

# Human Endothelial Colony-Forming Cells

Juan M. Melero-Martin

Department of Cardiac Surgery, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

Correspondence: [juan.meleromartin@childrens.harvard.edu](mailto:juan.meleromartin@childrens.harvard.edu)



Endothelial colony-forming cells (ECFCs) are progenitor cells that can give rise to colonies of highly proliferative vascular endothelial cells (ECs) with clonal expansion and in vivo blood vessel-forming potential. More than two decades ago, the identification of ECFCs in human peripheral blood created tremendous opportunities as having a clinically accessible source of autologous ECs could facilitate meaningful therapies with the potential to impact multiple vascular diseases. Nevertheless, until recently, the field of endothelial progenitor cells has been plagued with ambiguities and controversies, and reaching a consensus on the definition of ECFCs has not been straightforward. Moreover, although the basic phenotypical and functional characteristics of cultured ECFCs are now well established, some fundamental questions such as the origin of ECFCs and their physiological roles in health and disease remain incompletely understood. Here, I highlight some critical studies that have shaped our current understanding of ECFCs in humans. Insights into the biological attributes of ECFCs are essential for facilitating the clinical translation of their therapeutic potential.

## DEFINITION OF HUMAN ECFCs

Human endothelial colony-forming cells (ECFCs) are a rare population of progenitor cells that circulate in peripheral blood and that, in culture, give rise to colonies of highly proliferative cells with an incontrovertible endothelial phenotype (Yoder 2012; Hebbel 2017). ECFCs are generally considered true progenitors of endothelial cells (ECs), and as such, consensus has formed in that ECFCs must exhibit the following specific characteristics (Yoder 2012; Medina et al. 2017; Smadja et al. 2019): (1) ability to restrictively differentiate to the endothelial lineage, (2) robust clonal proliferative potential, and (3) capacity to form and line the lumens of stable, functional blood vessels in vivo. Collectively,

these three criteria constitute a minimal definition of ECFCs (Fig. 1).

The first criterion, the ability to differentiate into cells with a bona fide endothelial phenotype, is essential for considering ECFCs as true EC progenitors. Indeed, once isolated in culture, ECFCs are fundamentally ECs and do not exhibit ambiguous nonendothelial characteristics (Ingram et al. 2004; Melero-Martin et al. 2007; Yoder et al. 2007). Importantly, ECFCs are distinct from other blood outgrown cell types originating from hematopoietic lineages, such as myeloid angiogenic cells (MACs) (Fig. 1). The differences between cultured ECFCs and MACs are transcriptionally and functionally evident (Gulati et al. 2003; Rehman et al. 2003; Case et al. 2007; Yoder et al. 2007; Medina et al. 2010b).

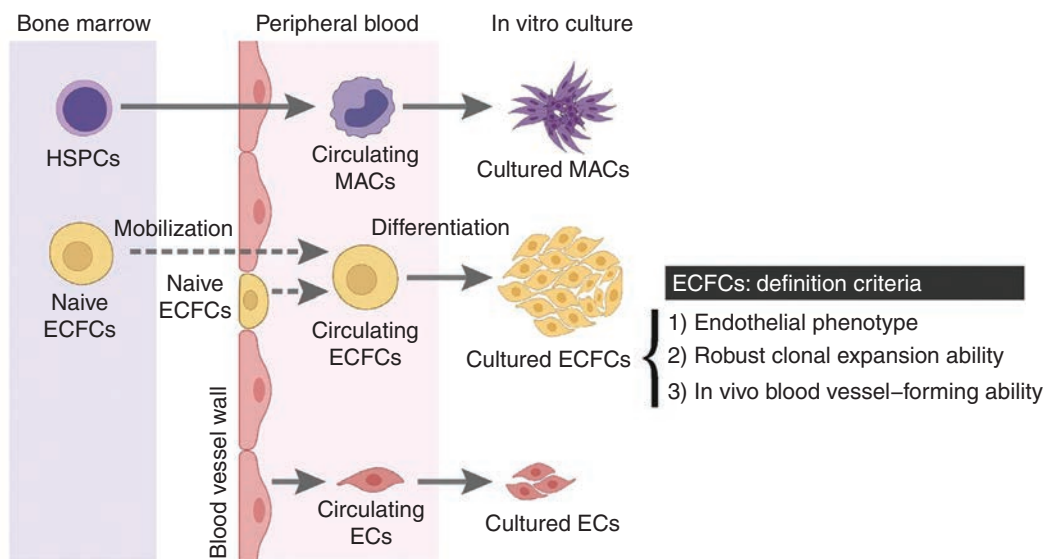
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**Figure 1.** Definition, origin, and culture of human endothelial colony-forming cells (ECFCs). In vivo, human ECFCs are present in their original niche and circulating in peripheral blood (herein referred to as naive ECFCs and circulating ECFCs, respectively), whereas in vitro, cultured ECFCs are manifested as colonies of highly proliferative endothelial cells (ECs). Operationally, the criteria used to define ECFCs are only testable on cultured ECFCs, once the cells are isolated in vitro. Thus, the definition of ECFCs is intimately associated with their appearance in vitro as cultured ECFCs. (MACs) Myeloid angiogenic cells, (HSPCs) hematopoietic stem/progenitor cells.

The second criterion refers to the fact that, as progenitors, ECFCs are endowed with the potential for clonal expansion (Hirschi et al. 2008; Yoder 2012). This clonogenic ability distinguishes ECFCs from other mature ECs, including circulating ECs (CECs) that are occasionally dislodged from the endothelial monolayer. The clonal expansion potential of human ECFCs is exceedingly robust (i.e., a single cell can form colonies of >2000 cells within 10 d in culture), although not unlimited. The remarkable expansion ability of ECFCs is manifested in culture and correlates with a high level of telomerase activity (Lin et al. 2000; Ingram et al. 2004; Melero-Martin et al. 2007).

Last, the third criterion refers to the notion that ECFCs must form functional vascular networks in vivo (Melero-Martin et al. 2007). This ability, however, is inherent to all ECs, and not exclusive of ECFCs. Indeed, the blood vessel-forming ability requirement is somewhat redun-

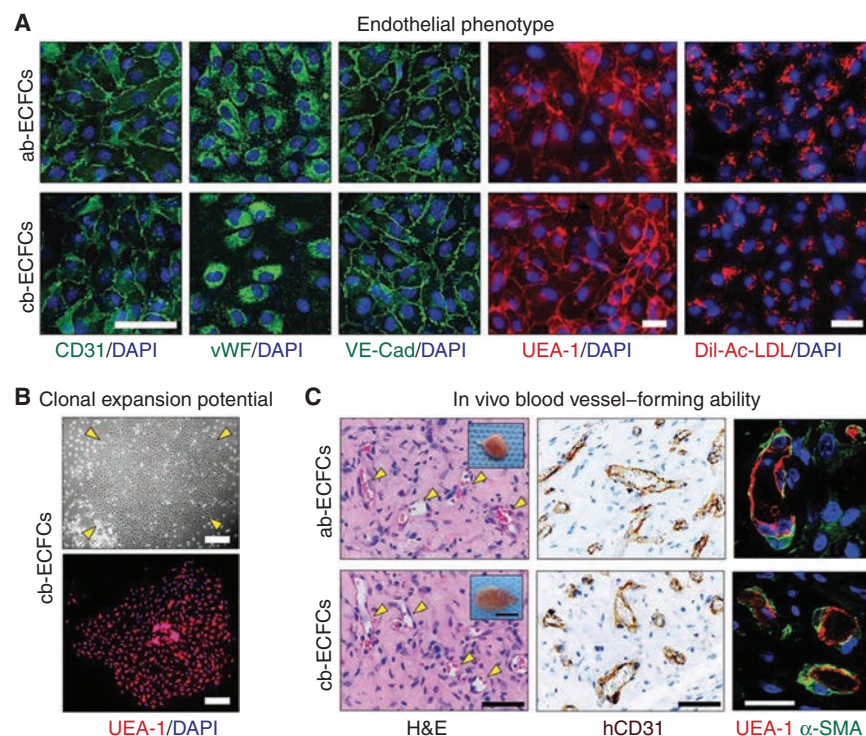
dant considering the first criterion (i.e., endothelial phenotype). Nonetheless, because some other blood-derived cells occasionally exhibit EC-like attributes in culture, a consensus has emerged that the formation of functional vasculature in vivo should be a requirement to distinguish ECFCs from non-ECs derived from other putative circulating progenitors (Yoder 2012).

