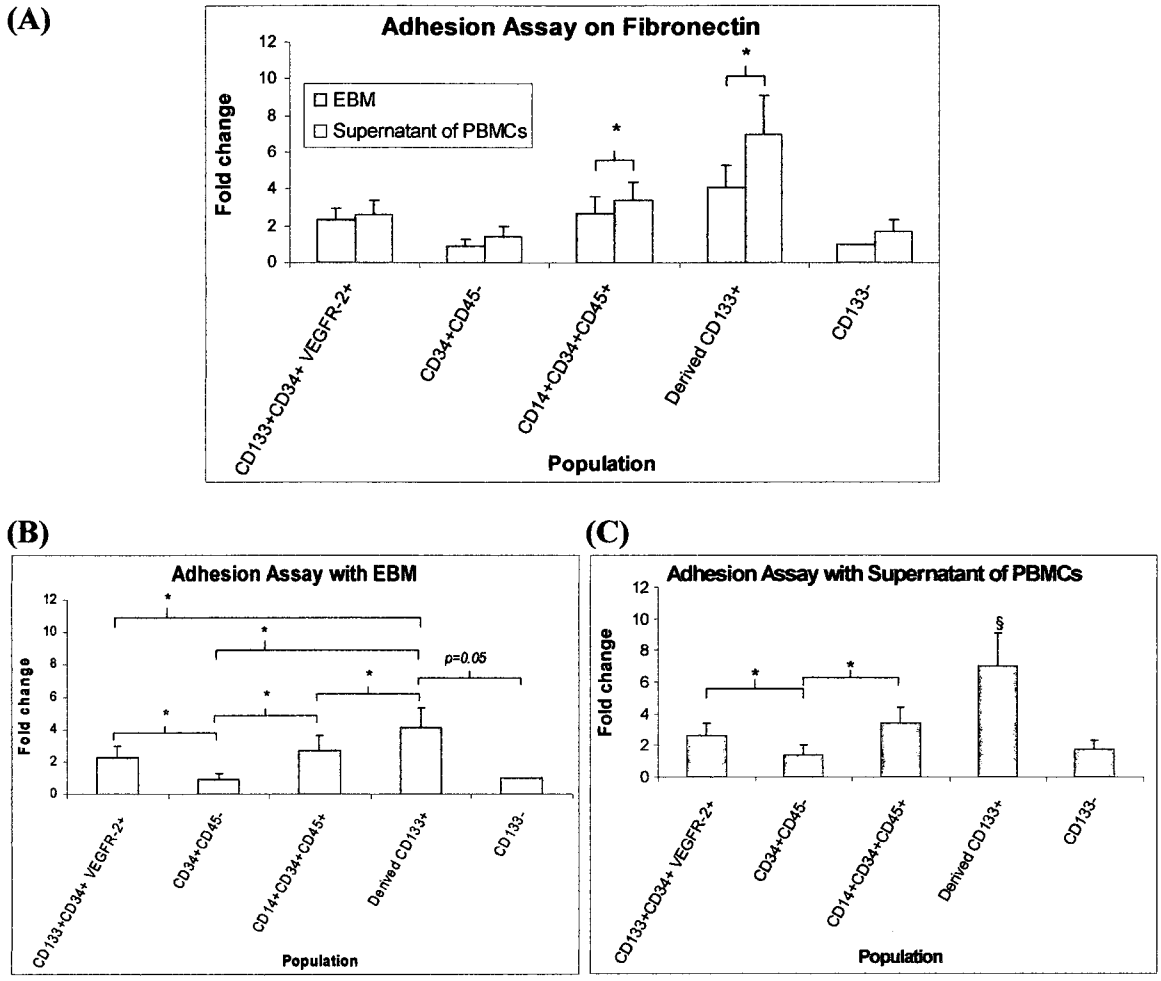


Figure 6: Static adhesion assay on fibronectin (n=6). **(A)** Comparison of the cell populations' adhesiveness on fibronectin when supplemented with EBM or donor specific supernatant of PBMCs. **(B)** Comparison between cell populations supplemented with EBM. **(C)** Comparison between cell populations supplemented with donor specific supernatant of PBMCs. $*p < 0.05$ and $^{\S}p < 0.05$ versus all cell populations.

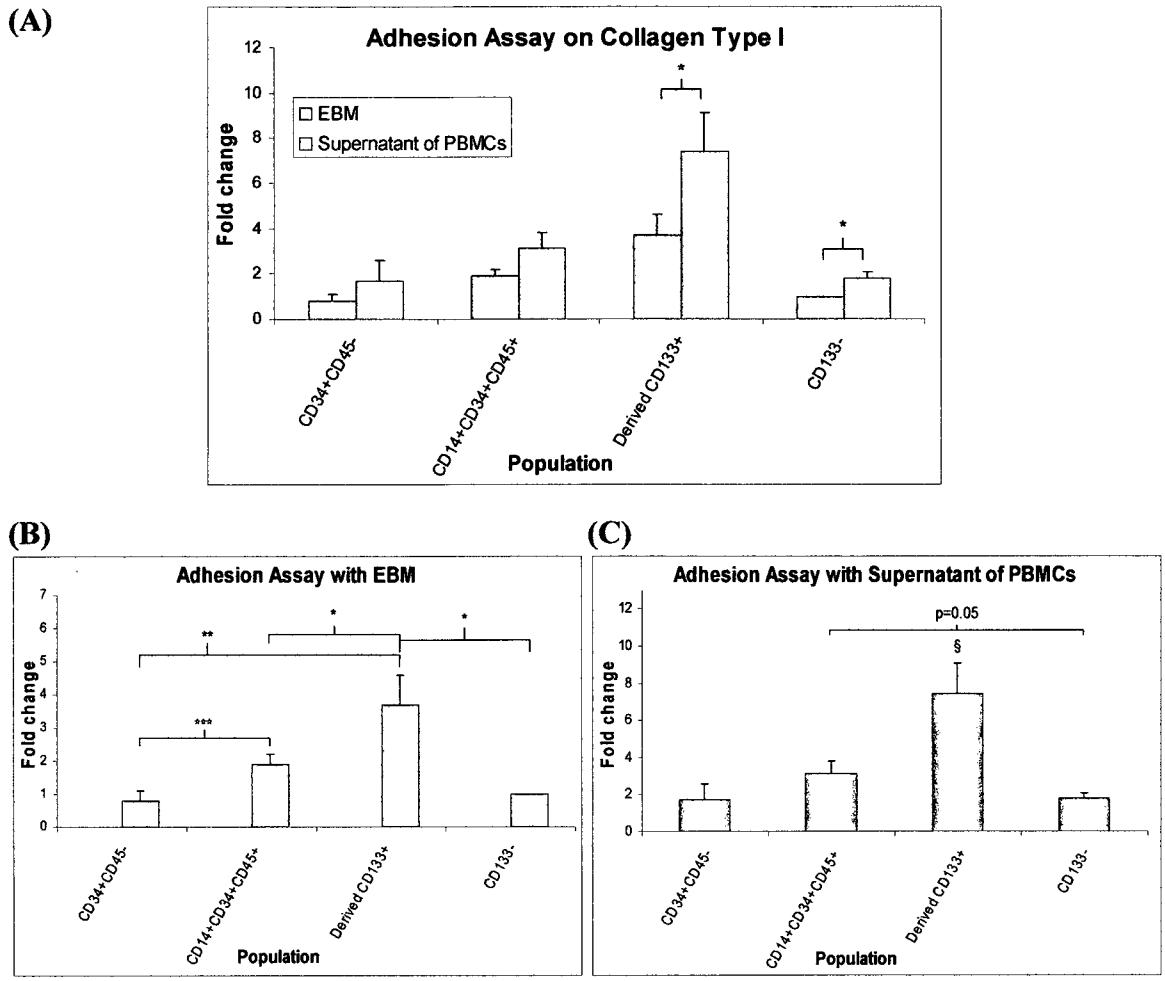
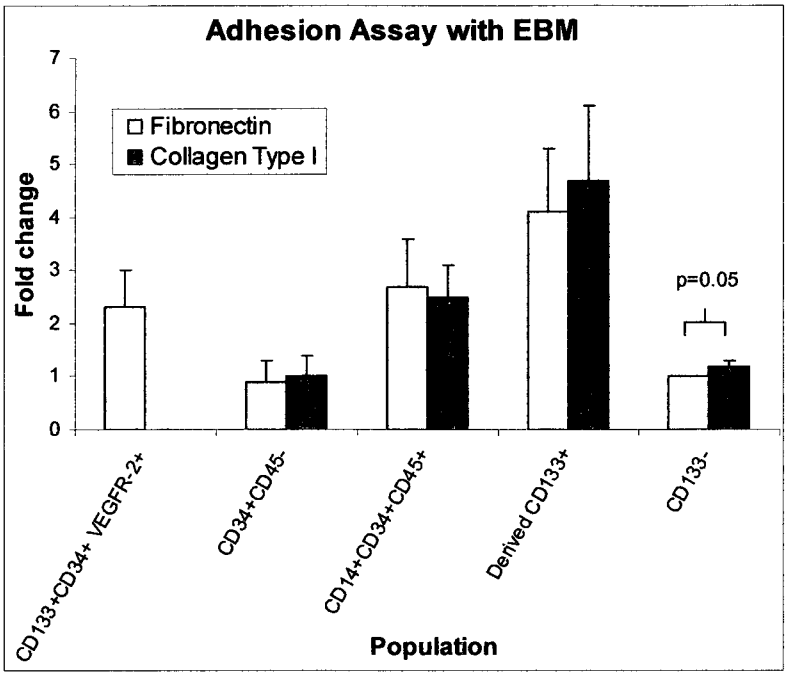


Figure 7: Static adhesion assay on collagen type I (n=6). **(A)** Comparison of the cell populations' adhesiveness on collagen type I when supplemented with EBM or donor specific supernatant of PBMCs. **(B)** Comparison between cell populations supplemented with EBM. **(C)** Comparison between cell populations supplemented with donor specific supernatant of PBMCs. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ and $^{\S}p < 0.05$ versus all cell populations.

