Endothelial Stem and Progenitor Cells for Regenerative Medicine

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Vascular endothelial stem cell (VESC); Endothelial colony-forming cell (ECFC); Endothelial progenitor cell (EPC)

Human and Animal Rights

This article is a review and does not contain any unpublished studies with human or animal subjects. All of the published works referenced contain notation on proper conduct of animal or human subjects from their institutions.

Abstract

Purpose of Review：Vascular endothelial stem (VESC) and progenitor cells are emerging as local resident regulators of vascular endothelial repair and replacement in mammalian subjects. However, widely recognized and accepted standard measures of stem cell function have yet to be published and thus, we summarize some recent evidence that VESCs demonstrate stem cell properties in the process of EC lineage emergence, repair, and regeneration.

Recent Findings：Some rare resident ECs have been identified that are quiescent and reside within blood vessels, but are activated and proliferate in response to injury. Transcriptome analyses of these ECs at a single cell level are providing new insights into VESC identity, including tissue specific EC heterogeneity.

Summary：Blood vessels and circulating blood contain rare immature EC that display stem cell potential. Continuous efforts to define their precise location, origin, surface marker and molecular signatures would enhance current approaches for purification of cells that would enable us to build new vessels for regenerative medicine.

Introduction

Endothelial cells (ECs) form the inner cellular lining of blood vessels. They are not merely a tubular conduit for blood cells and plasma. EC phenotypes are differentially regulated in space and time, giving rise to EC heterogeneity within tissues and organs [1]. Accumulating evidence indicates some rare ECs in a variety of tissues, display high proliferative potential in mouse, rat and human subjects [2-6]. These EC give rise to the endothelial proliferation identified within mammalian blood vessels during development and in response to injury. However, when, where and how this proliferation starts and distributes within the endothelium are still not clear. The concept that endothelial progenitor cells (EPCs) exist in adult animals and may participate in vascular repair gained great basic and translational interest from a report published by Asahara and colleagues in 1997 [7]. Over the past 2 decades, advances in methods and tools for lineage fate tracing, confocal microscopy, molecular signature identification, and transplantation into various mouse models of human disease have permitted clarification and delineation of the vascular EC hierarchy consisting of stem, progenitor, and mature endothelial states within the vasculature [8-10].

In this review, we first examine the emerging methods to identify stem cell potential for

the EC lineage. Second, we summarize the recent advances for identifying resident VESCs in tissue and circulation. Third, we provide overview of some current advances that derive proliferative EC via direct somatic cell reprogramming or via pluripotent stem cells (PSCs). Finally, we provide an update on limitations for translating the currently available VESC for regenerative medicine.

Vascular endothelial stem and progenitor cells

Stem cells are generally defined as undifferentiated clonogenic cells capable of producing differentiated daughter cells and retaining their stem cell identity by self-renewal [11, 12]. Lineage-specific stem cells maintain themselves by clonal proliferation and contribute to all of the cell types of a specific lineage within a particular tissue or organ niche [13]. Here, we propose that endothelial lineage stem cells need to meet the following stem cell criteria: a) self-renewal ability, b) *in vitro* clonal proliferation (that can be shown by EC colony-forming potential upon re-plating at a single cell level), and c) *in vivo* potential of functional vessel-formation (including arterial, venous and capillary ECs) in recipient hosts (Fig. 1) [8]. Additional data that functionally confirms the above criteria include; d) isolation of the primary perfused donor-derived vessels and secondary clonal EC colony formation *in vitro*, and e) secondary *in vivo* vessel-formation

by clonal transfer of the isolated primary donor-derived vasculature (Fig. 1). To delineate between VESC and vascular EPCs is not straight forward. We will present evidence and an emerging consensus that investigators need to be more prudent in using the term "EPCs" and restrict the use of the term for only those cells that display *in vitro* clonogenic (single cell colony-forming) potential and *in vivo* vessel-forming potential [14, 15].

