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A Systematic Review of Endothelial Colony-Forming Cells

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Abstract: Endothelial Progenitor Cells (EPCs) may play a crucial role in postnatal vasculogenesis which contribute to the development of vascular biology. So far, there has not a consensus definition of EPCs. In 2004, Ingram developed a single-cell clonogenic assay and identified a unique cell population based on their proliferative potential in human umbilical cord blood and adult peripheral blood and named this cell population as Endothelial Colony-Forming Cells (ECFCs). Accumulating data suggest that ECFCs possess all the characteristics of EPCs including robust clonal proliferative capacity and the ability to form *de novo* blood vessels with a lumen *in vivo* and capillary-like tube *in vitro*. Therefore, ECFCs may be true EPCs and have been highly concerned as a superior type of cells to treat patients with vascular diseases. This review will focus on the characteristics, hierarchy, expansion, functions of ECFCs. Researchers attempt to provide a comprehensive understanding of the cells to promote the development of cell-based therapy for patients suffering from vascular diseases.

Key words: Endothelial colony-forming cells, endothelial progenitor cells, neovascularization, hierarchy, expansion

INTRODUCTION

Endothelial Progenitor Cells (EPCs) were first described by Asahara *et al*. (1997) in vasculogenesis which challenged the traditional conception that neovascularization occurs exclusively via the proliferation of pre-existing resident vessel wall endothelial cells. So far, there has not been a uniform definition of EPCs which at least have a robust clonal proliferative capacity form secondary endothelial cell colonies on replating and form blood vessels *in vivo* (Yoder *et al*., 2007). Recently, putative EPCs have been classified as three different cell populations including CFU-Hill cells (also termed as CFU-ECs), Circulating Angiogenic Cells (CACs) and Endothelial Colony-Forming Cells (ECFCs) which can be isolated from human Mononuclear Cells (MNCs) by three different assays (Prater *et al*., 2007; Hirschi *et al*., 2008). CFU-Hill cells and CACs mainly composed of both immune cells (T-and B-cells) and myeloid cells are also referred to as early outgrowth EPCs (Yoder *et al*., 2007; Hirschi *et al*., 2008) which have the characteristics of both proangiogenic macrophages and hematopoietic cells and never show the whole characteristics of EPCs. In contrast, ECFCs, also termed as late outgrowth EPCs (Prater *et al*., 2007; Hirschi *et al*., 2008), blood outgrowth endothelial cells (Lin *et al*., 2000; Hur *et al*., 2004) and Bone Morphogenetic Proteins (BMP)-positive endothelial precursors (Smadja *et al*., 2008) are endothelial cell origin and show all the properties of EPCs (Hirschi *et al*., 2008; Critser and Yoder, 2010). ECFCs possess high proliferative potential and have a capacity for self-renewal and spontaneously form functional human-murine chimeric blood vessels on implantation in immunocompromised mice (Ingram *et al*., 2004; Rohde *et al*., 2007; Yoder *et al*., 2007; Hirschi *et al*., 2008). The endothelial cell potential of ECFCs can be revealed by tube formation with a lumen *in vitro* and *in vivo* engraftment into functional blood vessels which can distinguish ECFCs from hematopoietic cells including CFU-Hill cells and CACs. Some studies have shown that CFU-Hill cells are not EPCs whereas ECFCs may be considered of true EPCs that can clonally give birth to endothelial progeny form endothelial tubes with a lumen *in vitro* and account for the functional lining of vascular structures *in vivo* (Ingram *et al*., 2004; Yoder *et al*., 2007; Hirschi *et al*., 2008; Critser and Yoder, 2010). Thus, ECFCs may serve as potential cell therapy products to vascular regeneration and tissue repair.