(A)



(B)

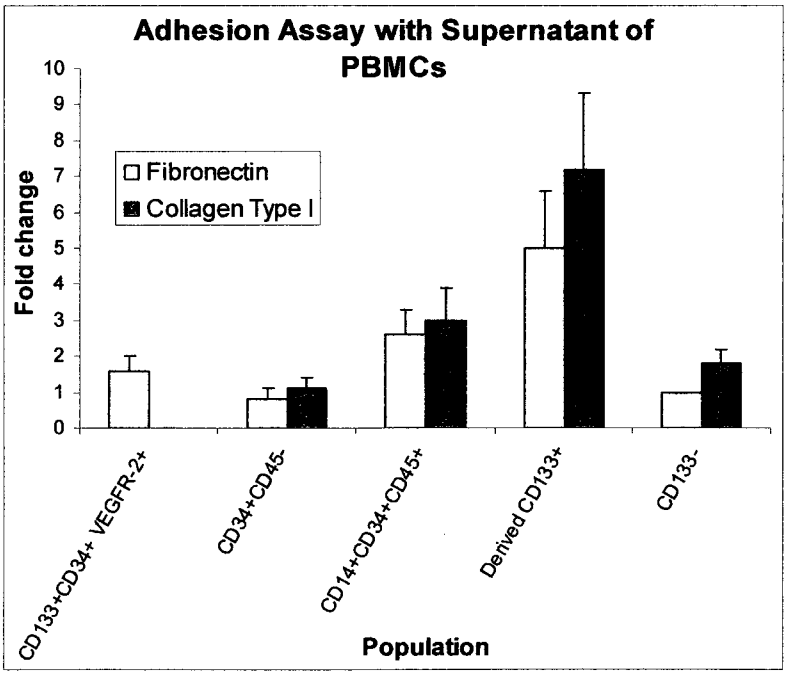


Figure 8: Static adhesion assay on fibronectin and collagen type I (n=6).

(A) Comparison of the cell populations' adhesiveness when supplemented with EBM.

(B) Comparison of the cell populations' adhesive potential when supplemented with donor specific supernatant of PBMCs.

Migration Assay

The migratory potential of the cell populations investigated was evaluated in response to VEGF and possible cell-cytokine interactions. Overall, the different cell populations showed a considerable enhancement in migratory potential when supplemented with donor specific supernatant of PBMCs. Fold increases of 2.3±0.4 ($CD133^+CD34^+VEGFR\text{-}2^+$; p=0.023), 2.6±0.4 ($CD34^+CD45^-$; p=0.018), 2.4±0.5 ($CD14^+CD34^+CD45^+$; p=0.045), 3.1±0.4 (derived $CD133^+$; p=0.045) and 3.7±0.9 ($CD133^-$; p=0.038) were observed **(figure 9a)**. In addition, significant differences were observed between the different CPC populations **(figures 9b-c)**. Compared to the other cell populations, the derived $CD133^+$ cells demonstrated greater migration when cultured with supernatant of PBMCs (13.6±4.8 fold), and with the EBM treatment (4.5±1.5 fold).

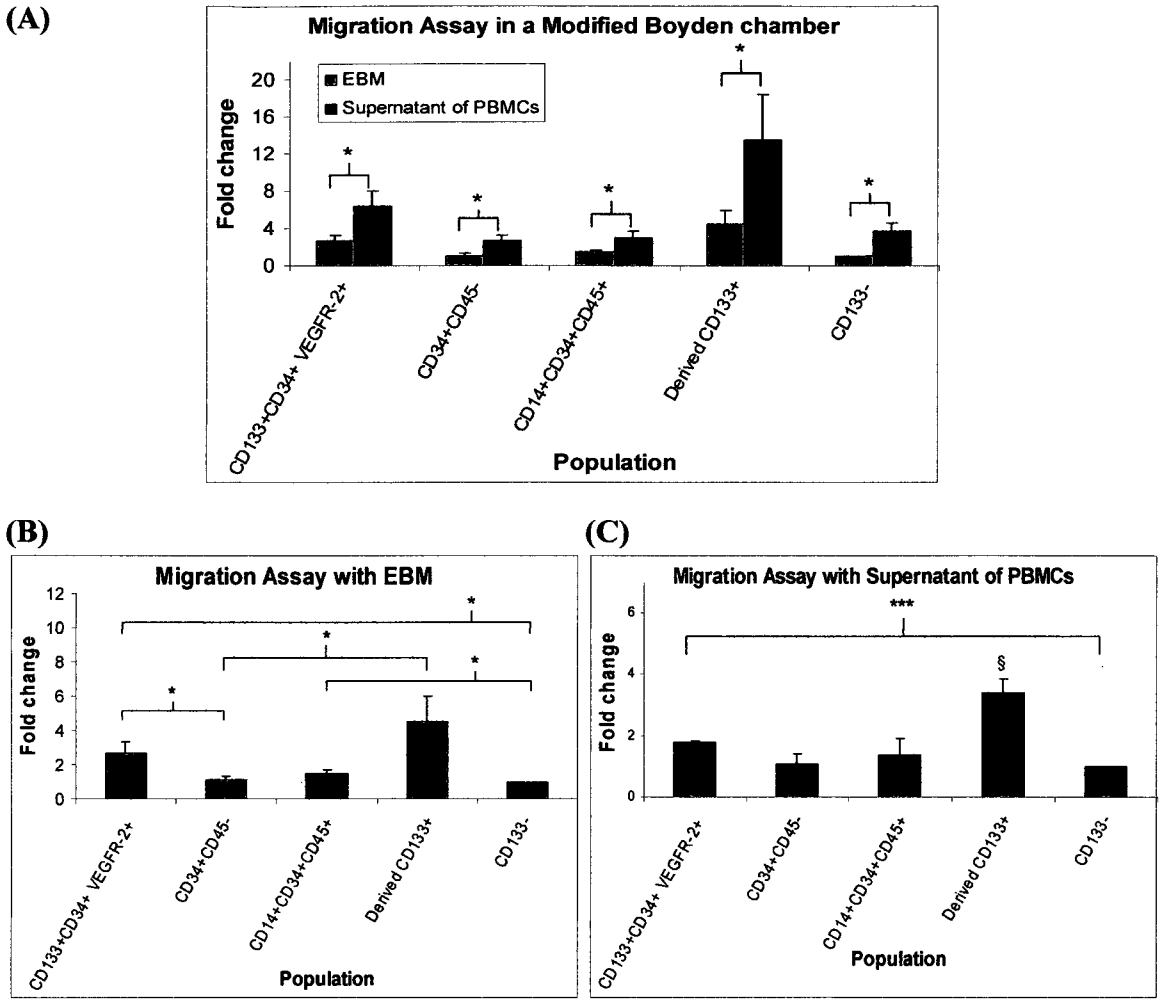


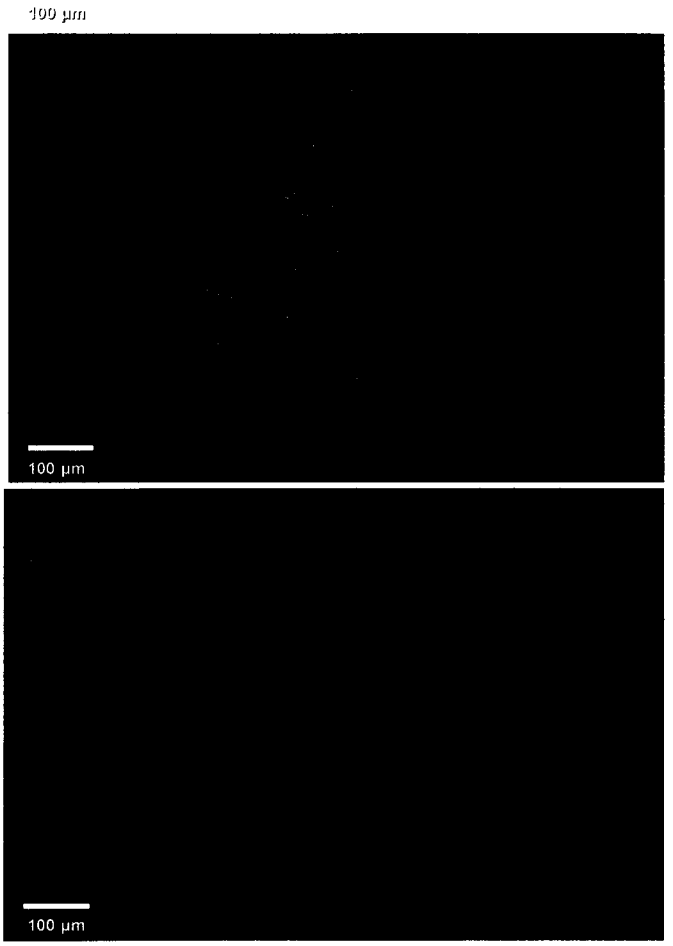
Figure 9: Migration assay using a modified Boyden chamber (n=6). **(A)** Comparison of the cell populations' migratory potential when supplemented with EBM or donor specific supernatant of PBMCs. **(B)** Comparison between cell populations supplemented with EBM. **(C)** Comparison between cell populations supplemented with donor specific supernatant of PBMCs. $*p < 0.05$; $***p < 0.001$ and $^{\S}p < 0.05$ versus all cell populations.

