INTRODUCTION

Endothelial progenitor cells (EPCs) are a heterogeneous group that exist in a variety of developmental stages ranging from hemangioblasts to fully differentiated endothelial cells. The immature EPCs can be characterized by the expression of CD133, CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2) ***(Hristov et al., 2003).***

Circulating human $CD34^{+}/VEGFR2^{+}$ cells constitute a phenotypically and functionally distinct population that may play a role in neo-angiogenesis ***(Peichev et al., 2000)***. These cells promote vascular health by facilitating endothelial integrity and function and can be considered potential biomarkers to guide therapeutic interventions. Decreased level of $CD34^{+}/VEGFR2^{+}$cells have been associated with increased cardiovascular risk in patients with chronic illness such as systemic lupus erythromatosis, diabetes mellitus and hypothyroidism ***(Di Meqlio et al., 2010)***.

It has been shown that circulating CD34+ cell numbers significantly correlated with left ventricular mass index (LVMI); however, they did not correlate with LV systolic function marker (LVFS). Left ventricular (LV) hypertrophy is a well-known predictor of cardiovascular events independent of coronary artery disease ***(Devereux and Reidchek, 1977)***.

In patients with β-thalassemia, increased level of circulating endothelial cells reflects endothelial damage and denudation. Repair of the denuded endothelial might be crucial for the restoration of endothelial function ***(Kyriakou et al., 2001).*** Patients receiving regular blood transfusions have increased iron load which has an impact on the thrombotic response to arterial injury, vascular production of reactive oxygen species (ROS), and endothelium-dependent vasoreactivity. Also, carotid atherosclerosis is positively associated with serum ferritin independently of traditional cardiovascular (CVD) risk factors ***(Wolff et al., 2004)***.

The importance of arterial dysfunction in patients with β-thalassemia is now recognized, with reduced brachial flow mediated dilation and increased stiffness of the carotid artery ***(Cheug et al., 2002).*** The exact mechanism of impaired cellular proliferative capacity remains speculative. Induction of heme oxygenase-1 secondary to increased oxidative stress and iron overload in thalassaemia may play a role ***(Livrea et al., 1996).***

Increased serum erythropoietin documented in thalassemia patients despite repeated blood transfusion is associated with increase in the mobilization of circulating EPCs in human subjects ***(Heeschen et al., 2003)***. This might be considered an intrinsic homeostatic mechanism to restore endothelial function ***(Cheung et al., 2012).***

AIM OF THE WORK

The aim of this work is to determine the quantity of circulating $CD34^+/VEGFR2^+$ cells in young patients with β-thalassemia major as a potential risk marker of vascular dysfunction in those patients and assess their relation to the clinicopathological characteristics of patients including iron overload, efficacy of chelation therapy and the level of erythropoietin.

THE ENDOTHELIAL CELLS

The endothelium is the thin layer of cells that lines the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of blood vessel .The endothelium was considered to be inert, described as a layer of nucleated cellophane, with only non-reactive barrier properties ***(Galley and Webster, 2004; Berrich et al., 2012).***

The differentiation of mesodermal cells into hemangioblasts which leads to formation of first vascular structures that called primitive blood islands. The hemangioblasts form the center of the islands give rise to the hematopioteic stem cells whereas, the peripheral differentiate into angioblasts, the precursors of mature endothelial cells under influence of vascular endothelial growth factor ***(Risau and Flamme, 1995).***

Endothelial cells form multiple junctional complexes with their neighbours. Adherens junctions are protein complexes that mediate calcium dependent adhesion through homophilic binding of extracellular domains of transmembrane proteins called cadherins predominantly vascular endothelial cadherin (VE-cadherin/cadherin-5) ***(Takeichi, 1995).***

1- Circulating Endothelial Cells (CECs):