This cautious restriction for the use of the term EPC to only those cells that display clonogenic and vessel forming potential is required if one follows the criteria for identifying stem and progenitor cells for the EC lineage [11-13, 15, 16]. Historically, EPCs have been isolated by cell sorting or *in vitro* cell culture [15, 17]. Although CD34 is frequently used as a cell surface marker for isolating EPCs, CD34+VEGFR-2 (KDR)+CD133+ cells are highly enriched in hematopoietic progenitor activity and do not give rise to any *bona fide* endothelial cell colonies *in vitro* [18, 19]. There is still debate about the most appropriate markers that define an EPC in the blood stream, with some of the discussion constrained by the lack of consensus on EPC definition [15, 20-22]. Cell culture is an alternative approach to obtain EPCs, enables expansion of cell numbers, and is based upon cell adhesion to specific substrates in specialized media [23]. Using this *in vitro* approach, at least two distinct types of "EPCs" with different angiogenic

properties have been identified: “early EPCs” (or myeloid angiogenic cells [MACs]) and “late EPCs” (or endothelial colony-forming cells [ECFCs]) [24-27]. While “early EPCs” represent hematopoietic cells that support endothelial repair and regeneration in injured vessels through largely proangiogenic paracrine mechanisms [15, 28, 29], “late EPCs ” (ECFCs) are committed to an endothelial lineage fate and have significant proliferative and de novo vasculogenic potential [30-34]. Only “late EPCs” (ECFCs) can display *in vitro* clonogenic potential and *in vivo* vessel-forming potential [30-34]; functional capacities that “early” EPCs lack. Therefore, the only population that has all the characteristics of a *bona fide* “EPC” is the ECFCs [2, 31, 33, 35-38].

Although there are increasing number of clinical trials using the common term “EPC” as a therapeutic agent, clinicians need to be aware that methods and cell types used in the clinical studies often vary significantly [39] and none of the studies have been conducted with ECFC (that display clonal proliferative potential and in vivo vessel formation). A recent search for clinical trials using human EPC [39] on www.clinicaltrials.gov registry (22/09/05-12/04/17) identified 341 clinical trials: most were non-interventional observational trials to measure EPC levels across various clinical states, but 26 trials utilized EPC as a therapeutic agent. Half of the trials (12) were listed as complete and

the other trials were either terminated, active, recruiting, or unknown. A total of 317 participants were registered and completed in 8 trials, and 9 papers were published [40-48]. Some clinically beneficial improvements in the EPC treatment group were observed in 7 out of 8 published trials and most of the registered participants showed no severe complications [40-48]. However, none of the studies were powered to provide sufficient evidence for conduct of further larger clinical trials leading to recommendations for changes in clinical practice. When searching PubMed and Web of Science databases (01/05/2008-01/05/2018), 5 additional relevant clinical trials using "EPC" as a test cell therapy were carefully reviewed by Keighron et al [39, 47, 49-52]. They found the cell types used in all the five published trials differed in the methods of cell isolation and culture, as well as the cell surface markers used for phenotypic characterization. Four of the five trials were single arm early phase studies designed to show safety and feasibility of EPC therapy. All five studies reported that EPC therapy was safe and some identified improvements in treated patients. However, the cell dose and the route of administration was also different between these trials [39, 52, 49-51, 47]. These differences made it difficult to compare efficacy and approach among the trials. Keighron et al. [39] called for a more detailed definition of "EPC" to be used in future trials, as recently published by Medina et al [15], if a better understanding of the potential clinical benefit of an "EPC"

based therapy is to be gained.

Resident endothelial stem and progenitor cells

In many organs and tissues, lineage-specific stem cells have been identified to reside in the tissue and to produce differentiated daughter cells and to retain their stem cell identity by self-renewal [53-56]. These resident stem cells survive in a quiescent state and begin to proliferative in response to tissue damage [57]. A similar idea may also be applicable for the endothelial-lineage. Over 4 decades ago, some studies already indicated non-random proliferating cell clusters were present in vessels even at homeostasis, though the overall basal rate of endothelial cell replication is negligible in adult animals [58, 59]. The responses of large arteries or veins to denudation injury have been examined and the intimal injury was repaired via viable endothelial cells from the edge of the wound with subsequent endothelial cell expansion within the wound site [60, 61]. However, detailed cellular and molecular dynamics during this regenerative process have been lacking. McDonald et al. recently addressed some of these questions through a combination of multi-color lineage tracing, parabiosis, and single-cell transcriptomics analysis of aortic endothelial cells [9]. They utilized Cdh5 promotor driven tdTomato-expressing mice that were induced immediately prior to injury and showed that labeled

ECs post-injury gave rise to essentially all of the regenerated endothelial lining. To prove whether these ECs are derived from resident cells or via the circulation from distant sites, they used pairs of GFP+ and GFP- mice that were surgically parabiosed to share a chimeric circulation and observed that the regenerated aortic endothelial lining contained no contributions from distant sites. These findings clearly revealed that local resident ECs, but not circulating bone marrow derived "EPCs", contribute to regenerate EC lining in the aorta of their adult mice model. Intriguingly, they found there are at least two EC populations which differed in proliferative potential and thus implicated the existence of an EC hierarchy in the resident tissue. In their single cell RNA-seq (scRNA-seq) analysis of aortic EC in unwounded young and old mice, Atf3-positive cells that were enriched in the young endothelium correlated with higher expression of *Fox*, *Jun*, *Egr1*, *Klf4*, and *Klf2*, suggesting this gene may play a role to activate quiescent ECs to re-enter cell cycle for the injury regeneration response.