***In vitro* Angiogenesis Assay**

The CPCs' angiogenic potential was tested either alone or in combination with a mature endothelial cell population (HUVECs), and the effect of possible cell-cytokine interactions was investigated. *In vitro* angiogenesis was absent when cell populations were cultured alone, as no formation of tubule or capillary-like structures were observed **(figures 10a-c)**. However, when seeded with HUVECs, CPCs contributed to the capillary-like structure formation **(figures 11a-c)**. It was anticipated that when cells were co-cultured with donor specific supernatant of PBMCs, an enhancement in their contribution to angiogenesis would be observed. However, the results obtained did not support the hypothesis and no significant changes, for the exception of the $CD14^+CD34^+CD45^+$ population ($p=0.013$) were observed between the groups cultured with EBM or supernatant of PBMCs **(figure 12a)**. In both treatments, the $CD133^+CD34^+$VEGFR-2^+ and derived $CD133^+$ cell populations tended to contribute the most to the *in vitro* angiogenesis' structure **(figures 12b-c)**. Compared to the $CD133^-$ control, fold changes of 2.44±0.62 and 3.60±0.51 for the derived $CD133^+$, and 2.40±0.50 and 3.10±0.72 for the $CD133^+CD34^+$VEGFR-2^+ were observed when the populations were supplemented with EBM or donor specific supernatant of PBMCs, respectively. Subsequently, the overall density of capillary-like structures demonstrated some differences between the two treatments and the different cell populations **(figures 13a-c)**. Significant increases were observed for the HUVECs control (1.2±0.1; $p=0.012$), $CD14^+CD34^+CD45^+$ (1.1±0.1; $p=0.018$) and derived $CD133^+$ (1.6±0.3; $p=0.038$) populations when submitted to supernatant of PBMCs. In comparison to the $CD133^-$ population, the other CPC populations possessed greater angiogenic potential by

increasing the density of capillary-like structures **(figure 13b-c)**. Despite the lack of significance, the derived $CD133^+$ population demonstrated a tendency to enhance the density of the vascular-like structures when supplemented with donor specific supernatant of PBMCs (26.8±4.0%) compared to the others **(figure 13c)**. However, the percentages observed in the EBM treatment were comparable for most cell populations **(figure 13b)**.

(A)



(B)

(C)

Figure 10: Representative microscopic view (100X) of the *in vitro* angiogenesis assay. CPCs cultured alone failed to form capillary-like structures. CPCs labelled with CellTracker orange CMTMR and DAPI were observed under bright field **(A)**, red fluorescence **(B)** and blue fluorescence **(C)** microscopy.

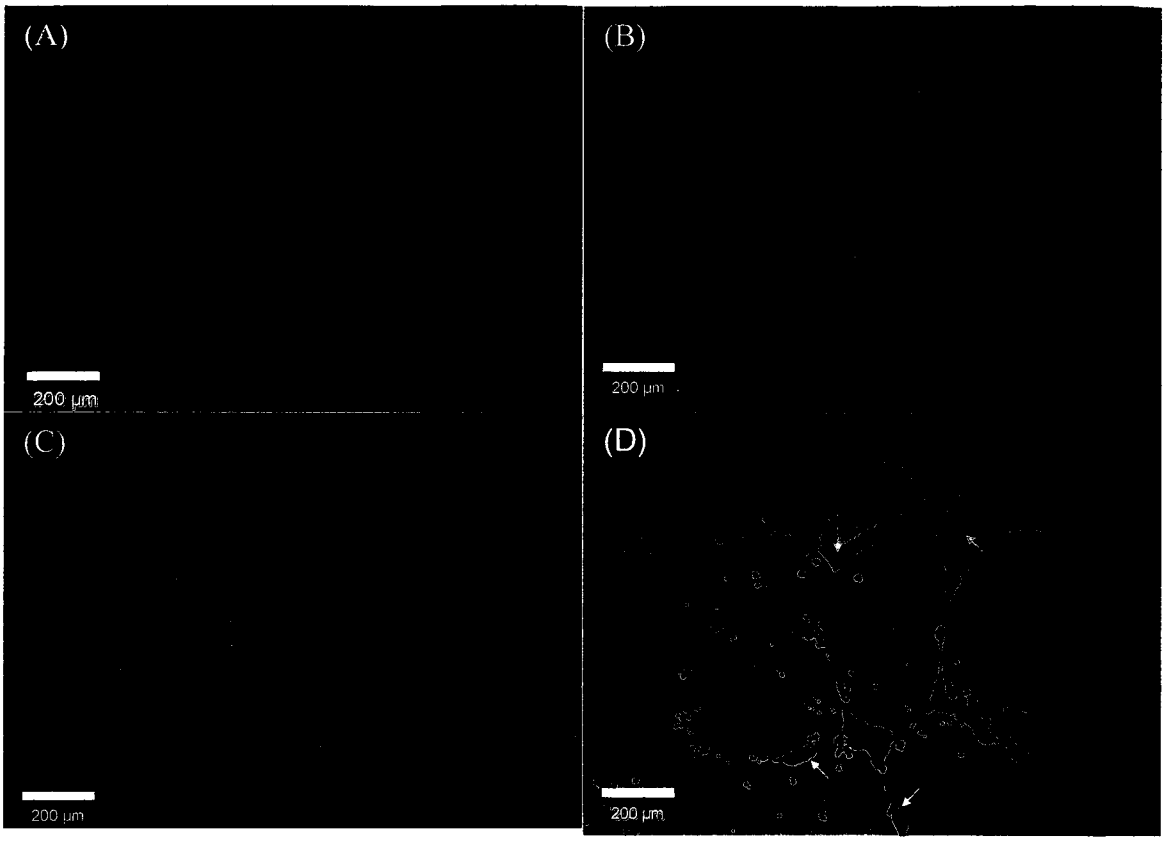


Figure 11: Representative microscopic view (100X) of the *in vitro* angiogenesis assay. Contribution of CPCs to capillary-like structures when co-cultured with HUVECs. HUVECs labelled with CellTracker orange CMTMR and CPCs labelled with CellTracker orange CMTMR and DAPI were observed under bright field **(A)**, red fluorescence **(B)** and blue fluorescence **(C)** microscopy. Image analysis was done by superposing the three panels; the arrow indicates the CPCs contributing to the angiogenesis' structure **(D).**