The CECs represent the desquamated but not apoptotic endothelial cells in circulating blood a useful marker of endothelial damage, they are rarely found in normal healthy individuals, in the order of 3 cells/ml. The increased levels of circulating endothelial cells in patients with vascular inflammation suggest a direct relationship between the number of these cells in the peripheral circulation and the extent of endothelial injury ***(Boos et al., 2006; Deanfield et al., 2007).***

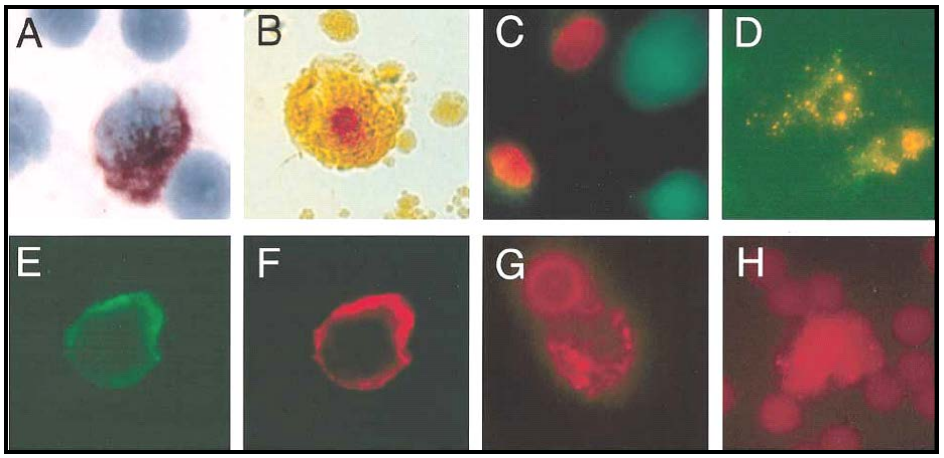

Figure (1): Examples of immunohistochemical and immunofluorescence analyses of circulating endothelial cells (CECs). A,a single CEC stained with alkaline phosphatase–conjugated P1H12 (red staining) and nuclei were counterstained with hematoxylin B, shows a CEC that is staining for both P1H12 (red area) and intracellular von Willebrand factor (brown area). C, shows live HUVEC (green-stained cytoplasm) and dead HUVEC (red-stained nuclei)).D, shows live CECsafter 11 days in cell culture (×600). Live circulating endothelial cells stain with both P1H12 (green area) and the cytoplasmic dye (orange areas). A cell identified as an endothelial cell by staining with fluorescein isothiocyanate–conjugated P1H12 (Panel E; ×1000) is also positive for CD36 (Panel F; ×1000). G shows a CECs with the punctate pattern of expression of intracellular P-selectin that is typical of unstimulated cells)The out-of-focus round object with the halo one of the immunomagnetic beads used to isolateCECs and H shows the diffuse pattern of expression of surface P-selectin that is typical of activated cells (×900) with numerous beads ***(Solovey et al., 1997).***

2- Circulating Endothelial Progenitor Cells (CEPCs):

A related circulating cell population is EPCs which originate from the bone marrow, rather than from vessel walls. Seen in small numbers in healthy individuals, their numbers tend to increase following vascular injury ***(Gill et al., 2001).***

Number of characteristic properties of EPCs have been proposed, notably positivity for CD34, kinase insert domain receptor (KDR) the receptor for vascular endothelial growth factor and CD133 (originally called AC133) which appears to be the most specific marker for early common EPCs. The loss of CD133 expression and the acquisition of other endothelial characteristics such as von Willebrand factor (vWF) reflect the transformation from anearly progenitor to a more committed or mature endothelial-like cell ***(Peichev et al., 2000; Hill et al., 2003).***

