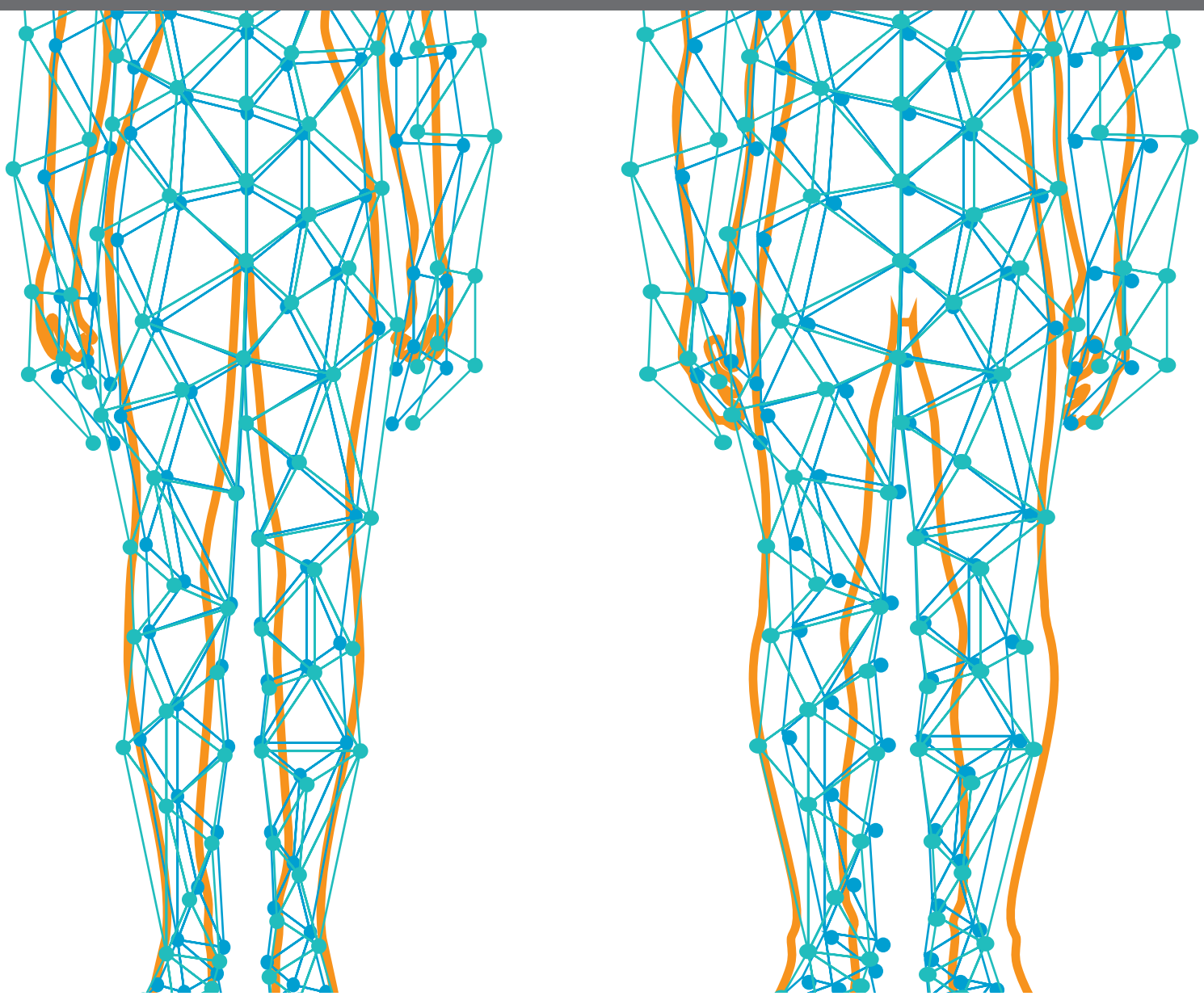
RECENT ADVANCES IN ENDOTHELIAL PROGENITOR CELLS TOWARD THEIR USE IN CLINICAL TRANSLATION

EDITED BY: Reinhold J. Medina and David M. Smadja

PUBLISHED IN: Frontiers in Medicine



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frontiers

ISSN 1664-8714
ISBN 978-2-88963-459-0
DOI 10.3389/978-2-88963-459-0

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RECENT ADVANCES IN ENDOTHELIAL PROGENITOR CELLS TOWARD THEIR USE IN CLINICAL TRANSLATION

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Since endothelial progenitor cells (EPCs) were first described in 1997, there has been significant debate surrounding their definition and roles; but also agreement in their potential to develop as biomarkers and cytotherapies. EPCs participate in vascular repair and postnatal angiogenesis by differentiating into endothelial cells or by producing pro–angiogenic growth factors. Various subtypes of EPCs have been studied, such as CD34+VEGFR2+ blood circulating cells, cultured endothelial colony forming cells (ECFCs), and myeloid angiogenic cells (MACs). EPCs have therapeutic potential for revascularisation and vascular repair in ischemic diseases such as myocardial infarction and diabetic vascular complications. In this eBook, we compile evidence to enable their translation including strategies to enhance the number, homing ability to the injury site and function of EPCs.

Citation: Medina, R. J., Smadja, D. M., eds. (2020). Recent Advances in Endothelial Progenitor Cells Toward Their Use in Clinical Translation. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-459-0

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frontiers in Medicine

EDITORIAL
published: 08 January 2020
doi: 10.3389/fmed.2019.00290

Editorial: Recent Advances in Endothelial Progenitor Cells Toward Their Use in Clinical Translation

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Keywords: endothelial progenitor, endothelial progenitor cell, endothelial colony forming cell, ECFC, vascular repair, therapeutic angiogenesis, angiogenesis

Editorial on the Research Topic

Recent Advances in Endothelial Progenitor Cells Toward Their Use in Clinical Translation

Cardiovascular disease remains the number one cause of death worldwide, by myocardial infarction, stroke, and critical limb ischemia. The common denominator in these diseases is the lack of blood vessels due to obstruction or degeneration. Therefore, the significant efforts for developing therapies based on endothelial progenitor cells (EPCs) for the regeneration of blood vessels in ischemic tissues. While preclinical evidence was convincing, results did not successfully translate into positive outcomes at clinical trials. Nevertheless, these first-in-man trials demonstrated safety and feasibility. The lack of efficacy is, to a certain extent, due to the poor cellular and molecular characterization of cells used, most of which were bone marrow mononuclear cells and described as enriched for endothelial progenitors. This fast clinical translation with ill-defined cell populations and negative results has damaged the field by dampening enthusiasm and hope from patients and clinicians.

