



# Recent Advances in Endothelial Colony Forming Cells Toward Their Use in Clinical Translation

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The term “Endothelial progenitor cell” (EPC) has been used to describe multiple cell populations that express endothelial surface makers and promote vascularisation. However, the only population that has all the characteristics of a real “EPC” is the Endothelial Colony Forming Cells (ECFC). ECFC possess clonal proliferative potential, display endothelial and not myeloid cell surface markers, and exhibit pronounced postnatal vascularisation ability *in vivo*. ECFC have been used to investigate endothelial molecular dysfunction in several diseases, as they give access to endothelial cells from patients in a non-invasive way. ECFC also represent a promising tool for revascularization of damaged tissue. Here we review the translational applications of ECFC research. We discuss studies which have used ECFC to investigate molecular endothelial abnormalities in several diseases and review the evidence supporting the use of ECFC for autologous cell therapy, gene therapy and tissue regeneration. Finally, we discuss ways to improve the therapeutic efficacy of ECFC in clinical applications, as well as the challenges that must be overcome to use ECFC in clinical trials for regenerative approaches.

**Keywords:** endothelial progenitors, blood outgrowth endothelial cells, vascular regeneration, cell therapy, gene therapy, tissue bioengineering

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## INTRODUCTION

The search for endothelial progenitors started in the late 1990s, when Asahara et al. reported the existence of circulating cells with endothelial surface markers and repair capacity, and labeled them “endothelial progenitors” (1). Shortly after, Lin et al. reported the presence of circulating cells which could differentiate into cells with the phenotypic characteristics of vascular endothelium (2). These studies triggered what was to become a decade-long debate on the existence and nature of “endothelial progenitor cells.” The controversy sadly damaged the “brand” EPC, so that the acronym is no longer in favor. EPC have been defined as circulating cells with the ability to differentiate into mature endothelial cells and contribute to postnatal vasculogenesis and endothelial repair at sites of vascular damage (3). We now know that some of the populations originally defined as EPC do not fulfill this definition. These include myeloid angiogenic cells (MAC), also called circulating angiogenic cells (CAC) or “early” EPC, which in fact are of hematopoietic origin and promote angiogenesis through paracrine mechanisms, but cannot give rise to mature endothelial cells (4–8). The population of circulating cells, which show clonal potential, ability to give rise to mature endothelial cells and promote vascular formation *in vitro* and *in vivo*, have been called endothelial colony forming cells (ECFC), blood outgrowth endothelial cells (BOEC), or late outgrowth EPC (4). For years, different groups have used their favorite acronym; it was recently suggested that the term “ECFC” should be used to harmonize the literature (4).

ECFC are more frequently isolated from cord blood or peripheral blood (2, 9) but can also be isolated from tissue-resident vascular endothelium, including human umbilical cord (9, 10), pulmonary artery endothelium (11), lung tissue (12), placenta (13), or can be derived from induced pluripotent stem cells (iPSC) (14).

The origin of circulating ECFC still remains unclear. The first study from Lin et al. on ECFC from circulating blood of patients undergoing bone marrow transplantation suggested a bone-marrow origin (2). Subsequent studies were unable to confirm these findings and proposed that ECFC are more likely to originate from tissue vascular niches (15). This is in line with recent findings suggesting the existence of vascular resident endothelial cells with clonal potential that contribute to postnatal angiogenesis *in vivo*, which is one of the main characteristics of ECFC (16–18). Transcriptome analysis of peripheral blood ECFC samples suggests a profile close to microvascular endothelial cells (19). Regardless of their origin, ECFC have attracted significant attention because of their potential for translational studies, gene therapy and vascular regeneration. In this review, we will discuss current applications and future perspectives of the use of ECFC in clinical translation (Figure 1).

## ECFC FOR THE INVESTIGATION OF ENDOTHELIAL CELL BIOLOGY IN DISEASE

ECFC are an invaluable tool to study molecular endothelial dysfunction in disease, giving access to endothelial cells from patients and control groups in a non-invasive way. ECFC have been used for functional and molecular studies in disease (20). Differences in terms of ECFC phenotype and functional abilities have been attributed in many cases to genetic or epigenetic dysfunction. Moreover, the number of ECFC colonies can vary between different groups, although the significance of this parameter is still unclear. It is also unclear whether ECFC directly contribute to the pathogenesis of diseases. These studies open the opportunity for personalized approaches to characterize molecular and phenotypic defects in diseases with heterogeneous clinical presentations. The main published literature is summarized below. Of note, interpretation and comparison across studies should be made with caution, as the ECFC isolation and culture methods vary between different laboratories, and this might affect the cells' proliferative capacity and possibly phenotype.

### Hematological Disorders

#### Von Willebrand Disease (VWD)

ECFC have been used to study endothelial dysfunction in patients with von Willebrand Disease (VWD), the most common genetic bleeding disorder. VWD is caused by a defect or deficiency in von Willebrand factor (VWF), an endothelial and platelet protein essential for haemostasis. The disease is genetically heterogeneous, mostly due to mutations in the VWF gene; clinically, three main subtypes have been identified: type 1 and 3 with quantitative defects, and type 2 with qualitative defects (21).