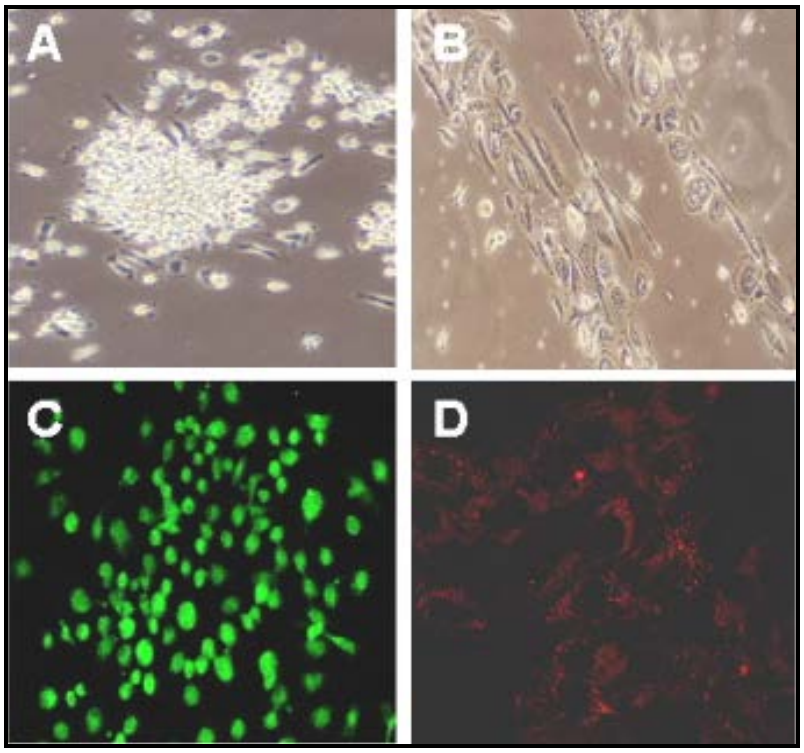

Figure (2): Isolation and expansion of endothelial progenitor cells (EPCs). Representative photomicrographs showing characteristic endothelial colony (A) and capillary-like structure (B) grown in vitro from human peripheral blood-derived EPCs.(C) Primary EPC cultures expressing CD34 and (D) uptaking LDL-cholesterol are also shown ***(Roura and Bayés-Genis, 2009).***

EPCs are not only involved in physiological neovascularization but also involved in wound healing, tissue regeneration in ischemia (e.g. myocardial ischemia, limb ischemia), tissue remodelling (diabetes mellitus and heart failure) and neovascularization and growth of tumors ***(Asahara et al., 2000).***

The principal mechanism of EPCs mobilization from bone marrow seems to depend on the activation of endothelial nitric oxide synthase (eNOs) in the presence of several mobilizing factors such as vascular endothelial growth factor (VEGF) and placental growth factor ***(Li et al., 2006).***