We believe that cell therapies to regenerate blood vessels will be effective in future trials, but in order to achieve this, it is essential to clearly and unequivocally define the cells of interest, as required by next generation cytotherapies. Moreover, EPCs, more than a cell therapy product, have been proposed as a liquid biopsy to investigate endothelial dysfunction in patients with vascular diseases. This Research Topic presents 4 original research articles and 5 review articles aiming to update scientists surrounding our current understanding of endothelial progenitors, with emphasis on a well-defined subtype known as endothelial colony forming cells (ECFCs).

Keighron et al. provide a comprehensive up-to-date review of clinical trials using EPCs for diseases such as peripheral artery disease (PAD), coronary artery disease (CAD), ischemic stroke, and pulmonary artery hypertension (PAH). They concluded that EPCs used in clinical trials were highly heterogeneous cell populations and highlighted the need for better-defined cell populations such as ECFCs.

O'Neill et al. summarize preclinical evidence from various disease models to demonstrate the therapeutic potential of ECFCs for vascular repair in ischemic tissues.

OPEN ACCESS

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *23 September 2019*
Accepted: *25 November 2019*
Published: *08 January 2020*

Citation:
Medina RJ and Smadja DM (2020) Editorial: Recent Advances in Endothelial Progenitor Cells Toward Their Use in Clinical Translation. Front. Med. 6:290. doi: 10.3389/fmed.2019.00290

The study by Tasev et al. explores in detail the response of ECFCs to hypoxia and their evidence indicates that 1% O_2 impairs isolation, growth, and function in ECFCs.

Boisson-Vidal et al. report the role of Osteoprotegerin in enhancing ECFC differentiation from cord blood CD34+ cells.

Carolina et al. present results to underscore the detrimental effects of the glucocorticoid Dexamethasone on ECFCs by diminishing their wound healing ability through reduction of CXCR4.

The study by McLoughlin et al. identifies the optimal reference gene panel for PCR when investigating cellular senescence in ECFCs.

Rossi et al. summarize how Endoglin interfere with ECFCs vasculogenic properties and how it allows ECFCs involvement with inflammatory cells and hemostasis.

The review by Edwards et al. describe ECFCs and myeloid angiogenic cells (MACs) in inflammatory disorders including diabetes, rheumatoid arthritis, and systemic lupus erythematosus, with important insights into molecular mechanisms responsible for cellular dysfunction.

Pashalaki and Randi outline current uses of ECFCs from their application as tools to study disease such as von Willebrand disease, PAH, and diabetes, to their utilization to develop cell and gene therapies.

All the manuscripts presented in this Research Topic recognized the importance of harmonizing definitions and protocols, as well as advancing our knowledge in relation to the molecular understanding of ECFC biology, which is critical for successful translation into clinics. Better in-depth knowledge and detailed molecular characterization of ECFCs will enable our scientific community to finally build successful multi-centric clinical studies for biomarkers or therapeutic approaches, after more than 20 years of basic scientific discoveries.

AUTHOR CONTRIBUTIONS

RM and DS wrote and approved this editorial for publication.

ACKNOWLEDGMENTS

The associate editors thank the authors, and all the reviewers and editors who contributed to this Research Topic.

Conflict of Interest: 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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REVIEW
published: 10 July 2018
doi: 10.3389/fmed.2018.00200

Endothelial Progenitor Cells: New Targets for Therapeutics for Inflammatory Conditions With High Cardiovascular Risk

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Over the past decade, we have witnessed an exponential growth of interest into the role of endothelial progenitor cells (EPCs) in cardiovascular disease. While the major thinking revolves around EPC angiogenic repair properties, we have used a hypothesis-driven approach to discover disease-related defects in their characteristics and based on these findings, have identified opportunities for functional enhancement, which offer an exciting avenue for translation into clinical intervention. In this review, we focus on two groups; circulating myeloid angiogenic cells (MACs) and late outgrowth endothelial colony forming cells (ECFCs), and will discuss the unique properties and defects of each population, as new insights have been gained into the potential function of each sub-type using current techniques and multiomic technology. We will discuss their role in inflammatory disorders and alterations in mitochondrial function. In addition, we share key insights into the glycocalyx, and propose this network of membrane-bound proteoglycans and glycoproteins, covering the endothelium warrants further investigation in order to clarify its significance in ECFC regulation of vascularization and angiogenesis and ultimately for potential translational therapeutic aspects.