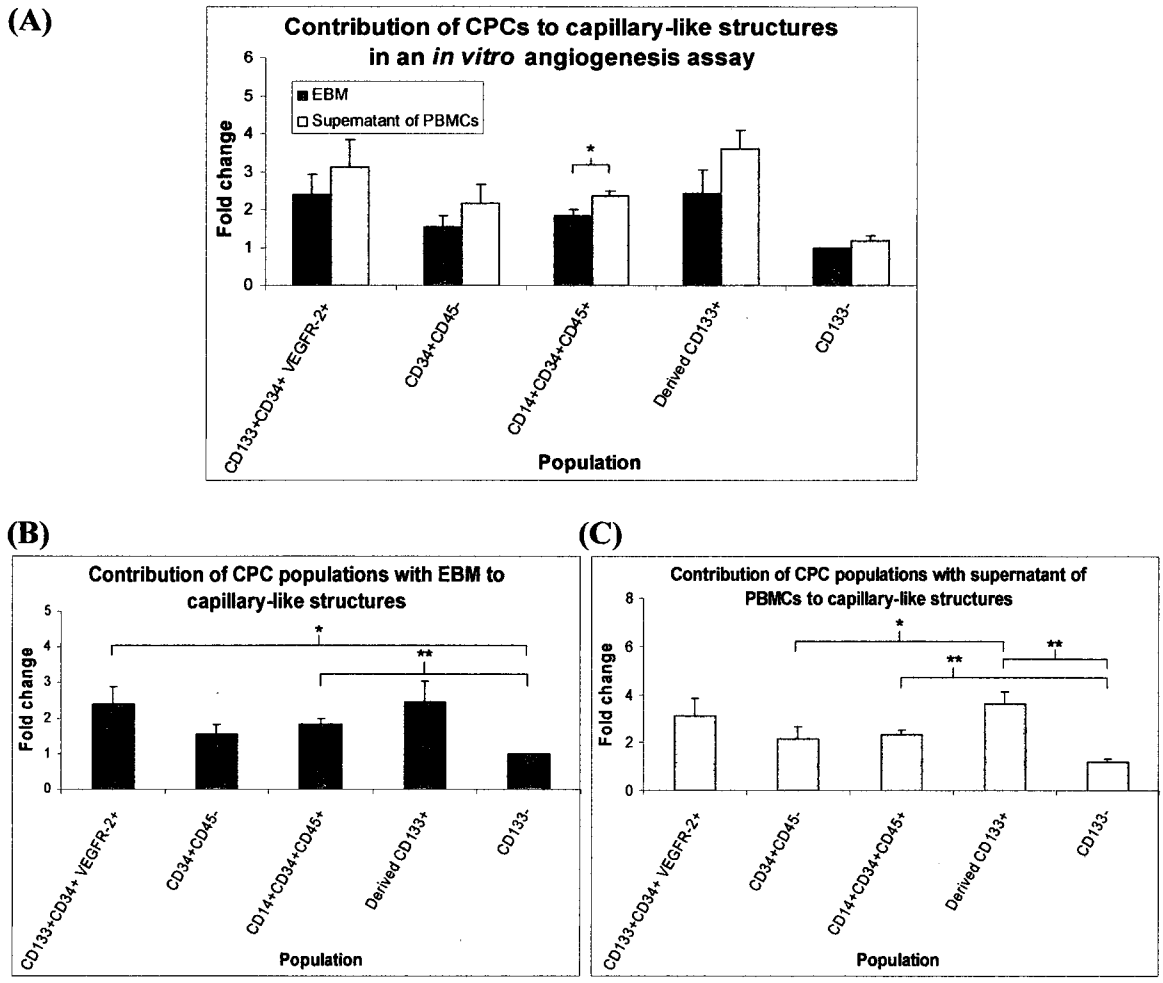


Figure 12: *In vitro* angiogenesis assay (n=5). **(A)** Comparison of the cell populations' contribution to capillary-like structures when supplemented with EBM or donor specific supernatant of PBMCs. **(B)** Comparison between cell populations supplemented with EBM. **(C)** Comparison between cell populations supplemented with donor specific supernatant of PBMCs. $*p < 0.05$ and $**p < 0.01$.

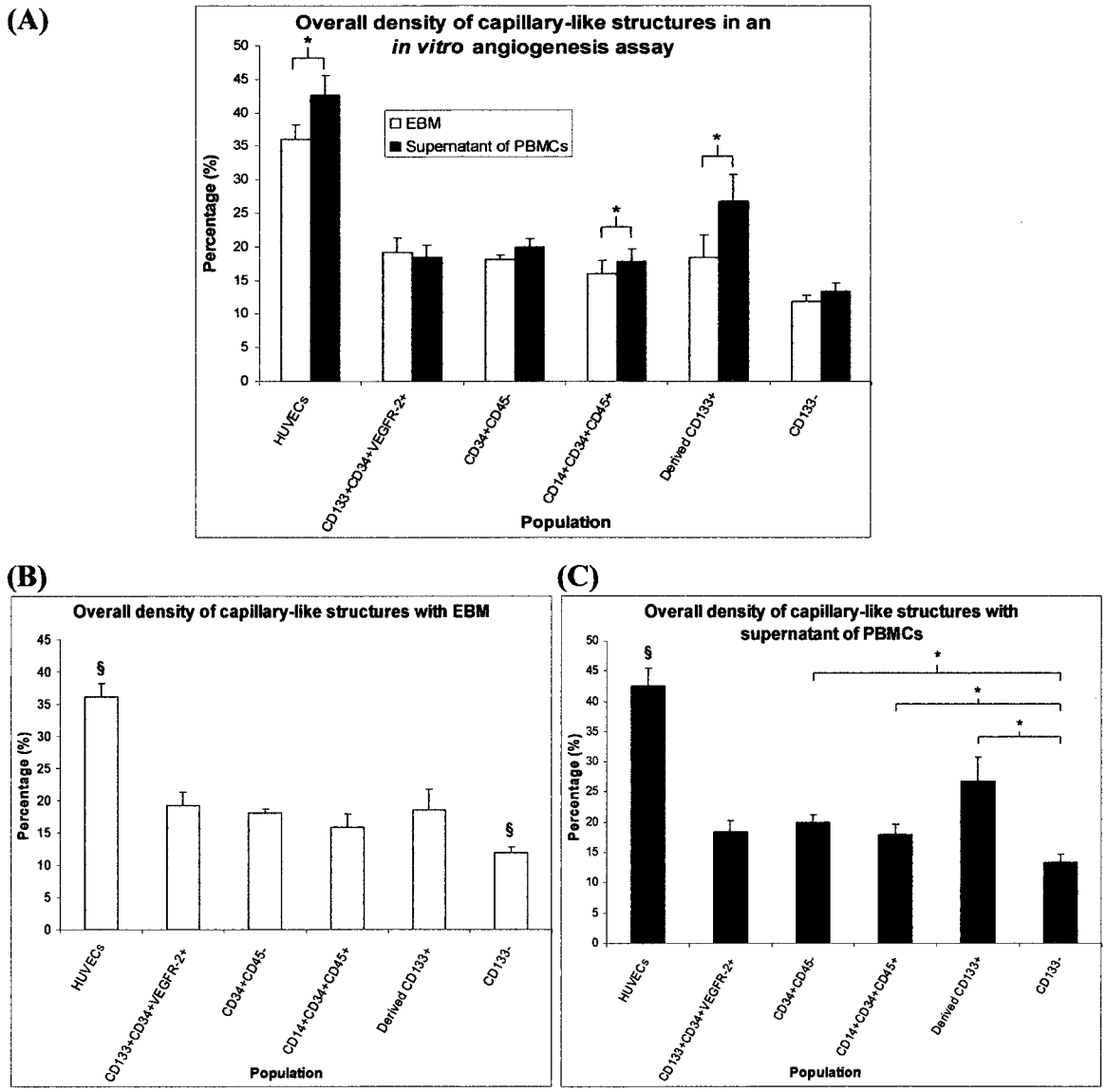


Figure 13: Overall density of capillary-like structures in *in vitro* angiogenesis assay (n=5). **(A)** Comparison of the cell populations' angiogenic potential when supplemented with EBM or donor specific supernatant of PBMCs. **(B)** Comparison between cell populations supplemented with EBM. **(C)** Comparison between cell populations supplemented with donor specific supernatant of PBMCs. $*p < 0.05$ and $^{\S}p < 0.05$ versus all cell populations.

Differentiation of CPCs in the *in vitro* Angiogenesis Assay

After an *in vitro* angiogenesis assay, the angiogenic matrix was digested to evaluate whether CPCs were differentiating into endothelial cells, as identified by specific endothelial cell surface markers (CD31 and VE-Cadherin), and taking a physical role in the angiogenesis process. The levels of expression for VE-cadherin were similar for all populations **(figure 14)**. Conversely, some significant differences were observed for antigen CD31 between the following populations; $CD133^{+}CD34^{+}VEGFR\text{-}2^{+}$ (12.5±1.6%), $CD34^{+}CD45^{-}$ (28.5±4.1%) and $CD34^{+}CD14^{+}CD45^{+}$ (21.5±3.6%).