It is important to note that in vivo human ECFCs are present in at least two locations: their original niche and circulating in peripheral blood (Hebbel 2017) (herein referred to as naive ECFCs and circulating ECFCs, respectively) (Fig. 1). However, the three defining criteria are only testable once the cells are isolated in vitro. Although the phenotypes of both naive and circulating ECFCs remain incompletely understood, it is unclear that ECFCs possess a competent endothelial phenotype at those stages. Thus, the definition of ECFCs is intimately associated

with their manifestation in vitro as cultured ECFCs. In any case, for simplicity, all naive, circulating, and cultured ECFCs are commonly referred to as ECFCs in the scientific literature, and one should discern, based on contextual information, the stage of ECFCs that is being discussed in each particular study.

It is also worth noting that ECFCs are not defined by a specific set of cellular markers—at

least not exclusively (Medina et al. 2017). Unfortunately, identifying a unique cell-surface marker that would permit prospective isolation and enrichment of human ECFCs remains elusive, although the recent identification of the distinct expression of neuregulin-1 (NRG1) in ECFCs is promising (Hong et al. 2021). Nevertheless, upon differentiation in culture, ECFCs display phenotypic features of vascular ECs



**Figure 2.** Phenotypic characterization of human cultured endothelial colony-forming cells (ECFCs). ECFCs were isolated from umbilical cord blood (cb-ECFCs) and adult peripheral blood (ab-ECFCs). (A) Indirect immunofluorescence of cultured ECFCs grown in a confluent monolayer showing positive staining for CD31, von Willebrand factor (vWF), and VE-cadherin. Cell nuclei counterstained with DAPI (scale bar, 100  $\mu$ m). Binding of UEA-1 lectin to a monolayer of ECFCs (scale bar, 50  $\mu$ m). ECFCs uptake of fluorescently labeled acetylated low-density lipoproteins (Dil-Ac-LDL) (scale bar, 50  $\mu$ m). (B) Cloning-forming ability of cultured ECFCs. Phase contrast micrograph of a cb-ECFC colony. Arrowheads delimitate the border of the colony (scale bar, 200  $\mu$ m). The endothelial nature of the colonies confirmed by binding of UEA-1 lectin (scale bar, 500  $\mu$ m). (C) In vivo blood vessel-forming potential of cultured ECFCs. ECFCs were combined with human mesenchymal stem cells (MSCs), and the mixture subcutaneously injected into nude mice using a collagen/fibrin-based hydrogel. H&E from representative explants at day 8 with numerous blood vessels containing RBCs (yellow arrowheads; scale bar, 50  $\mu$ m). *Insets* represent the macroscopic views of the explants (scale bar, 5 mm). hCD31 immunohistochemistry showed human specific lumens (scale bars, 50  $\mu$ m). Perivascular coverage assessed by double immunofluorescent staining of explants with UEA-1 (red) and  $\alpha$ -SMA (green). Nuclei counterstained with DAPI (scale bars, 50  $\mu$ m). (Panels A and C adapted from Lin et al. 2013; reprinted, with permission, from Springer © 2013. Panel B adapted from Moreno-Luna et al. 2014; reprinted, with permission, from Elsevier © 2014.)

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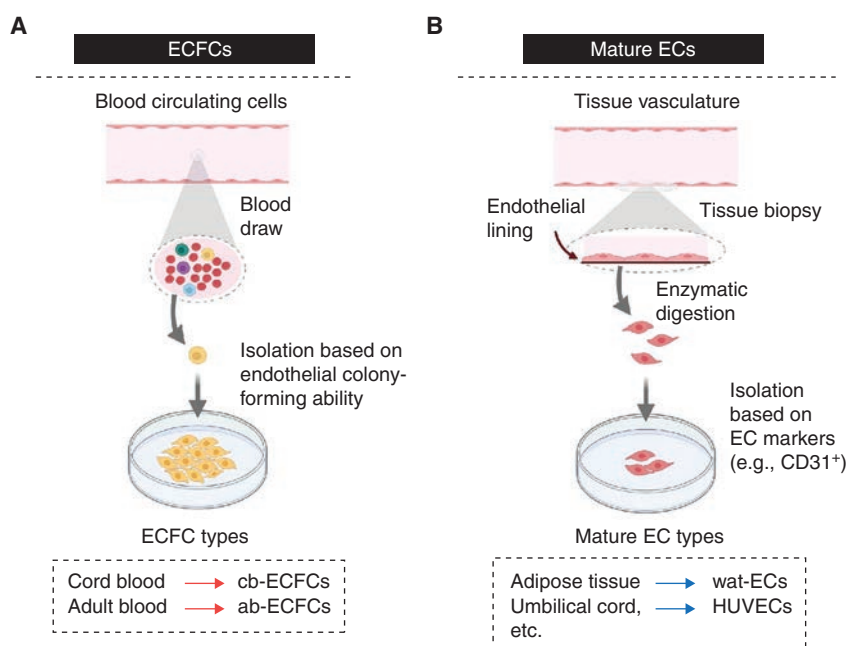
(Fig. 2). Therefore, they can be operationally characterized by the expression of CD31, VE-cadherin, von Willebrand factor (vWF), CD146, and VEGFR2. Importantly, ECFCs lack hematopoietic cell markers such as CD45 and CD14 (Case et al. 2007; Melero-Martin et al. 2007; Timmermans et al. 2007; Mund et al. 2012; Medina et al. 2017), a characteristic that has become critical to distinguish ECFCs from a number of other circulating subpopulations of myelomonocytic progenitors that participate in various angiogenic processes but that do not give rise to EC progeny per se.

A singular characteristic of ECFCs is that they can be obtained from cultures of blood (Fig. 3). Human ECFCs can be isolated from circulating mononuclear cells (MNCs) at different stages in life as well as from fetal blood, umbilical cord blood, and peripheral blood in

adults. However, ECFC levels do decrease progressively from birth to adulthood (Smadja et al. 2019), although the rate at which this decline occurs and the mechanisms that regulate their abundance in vivo remain poorly understood. Notwithstanding this uncertainty, the accessibility of ECFCs from peripheral blood creates tremendous opportunities as having a clinically accessible source of autologous ECs could facilitate meaningful therapies with repercussions to multiple vascular diseases.