Keywords: endothelial progenitor cells, glycocalyx, cardiovascular, mitochondrial function, inflammation, lupus, rheumatoid arthritis, diabetes

OPEN ACCESS

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *18 April 2018*
Accepted: *21 June 2018*
Published: *10 July 2018*

Citation:
Edwards N, Langford-Smith AWW, Wilkinson FL and Alexander MY (2018) Endothelial Progenitor Cells: New Targets for Therapeutics for Inflammatory Conditions With High Cardiovascular Risk. Front. Med. 5:200. doi: 10.3389/fmed.2018.00200

ENDOTHELIAL DAMAGE AND REPAIR IN HIGH-RISK DISEASE CONDITIONS

Endothelial Damage

Endothelial dysfunction is a preceding factor in the development of cardiovascular disease, with vascular damage leading to atherogenesis, and plaque formation (1). Endothelial damage may be mediated by a number of biological stimuli, such as inflammatory mediators, and hypoxia (2). Indeed, many conditions characterized by inflammation, of either a chronic or transient nature, are associated with a high risk of endothelial dysfunction. These "high risk" conditions include autoimmune diseases such as systemic lupus erythematosus (SLE) (3) and rheumatoid arthritis (RA) (4), as well as chronic conditions such as type 2 diabetes mellitus (T2DM) (5). The association between cardiovascular and autoimmune disease has been recognized for a number of years (6), where it is known that cardiovascular risk in young women with SLE is increased

50 fold (7, 8). These patients experience endothelial dysfunction prior to accelerated subclinical atherosclerosis, possibly due to the sustained activation of the immune response, including production of pro-atherogenic hormones and immune complexes (9), and research has shown that endothelial progenitor cells (EPCs) are likely to play a role in this process (10). In addition, T2DM patients likewise experience endothelial dysfunction due to the accumulation of advanced glycated end products, oxidized low density lipoprotein (oxLDL), and oxidative stress, making the diabetic patient prone to atherogenesis, and increased cardiovascular risk (5), with recent evidence suggesting this could be influenced by a decrease in the number and function of EPCs (11).

Endothelial Homeostasis and Repair

EPCs are important for vascular homeostasis and repair, where differences in their number and function in health and disease are apparent (12). It is well recognized that during stress or endothelial activation, EPCs can be mobilized, with their numbers being increased in the circulation (13). Growing evidence suggests that EPCs could be a link between a defective homeostatic or endogenous repair mechanism and vascular dysfunction (14) and in this regard could have a future impact as a biomarker of atherosclerosis and vascular disease (15). Characteristics of the late outgrowth endothelial colony forming cell (ECFC) phenotype, including cell-surface markers necessary for adhesion to the vascular endothelium, and their angiogenic capacity, support the suggestion that ECFCs are a key sub-population of EPCs involved in vascular repair (16, 17). Tissue injury studies in animal models have demonstrated mobilization and migration of EPCs from the bone marrow niche, followed by homing to the site of vascular damage, where they modulate repair through angiogenesis, neovascularization, and endothelial cell replenishment, all of which has been elegantly reviewed elsewhere (18, 19). Although the evidence for the role of EPCs in atherosclerosis has met with some discrepancy, it is likely due to differences in the particular EPC phenotype investigated, their distinctive functional effects, as well as initiating factors triggering their action. A recent report used EPCs, defined by their combined expression of $CD34^+$, $CD133^+$, and KDR^+, and demonstrated an association between a high EPC count with less coronary plaque burden of a stented vessel segment, which adds to previous findings of their protective role in atherosclerosis (20). In addition, it has been reported that factors released from atherosclerotic plaques *ex vivo,* induce, not only mobilization of EPCs, but also EPC expression of angiogenic factors (21).

In parallel, there is evidence that MACs are also of utmost importance in their contribution to angiogenesis, tissue regeneration and endothelial repair (22, 23), where these precursor cells exert paracrine and trophic effects that influence the host microenvironment (24, 25). The following sections will describe mechanisms underpinning vascular dysfunction in inflammatory conditions with high risk of cardiovascular disease (CVD) and consider potential therapeutic options aimed at improving progenitor cell reparative function (26, 27) as a novel approach to exploit endogenous repair processes.

DEFECTIVE EPCS IN AGING AND DIABETES

Aging

It would appear that numbers of circulating ECFCs reduce over time, such that older volunteers are found to have fewer ECFCs than their younger counterparts (28, 29). This is mirrored in coronary heart disease, reinforcing the connections between ECFC dysfunction, aging, and cardiovascular risk (28). However, there are also reports that bone-marrow derived progenitor cell numbers remain stable, suggesting that, in some cases, the decline in reparatory ability may be due to cellular impairments, in their homing, angiogenic capacity or their propensity for senescence and premature cell death (28, 30, 31). Studies have shown that late ECFCs from elderly volunteers demonstrate impaired migration, proliferation and adhesion properties compared to those from young participants (29, 32, 33), and show a reduced capacity for re-endothelialization and incorporation into a damaged vasculature (29).

ECFCs isolated from older individuals develop a decline in their response to signaling pathways. Among the many mechanisms that may underpin the impaired function of EPCs in disease, our own studies have demonstrated a reduction in 6-O-sulphation of heparan sulfate in aging ECFCs, suggesting the glycocalyx may play a role in the aging decline of vascular health (33). In addition, Kushner et al. report that ECFCs from older subjects, compared to their younger counterparts, have an increased sensitivity to apoptotic stimuli and demonstrate an increased level of intracellular caspase-3, along with accelerated senescence, which was linked to a loss of telomerase, and a pro-thrombotic phenotype (34). Xia et al. have shown altered CXCR4/JAK signaling in the elderly is linked to a reduced capacity for ECFC homing and re-endothelialization (29), which may concordantly induce anti-atherosclerotic EPC activity and up regulate expression of vascular endothelial growth factor (VEGF) receptors, as discussed elsewhere (35). Interestingly, Heiss et al. found increased concentrations of VEGF in the blood of older individuals although ECFC responses to the protein were muted (32, 36, 37), thus implying an increased effort to mobilize ECFCs and effect vascular repair (32).

Studies have been carried out with MACs also, where young patients with type 1 diabetes mellitus have shown significantly higher levels of MACs compared to adult patients, and where a direct correlation was found between MAC number and disease duration, when greater than 10 years (38). The authors propose that the high levels of MACs in the young patients might protect vessels against endothelial dysfunction and damage and such protection would be less effective in older subjects, who had lower EPC numbers (38). In addition, older MACs were shown to be more susceptible to oxidative stress due to reduced activity of antioxidant proteins such as GPX1, thus rendering them vulnerable to apoptosis (38, 39).