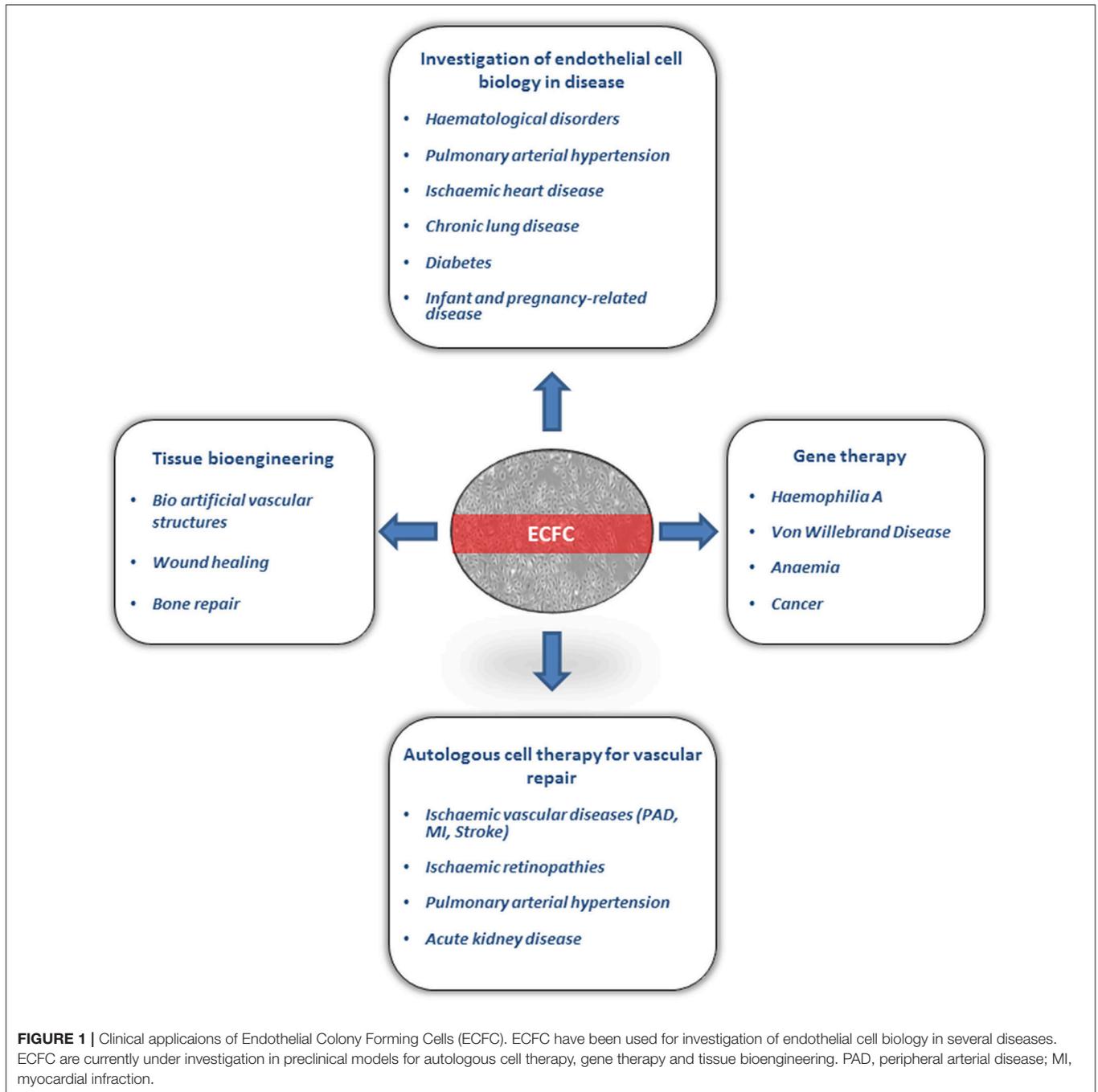
The classification can influence the therapeutic strategy, given that patients with type 1 VWD and cellular stores of VWF can be treated with an agent inducing VWF endothelial release (22), whilst patients with complete lack or dysfunction may require replacement therapy with plasma products. Using ECFC, it was possible for the first time to profile the synthesis and storage of VWF in endothelial cells from individual patients (23, 24), information which could be useful to influence the therapeutic choice. Besides its role in haemostasis, VWF has multiple vascular functions, including modulation of inflammation and angiogenesis (25). ECFC studies have confirmed that VWF plays a role in angiogenesis. In 2011, Starke et al. studied the *in vitro* angiogenic potential of ECFC isolated from type 1 and type 2 VWD patients and found overall significant enhancement of *in vitro* tube formation, proliferation and migration (26). Other studies have identified distinct defects in *in vitro* angiogenesis in ECFC from different VWD subtypes, and a significant degree of variability (27, 28). These studies highlight the great potential of ECFC to investigate processing and function of VWF in endothelial cells, and the need to standardize methods to be able to compare findings across laboratories.