## CONTROVERSIES

Reaching a consensus on the definition of ECFCs has not been straightforward (Yoder 2012; Medina et al. 2017; Smadja et al. 2019). Indeed, the field of endothelial progenitor cells (EPCs) has been plagued by ambiguities and controversies



**Figure 3.** Methods to derive endothelial colony-forming cells (ECFCs) and mature endothelial cells (ECs). (A) ECFCs are isolated from circulating mononuclear cells (MNCs) based on their robust endothelial colony-forming ability, whereas mature ECs are typically isolated based on their expression of definitive EC markers. Conceptually, ECFCs are different from mature ECs in that cultured ECFCs are differentiated, ex vivo, from circulating progenitors, and it is thus unclear whether they have previously been part of a functional blood vessel in vivo. In contrast, mature ECs are derived from the lining of blood vessels and thus have previously functioned as ECs in vivo. Despite this conceptual difference, ECFCs and mature ECs exhibit a wide range of phenotypical and functional similarities once in culture.

for over two decades (Hebbel 2017). Initially, the existence of postnatal circulating endothelial progenitors was suggested based on observations of the endothelium in transplanted organs. For instance, Kennedy and Weissman (1971) observed that an appreciable number of the ECs lining the coronary arteries in transplanted human cardiac allografts belonged to the recipient and not the donor; with no endogenous host endothelium adjacent to the allografted vessels, the presence of recipient ECs suggested a possible origin from circulating precursors. Nevertheless, the notion of circulating endothelial precursors in humans was not seriously considered at the time. The consensus was that the formation of ECs from mesodermal progenitors or angioblasts occurs only during embryonic development and not postnatally (Risau et al. 1988; Risau and Flamme 1995).

This long-held notion began to change in 1997 when Asahara and colleagues described the isolation of putative endothelial progenitors from peripheral blood (Asahara et al. 1997). In this seminal study, the authors showed that a subset of CD34<sup>+</sup> VEGFR2<sup>+</sup> circulating progenitors exhibited the capacity to differentiate, *ex vivo*, into cells with endothelial characteristics, including the ability to form distinct colonies that incorporated acetylated LDL, expressed PECAM-1, and Tie-2 receptor, and produced nitric oxide upon VEGF stimulation (Asahara et al. 1997). The cells were named putative EPCs and were later shown to participate in neovascularization processes in various animal models of induced ischemia (Isner and Asahara 1999; Takahashi et al. 1999; Kalka et al. 2000). In subsequent years, numerous studies addressed different aspects of these putative EPCs, from their fundamental biological attributes to their roles in health and disease and their potential for therapies (for review, see Fadini et al. 2012). Unfortunately, since Asahara et al. coined the term putative EPCs (Asahara et al. 1997), these cells have been referred to by several different names in the scientific literature, causing considerable confusion in the field (for review, see Medina et al. 2017). This confusion was intensified by multiple studies in mice that did not correlate with equivalent observations in humans.

The implications of the existence of putative EPCs were enormous as it suggested the possibility of postnatal vasculogenesis and the prospect of a clinically accessible source of cells with potential for therapeutic vascularization (Raffi and Lyden 2003). However, comprehensive investigations from multiple laboratories eventually revealed that these putative EPCs are not true EC precursors (for review, see Yoder 2012). EPCs participate and have a crucial role in multiple neovascularization processes, but they do not contribute to functional endothelium. Instead, putative EPCs are bone marrow-derived myelomonocytic progenitors that exhibit paracrine proangiogenic characteristics that aid in the formation of new blood vessels (Rehman et al. 2003; Yoon et al. 2005; Yoder et al. 2007). Hence, despite sharing several properties with ECs (e.g., the expression of CD31 and Tie-2 receptor; the ability to produce nitric oxide), EPCs are not EC precursors. Moreover, with the realization that EPCs do not give rise to EC progeny, the term “EPC” became problematic, and a consensus is emerging around the use of alternative nomenclature to refer to these myelomonocytic cells, including the term myeloid angiogenic cells or MACs (Fig. 1; Medina et al. 2017). Nevertheless, the term EPC and its association with various subpopulations of myeloid precursors is widespread and will likely persist in the scientific literature for years to come.

It is essential to recognize that putative EPCs (or MACs) and ECFCs are two distinct cell populations with entirely different biological and functional attributes (for review, see Yoder 2012) and that only ECFCs fulfill the three above-mentioned criteria for EC progenitors. The identification of ECFCs in human blood, defined as cells with the capacity to give rise to EC progeny, was contemporaneous but largely independent from the human and murine studies that led to the discovery of the putative EPCs. Unfortunately, ECFCs were also initially referred to by various terminologies, including the term EPC, which added enormous confusion to the field (for review, see Medina et al. 2017). Over the last few years, our collective understanding of ECFCs has improved significantly and despite occasional disagreements over nomenclature,

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the distinction between ECFCs and putative EPCs is no longer controversial.

### ORIGIN OF CIRCULATING HUMAN ECFCs

Human ECFCs are obtained as highly proliferative ECs that arise from peripheral blood MNC culture (Lin et al. 2000; Gulati et al. 2003; Hur et al. 2004; Ingram et al. 2004; Melero-Martin et al. 2007). Therefore, operationally, ECFCs are derived from blood. However, the exact origin of ECFCs within the body has been the subject of intense debate for the last two decades, and it remains somewhat unclear (Yoder 2012; Hebbel 2017).

One of the first and most compelling pieces of evidence for a possible ECFC origin came from a clinical investigation by Lin et al. (2000) in which the authors cultured MNCs from blood samples obtained from bone marrow transplant recipients who had received gender-mismatched transplants several months earlier. This study used fluorescence in situ hybridization (FISH) analysis to demonstrate that the majority of the highly proliferative EC colonies that had appeared in culture (i.e., what later came to be defined as ECFCs) were of donor genotype, strongly suggesting a bone marrow origin. (Note that at the time, ECFCs were referred to by other names, including blood outgrowth ECs, OECs, or BOECs, nomenclature that is still used in the literature.) This seminal study helped establish several important distinctions between ECFCs and other mature ECs that are occasionally found circulating in peripheral blood (referred to as circulating ECs or CECs). Indeed, the EC outgrowth from ECFCs typically appeared in culture slowly over time (~1 mo) and then exhibited a robust proliferative potential (~1000-fold expansion). In contrast, the colonies from CEC emerged significantly earlier (within the first week) but had a modest expansion potential (~20-fold) after that. Notably, the outgrowths from CECs were predominantly of the recipient genotype, suggesting the endothelium of host blood vessels as their most likely origin. Together, these observations implied that (1) ECFCs are derived from transplantable bone marrow-derived cells (hence the likelihood of a bone mar-

row origin), and (2) ECFCs are distinct from mature CECs and exhibit delayed outgrowth and robust proliferative rates in culture, intrinsic characteristics that are consistent with the definition of progenitor cells.

Over the last two decades, we have come to appreciate that human ECFCs are not related to other putative EPCs of myelomonocytic origin (Gulati et al. 2003; Rehman et al. 2003; Case et al. 2007; Yoder et al. 2007; Medina et al. 2010b). Nevertheless, this distinction was initially ambiguous, and thus our understanding of ECFCs at the time was often confounded by studies and observations that were made with EPCs. Indeed, the evidence that putative EPCs were originated from the bone marrow was scientifically compelling (Shi et al. 1998; Peichev et al. 2000), which contributed to perpetuating the notion that all endothelial progenitors, including ECFCs, originate from the bone marrow. Also, the fact that ECFCs were successfully isolated from blood MNCs (and later from CD34-enriched circulating cells) contributed to the impression of a possible association between ECFCs and the hematopoietic cell system and thus the bone marrow.

However, besides the persuasive study by Lin et al., evidence for a bone marrow origin of human ECFCs was scarce, and the counterargument that a transplantable bone marrow-derived cell population might contain circulating cells that are not resident in the bone marrow remained a limitation. Moreover, although ECFCs can surely be isolated from umbilical cord and peripheral blood, there was no conclusive evidence that they could also be obtained from the culture of bone marrow cells (Tura et al. 2013).