ISOLATION, CHARACTERISTICS AND HIERARCHY OF ENDOTHELIAL COLONY-FORMING CELLS

ECFCs were first isolated and characterized by *in vitro* colony-forming cell assays in 2004 (Ingram *et al*., 2004). Specifically, peripheral or umbilical cord blood-

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derived mononuclear cells were isolated and seeded onto tissue culture plates precoated with type I rat tail collagen in Endothelial Growth Media (EGM-2). After 24 h of culture, non-adherent cells and debris were discarded, adherent cells were washed with complete EGM-2 medium and medium was changed daily for 7 days and then every other day until the first passage. Well-circumscribed monolayers of cobblestone-like colonies appeared between 5 and 10 day for cord blood and day 14 and 22 day for peripheral samples. ECFC colonies were derived from adherent cells. ECFCs show high proliferative capacity, achieve at least 100 population doublings form at least secondary and tertiary colonies upon replating and retain high levels of telomerase activity. ECFCs express a profile of endothelial cell-specific surface markers which are not specific to ECFCs such as CD31, CD105, CD144, CD146, von Willebrand Factor (vWF), kinase insert domain-containing receptor (also known as vascular endothelial growth factor 2 receptor) and Ulex Europeus Agglutinin-1 (UEA-1) (Ingram *et al*., 2004; Yoder *et al*., 2007; Critser and Yoder, 2010). In addition, ECFCs also express the markers of BMP2/4 which appear to be specific (Smadja *et al*., 2008). ECFCs do not express hematopoietic and monocytes/macrophage cell surface proteins CD14, CD45 and CD115. ECFCs can incorporate Acetylated-Low Density Lipoprotein (Ac-LDL) and upregulate Vascular Cell Adhesion Molecule (VCAM-1) (Yoder *et al*., 2007).

The most important properties of progenitor cells are their clonogenic and proliferative potential (Zon, 2001). Based on this paradigm, ECFCs are organized in a Hierarchic Model of progenitor cells using proliferative potential as a defining aspect in human blood and blood vessel (Ingram *et al*., 2005a, b). Similarly, a hierarchy of ECFCs exists in the peripheral blood of the rhesus monkey (Shelley *et al*., 2012). A single cell assay developed by Ingram *et al*. (2004) makes it possible to determine the clonogenic and proliferative potential of ECFCs derived from umbilical cord or adult peripheral blood. The studies have shown that adult peripheral blood-derived ECFCs have the ability to expand for 20-30 population doublings, termed Low Proliferative Potential Endothelial Colony-Forming Cells (LPP-ECFCs), colonies from which contain >50 cells. Umbilical cord blood-derived ECFCs however are able to expand for 100 population doublings without senescence and are capable of forming secondary and tertiary colonies on replating, termed High Proliferative Potential Endothelial Colony-Forming Cells (HPP-ECFCs), colonies from which contain between 1000 and 2000 cells in a 14 days culture period. Clonal progeny of HPP-ECFCs contain higher telomerase activity than those of LPP-ECFCs (Ingram *et al*., 2005b). Both HPP-ECFCs and LPP-ECFCs can differentiate into mature endothelial cells. HPP-ECFCs can give rise to at least secondary HPP-ECFCs and differentiate into LPP-ECFCs; however, LPP-ECFCs can not give rise to secondary LPP-ECFCs moreover, HPP-ECFCs derived cells have more higher expansion potential than those of LPP-ECFCs (Ingram *et al*., 2004, 2005a). Some studies have demonstrated that HPP-ECFCs *in vitro* preferentially account for vascular tubule formation in low oxygen tensions (Zhang *et al*., 2009). Thus, the existence of hierarchic manner of ECFCs provides a much better choice for cell therapy products using the optimal HPP-ECFCs which may play a key role in neovascularization and tissue repair.