#### Myeloproliferative Neoplasms (MPN)

In patients with Philadelphia chromosome positive chronic myeloid leukemia (CML), a nine-fold increase in the number of ECFC colonies and increased proliferative potential was described compared to healthy donors (29). Analysis of *BCR/ABL1* revealed that all ECFC clones were *BCR/ABL1* negative and therefore were not clonally related to *BRC/ABL1*<sup>+</sup> hematopoietic progenitors (29). Initial studies in patients with Philadelphia-negative myeloproliferative neoplasms identified the Janus kinase 2 V617F (JAK2<sup>V617F</sup>) mutation only in hematopoietic cells and not in ECFC (30). However, subsequent studies revealed a subgroup of patients with the JAK2<sup>V617F</sup> mutation in ECFC, who had increased thrombotic events compared to patients without the mutation, suggesting that ECFC might be a useful tool to evaluate the thrombotic risk in these patients (31). Mechanism studies showed that the presence of the JAK2<sup>V617F</sup> mutation in MPN ECFC was associated with hyper-phosphorylation of signal transducer and activator of transcription 3 (pSTAT-3) and STAT-5 (pSTAT-5), indicating abnormal activation of the JAK/STAT pathway in these cells. In addition, JAK2<sup>V617F</sup>-mutated ECFC showed increased adhesion to normal mononuclear cells (31). Further studies are required to determine whether JAK2<sup>V617F</sup> mutated ECFC contribute to the increased risk of thrombosis in these patients or simply represent a biomarker of disease.

#### Sickle Cell Anemia

In children with sickle cell anemia, studies on ECFC were performed to investigate whether genetic differences affecting endothelial function could underlie the development of inflammatory vasculopathy causing stroke (occlusive disease at the circle of Willis) (32). Gene expression profiling of ECFC from children with vasculopathy demonstrated exaggerated responsiveness to inflammatory stimuli compared to ECFC from children without vasculopathy. Specifically, ECFC from the



higher risk group showed increased RelA activation in response to stimulation with interleukin-1 $\beta$ /tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (32). It would be interesting to determine whether these alterations are genetic or epigenetic, and whether this abnormal inflammatory response is ECFC-specific or a general response also affecting circulating immune cells.

### Hereditary Haemorrhagic Telangiectasia (HHT)

Hereditary hemorrhagic telangiectasia (HHT) is caused by mutations in endoglin or activin receptor-like kinase-1

(ACVRL1/ALK1) genes and is an autosomal dominant vascular disorder. Isolation of ECFC from patients with HHT demonstrated reduced endoglin expression, impaired TGF- $\beta$  signaling, disorganized cytoskeleton, and impaired angiogenic ability in a Matrigel assay compared to healthy controls (33). Further studies on ECFC from HHT patients revealed impaired ALK1- and ALK5-dependent TGF- $\beta$  signaling, which promotes fragility of small vessels and vascular lesions; these may contribute to the clinical symptoms associated with this disease (34, 35).

## Venous Thromboembolic Disease (VTD)

Two studies in patients with recurrent and unprovoked VTD have described dysfunctional ECFC; this might be linked to the increased risk of thrombotic events. The number of the ECFC colonies was increased in patients with VTD compared to control groups; moreover, electron microscopy studies revealed abnormalities of mitochondrial membrane, suggesting mitochondrial dysfunction (36). An increased pro-inflammatory cytokines profile was also found in the supernatants of cultures of ECFC from VTD patients (36). Further studies showed that ECFC from patients with VTD exhibit reduced proliferation, increased senescence, increased levels of reactive oxygen species (ROS) and impaired expression of ephrin-B2/Eph-4 genes, markers of venous and arterial endothelium involved in vascular regeneration (37). The abnormal inflammatory response, increased oxidative-stress and senescence detected in ECFC from patients with VTD might contribute to a defective response of the endothelium to vascular injury in these patients, possibly leading to recurrent thrombotic events. However, further studies are required to determine whether dysfunctional ECFC are involved in driving abnormal thrombus formation in these patients, or ECFC dysfunction in VTD is predominantly a biomarker.