Diabetes

As with aging, T2DM is associated with a reduction in circulating ECFCs, and also shows an impaired VEGF-driven mobility (38, 40–42), as well as major deficits in vital functions

such as differentiation and proliferation. The effects of a hyperglycaemic environment on ECFC number and function are comprehensively reviewed by Kang et al. (43). Furthermore, the reduced numbers of ECFCs in T2DM have been associated with poor glycaemic control, and increased arterial stiffness (41). Hyperglycaemia may also enable uncoupling of intracellular eNOS, rendering ECFCs susceptible to ROS and further migratory incapability (42), although of note, function can be restored following improved glycaemic control (40), indicating the potential for lifestyle and therapeutic options to improve vascular repair. This is particularly important in terms of peripheral vascular disease and poor wound healing, which often results in diabetic foot ulceration and amputation (44). The severely diminished ECFC number and function, which is apparent in T2DM, also correlates to the prevalence of atherosclerosis in the lower limbs (44, 45). In this case, the ECFCs demonstrate impaired clonogenicity and adhesion (45), which, when coupled with impaired homing, may contribute to the delayed wound healing observed in diabetes. As an added complication in a diabetic microenvironment, ECFCs appear to concurrently undergo a pro-calcific shift, expressing osteocalcin, and bone alkaline phosphatase, thus promoting the drive toward vascular calcification, which is so prevalent in diabetic vasculopathy (46). This phenomenon renders ECFCs not only important in endothelial dysfunction, but also in smooth muscle cell osteogenic differentiation.

MECHANISMS OF EPC DYSFUNCTION IN AGING AND DIABETES

EPCs and the Glycocalyx

Previous research by our group supports the theory of a decline in function with age, where we demonstrated structural changes in heparan sulfate within the glycocalyx of aged ECFCs, compared to those isolated from younger volunteers and cord blood. Our findings also demonstrate an association with reduced sensitivity to VEGF (33). Since heparan sulfate is indeed a ligand for VEGF, we suggest that aged ECFCs may be less sensitive to damage signals through reduced protective/reparative ligand-binding (33, 38, 47). Impairments in syndecan 4, another member of the heparan sulfate proteoglycan receptor family and involved in SDF-induced cell migration, has been shown to contribute to impaired ECFC function. The extracellular domain of syndecan 4 is shed from the cell surface of ECFCs in response to ROS-induced accumulation of advanced glycation end products, leading to impaired migration of the syndecan4 deficient ECFCs (48).

In other cell types, the glycocalyx also has a role in immune regulation, mediated by the binding of complement factor H to specific, age-related alterations in the sulphation patterning of heparan sulfate (49, 50). Furthermore, these age-related changes that we have identified in the glycocalyx of ECFCs (33, 49) could be caused by the accumulation of metal ions, including cadmium within the matrix, resulting in ROS-mediated damage to the glycocalyx, and mitochondrial dysfunction (51), however links between metal ions and ECFC regulation remain to be further investigated.

EPCs and Mitochondria

EPCs have previously been demonstrated to form cell-to-cell connections and, via tunneling nanotubes (TNT), transfer mitochondria and other organelles to endothelial cells. This TNT mitochondrial transfer can rescue senescent endothelial cells and change cell fate (52, 53). Further details of TNT mitochondrial trafficking in health and disease can be found in other reviews (54, 55). The energy required for the normal function of most endothelial cell phenotypes is primarily by glycolysis, however, the energy requirements for repair and angiogenesis are considerable and thus require the activation and proliferation of mitochondria (56, 57). In light of this role in angiogenesis, mitochondria are key integrators of environmental and disease signals (58), and they are crucial to the mechanism underpinning many other factors that influence EPC behavior discussed in this review. For example, among the many cellular processes which influences mitochondrial function and metabolic homeostasis are different shear stress conditions (58), in both mediating and also causing inflammatory responses (59, 60) and in diabetes (61). Of note, we have recently identified impaired angiogenic function and altered mitochondrial activity in ECFCs isolated from patients with diabetes and foot ulcers (62).

There is also a dynamic interplay between mitochondrial function, the glycocalyx and extracellular cell matrix; for example, the glycocalyx can be damaged/changed by ROS produced in the mitochondria (63) in certain conditions, while the stiffness of the matrix can also affect mitochondrial function in other cases (43, 64). Although many of these mechanisms have not been demonstrated in EPCs it is likely they also have a role, however, further work is warranted to enhance our understanding of the complex interplay between EPC function, the glycocalyx/matrix, mitochondria, and other disease and aging stimuli.

EPCs and Shear Stress

CD31, or platelet endothelial cell adhesion molecule-1 (PECAM-1), is a 140kDa type I integral membrane glycoprotein often used as a marker of EPCs as well as the more mature endothelial cells and is known to play various roles in vascular biology, including angiogenesis, platelet function, and thrombosis. It is also a mechanosensor of the endothelial cell response to fluid shear stress. It is thought that ECFC filopodial processes may play a role in cellular communication, and regulating cell to cell contact by allowing a sensory response to circulatory or sheer stress. Further work is required to gain insight into the effects of sheer stress on MAC and ECFC function. Enhanced signaling and re-endothelialization has been shown to be restored in elderly ECFCs, following shear stress treatment (29). However, there is little understanding of the influence of flow stress changes within the glycocalyx and extracellular matrix and how this might influence MAC or ECFC behavior, providing the impetus for further studies into novel mechanobiological studies of how these cells respond to changes in physiological or turbulent flow.