Although the study of McDonald et al [9] was primarily focused on mouse aorta EC response to injury, others have begun to identify vascular resident stem cells (VESC) in several different tissues and organs. Naito et al. reported the identification of resident vascular stem/progenitor cells in the side population (SP) of lung, liver, heart, and hind

limb muscle cells [5]. The SP as a marker of stem cells was first identified by Goodell et al.[62] as bone marrow cells effluxing Hoechst 33342 dye through ATP-cassette transporter receptors and this subset contained dormant bone marrow hematopoietic stem cells. Naito et al. showed that resident quiescent EC stem/progenitor cells are found within SP cells by in vitro single cell assays for clonogenic potential and in vivo vessel-formation assay [5,8]. Their group subsequently identified CD157 as a cell surface marker of tissue-resident VESC in large arteries and veins of several mouse organs [8]. Starting from the evidence that endothelial-SP cells are enriched with CD157 and CD200 expression, they revealed that liver CD157+CD200+ ECs exhibit a high proliferative potential in vitro and reconstitute portal vein, central vein, and sinusoid as well as capillaries after in vivo transplantation in a rodent liver injury model. They also performed donor cell transplantation at a single cell level and 3 of 350 transplanted CD157+CD200+ cells could generate functional donor-derived blood vessels, and EC progeny contained some CD157+CD200+ cells implicating their self-renewal potential.

Other cell surface markers including c-Kit (CD117) [6] and Protein C Receptor (Procr, CD201) [63] have also been investigated as VESC markers. Fang et al. found that c-Kit positive ECs from mouse lung tissue display 10-fold greater colony-forming potential

compared to c-Kit negative ECs. They also observed that all EC colonies originated from vessel wall c-Kit+ ECs and are not produced by hematopoietic stem or progenitor cells. However, no fate mapping studies to identify the contributions of the c-Kit+ ECs within various vascular beds was presented and this marker seems to be not highly specific, since overall colony-formation efficiency was low. Yu et al. [63] identified that Procr positive ECs reside as VESC in murine mammary fat pad, skin, and retina. Procr+ VESCs exhibit robust clonogenicity in culture, high vessel reconstitution efficiency in transplantation, and long-term clonal expansion in lineage tracing studies *in vivo*.

The different cell surface protein candidates selected for enriching VESCs in the above studies [6, 8, 63] may reflect tissue specific EC heterogeneity since each study mainly focused on an individual organ, such as, mouse lung [6], mammary fat pad [63], or liver [8], respectively. This notion is supported by some recent scRNA-seq analyses for several organs [64, 65], indicating tissue-specific gene regulatory architecture of each EC subtype from brain, liver, lung, and kidney [65]. Taken together, all of these recent advances in this field are paving the way for providing tools to identify VESC and providing models through which the stem cell properties may be distinctly identified.

Circulating endothelial stem cells (circulating ECFCs)

As noted above, accumulating evidence indicates that many of the putative circulating marrow-derived EPCs contribute to neovascularization by a paracrine manner and fail to display vasculogenic activity by themselves. Rather, this property is reserved for circulating ECFCs, a rare population of viable endothelial cells with colony-forming cell ability in the blood stream [15, 28, 33, 36, 66]. Cord blood and peripheral blood ECFCs are well-known circulating ECFCs and can be derived from blood mononuclear cells and possess robust proliferative potential and capacity to form new blood vessels in vitro and in vivo [2, 31, 32, 37, 67].