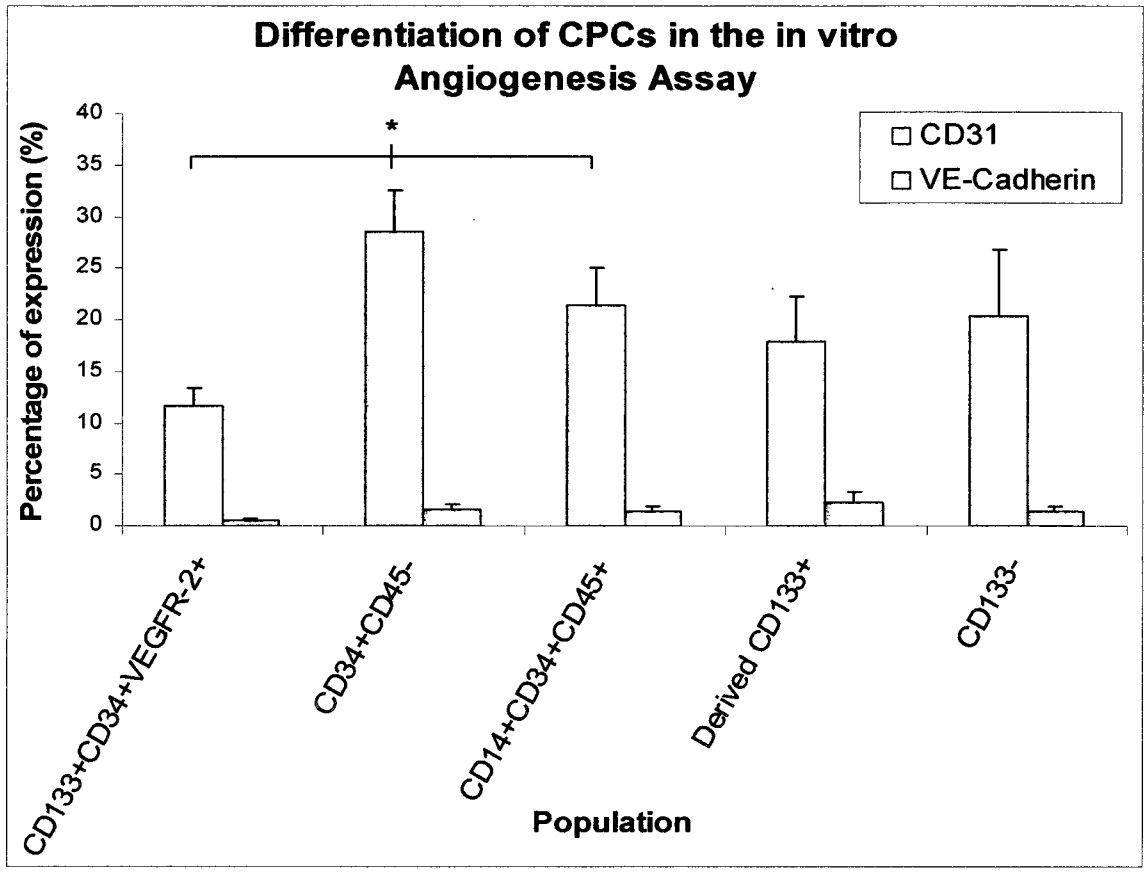


Figure 14: Differentiation of CPCs in the *in vitro* angiogenesis assay (n=5). The level of expression (%) on the CPC populations for the specific endothelial cell surface markers CD31 and VE-Cadherin. $*p < 0.05$

Cytokine Antibody Array

A human cytokine antibody array was performed to quantify the levels of growth factors and cytokines expressed by the different CPC populations in culture media. Compared to EBM, the different cell culture supernatants showed a tendency for increased levels of the following factors **(figure 15)**: IL-4, IL-8, IL-16, MCP-1, Oncostatin M, VEGF and TGF- β2. Their potential functions are summarized in **Table 3**. As previously demonstrated, supernatant (or conditioned medium) contains increased levels of factors that play a crucial role in adhesion, migration, angiogenesis, proliferation and others.[47] This correlates with the observed trend where functional activities, notably migration, were increased when cell populations were treated with supernatant of PBMCs.

GROWTH FACTORS/ CYTOKINES	ABBREVIATION	POTENTIAL FUNCTION
INTERLEUKIN-4	IL-4	PROMOTES THE PROLIFERATION AND DIFFERENTIATION OF ACTIVATED B-CELLS
INTERLEUKIN-8	IL-8	CELL MIGRATION; ANGIOGENESIS AND INCREASED EXPRESSION OF ADHESION MOLECULES
INTERLEUKIN-16	IL-16	CELL MIGRATION; CYTOPROTECTION
MONOCYTE CHEMOATTRACTANT PROTEIN-1	MCP-1	MONOCYTE MIGRATION; CELL ACTIVATION
ONCOSTATIN M	OSM	VEGF INDUCTION; CELL PROLIFERATION AND CYTOPROTECTION
VASCULAR ENDOTHELIAL GROWTH FACTOR	VEGF	CYTOPROTECTION; PROLIFERATION; MIGRATION AND ANGIOGENESIS
TRANSFORMING GROWTH FACTOR-BETA-2	TGF- B2	VESSEL MATURATION; CELL PROLIFERATION

Table 3: Growth factors and/or cytokines secreted by CPCs

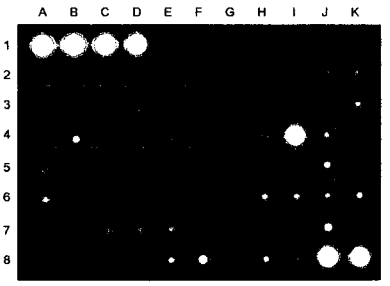
(A)

RayBio® Human Cytokine Antibody Array 5 Map

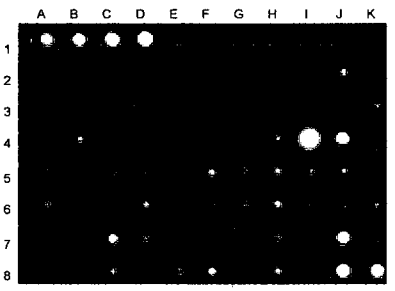
	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α
2	I-309	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12 p40p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β
4	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1	TNF-α	TNF-β	EGF	IGF-I	Angiogenin
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	6Ckine	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
8	NT-4	Osteopontin	Osteoprotegerin	PARC	PIGF	TGF-β2	TGF-β3	TIMP-1	TIMP-2	Pos	Pos

Note: GRO detects CXCL1, CXCL2, CXCL3; GRO-α detects only CXCL1
Note: IL-12 p40p70 detects both IL-12 p40 and IL-12 p70
Note: TGF-β1 detects only active form
Note: VEGF detects VEGF-165 and VEGF-121

(B)



(C)



(D)

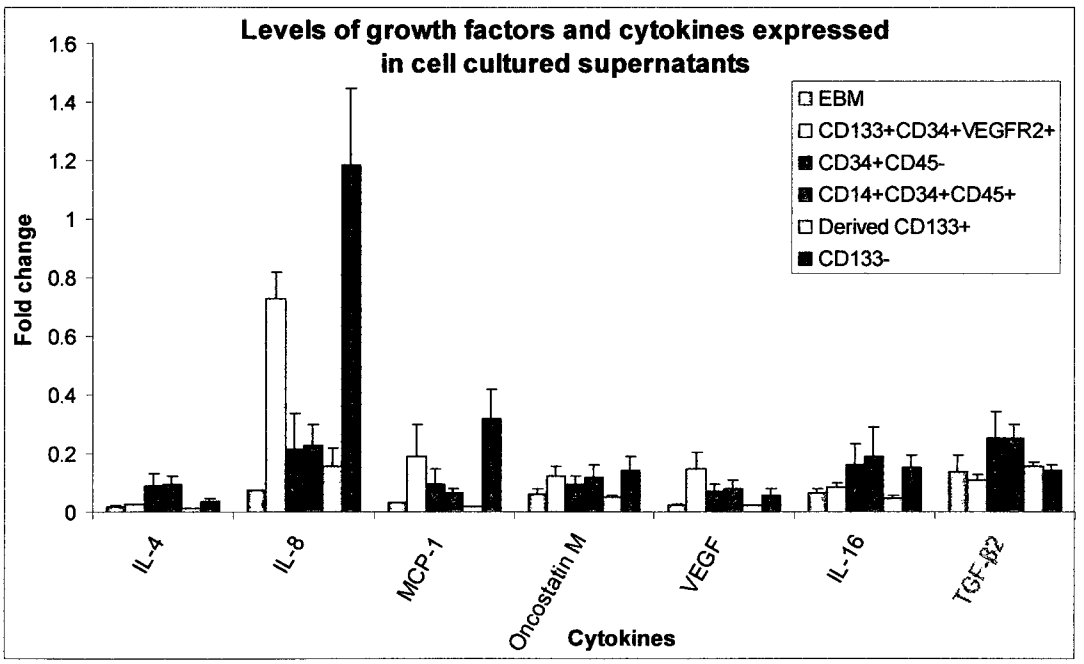


Figure 15: Cytokine antibody array (n=3). **(A)** Cytokines and growth factors map of array membrane provided by RayBiotech, Inc. **(B-C)** Representative images of cytokine antibody array membranes for: **(B)** EBM and **(C)** supernatant of $CD133^-$ cells. **(D)** The levels and intensity of particular cytokines/growth factors are expressed as fold changes and were determined by means of internal controls.

DISCUSSION

Endothelial progenitor cells (EPCs), a subset of circulating progenitor cells, have been studied over the years primarily for their capacity to regenerate the endothelial vasculature.[11,12,13] EPCs have been described by many studies as a heterogeneous population, varying in its potency, plasticity and more problematically in its phenotypic definition.[40] Several phenotypes have been associated with this controversial cell population, and functional comparisons between those different phenotypes are needed. The question remains as to which cell type should be use in cell-based therapy for optimal efficacy. Consequently, the main objective of this study was to compare the functional properties of different CPC subpopulations from the peripheral blood *in vitro*. The adhesive, migratory and angiogenic potential of five enriched isolated populations were tested.

CPC Adhesion *in vitro*

The population with the greatest adhesive potential was the derived $CD133^+$. In general, derived $CD133^+$ cells adhered better to fibronectin and collagen type I compared with the other four populations. Earlier data from our laboratory have shown that derived $CD133^+$ cells have greater adhesive potential compared to freshly isolated $CD133^+$ and combined $CD133^{+/-}$ cells.[47] Increased adhesiveness was previously demonstrated when cells are cultured on collagen type I compared to fibronectin, particularly for the derived $CD133^+$ cells.[47] For this study, a similar trend was observed with some of the cell populations, especially when cells were supplemented with the supernatant of PBMCs.