PROGENITOR CELL IMPAIRMENTS IN INFLAMMATORY RHEUMATIC DISEASES

Rheumatoid Arthritis

Reports pertaining to progenitor cell numbers in autoimmune rheumatic conditions are conflicting due to the different methods of progenitor cell characterization and patient inclusion criteria used by various groups. While some studies suggest a decrease in $CD34^+$ cells in rheumatoid arthritis (RA) (65), other studies demonstrate increased levels (66), or indeed no change at all in number (23). Although reports of EPC number in RA are inconsistent, low levels of $CD34^+/KDR^+$ cells have been associated with carotid atherosclerosis in patients (67), suggesting that a reduction in number may be more representative of vascular dysfunction than inflammatory activity (68). Furthermore, it has been suggested that ECFC depletion is associated with disease progression, as patients experiencing long-term disease appear to show a decline in ECFC numbers, regardless of age, compared to those with recent disease onset, whose ECFC numbers match those of healthy participants (69). Although discrepancies exist in respective RA studies because of differences in MAC and ECFC isolation methods (70), including their seeding density, the matrix used for coating culture dishes, the markers in use for characterization, and the potential variances in drug regimens of study participants before isolation of their cells, it is clear that both MACs and ECFCs do have potential to act as targets for therapeutic improvement in disease (68).

Systemic Lupus Erythematosus

Patients with systemic lupus erythematosus (SLE) have an elevated vascular risk due to an early onset of atherosclerosis, which appears to be independent of traditional CVD risk factors and associated with an altered interferon-α (IFNα) signaling pathway. It has been shown that IFNα alters the balance between endothelial cell apoptosis and vascular repair which is governed by both ECFCs and MACs (71, 72). When focusing on $CD34^+$ cells, it becomes clear that the majority of studies find decreased levels of circulating MACs in SLE patients (68). A reduction in numbers of $CD34^+/KDR^+$ MACs in SLE patients, has been attributed to increased apoptosis, which is also reported in patients with stable disease in remission, supporting the proposal of chronically decreased levels throughout the disease, rather than solely during a disease flare (73). Moonen et al. described MACs with unusual morphology (74), while Denny et al. found decreased ability to express pro-angiogenic cytokines such as VEGF (75), which they correlated with impaired VEGF-driven migration (76), and was supported by a subsequent study in our group by Williamson et al. (33). SLE ECFCs have also been shown to have fundamental impairments in critical functions such as colony forming ability and proliferation (76), as well as reduced migration and tube forming capabilities (75). These findings are strengthened by Deng et al. who found that while ECFCs isolated from patients with SLE are highly activated and have elevated expression of interleukin-6 (IL-6) and intracellular adhesion molecule-1 (ICAM-1) compared to control subjects, they are impaired in their basic physiological function (77).

INFLAMMATORY SIGNALING AND RESTORATION OF ECFC FUNCTION

Cytokine-Induced Endothelial Damage

The inflammatory environment plays a vital role in ECFC function and maturation; IFNα is most often associated with SLE but may also be elevated in RA and demonstrates striking correlations with ECFC number and function, suggesting a role in the induction of differentiation of the ECFC population (23). One theory states that IFNα drives premature differentiation of ECFCs to a more mature phenotype, with little reparatory potential, therefore, even if the cells are found at healthy levels, their ability to repair vascular damage is severely limited (69). Impairments in ECFC maturation and function are likewise linked to IFN signaling in a type I IFN receptor knockout murine model of SLE, where Thacker et al. demonstrated increased ECFC number and function, with improved neoangiogenesis and differentiation (78). It was suggested that type 1 IFN receptor activation causes the impairment by transcriptional repression of IL-1β, upregulation of inflammasome components, such as caspase-1 and a skew toward pro-inflammatory IL-18. Indeed, blockade of both caspase-1 and IL-18 enhance differentiation of progenitor cells (79). Denny et al. support the damaging effects caused by an altered IFNα signature in an *in vitro* SLE model, where they demonstrate increased production of IFNα by both MACs and ECFCs, which become cytotoxic to the cells, supporting apoptosis and preventing growth of a confluent monolayer (75). Administration of IFNα was shown to enhance thrombosis and platelet activation in a lupus-prone mouse model (78) and high IFNα levels have been suggested as an independent risk factor for cardiovascular disease in both SLE and RA (69, 80). In addition, IL-18 has been associated with vascular stiffness and plaque instability, acting as an independent predictor of cardiovascular mortality in patients with subclinical atherosclerosis (79).

A further hallmark of inflammation is elevated expression of systemic or tissue tumor necrosis factor α (TNFα), which is another key cytokine elevated in autoimmune rheumatic disease (81); accordingly, treatment of harvested healthy ECFCs with TNFα has been shown to impair proliferation, migration and tube formation in these cells, and increase apoptosis *in vitro* (82). As with increased levels of IFNα contributing to ECFC dysfunction, so the increased levels of TNFα and subsequent damage to MACs and ECFCs, may contribute to a poor vascular repair in these patients. Additional members of the TNF family may assert detrimental effects on an altered differentiation programme of progenitor cells. For example, osteoprotegerin (OPG), which inhibits osteoclastogenesis and is a marker of vascular calcification (83), has been shown to be inversely correlated with ECFC numbers in SLE patients, and linked to an increased rate of OPG-stimulated apoptosis compared to those from healthy participants, suggesting that the apoptotic cells could act as a nidus for calcified matrix progression. The same study demonstrated that ECFCs increased basal production of ROS, suggesting that the increased inflammation and exposure to apoptotic stressors associated with SLE increased the likelihood

of both ECFCs and MACs becoming exhausted and succumbing to apoptosis (84).

miRs and Microvesicles: Their Effects on EPCs

MicroRNAs (miRs) are critical players in posttranscriptional regulation of almost all genes influencing cellular processes, cell fate decisions, regulating epigenetic changes and contributing to the disease process, details of which are outside the scope of this review, but are elegantly reviewed elsewhere (85–87). Elucidation of the regulatory mechanisms controlled by miRs is an important step toward development of a novel therapy for cardiovascular disease and the co-morbidities associated with it. A study by Khoo et al. describes how differential expression of miR-193a-3p by ECFCs reduces proliferation, migration and tube forming ability by interacting with novel targets such as high mobility group box-1 (88). A further consideration is the reversal of this pathway, in which microvesicles (MVs) and exosomes derived from EPCs act upon the endothelium (89); indeed, circulating ECFC-MVs have been found to stimulate a pro-angiogenic effect upon endothelial cells, which is mediated by the transfer of mRNA carried within the MVs (90). Ranghino et al. expanded on this by establishing a connection between specific miRNA, such as miR-126, and neoangiogenesis, through the use of ECFC-MVs for resolution of hind limb ischaemia (91).