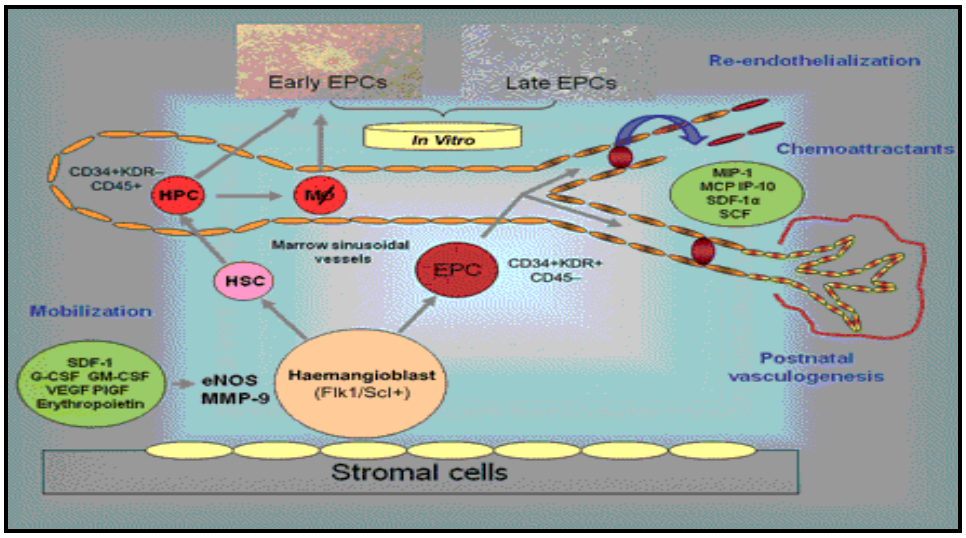

Figure (3): Mechanism and mediators of EPC mobilization. Adult bone marrow contains the haemangioblast that are mobilized by several factors mainly through NO and MMP-9-mediated mechanisms, share common antigens, like the CD34, and differ for the expression of CD45, the common leucocyte antigen that lacks on true circulating endothelial progenitor**.**eNOS-derived nitric oxide(NO),stromal-derived factor-1 (SDF-1)placental growth factor (PIGF),vascular endothelial growthfactor (VEGF),granulocyte colony-stimulatingfactor (G-CSF)**,** granulocyte monocyte colony-stimulating factor (GM-CSF)**,**matrix metalloproteinase-9 (MMP-9) ***(Leone et al., 2009).***

EPCs release from bone marrow is mediated by eNOs-derived Nitric oxide (NO) produced by the regulatory components of bone marrow micro-environment, osteoblasts and endothelial cells. Substances that increase NO bioavailability like growth hormone and insulin like growth factor increase EPC levels in contrast, higher levels of endogenous substances that impair NO bioavailability are associated with lower levels of EPCs and inhibit mobilization and differentiation ***(Thum et al., 2005; Thum et al., 2007).***

Activation, proliferation and migration of EPCs mediated by VEGF glycoprotein which synthesized by normal cells and up-regulated by hypoxia. Transcription factors like hypoxia inducible factor 1 are activated leading to increased transcription of VEGF which stimulates VEGFR1 and VEGFR2 present on endothelial cells and haematopoietic stem cells ***(Nakamaura et al., 2004; Dery et al., 2006).***

Physiological factors mobilizing EPCs from bone marrow includes physical exercise and estrogens which lower incidence of cardiovascular events in pre-menopausal women. While, suppressive factors as age, family history of coronary artery disease (CAD), hypertension, cholesterol level, increase oxidative stress and reduce NO bioavailability resulting in depletion of EPCs ***(Strehlow et al., 2003; Michaud et al., 2006).***

In growing tumors a number of other factors like fibroblast growth factor (FGF), stromal derived factor 1 (SDF-1), osteopontin, monocyte chemoattractant protein-1 (MCP-1/CCL2) and chemokine eotaxin (CCL5) help in EPC mobilization. EPCs are then released into circulation by activation of matrix metalloproteinase-9 (MMP-9) which degrades the extracellular matrix ***(Heissiget al., 2002; Liu and Velazquez, 2008)***.