ENDOTHELIAL COLONY-FORMING CELL EXPANSION UNDER HUMANIZED CONDITIONS

ECFCs have been successfully isolated from peripheral blood of both adult and infant and human umbilical cord blood including term and preterm infants but the frequency of ECFCs is low (Ingram *et al*., 2004, 2005b; Yoder *et al*., 2007; Javed *et al*., 2008; Hoesli *et al*., 2010). In order to study ECFC functions *in vivo* and *in vitro*, it is necessary to propagate ECFCs. The conventionally established ECFC propagation methods begin with density-gradient separation and rely on Fetal Bovine Serum (FBS) however, the prerequisite of the methods is ECFCs adherence to culture surface coated by collagen mostly of animal origin (Prasain *et al*., 2012). As far as transplantation purposes are concerned, the methods of ECFCs expansion under FBS and animal origin matrix protein conditions are liable to xenoimmunization and transmission of pathogens. Thus, the abandonment of animal-derived surrogates is deemed to be a pivotal requirement for standardized clinical large-scale enrichment of human ECFCs. Pooled Human Platelet Lysate (pHPL) has been found to replace FBS and can be prepared as an efficient supplement for human ECFC cultures based on a protocol (Reinisch *et al*., 2009). For the purpose of surmounting the problem in unmanipulated peripheral blood and umbilical cord blood current novel protocols of methods of ECFC propagation replace FBS with pHPL in all steps and leave tissue culture plates uncoated (Reinisch *et al*., 2009; Reinisch and Strunk, 2009; Hofmann *et al*., 2012). After long term enrichment and cryopreservation ECFC proliferative potential, phenotype, hierarchy, vessel-forming function and genomic stability seem to be maintained (Reinisch *et al*., 2009; Estes *et al*., 2010; Prasain *et al*., 2012). The creative isolation and enrichment approach under humanized conditions is a major breakthrough which is

more efficient and applicable than conventional techniques (Hofmann *et al*., 2012). Thus, larger numbers of ECFCs propagated in this way are suitable for further investigations and may become a source of cell therapy products in human subjects (Hofmann *et al*., 2009, 2012).

CAPACITY OF FORMING BLOOD VESSEL AND INFLUENCING FACTORS OF ENDOTHELIAL COLONY-FORMING CELLS

A growing body of evidence indicates that neovascularization does not exclusively rely on proliferation of local endothelial cells but also involves bone marrow-derived circulating stem cells (Asahara *et al*., 1997). Tube formation appears to be specific to endothelial cells and tube formation potential of endothelial progenitor cells can be reliably tested in 3-dimension culture systems mainly composed of fibrin matrix. In contrast other cell types typically do not form tubes in such matrices (Hirschi *et al*., 2008). ECFCs have been classified as EPCs that exhibit essential progenitor characteristics including tube formation (Ingram *et al*., 2004, 2005a, b). ECFCs have been demonstrated to directly participate in neovascularization. Using a well-established *in vivo* vasculogenesis assay (Schechner *et al*., 2000), ECFCs were suspended in a mixed solution containing rat tail collagen I, human fibronectin, sodium bicarbonate, FBS, complete EGM-2, after polymerizing and incubating, gels were bisected and implanted into the flank of NOD/SCID mice. At 2-4 weeks, the grafts were analyzed for chimeric blood vessel formation. This assay showed that ECFCs formed chimeric vessels perfused with mouse red blood cells (Yoder *et al*., 2007). Additional investigators have also shown that ECFCs implanted into NOD/SCID mice can form chimeric vessels (Melero-Martin *et al*., 2007; Au *et al*., 2008). Perhaps, implantation of the intended cells and quantitation of the formation of blood vessel may be the most efficient strategy to assess the capability of the cells as postnatal vasculogenic cells and based on this paradigm, ECFCs may be the only cell with this activity (Yoder *et al*., 2007).