Endothelial microvesicles (EMVs) are membrane-bound, cellular-derived vesicles that exert paracrine or endocrine effects through the intercellular transfer of contents such as lipids, proteins, mRNA and microRNA (miRNA), and are thus intricately linked to endothelial dysfunction (92). Elevation in EMV levels is associated with coronary artery disease (93), plaque instability (94), cardiovascular risk (95, 96), and is also apparent in autoimmune rheumatic diseases, such as SLE, where EMVs are also associated with vascular dysfunction and poor disease control (97, 98). A small number of studies have demonstrated how EMVs produced by endothelial cells following induced inflammation are able to induce functional defects in EPCs, such as impaired angiogenesis (99, 100). miRNAs may also be present in EMVs released from activated cells compared to those from untreated cells, and could be involved in eliciting these effects (99).

Epigenetic Influences on EPC Behavior

ECFCs isolated and expanded in culture maintain a phenotype related to the age, environment and pathologies of the individual donor; these epigenetic changes make these cells invaluable in the understanding the ECFC functionality in different conditions. A more detailed discussion of the histone modification and miR mechanism behind the epigenetic regulation in diabetes and other diseases can be found in other reviews (101, 102). It has also been identified that even ECFCs isolated from cord blood are epigenetically limited in their repair potential; Fraineau et al. recently identified a balance between histone modifications that increase gene expression (histone H3 lysine 4 trimethylation; H3K4me3) and those that inhibit it (histone H3 lysine 27 trimethylation; H3K27me3). Utilizing an inhibitor of the methyltransferase EZH2, that establishes the repressive H3K27me3 marks, Fraineau et al. demonstrated an increase in the expression of multiple pro-angiogenic pathways and an increase in vasculogenesis and blood-flow recovery in a hindlimb ischemia mouse model (103). Previously, a less targeted inhibition of histone deacetylases by Trichostatin A has also been shown to improve vasculogenesis in the hindlimb ischemia mouse model (104). Therefore, the pharmacological targeting of epigenetic modifications could be a promising strategy to improve the repair capacity of ECFCs *ex vivo* before transplantation (11).

Future Mechanistic Clinical Consideration

In light of the defective progenitor cell function in the presence of an inflammatory environment described above, one could hypothesize that anti-inflammatory treatment might improve EPC number and function. However, progenitor cell impairments appear to be exacerbated by immunomodulatory treatments such as methotrexate and rapamycin, which have been shown to increase ECFC apoptosis *in vitro* (65, 105). These observations suggest that one of the clinical effects of anti-inflammatory treatment in humans may target the protective properties of MACS or ECFCs. This has particular relevance in SLE and RA patients treated with chronic high-dose immunosuppressants to counteract autoimmune disease flares (106). Therefore, it is critical to consider the long-term side-effects of these anti-inflammatory medications on vascular repair and the cells responsible for it and provides the impetus to study the effects of anti-inflammatory treatment on these reparative cells *in vivo*.

THERAPEUTIC STRATEGIES FOR IMPROVED ECFC FUNCTION

Anti-inflammatory Agents

Improved understanding of progenitor cell subsets and the mechanistic problems surrounding disease-specific defects will enable development of targeted therapies to improve a patient's natural population of reparatory cells. Some current available therapies have potential for recovery of progenitor cell function; glucocorticoids and TNF-blocking treatments appear to boost progenitor cell numbers in RA patients, while antimalarial drugs, often prescribed to SLE patients, may also increase levels of ECFCs (23, 107). A number of monoclonal antibody therapies targeting cytokines have been approved for use in autoimmune conditions, including both anti-TNFα and anti-IFNα (108, 109), although little research has investigated their direct effects on progenitor cell function, and may be an area worthy of further study.

Anti-hypertensive Agents

Prostanoids may be another potential therapeutic target agent relevant to EPFC or MAC function; Iloprost, a prostacyclin analog and vasodilator, has been shown to increase ECFC numbers in systemic sclerosis, another autoimmune connective tissue disease. Following Iloprost infusion, cells demonstrate enhanced inhibitory regulation of apoptotic genes and increased VEGF expression, facilitating improved mobilization (110). This

may be supported by increased presentation of adhesion factors by the endothelium, alongside increased release of ECFCs from the bone marrow, as described by Coppolino et al. (111) using a population of uraemic patients undergoing revascularization for peripheral limb ischaemia. Indeed, prostanoids such as Iloprost, have been proposed to improve ulcer healing and reduce the need for major amputation (112), although further work is required to link this to MAC or ECFC function.

Glycomimetics as Novel Small Molecule Drugs

A promising approach is to target the action of proteoglycans such as heparan sulfate, which is present on the surface of ECFCs, and plays a vital role in processes including angiogenesis and wound healing through its varied sulphation patterning, regulating interactions with growth factors such as VEGF, as described above (33, 113). The synthesis of small molecule glycomimetic compounds removes the complexity of larger carbohydrates, enabling the study of such glycosaminoglycans (114) and their effect on cell fate and function. Of note, we have previously discovered a group of glycomimetics, which restore NO production and antioxidant activity in an *in vitro* model of lipid-induced endothelial dysfunction (113), and our preliminary data suggest the same glycomimetic compounds improve the function of ECFCs (62). Another mimetic, used by Chevalier et al. was shown to improve colony formation, proliferation and migration of ECFCs (115). Furthermore, Tong et al. developed a glycomimetic that accelerated wound healing and angiogenesis in a murine model of diabetes, demonstrating promising future options using glycomimetics to improve endogenous ECFC or MAC function for vascular repair, particularly in T2DM foot ulceration.