## Pulmonary Arterial Hypertension (PAH)

Pulmonary arterial hypertension (PAH) is a condition characterized by the formation of plexiform lesions and concentric intimal fibrosis in small pulmonary arteries, with increased blood pressure in the arteries of the lungs. Hereditary PAH (HPAH) is often caused by mutations in the bone morphogenetic protein receptor type 2 gene (BMPRII). ECFC have been extensively used to investigate the pathogenesis of PAH. ECFC from PAH patients with BMPRII mutations demonstrated a hyperproliferative phenotype with impaired ability to form vascular networks (38), suggesting a pathogenetic role in the observed vascular remodeling. ECFC from patients with BMPRII mutations show a compromised signaling in response to the endothelial BMPRII ligand BMP9, which can be restored by agents such as chloroquine (39). BMP9 has been described as the preferred ligand for preventing apoptosis and enhancing monolayer integrity in ECFC from patients with BMPRII mutations (40), and has been used to reverse established PAH in preclinical models, suggesting new potential therapeutic approaches for inherited PAH. Proteomic screening in ECFC of HPAH patients with BMPRII mutations compared to healthy control subjects revealed translationally controlled tumor protein as a key mediator of endothelial prosurvival and growth signaling in PAH (41). MicroRNA and proteomic profiling of ECFC from patients with HPAH and idiopathic PAH demonstrated metabolic abnormalities, confirming a switch from oxidative phosphorylation to aerobic glycolysis (42). This is possibly due to down-regulation of miR-124 and its targets of Polypyrimidine Tract Binding Protein and Pyruvate Kinase M2 in PAH (42). Increased expression of chloride intracellular channel 4 (CLIC4) has been reported in ECFC from patients with idiopathic PAH, in line with evidence in pulmonary vessels (43). Increased CLIC4 expression activates nuclear factor- $\kappa$ B, followed

by stabilization of hypoxia-inducible factor-1 $\alpha$  and increased production of vascular endothelial growth factor (VEGF) and endothelin-1, contributing to endothelial dysfunction in PAH (43). Studies on ECFC have also supported the concept of a pathogenetic role for type I interferon in PAH. ECFC from PAH patients treated with interferon  $\alpha$  show an abnormal increased release of interferon  $\gamma$  inducible protein 10 compared to healthy controls (44).

## Ischemic Heart Disease

ECFC studies in ischemic heart disease have multiple potential applications: as well as providing insights into the molecular mechanisms leading to endothelial dysfunction and how they can be targeted pharmacologically, ECFC are also being considered for autologous cell therapy for vascular regeneration (discussed later). The studies focusing on ECFC phenotype and/or function have so far yielded mixed findings, possibly because of the heterogeneity of this disease group. ECFC isolated from patients with stable ischemic heart failure demonstrated similar growth kinetics and neovascularization potential (*in vitro* and *in vivo*) compared to ECFC from control groups (young volunteers and age-matched controls) (45). In a model of hindlimb ischemia in nude mice, intramuscular injections of ECFC from patients with ischemic cardiomyopathy resulted in increased arteriogenesis similar to that observed with ECFC from healthy controls (45). Similar findings were confirmed in patients with premature coronary artery disease (46, 47). ECFC isolated from peripheral blood of patients with premature coronary disease showed no differences in the number of colonies or in the phenotype, function or microRNA expression profile, compared to healthy age and gender-matched controls (46). On the contrary, endothelial cells isolated from the vessel wall of the same patients exhibited impaired proliferation, adhesion and migration, and significantly reduced expression of microRNAs known to regulate endothelial function compared to controls (46), suggesting that in these patients circulating ECFC do not reflect the vascular dysfunction. Other studies focused on ECFC colony numbers. In patients referred for coronary angiography, higher numbers of ECFC colonies were associated with the presence of significant coronary artery disease, and ECFC number correlated with maximum angiographic stenosis severity (48). Interestingly, the onset of acute myocardial ischemia in a swine model resulted in rapid increase of ECFC numbers in the circulation compared to baseline levels, and mobilization of highly proliferative ECFC subpopulations (49). These findings suggest that ECFC could be acutely released in the circulation either from the damaged vascular tissue or in response to mobilizing factors secreted in the circulation due to myocardial ischemia (49). A subsequent study in patients with acute myocardial infarction (MI) vs. healthy controls confirmed increased ECFC numbers in the circulation within 3 h from the onset of symptoms (50). More recently, successful isolation of ECFC colonies in patients within 12 h from the onset of acute MI (51) was correlated with outcome. The presence of ECFC colonies was associated with reduced microvascular obstruction, infarct size and left ventricular remodeling, suggesting that circulating ECFC may be a marker of preserved microvascular integrity in

patients with acute MI (51). In these two studies, molecular and functional analysis of ECFC was not performed. Future studies on targeted patient populations will be required to understand the significance and possible role of ECFC in ischemic heart disease.

## Chronic Lung Disease in Adults

### Chronic Obstructive Pulmonary Disease (COPD)

COPD is a chronic inflammatory lung disease that results in largely irreversible and progressive airflow limitation. It is caused by long-term exposure to irritating gases, most often from cigarette smoke. We isolated ECFC from smokers and patients with COPD to study endothelial dysfunction in these groups that have increased risk of cardiovascular disease. ECFC from smokers and COPD patients exhibited accelerated aging due to epigenetic dysfunction (52). This involved reduction of sirtuin-1 (SIRT1), a protein deacetylase that protects against DNA damage, due to activation of ataxia telangiectasia-mutated (ATM) kinase, which plays a key role in DNA damage response. Impaired angiogenic ability of ECFC from COPD patients was shown in an *in vivo* Matrigel assay in nude mice. Importantly, targeted pharmacologically treatment was able to reduce the increased senescence in ECFC from COPD patients (52). Further studies demonstrated microRNA dysregulation in ECFC from COPD, affecting the miR-126-3p, a critical microRNA in vascular development, endothelial homeostasis and inflammation (53). We found that miR-126-3p is downregulated in ECFC from smokers and COPD patients and promotes an augmented DNA damage response through activation of ATM, contributing to endothelial senescence and dysfunction in these groups (54).