The $CD34^+CD14^+CD45^+$ and $CD133^+CD34^+VEGFR\text{-}2^+$ populations showed a trend towards possessing better adhesive potential compared to the $CD34^+CD45^-$, which

had the lowest number of adherent cells. Recent studies have argued that late outgrowth endothelial cells are derived from the $CD34^+CD45^-$ population and not from $CD45^+$ or $CD133^+$ populations.[43,44] On the other hand, it has been shown that both populations, $CD45^+$ and $CD133^+$, demonstrate endothelial cell-like characteristics proper to early EPCs, found to arise in short-term (4 to 7 days) culture assays.[44] Considering that late OECs make their appearances later in culture (7 to 21 days), this could partially explain the lower adhesiveness of $CD34^+CD45^-$ in this assay.[5] In addition, the higher expression of the cell surface antigen CD45 on the populations of cells with better adhesive potential evokes its role in the adhesion process. CD45 has been found to regulate the adhesion process of lymphocytes, and in particular, the T cell.[85,86] However, studies have also shown that the absence of CD45 increases the adhesiveness of lymphocytes (and leukocytes in general) to fibronectin, which does not correlate with our data.[85,87] Leukocytes are derived from hematopoietic precursors which are likely to be present in this study considering their markers overlap with EPCs, and our populations were heterogeneous. However, the role of CD45 in relation with angiogenesis in ischemic conditions or in an endothelial environment was not studied. Furthermore, CD45's relationship with the adhesive molecule CD34 might be interesting to pursue considering the investigated cell populations.

CPC *in vitro* Migratory Potential towards VEGF

From all five populations investigated, the greatest migratory potential, in response to VEGF, was observed for the derived $CD133^+$ cells in the presence of PBMC supernatant. The same tendency was observed with the EBM treatment, but statistical significance was not achieved. As described in the introduction, VEGF is a critical

chemokine during ischemic conditions. It is acknowledged that VEGF is up-regulated during ischemia, which activates the angiogenic progenitor cells (or EPCs) from the bone marrow to circulate through the peripheral blood and home to the site of ischemia.[10,30] Thus, in relation with cell-based therapy, the therapeutic population of cells chosen to be injected would be expected to follow the same fate. The increased number of migrated cells for a particular population might be directly linked to their expression level of one of the VEGF receptors; more specifically in this case VEGFR-2. VEGFR-2 in descending order of expression for the different populations was 57.1±10.9% ($CD133^+CD34^+VEGFR\text{-}2^+$), 35.1±4.7% ($CD14^+CD34^+CD45^+$), 26.1±2.6% ($CD34^+CD45^-$), 9.9±3.3% (CD133) and 0.8±0.4% (derived $CD133^+$) respectively. Aside from the derived $CD133^+$ population, a trend can be observed between the expression level of VEGFR-2 and the migratory potential of the cell population. Li *et al* have exploited the effect of the chemoattractant VEGF on EPCs' migration and their results partly support our observed data.[88] They showed that the migratory potential of EPCs was positively regulated in a VEGF concentration-dependent fashion and that inhibition of EPC's VEGF-R2 receptors negatively influenced their functional activity. As VEGF was found to influence multiple processes of angiogenesis, researchers have tried to exploit its function as a possible vascular therapeutic option. In parallel with cell-based therapy, genetically modified EPCs engineered to release specific proteins (for example VEGF) are available and could be used in a near future to optimize the benefits of this treatment.[89,90] The genetically modified EPCs could increase the up-regulation of VEGF at the site of ischemia, hence, optimizing the processes of angiogenesis.[8]

With regards to the low expression of VEGF from the derived $CD133^+$ population, its low level of expression for all antigens could be related to its lower viability. Flow analysis resulted in high levels of non-specific binding (IgG controls) which decreased the level of expression for the antigens investigated. A possible explanation could be linked to the derived $CD133^+$ handling and storage techniques. Therefore, high IgG controls could be the result of debris acquired from the derived $CD133^+$ culture protocol followed by a long storage period. Another justification could be related to the magnetic separation of the positive population; the technique and related protocol may have perturbed the cell membrane integrity resulting in low cell viability.[82] For future reference, a viability stain, like propidium iodide or Hoechst per example, should be used in order to discriminate the damaged cells from the viable ones.[91] Nonetheless, comparison (based on their CD133 expression) between $CD133^+CD34^+VEGFR\text{-}2^+$ and derived $CD133^+$ populations showed that more of the derived $CD133^+$ cells (89.4±2.6%) simultaneously co-expressed CD34 and VEGFR-2 compared to the $CD133^+CD34^+VEGFR\text{-}2^+$ (40.4±4.7%). This could partly explain its greater functional activity. Very little studies have been completed on $CD133^+$ cells derived from the $CD133^-$ fraction of PBMCs; therefore, further research will hopefully help us elucidate the derived $CD133^+$ cells' behaviour.

Characterization of Isolated CPC Populations by Flow Cytometry

The expression level of the following cell surface macromolecules was evaluated for the isolated populations investigated in this study; CD34, VEGFR-2, CD133, CD14 and CD45. The purity of the isolated populations was not absolute, which indicates that this study was conducted with populations enriched for specific markers. The fact that

significant differences were observed in the *in vitro* assays between the different populations suggests that enrichment for specific cell types may greatly affect the functional activity of the heterogenous population. This could be supported by studies of cultured EPCs, either early EPCs or late OECs, classified as heterogeneous or homogenous populations, which are based on populations expressing a variety of antigens at various levels.[18,92] The functional properties of both populations were intensively studied in the past years, and although their antigen expression may vary from one laboratory to another, their role in angiogenesis seems important.

CPC *in vitro* Angiogenic Capacity

Adhesion and migration were investigated in this study, since they are fundamental processes involved in angiogenesis. In the last few years, the role of EPCs in the angiogenesis process was debated. The angiogenic potential of the $CD133^+CD34^+VEGFR\text{-}2^+$ population, generally recognized as EPCs, was recently questioned.[36,43] Subsequently, the investigated CPC populations' vascular morphogenesis potential, more precisely its ability to form tubules or capillary-like structures, was analysed by using a short term *in vitro* angiogenesis assay.[31] All CPC populations failed to form capillary-like structures when seeded alone; however, when seeded with a mature endothelial cell population (HUVECs), CPCs contributed to the capillary-like structure formation. This observation is supported by data from other groups.[46,93] Zhang et *al* tested cultured (day 4, 14 and 28) EPCs in a similar *in vitro* angiogenesis assay and reported no tube-like formation for either populations, as opposed to HUVECs, which successfully formed tubules.[93] The same observations using an *in vitro* Matrigel assay, for the cultured population $CD14^+CD45^+CD34^+$, was made by

Kuwana and colleagues.[46] Case *et al* also demonstrated no capillary-like structures formation with the $CD133^+CD34^+VEGFR\text{-}2^+$ seeded alone on Matrigel.[43] They did, however, observe capillary-like structure from cultured endothelial colony-forming cells (ECFC; also referred by other as late OEC[5,9]) enriched from the $CD34^+CD45^-$ population. Other studies have also illustrated the vessel-forming capacity *in vitro* of the late OECs.[32,94] Comparison with our data indicates that functional activities of freshly isolated $CD34^+CD45^-$ may not be comparable to cultured late OECs $CD34^+CD45^-$ population (a population previously described by others[43,44]). The late OECs obtained from particular cell cultures have most probably acquired more specific endothelial cell surface markers, therefore contributing to their different functional properties. Direct functional comparisons between the freshly isolated and the cultured enriched $CD34^+CD45^-$ populations would be interesting to pursue. In summary, the outcome of this angiogenesis assay showed that isolated CPCs rely on the support, either directly (cell-cell interactions) or indirectly (cell-cytokine interactions), of a more mature endothelial lineage for their contribution to angiogenesis.

The angiogenic potential of the different cell populations was more specifically examined by analysing their participation and effect on vascular formation. More precisely, the CPCs' direct incorporation to tubule formation as well as their influence on the overall density of capillary-like structures was quantified. Of the five cell populations investigated, none was demonstrated to possess a significantly greater angiogenic potential. However, the derived $CD133^+$ cells contributed considerably more to tubule formation compared to the $CD34^+CD45^-$ and the $CD133^-$ populations, when treated with the supernatant of PBMCs. Previously, our laboratory had shown that derived $CD133^+$

cells contributed significantly more to tubule formation, when compared to freshly isolated $CD133^+$ cells or $CD133^+$ cells in combination with $CD133^-$ cells.[47] In this study, it is rather the freshly isolated $CD133^+$ population co-expressing the antigens CD34 and VEGFR-2 that was investigated. Moreover, technical differences in the isolation and culture of the cell populations used in these two studies may explain the observed functional assay differences with our previous data. This study showed no functional differences between the two populations enriched for antigens CD133, CD34 and VEGFR-2. Moreover, their contribution to tubule formation was closely comparable; analyses obtained for $CD133^+CD34^+VEGFR\text{-}2^+$ and derived $CD133^+$ population, respectively, were 2.40±0.50 vs 2.44±0.62 (EBM; p=0.94) and 3.10±0.72 vs 3.60±0.51 (supernatant of PBMCs; p=0.42). However, as both HUVECs and CPCs were labelled with a cytoplasmic red dye with the previous intention of better characterizing the capillary-like structures under fluorescence microscopy, it was difficult to evaluate exactly which cells were physically incorporated in the vascular-like structures.