Several investigations eventually resolved the initial uncertainty of a possible ECFC origin from the hematopoietic cell system (Yoder et al. 2007; Piaggio et al. 2009). Of particular importance, Yoder et al. convincingly demonstrated that ECFCs are not clonally related to hematopoietic stem cells by examining ECFCs from polycythemia vera patients harboring a Janus kinase 2 (JAK2) V617F mutation in hematopoietic stem cell clones (Yoder et al. 2007). This seminal study also helped establish the notion that ECFCs are distinct from putative EPCs, which were proven to be hematopoietic-derived

progeny committed to the myeloid lineage. Nevertheless, the absence of a hematopoietic linkage did not rule out the bone marrow site as a potential niche for ECFCs.

More recently, Fujisawa et al. (2019) revisited the question of a possible bone marrow origin by examining ECFCs from male patients who had undergone allogeneic bone marrow transplants from female donors to treat hematological malignancies. ECFCs were comprehensively analyzed by FISH to detect the X and Y chromosomes. The study revealed that all highly proliferative ECFCs had male XY genotypes and therefore did not arise from the transplanted bone marrow-derived female cells, which implied that ECFCs in circulation do not originate from the bone marrow. These results are compelling, especially considering that the clonal expansion potential of ECFCs was validated at the single-cell level. However, these findings contrasted with those established by Lin and colleagues (2000), and therefore further studies are warranted before ruling out the bone marrow origin paradigm.

The question of origin was always intimately related to the actual definition of ECFCs. However, the lack of an initial uniform definition of ECFCs that distinguished them from other EPCs made the interpretation of most studies difficult. Eventually, a consensus emerged around a minimal definition based on common hallmarks of stem and progenitor cells. Indeed, as progenitor cells, ECFCs are expected to exhibit the ability to proliferate and give rise to functional progeny and clonal growth potential. Using these principles, Ingram et al. (2004) developed a single-cell assay to define ECFCs based on their proliferative and clonogenic potential. This study was enormously influential, and in addition to coining the term ECFCs, it revealed a hierarchy of endothelial progenitors based on their proliferative potential (Ingram et al. 2004). Multiple independent laboratories subsequently substantiated the robust clonogenicity and proliferative potential of ECFCs (Lin et al. 2013; Moreno-Luna et al. 2014; Ferratge et al. 2017; Toupance et al. 2020).

Nevertheless, the realization that ECFCs are EC outgrowths with robust clonal proliferative

potential did not clarify whether they originate from the bone marrow. Indeed, various human EC subsets derived from the wall of blood vessels are also highly proliferative in culture (~30–40 population doublings), including ECs from the umbilical vein, aorta, carotid artery, and the microvasculature of various organs (Yang et al. 1999; Bompais et al. 2004; Naito et al. 2012). This replicative capacity is similar to that of circulating ECFCs from adults (Hur et al. 2004; Ingram et al. 2004; Melero-Martin et al. 2007). Moreover, Ingram et al. (2005) demonstrated that both the aorta and the umbilical vein contain a complete hierarchy of highly proliferative ECs with equal colony-forming ability to blood-derived ECFCs. Likewise, Lin et al. (2013) showed that the vasculature of human white adipose tissue contains ECs with hierarchical clonogenic ability, expansion potential, and stable phenotype similar to ECFCs. Thus, tissue-specific endothelial heterogeneity appears within the blood vessel wall, which has also been reflected by recent single-cell transcriptomic analyses (Wakabayashi et al. 2018; Lukowski et al. 2019). Collectively, this endothelial diversity contributed to an emerging concept with implications for the possible origin of ECFCs, namely, that the walls of blood vessels throughout the body contain subsets of ECs that display stem- and progenitor-like properties similar to ECFCs derived from circulating MNCs (Yoder 2010). Nevertheless, whether the vessel wall-derived ECFCs are related to circulating ECFCs and play an equally significant role in endothelial repair and regeneration in response to injury remains unclear.

Unfortunately, the question of human ECFC origin may remain elusive for some time due to the absence of suitable animal models. Notably, the lack of mouse models (ECFCs are nonexistent or, at least, exceedingly rare, in postnatal murine peripheral blood) has impeded definitive lineage-tracing experiments that could address fundamental questions about ECFC origin. Notwithstanding these ongoing uncertainties, the question of origin remains relevant as the clinical interest in harnessing ECFCs to promote vascular regeneration continues. Over the last decade, autologous bone marrow cells have been deliv-

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ered to thousands of patients on the premise that these populations contain functional endothelial precursors. Perhaps the conflicting results and the general lack of benefit observed in these clinical trials (Nowbar et al. 2014) should serve as an indication that the bone marrow might not, after all, be the origin of circulating ECFCs. Certainly, ECFCs arising from alternative niches in the blood vessel wall would represent a paradigm shift that would require a reexamination of our approaches to harness EC progenitors for therapeutic vascularization.

### ISOLATION AND CULTURE OF HUMAN ECFCs FROM BLOOD

From a translational standpoint, the accessibility of blood has always been one of the main appeals of human ECFCs. Indeed, the presence of circulating ECFCs in peripheral blood has made their derivation readily accessible to laboratories worldwide. Unfortunately, several issues have hampered the general use of ECFCs—most notably the lack of consensus about the methods used to isolate, quantify, culture, and evaluate the cells. Nonetheless, several recent position papers have called for standardization of methods to isolate and culture ECFCs from peripheral blood (Medina et al. 2017; Smadja et al. 2019), which undoubtedly will facilitate their widespread use.

In essence, the most effective means to isolate ECFCs remains reasonably similar to the original methodology reported for endothelial outgrowth from peripheral blood two decades ago (Lin et al. 2000). This method involves collecting low-density MNCs from human blood (either adult peripheral or umbilical cord blood) and plating them in dishes coated with an appropriate extracellular matrix component in a commercial cell culture medium containing endothelial growth factors and fetal calf serum (Gulati et al. 2003; Bompais et al. 2004; Hur et al. 2004; Ingram et al. 2004; Yoon et al. 2005; Melero-Martin et al. 2007; Nagano et al. 2007; Timmermans et al. 2007; Au et al. 2008). Over a 2- to 4-wk period in culture, this methodology produces ECFCs identified as well-circumscribed colonies (>50 adherent cells/colony) of cobblestone-shaped endo-

thelial-like cells with a remarkable proliferative capacity.

Consensus is emerging on a standardized method to isolate ECFCs (Smadja et al. 2019). However, examination of recently published studies reveals that there are still notable variations for each of the specific parameters involved in the methodology. For instance, different laboratories have reported plating densities of MNCs, ranging from  $10^5$  to  $10^6$  cells per  $\text{cm}^2$ . However, it remains unclear whether the plating density significantly influences the number of ECFCs obtained (Mauge et al. 2014). Also, various extracellular matrices are used to plate MNCs—most notably fibronectin, type I collagen, and gelatin (Yoder et al. 2007; Melero-Martin and Bischoff 2008; Kim et al. 2020). Again, how much influence the matrix has on total ECFC yield remains unclear. There are also differences in the media composition used to isolate ECFCs, although commercially available EGM-2 is consistently the preferred culture medium, usually supplemented with 5%–10% fetal bovine serum (FBS). Of note, the percentage of FBS appears to produce no significant differences (Smadja et al. 2019), and both human plasma and platelet lysate appear to be adequate substitutes for animal serum (Reinisch et al. 2009; Huang et al. 2011; Kim et al. 2015; Tasev et al. 2015).