Vitamin D Supplementation

Natural solutions for progenitor cell therapy have also been considered. We have previously found that supplementation of SLE MACs with calcitriol, a vitamin D supplement, restores cell surface markers and angiogenicity, via reduced expression of CXCL10 (25). This supports other research, stating improvements in angiogenesis, proliferation and VEGF expression following vitamin D treatment (116). This is particularly relevant as reduced levels of vitamin D have been associated with low ECFC numbers, carotid intima-media thickness and arterial stiffening in rheumatoid arthritis (117). Vitamin D deficiency also results in impaired ECFC angiogenic capacity and interferon-stimulated genes in a murine model of SLE (118). In addition to deficiency, reduced ECFC expression of the vitamin D receptor has been linked to coronary artery

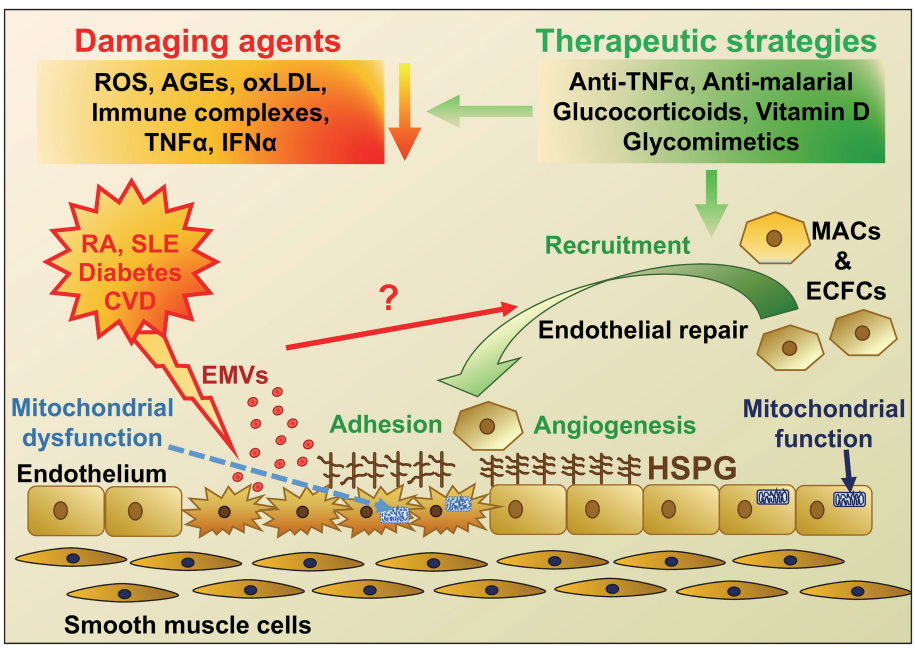

FIGURE 1 | Schematic diagram highlighting the agents that cause endothelial damage in diseases with a high risk of developing cardiovascular disease (CVD) and potential therapeutic strategies for endothelial repair. The main protagonists of endothelial damage in diseases including rheumatoid arthritis (RA), Systemic Lupus Erythematosus (SLE), diabetes and CVD are reactive oxygen species (ROS), immune complexes, advanced glycation end products (AGEs), oxidized LDL, tumor necrosis factor-α (TNFα), and interferon-α (IFNα). Endothelial damage and activation leads to an increase in adhesion molecules and inflammatory cell infiltration, mitochondrial damage, as well as release of endothelial microvesicles (EMVs), which may instigate recruitment of endothelial progenitor cells (EPCs; myeloid angiogenic cells [MACs] and endothelial colony forming cells [ECFCs]) for endothelial repair. These disease conditions, along with aging, are also thought to change heparan sulfate proteoglycan (HSPG) structure on the cell surface, resulting in altered cell signaling and adhesion of EPCs and defective repair. Various therapeutic strategies could be employed to reduce the initial damage but also novel approaches using mimics of HSPG to target and improve HSPG signaling and repair are under investigation.

disease; high glucose conditions also reduced vitamin D receptor expression in an *in vitro* diabetic cellular model (119). Moreover, calcitriol supplementation supported ECFC viability and colony forming ability in patients with T2DM (120), which could be exploited further as a potentially simple and cost-effective mode of enhancing the health of patients.

CONCLUSION AND FUTURE APPROACHES

Regenerative medicine is now becoming a realistic innovative treatment strategy that could be applied to a range of chronic inflammatory disorders. Validation of the regenerative potential of adult MACs and ECFCs will be a prerequisite step before application of cell therapy in the clinical setting and although still an embryonic field, this challenge holds great promise for the future. Growing evidence demonstrates that both MACs and ECFCs play a key role in vascular homeostasis and the repair of endothelial damage, which has been summarized schematically in **Figure 1**. There has been a rapid rise in the number of publications in EPC function, where proliferation, migration, differentiation, apoptosis, and angiogenic tube formation have been studied. More recent research is focused on signaling pathways involved in these cellular processes. The "omic" technologies have been used in combination with bioinformatic analyses to identify transcriptional switches, miR involvement and their potential targets in both MACs and ECFCs, which contributes to their compromised function in disease.