EPCs encompass different cell populations and therefore conflicting roles for EPCs in vessel formation have been described by many investigators (Rehman *et al*., 2003; Hur *et al*., 2004; Yoder *et al*., 2007). The early outgrowth EPCs and late outgrowth EPCs are mainly subpopulations of EPCs which seem to be considered to play different role in neovascularization. Currently, the roles of the two kinds of different subpopulations of EPCs in neovascularization may be that after the release of chemokines including Stromal Derived Factor-1 (SDF-1) and VEGF under certain stress conditions (Ceradini *et al*., 2004; Kleinman *et al*., 2007), the early outgrowth EPCs are recruited to the sites of neovascularization or impaired endothelium to initiate the process of neovascularization and release chemokines to recruit ECFCs. Subsequently, ECFCs derived from circulation, nearby endothelium and bone marrow migrate into the sites of impaired vessels which form *de novo* blood vessels by sprouting way and repair the impaired tissue (Ingram *et al*., 2005a; Yoder, 2009). The early outgrowth EPCs promote angiogenesis in a paracrine manner and can not form de-novo vessels *in vivo* (Rehman *et al*., 2003; Hirschi *et al*., 2008; Sieveking *et al*., 2008; Van Beem *et al*., 2008) whereas ECFCs directly participate in neovascularization via proliferation and contribution to the lining of the neovasculature. Furthermore, ECFCs are able to form *de novo* vessels *in vivo* and play an important role in tissue repair (Yoder *et al*., 2007; Sieveking *et al*., 2008) (Fig. 1).

Neovascularization induced by ECFCs is a complex process in which many influencing factors are involved. Hyperglycemia is characteristics of diabetes mellitus which is a common and frequently-occurring disease and its major complications are micro and macrovascular disorders. The primary causative factors for them are unclear. Hyperglycemia can activate protein kinase C which may be responsible for decreasing capacity of ECECs in neovascularization leading to micro-and macrovascular disorders (Ingram *et al*., 2008).

To test whether hyperglycemia affects ECFC functions, Ingram *et al*. (2008) using colony-forming assays, matrigel assays and xenograft transplant demonstrated that hyperglycemia reduced cord blood ECFC proliferation and impaired the capacity of forming

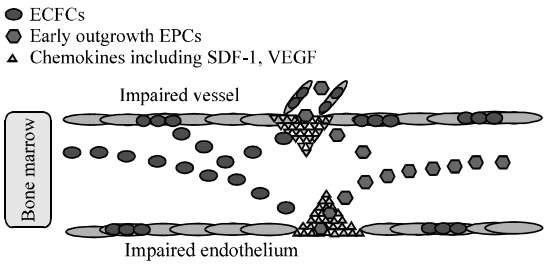

Fig. 1: The roles of two kinds of different subpopulations of EPCs in neovascularization and endothelium repair. Impaired vessels release chemokines which direct circulating early outgrowth EPCs moving into the sites of neovascularization or impaired endothelium to initiate and facilitate neovascularization. Subsequently, ECFCs recruited into the sites of impaired vessels under certain stress conditions directly participate in neovasularization and endothelium repair

blood vessel *in vitro* and *in vivo* which resulted from enhancing senescence of ECFCs, not apoptosis. The similar results of hyperglycemia-induced senescence of ECFCs were also confirmed by Chen *et al.* (2007).

Activated oxygen species, produced in ischemic tissues are responsible for apoptosis in many cells. To test whether ECFCs are sensitive to oxidant stress, Ingram *et al.* (2007) cultured ECFCs with increasing H_2O_2 in colony-forming assays and matrigel assays. The results showed that the experimental groups, compared with untreated controls, displayed significantly decreased clonogenic capacity and ability to form vessels *in vitro* and *in vivo* which correlated with activation of a redox-dependent stress-induced kinase pathway. Likewise, the laboratory strengthened the results that ECFCs treated with H_2O_2 showed reduced proliferative ability, impaired capability of migration and tube formation *in vitro* (Wei *et al.*, 2012). Numerous diseases before clinically significant vascular disorders, exhibit enhanced oxidant stress *in vivo* and enhanced Reactive Oxygen Species (ROS) production which are the hallmark of acute ischemia (Taniyama and Griendling, 2003; Loomans *et al.*, 2005). Some studies have also reported that hyperoxia-induced oxidative stress decreases growth of preterm ECFCs followed by diminished vasculogenesis (Baker *et al.*, 2009; Fujinaga *et al.*, 2009), the mechanisms of which may be associated with disruption of Vascular Endothelial Growth Factor-nitric Oxide (VEGF-NO) signaling (Fujinaga *et al.*, 2009). Ischemic tissue produce excessive activated oxygen species which are associated with impaired ECFC functions in vasculogenesis. Further investigation of the mechanisms by which oxidant stress causes ECFC apoptosis may reveal the optimal conditions for the neovascularization.