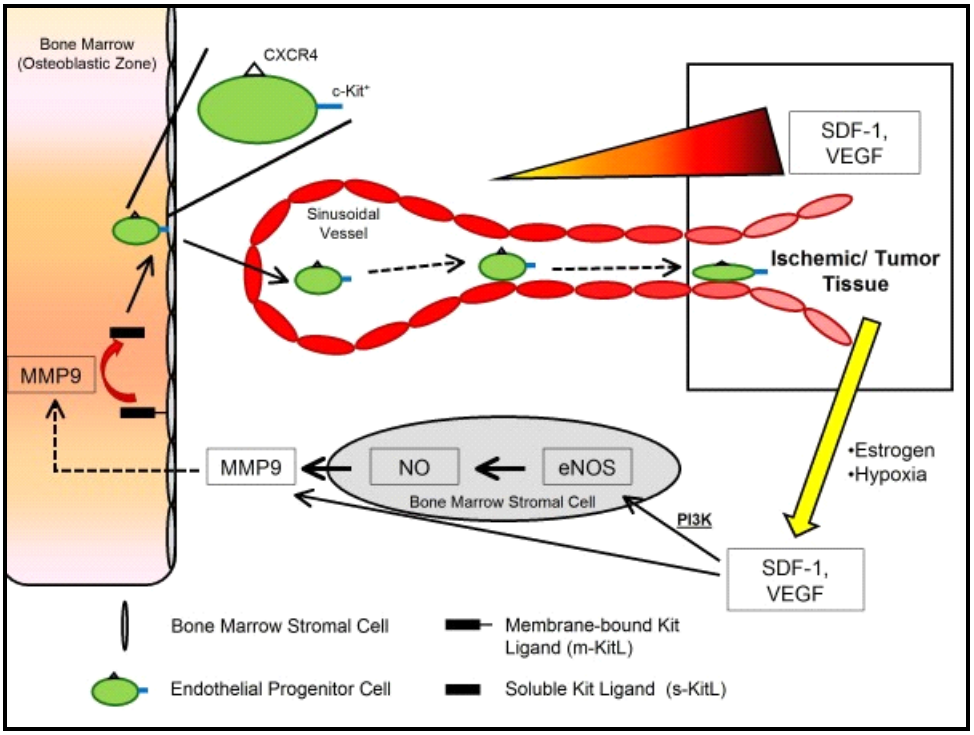

Figure (4): Trafficking of EPCs to ischemic/tumor tissues as directed by major cytokine/chemokine expression. Endothelial progenitor cell homing from the bone marrow niche to sites of neovasculogenesis is dependent a cytokine/chemokine gradient. chemokine receptors including (CXCR-4)eNOS-derived nitric oxide(NO),stromal-derived factor-1 (SDF-1), vascular endothelial growthfactor (VEGF),,matrix metalloproteinase-9 (MMP-9) ***(George et al., 2011).***

Function:

The physiological function of circulating EPCs is to maintain vascular integrity which is also crucial in the pathogenesis of various diseases with vascular insult. The vasculogenic potential of EPCs is also used by tumors to facilitate their growth and metastasis ***(Fang and Xiao-Qin, 2010).***

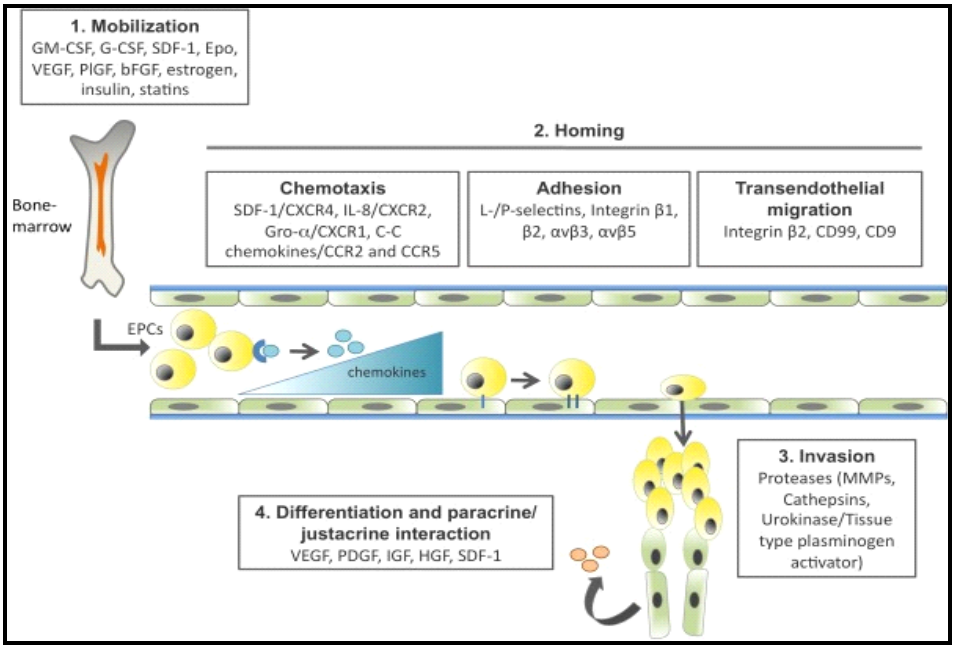

Figure (5): Molecular mechanism regulating the multiple steps of endothelial progenitor cell biology during postnatal vasculogenesis. Recruitment and incorporation of EPCs into angiogenic sites requires a coordinated multistep process including mobilization, chemoattraction, adhesion, endothelial transmigration, migration, tissue invasion, in situ differentiation and paracrine and/or juxtacrine factor production. The major molecular mechanisms that have been implicated in the distinct steps of EPC biology are indicated ***(Fusenig and Marmé, 2008).***