### Idiopathic Pulmonary Fibrosis (IPF)

IPF is a rare lung disease characterized by progressive scarring of the lungs and is associated with pulmonary vascular remodeling. ECFC isolated from patients with IPF compared to a control group showed no difference in terms of number of colonies or proliferative potential (55). However, subgroup analysis of the IPF patients showed increased number of ECFC colonies in IPF patients with significantly impaired gas transfer [Diffusing capacity of the lung for the carbon monoxide (DLco) <40%]. Also, ECFC proliferation was increased in patients with exacerbation compared to stable disease (55). Another study suggested that the vascular remodeling in fibrotic lung diseases might be regulated by cooperation of ECFC with fibrocytes, cells that coexpress hematopoietic and fibroblast markers and contribute to organ fibrosis (56). Further studies demonstrated increased microparticles released from ECFC isolated from IPF patients compared to controls; these exhibited increased plasminogen activation and could stimulate fibroblast migration (57), suggesting a pathogenetic role of ECFC-derived endothelial microparticles to pulmonary fibrogenesis.

## Diabetes

Most studies of ECFC in diabetes have investigated cord blood ECFC from gestational diabetes mellitus (GDM) pregnancies. The first study published by Ingram et al. demonstrated that cord blood ECFC from diabetic pregnancies exhibited premature

senescence and impaired proliferation *in vitro*, and reduced vasculogenic potential *in vivo* compared to cord blood ECFC from uncomplicated pregnancies (58). Microarray screen and validation of selected genes identified epigenetic dysfunction, involving DNA methylation of placenta-specific 8 as contributor to increased senescence and reduced proliferation of ECFC from GDM compared to control pregnancies (59). ECFC isolated from peripheral blood from patients with type 2 diabetes demonstrated reduced *in vitro* proliferation rate and importantly reduced neovascularization capacity *in vivo* compared to nondiabetic ECFC (60). Interestingly, pre-treatment of diabetic ECFC with globular adiponectin was able to improve neovascularization in a murine hind limb ischemia model (60).

## Premature Neonates, Pregnancy-Related Disease, and Associated Comorbidities

It is well established that ECFC from full-term cord blood emerge earlier in culture, proliferate faster and show enhanced vasculogenic ability compared to ECFC from adult peripheral blood (9, 61, 62). In contrast, numerous studies describe reduced numbers and dysfunction of ECFC isolated from low birth weight (LBW) or preterm (PT) infants, linked to associated comorbidities such as bronchopulmonary dysplasia (BPD) and increased risk of cardiovascular diseases in adulthood. The yield of ECFC colonies has been found reduced in PT infants (24–28 weeks) compared with term controls, whereas gestational age of 33–36 weeks yielded ECFC colonies at equivalent numbers to term infants (63). The angiogenic properties of ECFC have been found impaired in LBW preterm neonates *in vitro* and *in vivo* (64). Transcriptome profiling of LBW ECFC demonstrated an increased expression of antiangiogenic genes including thrombospondin 1 (64). The same group subsequently showed that ECFC isolated from PT infants display accelerated senescence due to reduced expression of SIRT1, and that SIRT1 overexpression could restore PT.

ECFC angiogenic ability *in vivo* (65). SIRT1 deficiency in PT ECFC was also found to regulate the biogenesis of microparticles, mediating a paracrine induction of senescence in naïve endothelial cells (66). These findings suggest a novel link between epigenetic dysfunction leading to ECFC senescence and increased cardiovascular risk in individuals born preterm.

Maternal characteristics and pregnancy related disease might also affect ECFC isolated from cord blood. For example, a positive correlation between pre-pregnancy maternal body mass index and ECFC yield has been described, which was independent of other obstetric factors (67). In preeclampsia, the number of cord blood ECFC was reduced compared to control pregnancies; however, ECFC function was similar between preeclampsia patients and controls, including the ability to form vascular networks *in vivo* (68). As mentioned earlier, gestational diabetes can also affect ECFC levels and function (58, 59).

## Bronchopulmonary Dysplasia (BPD)

BPD is a chronic lung disease mainly affecting low birth weight premature infants treated with supplemental oxygen and mechanical ventilation for respiratory distress syndrome. It associates with impaired vascular and alveolar growth. Several

studies suggest a pathogenetic role of ECFC in preterm infants. Increased number of ECFC colonies have been found in preterm cord blood; these cells exhibit increased growth but also increased apoptotic susceptibility to hyperoxia compared to ECFC from term cord blood (69). This effect might be caused to disruption of VEGF-nitric oxide signaling (70). Additional studies have demonstrated that the number of cord blood ECFC in preterm babies who developed moderate or severe BPD were significantly reduced (71, 72), suggesting that a low number of ECFC may contribute to abnormal vascular repair in preterm infants resulting in BPD.