The number and functions of ECFCs can also be modulated by other influencing factors such as collagen matrix physical properties and drugs. Recently, some studies *in vitro* have shown that vessel formation is modulated by certain physical properties of collagen matrices including fibril density and stiffness. ECFCs can be modulated by collagen matrices in the process of vessel forming *in vivo* (Sieminski *et al.*, 2004; Yamamura *et al.*, 2007; Critser *et al.*, 2010). These assays implanted ECFCs which were seeded into increasing collagen concentration matrices *in vitro* and then cultured for 18 h, bisected and each half was transplanted subcutaneously into a different immunodeficient mouse and opposite flank as described by Yoder *et al.* (2007). After 14 days, ECFC-seeded matrices were harvested and then ECFC functional blood vessels in matrices are investigated. The study showed that vessel areas became larger with increased collagen concentration but less of them whereas decreased collagen concentration resulted in higher densities of smaller area vessels. The underlying mechanisms by which the properties of collagen matrix induced changes in cellular behavior need to be further elucidated. Wu *et al.* (2009) used probucol and succinobucol to induce the formation of Heme Oxygenase-1 (HO-1) in wild type mice and found that the number of ECFCs was increased. Thus, there may exist a correlation between HO-1 and ECFCs but the mechanisms of which are not fully understood, enzyme activity and active products of HO-1 are possible responsible in part (Wu *et al.*, 2009). Some studies have demonstrated that antihypertensive drug nisoldipine induces ECFC mobilization in patients with essential hypertension (Benndorf *et al.*, 2007) which may provide some new information on the mechanisms of vasoprotection offered by this compound.

ENDOTHELIAL COLONY-FORMING CELLS IN DISEASE STATES

In physiological conditions, adult ECFCs obtained from healthy individuals are fewer and proliferate much more slowly than ECFCs obtained from cord blood including term or preterm infants (Ingram *et al.*, 2004). There exists a correlation between ECFC frequency and gestational age in cord blood (Borghesi *et al.*, 2009). It seems that the number of ECFCs decreases with decreasing gestational ages. Specifically, ECFC levels are lower at 24-28 weeks gestational age than those of higher gestational ages (Javed *et al.*, 2008). Cord blood from preterm infants with a gestational age of >28 weeks yielded significantly higher frequency of ECFCs than cord blood from preterm infants with a gestational age of <28 weeks (Borghesi *et al.*, 2009). The number of ECFCs at 33-36 weeks gestational age is equivalent to term infants (Javed *et al.*, 2008). However, it has been reported that ECFC colonies in preterm cord blood at 28-35 weeks gestation age are significantly more than term cord blood (Baker *et al.*, 2009).

In cord blood, Borghesi *et al.* (2009) showed that infants subsequently developed Bronchopulmonary Dysplasia (BPD) have significantly lower ECFCs than those of without BPD at birth and extremely preterm infants who display reduced circulating numbers of ECFCs at birth are liable to development of BPD which is not an effect of gestational age. This study has demonstrated that higher circulating numbers of ECFCs from premature infants in cord blood have a protective role from BPD. It has been reported that the frequency of ECFCs in peripheral blood of patients suffering from acute myocardial infarction increases significantly than that of

healthy controls (Massa *et al*., 2009). Similar result has been shown in the swine model of Acute Myocardial Infarction (AMI) (Huang *et al*., 2007). Guven *et al*. (2006) found that the concentration of ECFCs inversely correlates with the severity of cardiovascular disease (Guven *et al*., 2006), the underlying mechanisms of which may be associated with the upregulation of ECFC mobilization from bone marrow into the circulation. Patients with Polycythemia Vera (PV) were found to have reduced numbers of ECFCs, the mechanisms of which may be associated with disturbance of NO metabolism (Santilli *et al*., 2008). It is well known that NO is a regulator of EPC mobilization (Aicher *et al*., 2003), therefore, mobilization of ECFCs may be inhibited in PV.