Marker of endothelial progenitor cells:

EPCs as well as mature endothelial cells and other circulating cell types commonly express a great variety of endothelial lineage-specific markers, among which are vascular endothelial growth factor receptor (VEGFR)-2, vascular endothelial (VE)-cadherin, von Willebrand factor (vWF), CD117, Tie-2, E-selectin, CD146 ,CD31 and Sca-1 ***(Walenta et al., 2005).***

Immature bone marrow-derived EPCs are positive for CD133, VEGFR-2 and CD34 but do not express vWF and VE-cadherin. The EPCs found in peripheral circulation have lost CD133 and are positive for CD34 and VEGFR-2, whereas mature endothelial cells additionally show high expression of VE-cadherin and vWF ***(Nishikawa, 2001)***.

CD133 (originally called AC133) appears to be the most specific marker for early common EPCs. The loss of CD133 expression and the acquisition of other endothelial characteristics such as vWF reflect the transformation from an early progenitor to a more committed or mature endothelial-like cell, a process which takes place during the progress from native sources to target vascular sites ***(Hristov et al., 2003; Ingram et al., 2005).***

CD31 (platelet-endothelial cell adhesion molecule-1) (PECAM-1**)**. A transmembrane glycoprotein expressed by

endothelial cells, platelets, monocytes, neutrophils, and certain T cell subsets that plays a key role in removing aged neutrophils, tissue regeneration, neutrophil recruitment in inflammatory responses, transendothelial migration of leukocytes, as well as in cardiovascular development ***(De Lisser et al., 1994)***.

CD146 (Melanoma Cell Adhesion Molecule) A Ca2+-independent cell adhesion molecule belonging to the immunoglobulin superfamily, involved in heterophilic cell interactions. CD146 is also expressed on metastatic lesions and advanced primary tumours, and thus has been suggested to play an important role in tumour progression and metastasis ***(Boneberg et al., 2009).***

Tie2 A member of the receptor tyrosine kinase superfamily, expressed predominantly on endothelial and hematopoietic progenitor cells. Tie2 is essential for blood vessel formation and maintenance. It binds to angiopoietin-1 with high-affinity and induces the signaling pathway of both cell migration and cell survival via the related mediator such as growth factor receptor bound protein 2(GRB2) and nontransmembrane human protein-tyrosine phosphatase that contains two Src homology 2 (SH2) domains SH-PTP2. Deficiency in TI2 gene is associated with inherited venous malformations ***(Sato et al., 1998)***.

VE-Cadherin (CD144, CDH5) A classical cadherin from the cadherin superfamily with calcium-dependent activity. VE-Cadherin is required for maintaining a restrictive endothelial barrier and indispensable for proper vascular development. It also has a role in maintaining newly formed vessels ***(Breviario et al., 1995; Harris and Nelson, 2010).***

Von Willebrand factor (vWF) is a multimeric glycoprotein that is synthesized exclusively in endothelial cells and megakaryocytes**.** Most, if not all, circulating plasma vWF is derived from the endothelium. So, plasma levels of vWF should reflect endothelial function, and abnormal levels would indicate endothelial dysfunction and damage ***(Chong et al., 2003).***