## ECFC FOR AUTOLOGOUS CELL THERAPY FOR VASCULAR REPAIR

ECFC represent the most potent vascular reparative cell type among all putative “EPC,” the only cell population with clonal proliferative potential and ability to form blood vessels *in vivo* (9, 10, 73). Numerous preclinical *in vivo* studies have demonstrated the vascular reparative effect of ECFC in disease models (74). Several mechanism of action have been suggested: (a) direct incorporation of ECFC at sites of vascular damage and formation of blood vessels (61, 75), (b) paracrine effect, mediated by the release of pro-angiogenic growth factors or extracellular vesicles secreted from ECFC (76–79), (c) support of the reparative ability of other cells, such as mesenchymal progenitor cells (MPC) (80–82) or adipose stromal cells (ASC) (83). A combined mechanism of action has been suggested in several studies, showing direct incorporation of ECFC in the blood vessels and paracrine effect on other reparative cells (84, 85). However, in many studies the exact mechanism of action remains elusive. Below we review the evidence supporting the use of ECFC for autologous cell therapy and comment on the proposed mechanism of action.

ECFC have been used as cell therapy in several preclinical models of ischemic vascular diseases. They have been shown to promote neovascularization in numerous *in vivo* models of hind limb ischemia (84, 86–90) and MI (91). Direct incorporation of ECFC in the ischemic tissue has been demonstrated (84, 87, 88), and shown to be of significant importance for the functional recovery of the ischemic tissue (86). The co-implantation of ECFC with MPC further improved neovascularization of ischemic tissue compared to ECFC alone (84). However, whether this is due to direct involvement of ECFC and MPC in new blood vessels formation, or to ECFC and MPC-derived factors stimulating endogenous neovascularization is not clear (84). The use of ECFC in models of ischemic stroke (85, 92) and traumatic brain injury (93, 94) suggests that ECFC might be a promising treatment to improve tissue viability and function by promoting angiogenesis and neurogenesis. ECFC homing has been confirmed at sites of ischemic brain tissue (85, 92, 93). However, engraftment of ECFC into neovessels was limited and a therapeutic effect via a paracrine mechanism has been suggested (85).

ECFC cell therapy may also promote vascular repair and revascularization of ischemic retinopathies, such as diabetic

retinopathy and retinopathy of prematurity. Medina et al. using a murine model of retinal ischemia, demonstrated that ECFC could incorporate into the retinal microvascular tube network and promote vascular repair (95). ECFC might also exert a vasoregenerative effect in ischemic retinopathies via a paracrine way, mediated at least in part by extracellular vesicles containing microRNA that promote angiogenesis (96). Optimization studies of ECFC for cell therapy have identified a low dose of ECFC which was effective both by local intravitreal injection and systemic intracarotid delivery, without any significant toxicity (97).

ECFC autologous cell therapy might also exert vascular reparative effects in preclinical models of PAH and BPD (98). In a model of hyperoxia-induced BPD, intravenous administration of cord blood ECFC was able to reverse the alveolar growth arrest, preserved lung vascularity, and attenuated PAH (98). The observation of low ECFC engraftment and the protective effect of ECFC-derived conditioned media suggest a paracrine mechanism of action (98); this is also supported by other studies in experimental models of BPD showing prevention of PAH by ECFC-derived conditioned media (76).

ECFC cell therapy might also exert a therapeutic effect in acute kidney injury (AKI). Preclinical models have shown that use of ECFC can mitigate the severity of disease and preserve vascular function (77, 78, 99, 100). In most studies the effect is mediated by secreted factors from ECFC and not cell engraftment. In mice with ischemic AKI, intravenous administration of ECFC significantly attenuated increases in plasma creatinine, tubular necrosis, macrophage infiltration, oxidative stress and apoptosis, without cell engraftment in the kidneys (77). Administration of ECFC conditioned media or ECFC-derived exosomes also exerted a protective effect, indicating that the effects of cord blood ECFC are due to ECFC-derived exosomes (77, 78). Further studies demonstrated that ECFC exosomes were enriched in miR-486-5p, which targets the phosphatase and tensin homolog (PTEN) (79). In mice with ischemic kidney disease, infusion of ECFC exosomes reduced kidney injury via transfer of miR-486-5p targeting PTEN (79).

## ECFC FOR GENE THERAPY

ECFC have being used as cellular vehicle for gene therapy. Important advantages of ECFC compared to other cell types include non-invasive isolation and expansion from peripheral blood, genomic stability in cell culture and ease of genetic manipulation. Preclinical studies that have used ECFC as vehicles for gene therapy are the following.