The methodology to isolate ECFCs is similar for both umbilical cord and adult peripheral blood. However, there are notable differences regarding overall culture times and yield. For instance, the average time at which the first ECFC colonies appear in culture is much shorter for cord blood (~1 wk) than for adult blood (~3 wk) (Ingram et al. 2004; Melero-Martin et al. 2007; Smadja et al. 2019). Moreover, the abundance of ECFC colonies obtained is significantly different, with ~2–5 colonies/mL from human umbilical cord blood and 0.05 to 0.2 colonies/mL from adult peripheral blood. In addition, in healthy subjects, ECFC numbers vary with age, although the mechanisms that regulate their abundance in vivo remain poorly understood. Certainly, consensus holds that ECFCs are abundant in fetal blood (Javed et al. 2008; Baker et al. 2009; Ligi et al. 2011), umbilical cord blood at

birth (Ingram et al. 2004; Melero-Martin et al. 2007; Moreno-Luna et al. 2014), and in children (Levy et al. 2013). In contrast, ECFCs are rare in adult peripheral blood (Gulati et al. 2003; Ingram et al. 2004; Yoder et al. 2007), although it is unclear whether ECFC levels continuously decline during adulthood. A survey among experience laboratories revealed a success rate of ~70% in isolating ECFCs from peripheral blood of healthy adults (Smadja et al. 2019). Indeed, a recent study that analyzed blood samples from 116 participants corroborated that no ECFCs colonies were obtained from ~25% of the subjects (Toupance et al. 2020). Thus, unsuccessful isolation of ECFCs (i.e., “zero colonies”) is expected from some adult subjects, and it should be openly reported as such.

Some limitations are intrinsic to the current method used to isolate ECFCs. Relying on the growth of colonies in culture from a highly heterogeneous population of MNCs is inherently not precise. Moreover, this approach does not promote the emergence of only ECFC colonies. Indeed, numerous subpopulations of circulating MNCs (e.g., blood stem, progenitor, or committed cells) express integrin receptors for extracellular matrix molecules such as fibronectin and collagen and can readily attach to plates coated with these substrates and develop into colonies of various phenotypes. A notable example is monocytes, which are abundant in peripheral blood and can efficiently attach and produce colonies upon exposure to endothelial growth media. Moreover, some of the monocytic colonies can take up acetylated LDL (AcLDL), exhibit high affinity for lectin binding, and express several cellular markers shared with ECs (e.g., vWF, eNOS, CD31, VE-cadherin, and VEGFR2) (Hassan et al. 1986; Schmeisser et al. 2001, 2003; Yoder 2012), making ECFC detection and quantification challenging. In addition, platelets present in most MNC preparations can transfer some plasma membrane proteins shared by ECs to adherent cells in a nonspecific manner (Prokopi et al. 2009), further complicating the identification of ECFCs (for review, see Yoder 2012).

Notwithstanding these challenges, the isolation of ECFCs benefits from their unique proliferative potential. Indeed, the superior prolifera-

tive capacity of ECFCs over hematopoietic cells will generally outpace the growth of any other hematopoietic colony in the culture (Melero-Martin and Bischoff 2008). Nonetheless, in samples of adult blood, myeloid cells are much more abundant, and can adhere to and populate the culture plates such that ECFC colonies could be left with limited free space to expand. In these cases, it is customary to pick the ECFC colonies (either manually under a microscope or with cloning rings) and transfer the cells to new culture plates where they can be expanded free from the hematopoietic cells, thereby reducing the chances of contaminating cells.

An additional concern is the potential contamination with mesenchymal cells. Although rarer than the hematopoietic contaminants, circulating mesenchymal cells have been reported, mainly in umbilical cord blood samples (Kim et al. 2004; Lee et al. 2004; Ricousse-Roussanne et al. 2004; Melero-Martin et al. 2008). Thus, blood-derived mesenchymal cells constitute a potential contaminant that can easily adhere and proliferate in the culture plates. Fortunately, mesenchymal cells do not express endothelial-specific cell-surface markers such as CD31, and therefore they can be easily depleted from the culture via flow cytometry sorting or magnetic bead purification steps (Melero-Martin et al. 2008).

A long-standing goal of the field is to develop a reliable alternative methodology to identify and isolate ECFCs by flow cytometry or other immunological techniques based on cell-surface antigen expression. This approach, however, has turned out to be an elusive endeavor for several reasons. The first is the low abundance. ECFCs circulate at <5 cells/mL in human umbilical cord blood and <0.2 cells/mL in adult peripheral blood (Ingram et al. 2004), making it extremely challenging to sort circulating ECFCs directly from blood without an enrichment strategy. Second, it is unclear that ECFCs can be prospectively separated from the rest of MNCs based on a set of cellular markers. Initially, the focus was on their expected coexpression of the cell-surface antigens CD34, CD133, and VEGFR2. Indeed, some early studies reported successful ECFC enrichment by dual CD34 and CD133 selection

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using large volumes of cord blood (Wu et al. 2004). However, this strategy was abandoned as multiple studies demonstrated that these antigens are either not specific or not suitable altogether (for review, see Yoder 2012). For instance, Case et al. (2007) reported that purified CD34<sup>+</sup> CD133<sup>+</sup> VEGFR2<sup>+</sup> cells were highly enriched in hematopoietic progenitor activity but did not yield any endothelial colonies in vitro. Indeed, reports that later turned out to be only applicable to putative myelomonocytic EPCs and not ECFCs were rife in the early scientific literature. In addition, the expression of certain cell-surface molecules in cultured ECFCs (mostly common EC markers) do not guarantee that one could use these markers to isolate ECFCs from circulation. Unfortunately, the phenotypic characteristics of circulating ECFCs remain largely unknown. Perhaps, one of the few exceptions is the expression of the cell-surface marker CD34, which has consistently been reported to allow for enrichment of circulating ECFCs within MNCs (Timmermans et al. 2007; Lin et al. 2015).

### PHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF HUMAN ECFCs

Multiple independent laboratories have extensively characterized cultured ECFCs, and thus their phenotype is reasonably well understood. In culture, human ECFCs display attributes that are consistent with those of typical ECs. Indeed, cultured ECFCs express EC markers such as CD31, VE-cadherin, vWF, CD146, eNOS, and VEGFR2 at protein and mRNA levels (Fig. 2; Lin et al. 2000; Ingram et al. 2004; Melero-Martin et al. 2007). Moreover, genome-wide transcriptional profiling and ultrastructural analysis has confirmed that cultured ECFCs exhibit commitment to the endothelial lineage (Medina et al. 2010b; Wang et al. 2020). Currently, there is little controversy regarding the endothelial identity of ECFCs in culture, including their negative expression of hematopoietic markers such as CD45 and CD14 (Medina et al. 2017).

Early studies focused on the notion that human circulating ECFCs coexpress CD34, CD133, and VEGFR2 (Gehling et al. 2000; Peichev et al. 2000), although this premise has been

repeatedly challenged (Gulati et al. 2003; Rehman et al. 2003; Case et al. 2007; Yoder et al. 2007; Medina et al. 2010b). Indeed, the true phenotype of circulating ECFCs remains unclear. However, there is little uncertainty about these markers in culture. Isolated ECFCs consistently express VEGFR2 and CD34 (Lin et al. 2000; Ingram et al. 2004; Melero-Martin et al. 2007; Medina et al. 2010b; Wang et al. 2020) (although CD34 levels vary over time—highly expressed in early passages but expressed at lower levels as ECFCs are expanded), whereas there is no conclusive evidence for a meaningful expression of CD133 (Timmermans et al. 2007).

Conceptually, cultured ECFCs are different from mature ECs derived from existing vasculatures (Fig. 3). Cultured ECFCs are differentiated ex vivo from circulating progenitors and presumably have not been part of a functional blood vessel in vivo, with the caveat of a possible existence of naive ECFCs in the vessel wall. In contrast, mature ECs are derived from the lining of blood vessels and thus have previously functioned as ECs in vivo. However, despite this conceptual difference, once in culture, ECFCs and mature ECs exhibit a wide range of phenotypic and functional similarities. Indeed, identifying distinct attributes that distinguish cultured ECFCs from mature ECs remains elusive, and the search for unique human ECFC markers continues to be an area of intensive investigation.