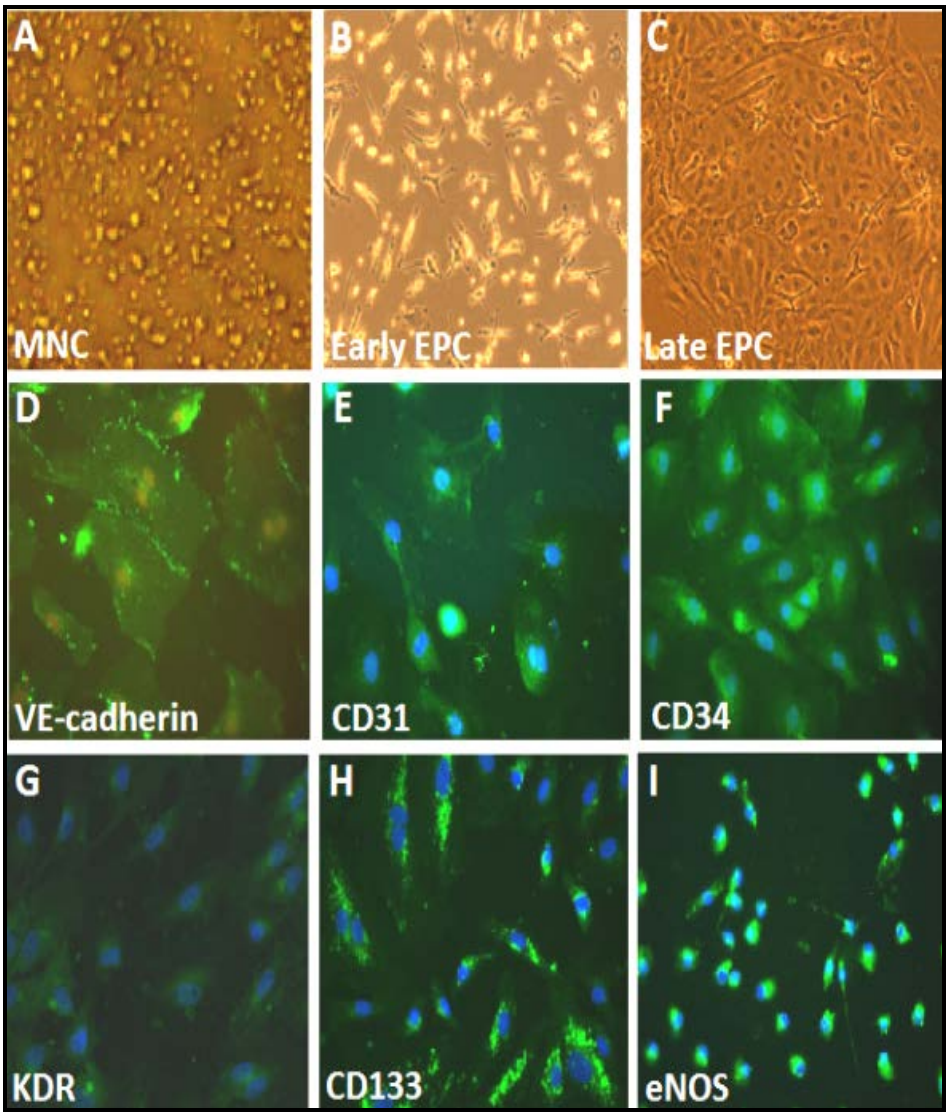

Figure (6): Morphology andcharacterization of human endothelial progenitor cells (EPCs) from peripheral blood**. (A)** Peripheral blood mononuclear cells (MNCs) were plated on a fibronectin-coated culture dish on the first day. (B) Four days after plating, adherent early EPCs with a spindle shape were shown. (C) Three weeks after plating, ECFCs with a cobblestone-like morphology were selected, reseeded, and grown to confluence. (D-I) ECFC characterization was performed by immunohistochemical staining. Most of the EPC expressed endothelial and hematopoietic stem cell markers, VE-cadherin, PECAM-1 (CD31), CD34, KDR, AC133, and eNOS, which are considered critical markers of EPCs. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for the nuclei (blue) ***(Chiang et al., 2012).***