The incorporation of EPCs or CPCs into vascular structures is a debatable topic. Previous work has reported that EPCs can incorporate to some extent within the tubule or capillary-like structure in *in vitro* angiogenic assays.[8,46,94] It has also been acknowledged that incorporation of late OECs into capillary-like structures compared to the early EPC was significantly superior.[94] However, others have conducted *in vivo* studies where EPCs did not incorporate into *de novo* vessels.[95,96] As a result, the role of EPCs in angiogenesis has been recently argued to be regulated through cytokine and growth factor secretion; a process referred to as the paracrine effect.[95] Further studies are needed in order to confirm if progenitor cells are physically incorporated into the vasculature to any

significant level, or if their contribution is through paracrine effects, or both. As for the overall density of capillary-like structures, the CPC populations showed enhanced angiogenic potential compared to the $CD133^-$ population in the EBM treatment. A similar trend was observed with the supernatant of PBMCs treatment. This observation could be explained by the notion that the $CD133^-$ population was deprived of a more primitive EPC population, which expressesed CD133, and therefore resulted in lower angiogenic abilities through paracrine effects. The derived $CD133^+$ cells showed a tendency to possess a greater angiogenic effect over all the populations investigated, in the supernatant of PBMCs treatment. Taken together, some populations seem to possess a better angiogenic potential compared to others which increases their physical or paracrine contribution to the endothelial vasculature formation.

The above discussed *in vitro* functional assays and the behavioural tendencies of the different CPC populations can be summarized in **table 4**. The derived $CD133^+$ population showed a tendency to have superior functional abilities, which was more pronounced in the supernatant of PBMCs treatment, while the $CD133^-$ population, which is not defined as an EPC population per se, translated in lower abilities.

Angiogenic CPC Differentiation into Endothelial Cells

As mentioned, direct incorporation of CPCs to *de novo* vessel formation is still questionable. Therefore, we further investigated the presence of CPCs in angiogenesis by quantifying the level of expression of specific endothelial cell surface markers that might have been acquired or altered during the process, through differentiation. The level of expression for CD31 and VE-cadherin of adherent CPCs in an *in vitro* angiogenesis assay

POPULATION	FUNCTIONAL ACTIVITY			
	ADHESION	MIGRATION	ANGIOGENESIS	
			Incorporation	Density of vasculature
CD133+CD34+VEGFR-2+	++	++	++/+++	++
CD34+CD45-	+	+	++	++
CD14+CD34+CD45+	++	++	++	++
Derived CD133+	+++	+++	++/+++	++/+++
CD133-	+	+	+	+
+ mild; ++ moderate; +++ highest				

Table 4: Representative comparison of the different isolated CPC populations' abilities in the *in vitro* adhesion, migration and angiogenesis assays.

were analysed. CD31 and VE-cadherin are both well known vascular cell adhesion molecules that are expressed on endothelial cells. It has been demonstrated previously that as circulating EPCs differentiate and lose their multipotency, their expression for CD31 and VE-cadherin increases. However, a low level of expression for both markers can be found on circulating EPCs.[97] Considering the level of expression detected by flow cytometry for all CPC populations investigated in this study, it was unlikely that any significant endothelial differentiation occurred. This statement is based on previous studies where CD31 and VE-cadherin expression of freshly isolated populations or of cultured 'early' EPCs, were evaluated. Studies on the early EPCs reported that CD31 expression was at a low to moderate level[18,32,94] and values between studies have been variable, ranging from 34.5±8% in one study[98], to 51.3±2.7% in another[18]. CD31 expression variability from one study to another may be due to donor variability and cell origin (peripheral blood vs bone-marrow cells derived). Our data showed lower CD31 expression for $CD133^+CD34^+VEGFR\text{-}2^+$ and derived $CD133^+$ populations, with

expression at 11.72±1.61% and 17.94±4.31% respectively. Alternatively, CD31 expression was significantly enhanced for the $CD34^+CD45^-$ population (28.52±4.05%; p=0.005) compared to the $CD133^+CD34^+VEGFR\text{-}2^+$. A plausible explanation could rely on the possibility that $CD34^+CD45^-$ population may be an endothelial precursor originating from a non-hematopoietic lineage which was found to generate late OECs *in vitro*.[43] Consequently, its level of expression for specific endothelial markers, namely CD31, could be higher compared to other early EPC populations such as $CD133^+CD34^+VEGFR\text{-}2^+$, which is defined as a hematopoietic derived cell population. As for VE-cadherin level of expression on early EPCs, studies are in agreement with our experimental data. A barely detectable to a low expression level was consistently reported regardless of the investigated population.[32,92,93,94] Taken together, it was unlikely that differentiation of CPCs to a more mature endothelial-lineage occurred. This supports the suggested hypothesis that EPC contribution to angiogenesis occurs mostly through paracrine effects.[35,56,98]

Effects of Cytokines/Growth Factors on CPC Function

As other studies postulated, the research of isolated populations for therapy may not be viable, due to lack of consideration of interactions between cells, cytokines, growth factors or other types of cells.[35,47,63] In support of this, the current study investigated whether interactions between subsets of CPCs and/or their cytokines were involved in mediating their function. For this, cells in *in vitro* functional assays were supplemented with conditioned medium, which we referred to as supernatant of PBMCs, and the effects were compared to the standard EBM culture medium. In particular, the derived $CD133^+$ cells were positively influenced when subjected to the donor specific

supernatant of PBMCs. More precisely, their functional activities (adhesive, migratory and angiogenic) were significantly enhanced when supplemented with cultured medium. The same trend was observed with the $CD34^+CD14^+CD45^+$ population. In addition, all CPCs demonstrated a greater migratory potential when subjected to donor specific supernatant of PBMCs. Similarly, Urbich and colleagues[63] observed increased migratory potential of mature endothelial cells and cardiac resident progenitor cells when exposed to the conditioned medium of EPCs. In addition, conditioned medium of EPCs was also found to increase functional activities of late OECs.[35] The present study concentrated on the supernatant of PBMCs, which may reflect more on the microenvironment found *in vivo,* and their affects on specific CPC subpopulations submitted to functional processes of angiogenesis. We therefore provide some insight on the cytokine release by 'early' EPCs, and hence, their role in angiogenesis through perhaps paracrine effects.[35,60] Furthermore, the cytokine and growth factors released by the five populations investigated in culture was evaluated. The cultured supernatants of each isolated CPC population had higher levels of expression for several cytokines/growth factors. Notably, some of these factors have been found to regulate processes involved in angiogenesis, such as IL-4, IL-8, MCP-1, Oncostatin M, VEGF, IL-16 and TGF-β2 (summarized in **table 3**).[56,99] Of these, the most studied in relation to EPCs and their role in angiogenesis are VEGF, MCP-1 and IL-8. IL-8 and MCP-1 (whose expression is significantly increased in response to ischemia), were described as strong chemoattractants for bone-marrow derived EPCs.[99,100] IL-8 secretion was previously demonstrated to be up-regulated by an 'early' $CD14^+$ EPC population and, through a paracrine manner, significantly enhanced late $CD14^-$ OECs angiogenic ablility.[35] Moreover, it was found to

increase endothelial cell permeability, as its activity was discovered to be closely associated to VEGF/VEGFR-2, and to regulate EPC homing to sites of ischemia.[99,101] As for MCP-1, its activity has been found to mediate EPC recruitment to sites of ischemia, and their subsequent adhesion and differentiation.[100,102] Altogether, our *in vitro* experimental data suggest that interactions between peripheral blood cell populations and/or their cytokines can regulate progenitor cells migratory function. Conversely, those interactions may have greater influences on particular progenitor cells and their functional potential. In general, elevated concentration of cytokines and growth factors, released by heterogeneous population of monocytes, increases the functional potential of CPCs.

Limitations

One of the major limitations of this study is the heterogeneity of the isolated CPC populations by FACS and autoMACS. Despite the facts that highly purified EPC populations remain somewhat heterogeneous because of the lack of specific markers to isolate those cells, the purity of the enriched populations investigated may have been suboptimal. The purity of the populations, especially the derived $CD133^{+}$, could have been increased by including a cell viability dye (ex. propidium iodide) with FACS separation, which would have lowered the expression of unspecific binding, as dead cells were acknowledged to non-specifically trap fluorochrome-conjugated antibodies.[103] Moreover, multiple sorts by FACS would have increased the phenotypic expression of a particular cell population.[82] It is not to forget that high levels of purity may be hard to achieve as EPC phenotypic characterization is considered labile (or unstable), and that a

simple experimental manipulation can alter its marker expression.[91] Also, the possibility of fluorochromes' endocytosis can not be dismissed.[104]

Conclusion and Future Considerations

This study established a functional comparison *in vitro* of five predominant phenotype populations and was found to may be relevant in angiogenesis. However, clinical research, by monitoring the faith of the CPCs, will indicate if our preliminary *in vitro* results will translate *in vivo*. It is acknowledged that two types of endothelial precursor can be obtained from *in vitro* culture: early EPCs and late OECs. This study did not address late OEC functional activity. However, to increase the success of revascularization in ischemic conditions, a combination of those cells may be considered as potential cells to administer in cell-based therapy. Perhaps the two cell populations could be independently injected at different time points or injection sites. The outcome would be interesting to investigate, as the two cell populations were shown to synergistically influence the angiogenesis process *in vitro* in a paracrine manner.[35]

In conclusion, this study compared the functional activities of different CPC subpopulations in three critical processes involved in myocardial revascularization: adhesion, migration and angiogenesis. The derived $CD133^+$ cells, a cell population generated from the $CD133^-$ cells, was demonstrated to potentially translate in superior outcome considering its tendency to possess enhanced functional abilities. Few studies have been reported on this novel cell population. However, this study, in addition to previous ones accomplished from our laboratory, provides some indication that this population, whose characteristics resemble that of early EPCs, warrants further investigation. All CPC populations failed to form capillary-like structures on their own;

however, when combined with a mature endothelial cell population, CPCs contributed to the formation of capillary-like structures. It was unlikely that this contribution occurred through differentiation, as significant endothelial marker expression was not observed. Moreover, functional abilities (particularly for migration) of the cells were up-regulated in the presence of monocyte cytokines/growth factors secretion, a process more commonly known as paracrine effect, and this mechanism may be necessary for CPCs to achieve optimal functional potential.