Recently, Hong et al. (2021) suggested that the constitutive expression of the cardioprotective growth factor NRG1 could serve as a distinct marker for human cultured ECFCs. Human ECFCs isolated from adult and umbilical cord blood exhibited high constitutive expression of NRG1, whereas NRG1 expression was notably absent in mature ECs derived from the wall of the umbilical cord and the vasculatures of adipose and myocardial tissues (Hong et al. 2021). Whether or not NRG1 expression can be used to isolate circulating ECFCs remains to be seen. Also, further studies are warranted to determine whether NRG1 can distinguish blood-derived ECFCs from other presumed sources of ECFCs, namely, those derived from the blood vessel walls in various tissues.

Cultured ECFCs and mature ECs differ in their ability to grow clonally. Although some mature ECs can certainly form colonies from single cells, ECFCs do so more robustly. Indeed, cultured ECFCs exhibit a hierarchy of clonal proliferative potential that is absent in mature ECs. This distinction includes highly proliferative ECFCs that can achieve at least 100 population doublings, replat into at least secondary and tertiary colonies, and retain high levels of telomerase activity (Ingram et al. 2004; Ferratge et al. 2017; Toupance et al. 2020). The enormous proliferative capacity of cultured ECFCs translates into a prolonged life span in vitro, which, in practical terms, is appealing to generate many ECs. The overall expansion potential of cultured ECFCs differs depending on the source—ECFCs from umbilical cord blood are significantly more proliferative than from adult peripheral blood (Ingram et al. 2004; Melero-Martin et al. 2007; Au et al. 2008). In any case, the clonal expansion potential, extended life span, and high telomerase activity are defining (and measurable) attributes of cultured ECFCs.

Functionally, cultured ECFCs are competent vascular ECs. For instance, ECFCs exhibit affinity for the uptake of AcLDL and for *Ulex europaeus* agglutinin I (UEA-I), a lectin that binds to glycoproteins and glycolipids containing  $\alpha$ -linked fucose residues on the surface of ECs (Ingram et al. 2004; Moreno-Luna et al. 2014). In vitro, ECFCs grown on top of a basement membrane extracellular matrix (e.g., Matrigel) can self-assemble into tube-like structures and can form vascular networks when seeded in three-dimensional scaffold (Wu et al. 2004; Sieminski et al. 2005; Fuchs et al. 2007; Melero-Martin et al. 2007; Wang et al. 2020). In addition, they respond to angiogenic stimuli by extending luminal sprouts (Sobrino et al. 2016; Wang et al. 2020). Cultured ECFCs display antithrombogenicity by promoting anticoagulant properties and counteracting platelet activation and aggregation (Cuccuini et al. 2010). If activated by vasoactive agents, ECFCs release vWF (Randi et al. 2018; Jong et al. 2019), which promotes binding of platelets and t-PA that, in turn, enhances control by fibrinolysis. Moreover, ECFCs can regulate specific inflammatory processes, including

leukocyte trafficking. Indeed, upon exposure to inflammatory cytokines (e.g., TNF- $\alpha$ , IL-1), cultured ECFCs up-regulate various leukocyte adhesion molecules (e.g., E-selectin, VCAM-1, ICAM-1), which facilitate the adhesion of blood leukocytes (Melero-Martin et al. 2007; Wang et al. 2020).

### VASCULOGENIC ABILITY OF HUMAN ECFCs

Another defining attribute of ECFCs is the capacity to form functional blood vessels in vivo (for review, see Nowak-Sliwinska et al. 2018). The vasculogenic ability of human cultured ECFCs has been repeatedly demonstrated using xenograft models with various strains of immunodeficient mice (Gulati et al. 2003; Bompais et al. 2004; Hur et al. 2004; Yoon et al. 2005; Melero-Martin et al. 2007, 2008; Nagano et al. 2007; Yoder et al. 2007; Au et al. 2008; Traktuev et al. 2009; Chen et al. 2010, 2012; Lin et al. 2014, 2017; Hong et al. 2021). In one of the earliest demonstrations, Shepherd et al. showed that cord blood–derived ECFCs could repopulate decellularized human skin substitutes; after transplanting the grafts into mice, ECFCs were shown forming perfused vessels that connected with incoming host vessels (Shepherd et al. 2006). In 2007, Melero-Martin et al. demonstrated that ECFCs could self-assemble into perfused vascular networks in vivo. Human ECFCs and saphenous vein smooth muscle cells were embedded in Matrigel and injected subcutaneously into immunodeficient nude mice. After 1 wk, the implants contained an extensive network of perfused blood vessels lined by the ECFCs, which indicated the formation of functional anastomoses with the host vasculature (Melero-Martin et al. 2007). Importantly, ECFCs from both umbilical cord blood and adult peripheral blood were shown to rapidly form perfused vessels in vivo (Melero-Martin et al. 2007), although subsequent studies suggested that only the vessels formed by cord blood–derived ECFCs are long-lasting (Au et al. 2008). Moreover, Yoder et al. (2007) demonstrated human ECFCs to form perfused blood vessels in vivo without supporting exogenous perivascular cells. Nevertheless, the microvascular density achieved with

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ECFCs in the absence of mural cells is notably less than that achieved with perivascular support (Au et al. 2008; Melero-Martin et al. 2008; Chen et al. 2010). Indeed, various sources of perivascular cells can facilitate the robust formation of vascular networks by human ECFCs, including smooth muscle cells (Melero-Martin et al. 2007), mesenchymal stem cells (MSCs) from both the bone marrow and adipose tissue (Melero-Martin et al. 2008; Traktuev et al. 2009; Lin et al. 2014), and dermal fibroblasts (Chen et al. 2010).

In summary, the phenotypical characteristics of cultured human ECFCs are well understood and consistent with those of typical ECs. ECFCs are competent ECs in many respects and display functional attributes that are inherent to ECs both in vitro and in vivo. In addition, cultured ECFCs exhibit distinct high clonal expansion potential, extended life span, and high telomerase activity. These ECFC characteristics have been experimentally demonstrated by multiple research groups and are not controversial. Importantly, human ECFCs maintain their endothelial identity through prolonged periods in culture, indicative of phenotypic stability (Melero-Martin et al. 2007).

### HUMAN ECFCs IN CLINICAL DISORDERS

The widespread access to peripheral blood has made human ECFCs a valuable tool to study endothelial dysfunction in various clinical disorders (Medina et al. 2012). In addition, there is growing interest in the diagnostic and prognostic potential of ECFCs in health and disease (for review, see Paschalaki and Randi 2018).

An area of particular interest has been the study of ECFC levels and function in pregnancy-related disease and associated comorbidities. ECFCs are abundant in fetal blood and have been postulated to contribute to the rapid formation of the fetal vasculature and to the maintenance of vascular integrity during the third trimester of pregnancy (Hirschi et al. 2008; Javed et al. 2008; Baker et al. 2009; Ligi et al. 2011; Moreno-Luna et al. 2014). Moreover, recent evidence suggests that adverse conditions during fetal life could impair ECFC levels and function. For instance, offspring of diabetic mothers have a reduced

number of circulating ECFCs with impaired functionality (Ingram et al. 2008; Acosta et al. 2011; Blue et al. 2015), possibly contributing to long-term cardiovascular complications. Similar observations have been reported in neonates with bronchopulmonary dysplasia (BPD) (Borghesi et al. 2009; Baker et al. 2012), intrauterine growth restriction (Sipos et al. 2013), and in pre-eclampsia (Muñoz-Hernandez et al. 2014). Collectively, these observations are intriguing, especially considering that numerous cardiovascular diseases have their origin during development (Barker et al. 1989). Nevertheless, whether variations in ECFC levels and function during pregnancy contribute to long-term cardiovascular pathologies in the offspring remains unclear.