### Hemophilia A

Autologous ECFC have been successfully used for gene therapy in animal models of hemophilia A (101, 102). ECFC isolated from canine blood were transduced with a lentiviral vector encoding for the canine B-domain deleted Factor VIII transgene and were implanted subcutaneously in a Matrigel scaffold or into the omentum. Therapeutic levels of Factor VIII persisted over a long period of time, more than 15 weeks in Matrigel scaffolds and

up to a year with omentum implantation, indicating ECFC as promising vehicle for gene therapy in hemophilia A (101, 103).

## VWD

Gene therapy using ECFC has been considered for treatment of VWD. Development of gene therapy for VWD has been hampered by the considerable length of the VWF cDNA and posttranslational processing of the protein. ECFC isolated from type 3 VWD dogs were transduced with a lentiviral vector encoding complete human VWF; this resulted in high-transduction efficiencies and expression of functional vector-encoded VWF (104).

## Anemia

In patients with advanced-stage chronic kidney disease, treatment of chronic anemia requires routine injections of recombinant erythropoietin (EPO). Using genetically engineered ECFC, Melero-Martin's group managed to express EPO under the control of a tetracycline-regulated system and generated subcutaneous vascular networks capable of systemic EPO release in immunodeficient mice bloodstream (105). After activation of EPO expression, erythropoiesis was induced in mice, a process that was completely reversible, indicating delivery of EPO by genetically modified ECFC as a potential therapeutic approach in patients requiring frequent EPO injections.

## Cancer

ECFC have been also considered as carriers for systemic cancer gene therapy. The principle is to use genetically engineered ECFC to deliver angiogenic inhibitors or other suicide genes directly into the tumor endothelium (106–108). Systemic delivery of ECFC expressing fms-like tyrosine kinase-1 and/or angiostatin-endostatin fusion protein was able to inhibit tumor growth, reduced tumor volume and increased survival in mice cancer models (106, 107), indicating ECFC as potential candidates for tumor-specific delivery of cancer gene therapy.

## ECFC FOR TISSUE BIOENGINEERING

Development of vascular networks is an essential goal for regenerative medicine and may be a future approach for the treatment of ischemic vascular disease. ECFC are currently being explored for tissue engineering strategies for tissue repair or regeneration.

### Bioartificial Vascular Structures

Artificial vascularization of engineered biocompatible scaffolds is essential, since a major challenge in tissue engineering is to supply bioengineered tissue transplants with sufficient amounts of nutrients and oxygen and to allow metabolite removal. ECFC is an attractive approach for engineering bioartificial vascular structures, due to their proven advanced vasculogenetic abilities (74, 81, 100). However, scaffold materials might have a significant effect on ECFC's functional abilities. For example, Critser et al. showed that the physical properties of collagen matrices influence ECFC-dependent vasculogenesis *in vivo* (109). There are several natural and biocompatible synthetic matrices able to support

the development of vascular networks and tissue regeneration with ECFC (110–117). These matrices which mimic the natural extracellular matrix have been proven safe for clinical use, and can be modulated by changing the biochemical and physiological properties of the scaffold or by using growth factors such as VEGF, Angiopoietin 1, or BMP-2, promoting the pro-angiogenic abilities of the cells (74).

### Wound Healing

Vascularization is critical for wound healing, a process that involves the interaction of multiple cells, including platelets, fibroblasts, keratinocytes, and endothelial cells. ECFC have been used to support vascularization during wound healing using different scaffolds and tissue-engineered human skin substitutes (118–120). ECFC seeded in appropriate scaffold were able to promote wound healing when compared with intradermal ECFC injection (118). Use of ECFC in tissue-engineered human skin substitutes promoted the formation of vascular conduits enabling perfusion and survival of human bioengineered tissues (119). Furthermore, integration of ECFC in dermal fibroblast sheets induced robust vasculogenesis, accelerated epithelial coverage and matrix organization of the wound bed and prevented wound contraction (120). The therapeutic effects were due to both active incorporation of ECFC into new vessels and to trophic stimulation of host angiogenesis by growth factors secreted by ECFC, such as placental growth factor (120).

### Bone Repair

ECFC have been also investigated for bone repair. Vascularization is crucial for successful bone regeneration and for successful use of tissue engineered bone constructs, as a rapid connection to the vascular network is required. Subcutaneous implantation of cord blood ECFC with human fetal MSC induced formation of chimeric human vessels and ectopic bone formation in immunodeficient mice after 12 weeks (113). Cord blood ECFC with biphasic calcium phosphate/BMP-2 bone tissue engineering constructs demonstrated *in vivo* vasculogenesis within the macropore space of the constructs, suggesting that scaffolds containing ECFC have significant potential for advanced neovascularization in bone defects (114). Subcutaneous application of co-culture of ECFC with human primary osteoblasts on starch polycaprolactone fiber meshes induced formation of ECFC-derived blood vessels integrated into the host tissue and anastomosed to the vascular supply (116). These studies suggest that ECFC might have an important role for bone regeneration.