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APPENDIX A

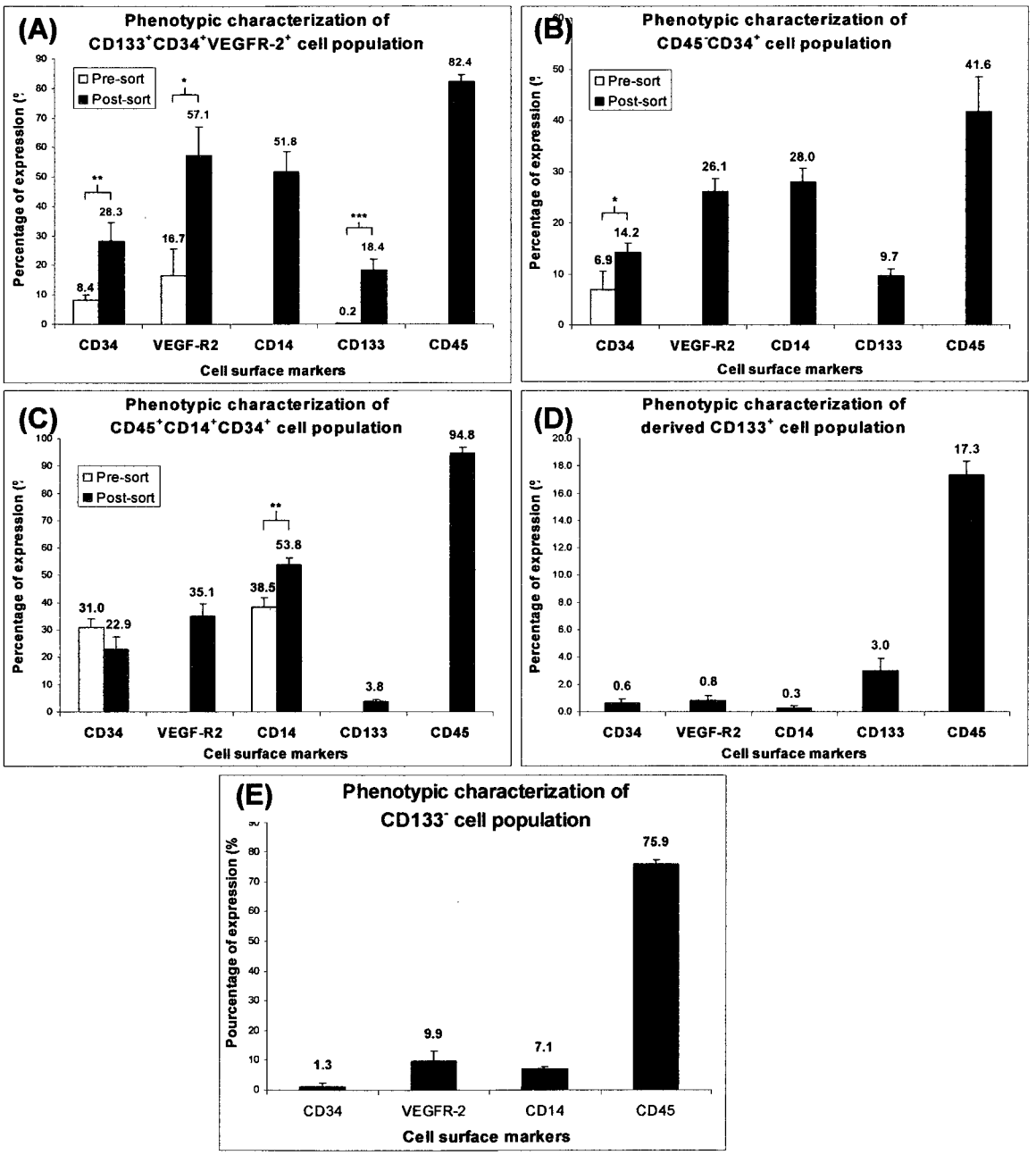

Figure 16: Flowcytometric phenotypic characterization (n=6). Percentage expression (%) for CD34, VEGFR-2, CD14, CD133 and CD45 antigens for the different isolated CPC populations before and after being sorted by FACS; $CD133^{+}CD34^{+}VEGFR2^{+}$ **(A)**, $CD45^{-}CD34^{+}$ **(B)**, $CD45^{+}CD14^{+}CD34^{+}$ **(C)**, derived $CD133^{+}$ **(D)**, and $CD133^{-}$ **(E)**.

APPENDIX B

(A) (B)

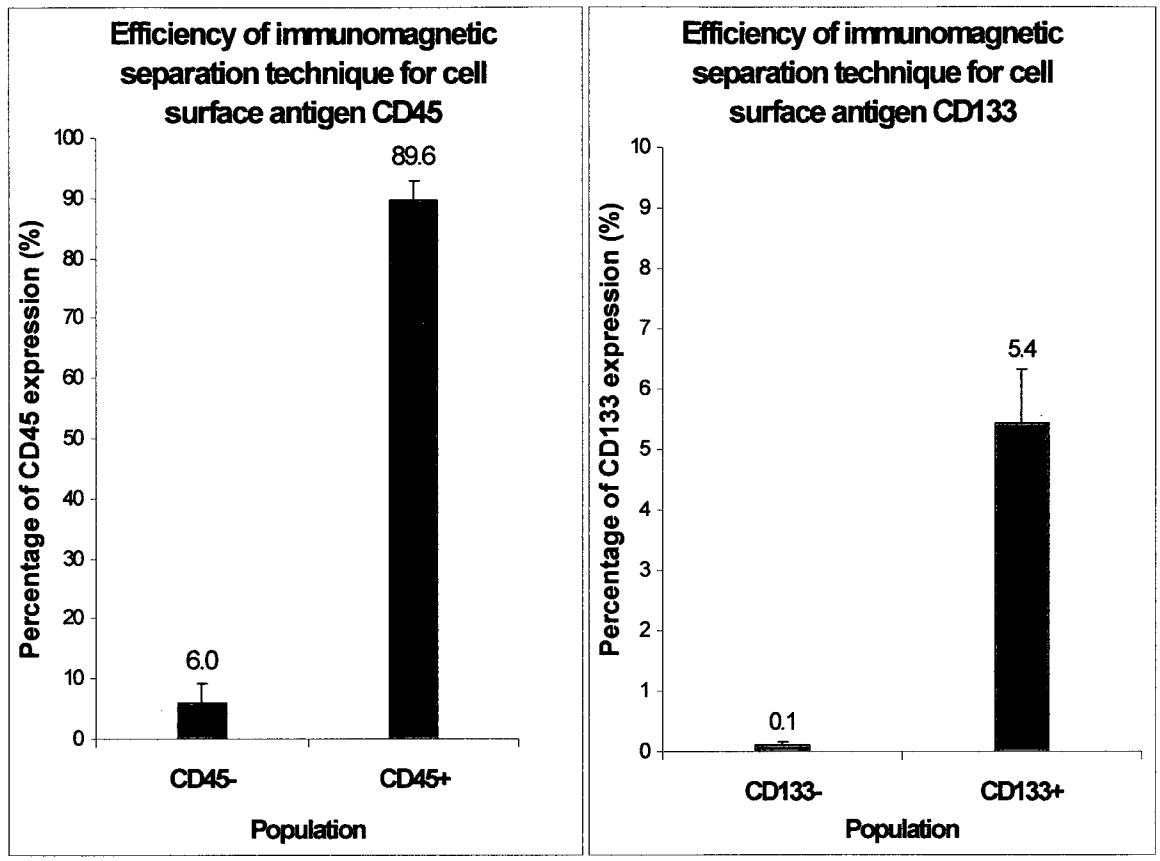

Figure 17: Immunomagnetic separation efficiency (n=3). Represent percentages of CD45 **(A)** and CD133 **(B)** expression for analogous antigen analyzed by flow cytometry after a magnetic separation with appropriate antigen-bound microbeads by autoMACS.