Since the resident VESCs are identified as the main players for repairing tissue damage in mammalian wounded aorta and liver [8, 9, 68], it is still elusive how the circulating ECFCs contribute to angiogenesis and vasculogenesis in physiologic and pathologic conditions. One obvious point, is that while there is no evidence that mouse circulating ECFCs are recruited to an injured site [9], a number of studies of human ECFC point to circulating ECFC as playing a role in health and disease [22, 23, 33, 34]. Campenelli et al [69]. measured the frequency and vasculogenic potential of circulating ECFC in infantile haemangioma (IH) patients, and showed that circulating ECFCs of untreated IH patients had a lower frequency than those of control subjects but displayed enhanced

capacity to form tube-like structures. After propranolol therapy, they had increased frequency and a reduction of their vasculogenic activity. These results suggest a recruitment of circulating ECFCs from peripheral blood to the site of lesion caused their reduced frequency at diagnosis, and involution of the tumor mass, decrease in ECFC recruitment function, and/or a direct action of propranolol might cause an increased frequency and diminished vessel-formation capacity of circulating ECFCs [69]. A contribution of circulating ECFC can be found in the studies of measuring ECFC levels in preterm infants with bronchopulmonary dysplasia (BPD) [70-72]. The number of CB-ECFCs in preterm babies who subsequently developed BPD were significantly decreased [70, 71]. Because impaired vascular growth has a main role in the pathogenesis of BPD, decreased ECFCs may contribute to abnormal vascular repair. Administration of CB-ECFCs into a rat pup model of BPD, shown to be deficient in ECFCs, reversed the disease phenotype dramatically through their contribution to lung neovascularization [72]. Taken together, circulating ECFCs are proposed to contribute to vessel formation both physiologically and pathologically, and giving external circulating ECFCs may be an effective treatment for building up the new vessels for tissue regeneration in subjects with dysfunctional vascular repair or regeneration.

Proliferative ECs via direct somatic cell reprogramming or via pluripotent stem cells

To generate high-proliferative EC by forced expression of transcriptional factor (s) or chemicals via direct somatic cell reprogramming or via differentiation from human pluripotent stem cells (PSCs) has become an increasingly important topic in the field of regenerative medicine. Direct reprogramming of fibroblasts into ECs was first reported by Margariti et al. [73]. They partially reprogrammed fibroblasts and let them differentiate into ECs that expressed EC cell surface markers and upon injection, improved mouse ischemic tissue recovery. Their study paved the way for many other approaches. Ginsberg et al [74]. reported that enforced expression of ETV2, FLI1, and ERG1 with TGFβ inhibition changed the fate of amniotic mesenchymal cells to EC-like cells. Their optimized protocol enabled the reprogrammed ECs to display single-cell clonogenic potential *in vitro* and form functional perfused vessels *in vivo*. Benefits for the use of ETV2 as the master regulator for EC fate conversion from other cell lineages has also been proposed by other groups [75, 76]. Two groups succeeded to derive reprogrammed ECs that upon injection, improved mouse hindlimb recovery after an experimentally induced ischemia injury, but the ideal duration and/or dose of ETV2 transgene expression to induce sufficient endothelium remains unknown [77, 78]. A better understanding and accurate tuning of ETV2 delivery, alone or with other

reprogramming molecules, should enhance the outcomes for the desirable direct reprogramming EC fate change. As one example, a recent very innovative approach to *in vivo* tissue reprogramming was reported by Gallego-Perez et al. [79]. This group developed a novel non-viral approach to topically reprogram tissues through a nanochannelled silicone ship through which a variety of molecules can be delivered to reprogram keratinocytes into neuron-like or EC-like cells. Fate mapping evidence confirmed that keratinocyte cells contributed to blood vessels and neurons within the tissues. The functional impact of the reprogramming was sufficient to rescue animals with experimentally induced wounds to heal those wounds significantly better than appropriate controls [79].

Prasain et al [80]. reported on a detailed culture protocol using BMP4, VEGF, and bFGF to differentiate human induced pluripotent stem cells (hiPSCs) towards ECs that molecular, phenotypic, and function features similar to CB-ECFCs. This protocol focused on isolation of CD31+Neuropilin-1 (NRP-1)+ after 12 days differentiation from hiPSCs. The derived ECs displayed clonal proliferative potential and *in vivo* vessel-forming ability, showing that functional human blood vessels were maintained in immunodeficient mice for up to 6 months [80]. Similar cytokine cocktails for EC