## IMPROVING ECFC THERAPEUTIC EFFICIENCY

As mentioned earlier, ECFC from patients with different diseases often present a dysfunctional phenotype with low proliferative potential, increased cellular senescence, a proinflammatory profile and reduced vasculogenic abilities, in many cases attributed to genetic or epigenetic dysfunction. Some of the key studies on how to improve ECFC therapeutic efficacy in view of their use for autologous cell therapy are summarized below.

## Epigenetic Activation

Recent studies have investigated the epigenetic profile of cord blood ECFC and found that key proangiogenic pathways are repressed, reducing ECFC's regenerative potential (121, 122). Importantly, these studies show that epigenetic drugs including HDAC inhibitors can lift this repression and activate proangiogenic signaling pathways, improving ECFC vasculogenic ability *in vivo* (89, 121, 122). Epigenetic dysfunction involving the histone deacetylase SIRT1 has been also demonstrated in disease, for example in COPD or ECFC from preterm infants, contributing to ECFC senescence and impaired angiogenic ability of them (52, 65). Targeting epigenetic dysfunction with *ex vivo* pre-treatment could partially restore ECFC senescence and dysfunction (52, 65), suggesting that *ex vivo* epigenetic activation of ECFC might improve their therapeutic efficacy if needed for autologous cell regenerative purposes.

## ECFC Pre-treatment With Bioactive Compounds/Agents

Numerous studies have demonstrated that pre-treatment of ECFC can improve their therapeutic efficacy. For example, pretreatment of dysfunctional ECFC from type 2 diabetes patients with globular adiponectin enhanced neovascularization in murine models of hindlimb ischemia, in both normoglycemic and hyperglycemic conditions (60). Priming of ECFC with EPO before intravenous injection increased revascularization in a hindlimb ischemia mouse model (123). Similarly, fucoidan-pretreatment was found to enhance survival, proliferation, incorporation and endothelial differentiation of senescent ECFC transplanted in a murine hind limb ischemia model (124). Transplantation of genistein-pretreated ECFC into myocardial ischemic sites enhanced neovascularization, decreased myocardial fibrosis and improved cardiac function (91). Implantation of ECFC with human platelet lysate promoted vasculogenesis and augmented blood vessel formation via diminishing apoptosis of the implanted ECFC *in vivo* (125). Transmembrane TNF- $\alpha$  expression is increased on the surface of highly proliferative ECFC, regulating their proliferative capacity, and might be another candidate pathway for enhancing ECFC angiogenic ability (126). For an extended list of compounds/agents having an effect on ECFC functional abilities please see reference from Tasev et al. (74).

## CHALLENGES THAT LIMIT ECFC CLINICAL APPLICATIONS

Current challenges that need to be addressed for the use of ECFC in clinical trials are summarized below.

### Time and Efficacy of Isolation and Expansion of ECFC From Peripheral Blood

ECFC cannot be successfully isolated from peripheral blood from all individuals. This is likely due to low number of circulating ECFC present in the blood, and this can be further reduced in several diseases, as described above. Currently there are no markers that uniquely identify human ECFC, which

could be used to enrich for these cells (62, 100). The recent identification of tissue-resident vascular endothelial stem cells, positive for CD157, that can be clonally expanded and contribute to angiogenesis and maintenance of blood vessels *in vivo*, is a promising achievement toward this direction (127).

## Replacement of Reagents Derived From Animals

Most protocols for ECFC isolation utilize reagents derived from animals, such as fetal bovine serum. Further optimization of protocols that use human platelet lysates (125, 128, 129) or autologous human serum (130) may provide the required alternative to generate ECFC compatible with good manufacturing practice (GMP) standards in cellular therapy facilities.

## Limited Availability of ECFC

Regenerative approaches usually require large numbers of cells, which in many cases is difficult to obtain from peripheral blood ECFC. Generation of iPSC-derived ECFC (14) could be used to obtain large amounts of autologous ECFC-like cells for vascular regeneration. However, the process of generating patient specific iPSC-derived ECFC is complex and time-consuming (14, 100). Moreover, no data is available yet on whether these cells recapitulate the functional and transcriptional profile of "true" ECFC and whether the "ECFC" phenotype is stable. Isolation of ECFC from white adipose tissue vasculature (131) might provide another practical alternative to obtain large amounts of autologous ECFC for vascular regeneration.

## CONCLUSIONS

It is evident from the current literature that significant progress has been achieved from the early days of "EPC" ambiguity, a term which included various cell populations of different origin. ECFC have been widely recognized as an important tool to study endothelial molecular dysfunction in disease and a promising tool for vascular regenerative approaches and gene therapy. There is plenty of evidence of the importance of these cells for multiple applications. Future studies will address current obstacles and help develop the use ECFC in clinical settings for regenerative approaches.

## AUTHOR CONTRIBUTIONS

KP: conception and design, manuscript writing. AR: conception and design, financial support, contributed to manuscript writing.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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