Postnatally, the association between ECFCs and disease has also been extensively examined in various pathological conditions, including hematological disorders (for review, see Paschalaki and Randi 2018). For instance, ECFCs have been used to study the mechanisms of synthesis and storage of vWF and EC dysfunction in patients with von Willebrand disease (vWD) (Starke et al. 2013; Wang et al. 2013). Studies with ECFCs from vWD patients have revealed that vWF plays a previously unknown role in regulating angiogenesis, showing the investigative potential of ECFCs (Starke et al. 2011; Groeneveld et al. 2015; Selvam et al. 2017). Other hematological disorders in which patients' ECFCs were used to study disease-specific EC dysfunctions include sickle cell anemia (Milbauer et al. 2008), hereditary haemorrhagic telangiectasia (HHT) (Fernandez-L et al. 2005, 2006a,b), venous thromboembolic disease (VTE) (Alvarado-Moreno et al. 2016; Hernandez-Lopez et al. 2017), and, more recently, COVID-19 (Alvarado-Moreno et al. 2021). Further studies are warranted to elucidate whether ECFCs contribute to the increased risk of thrombosis observed in these pathologies or simply represent a biomarker of disease.

ECFCs have also been examined in patients with myeloproliferative neoplasms. For example, studies have shown that patients with Philadelphia chromosome-positive chronic myeloid leukemia (CML) exhibit up to a ninefold increase in ECFC levels compared to healthy donors, and these ECFCs displayed an increased proliferative

potential upon isolation in culture (Otten et al. 2008), possibly reflecting the mobilization of EC precursors during these malignancies. Of note, CML patients harbor a molecular abnormality (e.g., JAK2V617F or MPLW515K mutations, SOCS gene hypermethylation) in their hematopoietic/granulocyte lineage and thus have been instrumental in helping to clarify the original lineage of ECFCs. Indeed, ECFCs from most patients with chronic myeloproliferative disorders lack the disease-specific molecular clonality marker, indicating that they are not clonally related to hematopoietic progenitors (Yoder et al. 2007; Piaggio et al. 2009). However, a subsequent study by Teofili et al. (2011) showed that in a subset of thrombotic patients with Ph-negative myeloproliferative neoplasms, ECFCs exhibit the JAK2V617F mutation, suggesting a complex and poorly understood relationship between the hematopoietic lineage, ECFCs, and the risk of thrombosis.

Human ECFC levels have also been analyzed in ischemic cardiovascular disease. For instance, clinical studies have shown an increased abundance of circulating ECFCs in the early phase of acute myocardial infarction (MI) (Massa et al. 2005, 2009). These observations were corroborated in a swine model of acute myocardial ischemia, which rapidly mobilized highly proliferative ECFCs into the animal's circulation (Huang et al. 2007). In addition, high ECFC levels were associated with the presence of significant coronary artery disease in patients referred for coronary angiography (Güven et al. 2006). Moreover, the presence of circulating ECFCs in patients within 12 hours from the onset of acute MI has been associated with a favorable outcome, including a reduced microvascular obstruction, infarct size, and left ventricular remodeling (Meneveau et al. 2011). Collectively, these findings suggest that ECFCs might be acutely mobilized into circulation upon myocardial ischemia, although the mechanisms that regulate this mobilization remain unknown. ECFCs may serve as markers of preserved microvascular integrity in patients with ischemic cardiovascular disease. However, it is not known whether the function of ECFCs is significantly altered in these patients. Dauwe et al. (2016)

showed that ECFCs isolated from patients with stable ischemic heart failure exhibited growth kinetics and neovascularization potential similar to those from healthy age-matched volunteers. Similarly, few differences were observed in ECFC levels and function between patients with premature coronary artery disease and healthy individuals (Stroncek et al. 2009; Brittan et al. 2015). Thus, ECFCs in culture might not reflect the vascular dysfunction commonly present in ischemic cardiovascular pathologies.

Cultured ECFCs have been instrumental in the study of the pathogenesis of pulmonary arterial hypertension (PAH). Toshner et al. (2009) showed that ECFCs isolated from patients with hereditary PAH caused by mutations in the bone morphogenetic protein receptor type 2 gene (BMPR-II) exhibited a hyperproliferative phenotype with impaired blood vessel-forming ability that was reminiscent of the pathological vascular remodeling typically observed in these patients. Additional studies have used ECFCs isolated from patients to demonstrate various aspects of endothelial dysfunction in PAH, including (1) the role of translationally controlled tumor protein (TCTP) as a mediator of endothelial prosurvival and growth signaling in PAH (Lavoie et al. 2014), (2) metabolic abnormalities, with a switch from oxidative phosphorylation to aerobic glycolysis in and idiopathic PAH (Caruso et al. 2017), (3) an increased expression of chloride intracellular channel 4 (CLIC4) in line with that observed in pulmonary vessels (Wojciak-Stothard et al. 2014), and (4) a pathogenetic role of type I interferon—ECFCs from the blood of PAH patients were more sensitive to the effects of type I IFN than those from healthy donors (George et al. 2014). Moreover, donor ECFCs have also been used to investigate possible therapeutic approaches to PAH. Long et al. (2015) isolated ECFCs from PAH patients with BMPR-II mutations and demonstrated that exposure to BMP9 could counteract the enhanced apoptosis and decreased vascular stability typical of the PAH pathogenesis. Indeed, even though BMP9 acts through BMPR-II, BMP9 protected BMPR2-mutant ECFCs, presumably due to signaling via the wild-type receptor (Long et al. 2015). Also, Dunmore et al. (2013) showed that

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the lysosomal inhibitor, chloroquine, can increase cell-surface BMPR-II levels and restores BMP9 signaling in ECFCs harboring BMPR-II mutations. Once again, ECFCs were proven instrumental in the search for novel potential therapeutics in PAH.

ECFCs isolated from patients with chronic obstructive pulmonary disease (COPD) have also enabled the study of EC dysfunction. For instance, Paschalaki et al. (2013) showed that cultured ECFCs from smokers and COPD patients exhibited accelerated aging due to epigenetic changes and displayed impaired ability to form robust vascular networks in vivo compared to ECFCs from healthy nonsmokers. Of note, this study showed that patients' ECFCs were susceptible to treatment with both the SIRT1 activator resveratrol and an ATM inhibitor (KU-55933), which rescued their dysfunctional phenotype (Paschalaki et al. 2013). In addition, Paschalaki et al. (2018) demonstrated that COPD patients' ECFCs exhibited microRNA dysregulation (specifically, miR-126-3p), affecting various aspects of endothelial senescence and dysfunction. Similarly, in idiopathic pulmonary fibrosis (IPF), studies have shown that patients with the exacerbated disease (i.e., low diffusing capacity of the lung for carbon monoxide <40%) exhibited both elevated ECFC levels and ECFCs with higher proliferative potential compared to patients with mild disease and healthy controls (Smadja et al. 2013), suggesting an adaptive response to disease severity. Meanwhile, Bacha et al. (2018) showed that compared to healthy controls, ECFCs isolated from IPF patients released more microparticles and exhibited increased plasminogen activation, producing a significant activation of fibroblast migration, thus implicating ECFCs in this pathology.

Taken together, it is clear that the use of blood-derived ECFCs has become a valuable research tool to study endothelial dysfunction in clinical disorders. Unfortunately, some of the methods to isolate and culture ECFCs vary considerably among laboratories, and comparison across studies should be made with caution. Certainly, the need to standardize methods remains a priority in the field (Medina et al. 2017; Smadja et al. 2019). Also, it is important to recognize that

most findings of ECFCs in clinical disorders involve their isolation in culture and thus are associative (i.e., causative roles of ECFCs remain unknown in most pathologies). In addition, our ability to study the actual involvement of human ECFCs in vivo, during physiological vascular homeostasis, or in pathological disorders, continues to be limited.