Altogether, the number and functions of ECFCs may change in some diseases such as AMI, myelodysplastic syndrome (Della Porta *et al*., 2008) and pulmonary arterial hypertension (Toshner *et al*., 2009). Thus, as CFU-Hill cells studied in cardiovascular disease (Vasa *et al*., 2001; Hill *et al*., 2003; Schmidt-Lucke *et al*., 2005), ECFCs may potentially be used as a predictive biomarker for disease severity or as a therapeutic strategy for some human diseases in the near future. Furthermore, determination of the number of ECFCs and exploration of functional changes of ECFCs in some diseases may provide insight into the pathogenesis of these diseases.

ENDOTHELIAL COLONY-FORMING CELLS AS CELL THERAPY PRODUCTS

The angiogenic ability of patients with vascular diseases is often impaired which results in impaired tissue repair. Current new concepts in vascular regenerative therapy are to deliver EPCs into impaired tissues or circulation to contribute to neovascularization in tissue repair. ECFCs have been shown to have a robust angiogenic potential in ischemic and physiologic oxygen tension (Decaris *et al*., 2009) and thus ECFCs may be a superior cell used to treat patients suffering from ischemic vascular diseases through cell therapy approaches.

Transplantation of human ECFCs into animal mode of stroke has been shown to reduce apoptotic cell number, increase capillary density and stimulate neurogenesis at the sites of injury (Moubarik *et al*., 2011). ECFCs transplanted into liver or intestine in fetal sheep model, regardless of administration route, seem to be organ-selective which preferentially home to or migrate into intestine. Similarly, ECFCs in liver preferentially locate in the perivascular regions rather than hepatic parenchyma. Thus, to a certain extent, ECFC-based cellular therapies may be more appropriate to treat patients suffering from intestine diseases rather than liver diseases (Wood *et al*., 2012). How to increase the number of ECFCs transplanted into the intended organ may be the challenge of cell-based treatment approaches. It should be pointed out that the transplantation of ECFCs may lead to excessive proliferation therefore, the security measures of cell therapy approaches should be taken to stop excessive proliferation of ECFCs, the application of suicide gene may be one of efficient solutions (Schwarz *et al*., 2012).

CONCLUSION

ECFCs possess all the properties of EPCs having robust proliferative potential giving birth to endothelial progeny, forming de-novo blood vessels *in vivo*. Based on these properties, ECFCs may be true EPCs which directly participate in neovascularization. In contrast, other cell populations of EPCs may initiate, facilitate and regulate the process of neovessel formation by indirectly way (Yoder *et al*., 2007). With the breakthrough of expansion methods of ECFCs under humanized conditions, a large number of ECFCs can be derived and used in human and animal trials *in vivo* and *in vitro*. The efficiency of ECFC induced neovascularization depends not only on the sufficient number of ECFCs but also on the efficient function of these cells. ECFC activities can be modulated by many influencing factors via different molecular and cellular mechanisms, a thorough understanding of which will reveal the optimal conditions for neovascularization. As cell therapy approaches, ECFC treatment in animal models has been successful (Moubarik *et al*., 2011; Jiga *et al*., 2012) however, there are less trials in human subjects and a large number of investigations need to be performed. A comprehensive understanding of this cell population will promote the development of effective cell-based therapy in vascular diseases given the potential usefulness of ECFCs as cell therapy products.

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