differentiation have been reported by other groups [81, 82]. Sriram et al. [81] derived arterial and venous ECs from human embryonic stem cells (hESCs) in serum-free conditions and showed that each of the the derived ECs expresses arterial-specific (NRP1, DLL4, CXCR4) or venous-specific (NRP2, EPHB4) gene expression, respectively. These cells largely maintained their respective phenotypes in vivo, as evidenced by the expression of EFNB2 and EPHB4, though some microvessels formed by arterial ECs acquired expression of venous markers and vice versa over time [81]. Harding et al. [82] also confirmed that the combination of BMP4, VEGF, and bFGF substantially induced proliferative ECs from hiPSCs. They revealed that all three MAPK and the PI3K pathway are responsible for induction of an EC fate and inhibition of the ERK pathway promoted smooth muscle cell differentiation [82]. Ohta et al. [83] utilized coating the tissue culture dishes with short peptide fragments of laminin 411 (LM411-E8), an ECM predominantly expressed in the vascular endothelial basement membrane, for EC differentiation from hiPSCs. They showed higher purity of ECs can be obtained by LM411-E8-coated tissue culture plates than by those coated with matrigel, LM511, type IV collagen or fibronectin [83]. Recently, Paik et al. [84] performed a large-scale single cell RNA-seq for differentiating iPSC-derived ECs at two time-points of differentiation. They revealed that transcriptional heterogeneity with four major subpopulations,

marked by the enrichment of CLDN5, APLNR, GJA5, and ESM1 genes respectively [84]. Additional single cell RNA-seq profiles in various EC derived by multiple induction methods from hiPSCs would guide us toward a better understanding for inducing proliferative ECs from hiPSCs that are comparable to VESCs identified in primary tissues.

Conclusions

Identification and understanding of tissue resident and/or circulating VESC is increasingly essential for us to provide those cells for vascular regenerative medicine. In this review, we proposed stem cell criteria for the endothelial lineage that might help to address this issue. Many recent advances, such as, precise cellular fate determination studies in the developing mouse embryo and in vitro differentiating mammalian cells by genome editing, state-of-the-art imaging techniques for those cells, and deep transcriptome analyses by scRNA-seq in vivo and in vitro studies, would help to provide the clues for unveiling VESCs in mammalian systems. Continuous efforts to identify VESCs and define their precise location, origin, surface marker and molecular signatures would lead to the purification of cells with the potential to address increasing clinical demands for tissue regeneration.

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Fig. 1

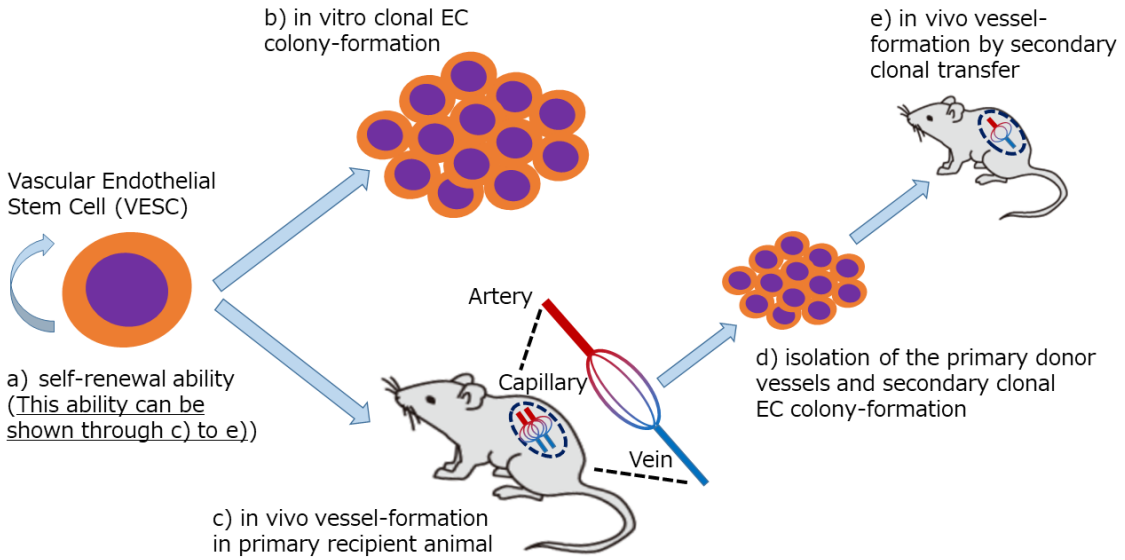

Fig. 1

Criteria for defining vascular endothelial stem cell (VESC) identity.

Endothelial lineage stem cells need to meet the following stem cell criteria: a) self-renewal ability, b) *in vitro* clonal proliferation (that can be shown by EC colony-forming potential upon re-plating), and c) *in vivo* potential of functional vessel-formation (including arterial, venous and capillary ECs) in recipient hosts. Additional data that functionally strengthens the above criteria include; d) isolation of the primary perfused donor-derived vessels and secondary clonal EC colony formation *in vitro*, and e) secondary *in vivo* vessel-formation by clonal transfer of the isolated primary donor-derived vasculature (confirming self-renewal of VESC within the primary vasculature).

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