## THERAPEUTIC POTENTIAL OF HUMAN ECFCs

The discovery of ECFCs in human peripheral blood over two decades ago was tremendously exciting because it created a promising opportunity to noninvasively obtain large quantities of highly vasculogenic autologous ECs for therapeutic vascularization. Indeed, the therapeutic potential of cultured human ECFCs has been repeatedly demonstrated in various disease models (for review, see Tasev et al. 2016; Paschalaki and Randi 2018). Nevertheless, it is essential to recognize that most evidence has come from preclinical animal studies, mainly using immunodeficient rodents as recipients.

Most studies on the therapeutic attributes of human ECFCs have focused on harnessing their blood vessel-forming ability and their capacity to incorporate at sites of neovascularization (i.e., ECFC direct contribution) including the use of cultured ECFCs in the revascularization of ischemic tissues. For instance, numerous studies have substantiated the capacity of ECFCs to promote the revascularization of ischemic hind limbs in mice, leading to functional recovery of blood flow in the affected tissues (Hur et al. 2004; Nagan et al. 2007; Smadja et al. 2008; Bouvard et al. 2010; Saif et al. 2010; Schwarz et al. 2011; Soler and Grosfeld 2014; Kang et al. 2017). Likewise, ECFCs promote vascularization in mouse models of MI (Kang et al. 2013; Lee et al. 2014; Hong et al. 2021), stroke and ischemic brain injury (Moubarik et al. 2011; Huang et al. 2013; Zhang et al. 2013; Ding et al. 2016), and ischemic retinopathy (Medina et al. 2010a; Dellett et al. 2017; Sakimoto et al. 2017; Reid et al. 2018; O'Leary et al. 2019). In most of these demonstrations, the mechanism of action entails the direct incorporation of ECFCs into the new vasculature, signif-

icantly improving blood flow and, in turn, restoring the function of the affected tissues to various degrees.

The robust vasculogenic properties of ECFCs have also been instrumental in tissue engineering research. Integrating functional vascular networks into bioengineered tissue constructs to enable adequate oxygenation, nutrient delivery, and removal of waste products upon implantation remains rate-limiting in many efforts in tissue engineering (for review, see Wang et al. 2019). Over the last two decades, researchers have resorted to exploiting the inherent blood vessel-forming ability of ECFCs to incorporate such vascular networks into multiple types of 3D scaffolds. Initially, models were simply focused on demonstrating that vascular networks engineered with ECFCs could stably engraft and exhibit durable function following implantation into immunodeficient mice (Melero-Martin et al. 2007, 2008; Nagano et al. 2007; Au et al. 2008; Traktuev et al. 2009; Chen et al. 2010, 2012; Lin et al. 2014, 2017). The engineered vascular networks could be explanted and reconnected into secondary mice, reestablishing perfusion and thus extending their potential in transplantable tissue-engineered constructs (Kang et al. 2011). Indeed, the ability to generate a functional vasculature with ECFCs have been applied to bioengineer more complex tissues, including myocardium (Riemenschneider et al. 2016; Schaefer et al. 2018; Hong et al. 2021), skin (Shepherd et al. 2006; Kung et al. 2008; Kim et al. 2009; Hendrickx et al. 2010), and bone (Fuchs et al. 2009; Levengood et al. 2011; Klotz et al. 2018; Kim et al. 2021).

In addition to their direct potential as vasculogenic cells, human ECFCs could also serve as indirect therapeutic agents. For example, studies have shown that cultured ECFCs provide paracrine reparative effects in models of PAH, BPD (Baker et al. 2013; Alphonse et al. 2014), and acute kidney injury (Burger et al. 2015; Viñas et al. 2016; Collett et al. 2017). Mechanistically, these regenerative effects are primarily indirect and mediated by specific paracrine growth factors or extracellular vesicles secreted from the transplanted ECFCs. Also, emerging evidence indicates that ECFCs can regulate regenerative

processes via paracrine production of trophic factors (Traktuev et al. 2009; Lin et al. 2012, 2014; Hong et al. 2021). Thus, using human ECFCs to provide trophic signals in support of specific stem cells in vivo has become an additional area of therapeutic interest (Tasev et al. 2016).

Another potential therapeutic application for human ECFCs is gene therapy. Indeed, ECFCs have been an appealing choice of autologous cells for genetic manipulation and gain-of-function studies due to their accessibility, expansion potential, prolong life span in culture, and robust ability to engraft. Preclinical applications in gene therapy have mainly entailed gain-of-function studies using genetically modified human ECFCs, including the overexpression of coagulation factor VIII to treat hemophilia A (Lin et al. 2002; Matsui et al. 2007), erythropoietin to treat anemia (Lin et al. 2011), and expression of tumor growth inhibitors in cancer (Dudek et al. 2007; Wei et al. 2007; Bodempudi et al. 2010). Studies have also included loss-of-function genetic modifications. For instance, Abrahimi et al. used clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) technology to selectively ablate ECFC expression of specific genes that encode proteins involved in T-cell activation (Abrahimi et al. 2015, 2016). This approach could become important for using ECFCs in future allogeneic therapeutic applications.

Notwithstanding the wealth of preclinical demonstrations using animal models, the number of clinical trials with human ECFCs over the last two decades has been quite low and has almost exclusively involved using ECFCs as biomarkers rather than as potential therapeutic agents (see [www.clinicaltrials.gov](http://www.clinicaltrials.gov)). This contrasts with the numerous trials that have been conducted with putative EPCs to treat various pathologies, including heart disease, diabetes, peripheral arterial disease, pulmonary disease, and cancer. Considering that ECFCs were identified almost two decades ago, the paucity of clinical trials is surprising considering that ECFCs can be derived by a simple blood draw, and thus could be readily accessible from almost any patient. However, several issues may explain the

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low number of clinical trials with ECFCs. For one, until recently, ECFC research was filled with controversies and confusion surrounding their definition, nomenclature, and origin. As well, the low frequency of ECFCs in adult peripheral blood and the lack of distinctive markers remain practical challenges (Ingram et al. 2004; Melero-Martin et al. 2007; Mund et al. 2012). Moreover, there are concerns regarding variability among donors and the absence of ECFCs in a substantial proportion of adult patients (Thill et al. 2008; Stroncek et al. 2009; Rignault-Clerc et al. 2013). Also, ECFCs require supporting perivascular cells to engraft robustly in vivo, complicating clinical translation (Loffredo and Lee 2008). Finally, the mechanism by which ECFCs are mobilized into circulation and how this process is regulated is not well understood in health and disease. While some of these concerns may remain for some time, as consensus grows regarding their identity (Medina et al. 2017), and with the standardization of isolation and culture methods (Smadja et al. 2019), the clinical use of ECFCs is likely to increase.

## CONCLUSIONS AND PERSPECTIVES

The vascular endothelium, which lines the lumen of all blood vessels, controls the passage of nutrients into tissues, maintains blood flow, and regulates the trafficking of leukocytes, among other functions. For decades, EC biology has been studied with cultures of mature ECs obtained from living vasculature. However, future vascular cell therapies will likely require a non-invasive source of autologous ECs with greater translational potential than mature ECs. Blood-derived ECFCs represent a robust population of progenitors that can generate autologous ECs with an enormous capacity to proliferate and the ability to form a bona fide functional endothelium in vivo. However, to date, most translational studies with human ECFCs are preclinical. While there has been years of debate and controversy concerning the exact definition, origin, and characteristics of human ECFCs, recent consensus over ECFC identity and the standardization of isolation and culture methods improves the prospect for clinical trials.

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