How to best exploit the properties of EPCs to prevent the downstream effects of endothelial damage in a disease setting is a key area that warrants further investigation. Several approaches are being interrogated for the exploitation and application of MAC or ECFC cellular therapy, such as the use of nanoparticles as carriers for a controlled release of EPC secretome (121); the use of hydrogels for delivery of EPCs into ischemic tissue, which has been shown to increase therapeutic efficiency and efficacy of repair in animal models (122) and also encapsulation of drugs or growth factors for slow release to enhance the differentiation of progenitor cells *in vivo*. Animal models are being used to understand signaling pathways involved in vessel repair and ways to increase endogenous EPC number and function. A few specific pharmacological strategies are being investigated to improve their vasculogenic properties before being re-administrated. However, at the moment the focus is toward myocardial ischemia and peripheral vascular disease, but has potential for a much broader range of diseases in the future. Questions remain to be answered over the use of MACs and ECFC for cell therapy in terms of their isolation, culture, survival, function, regulation and the timing and mode of administration into the tissue. Despite this, the concept of EPCs as a new therapeutic, or as part of the armamentarium for regenerative medicine is a new, dynamic area of research that will bring further insight in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 28 September 2018
doi: 10.3389/fmed.2018.00276

Glucocorticoid Impaired the Wound Healing Ability of Endothelial Progenitor Cells by Reducing the Expression of CXCR4 in the PGE2 Pathway

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Edited by:
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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: 16 June 2018
Accepted: 10 September 2018
Published: 28 September 2018

Citation:
Carolina E, Kato T, Khanh VC, Moriguchi K, Yamashita T, Takeuchi K, Hamada H and Ohneda O (2018) Glucocorticoid Impaired the Wound Healing Ability of Endothelial Progenitor Cells by Reducing the Expression of CXCR4 in the PGE2 Pathway. Front. Med. 5:276. doi: 10.3389/fmed.2018.00276

Background: Endothelial progenitor cells (EPCs) can be used to treat ischemic disease in cell-based therapy owing to their neovascularization potential. Glucocorticoids (GCs) have been widely used as strong anti-inflammatory reagents. However, despite their beneficial effects, side effects, such as impairing wound healing are commonly reported with GC-based therapy, and the effects of GC therapy on the wound healing function of EPCs are unclear.

Methods: In this study, we investigated how GC treatment affects the characteristics and wound healing function of EPCs.

Results: We found that GC treatment reduced the proliferative ability of EPCs. In addition, the expression of CXCR4 was dramatically impaired, which suppressed the migration of EPCs. A transplantation study in a flap mouse model revealed that GC-treated EPCs showed a poor homing ability to injured sites and a low activity for recruiting inflammatory cells, which led to wound healing dysfunction. Impairment of prostaglandin E2 (PGE2) synthases, cyclooxygenase (COX2) and microsomal PGE2 synthase 1 (mPEGS1) were identified as being involved in the GC-induced impairment of the CXCR4 expression in EPCs. Treatment with PGE2 rescued the expression of CXCR4 and restored the migration ability of GC-treated EPCs. In addition, the PGE2 signal that activated the PI3K/AKT pathway was identified to be involved in the regulation of CXCR4 in EPCs under the effects of GCs. In addition, similar negative effects of GCs were observed in EPCs under hypoxic conditions. Under hypoxic conditions, GCs independently impaired the PGE2 and HIF2α pathways, which downregulated the expression of CXCR4 in EPCs. Our findings highlighted the influences of GCs on the characteristics and functions of EPCs, suggesting that the use of EPCs for autologous cell transplantation in patients who have used GCs for a long time should be considered carefully.

Keywords: endothelial progenitor cells, glucocorticoids, CXCR4, wound healing, prostaglandin E2, HIF2α

INTRODUCTION

Glucocorticoids (GCs) have been widely used as extremely effective agents for suppressing inflammation associated with various kinds of diseases (1). However, despite its beneficial effects, GC treatment is associated with several deleterious effects, including impaired wound healing (2). This side effect happens either via the transrepression of pro-inflammatory cytokines, growth factors, matrix proteins and matrix protease or via direct inhibitory influence on genes that are important for skin regeneration (3). GCs act as the synthetic analogs of human natural endogenous GCs, which exert their effects by binding to GC receptors (GRs), thereby regulating the expression of target genes that play important roles in the wound healing process (4). In addition, GCs also cause the dysfunction of cells involved in wound healing (5, 6). We recently demonstrated that GC treatment impairs the wound healing function of adipose tissue-derived mesenchymal stem cells (MSCs) via the downregulation of stromal cell-derived factor 1 (SDF-1) (5).

Wound healing is a complex process that requires the contribution of many cell types (7). Central to this, MSCs and endothelial progenitor cells (EPCs) play crucial roles in promoting wound healing (7). While MSCs contributes to the wound healing process mostly via paracrine effects, secreting a mixture of growth factors and vesicles to recruit numerous of cells to the wound sites and support these cells' functions, EPCs are the key effectors of neovascularization and are involved in the formation of blood vessels and maintenance of the function of the vascular endothelium (8). EPCs possess the self-renewal potential, differentiation ability to endothelial cells, and neovascularization capability toward new blood vessel formation (9, 10). A previous study showed that EPCs accelerate wound healing not only as the endothelial substrate of blood vessels but also by migrating to the injured tissue, exerting their effects by excreting proangiogenic factors (10).

The definition of an EPC has been controversial. Originally, EPCs were identified as the cell population isolated from human peripheral blood that expresses the CD34 and Flk1 markers (11). However, recently studies have highlighted that positive with CD34 and Flk1 are insufficient to define EPCs *in vitro* (8). Several studies have reported the two distinct types of EPCs, early and late EPCs, which early EPCs are characterized by the expression of CD45 and CD14, together with some endothelial cell (EC) markers, and have a short lifespan of 3–4 weeks. Additionally, late EPCs are characterized by EC markers, such as CD31, CD34, VEGFR2, and VE-cadherin, but are negative for myeloid markers (12). We previously reported a novel method for isolating EPCs according to the aldehyde dehydrogenase (ALDH) activity which showed that the expression of CD34 declines during the culturing of DiI-Ac-LDL–positive/$CD45^-$/$CD31^+$ cells; whereas, ALDH activity was retained stably in EPCs in long term culture (13). Of note, EPCs with a low ALDH activity (Alde-low) possess a higher migratory ability toward damaged tissue and show a better recover ability in ischemic wound healing than Alde-high EPCs (13). Under hypoxic conditions, Alde-low EPCs are highly responsive and show the upregulation of hypoxic condition inducible factor 2α (HIF2α), which regulates the expression of CXCR4, a major chemokine receptor for cell migration in response to SDF-1 (13). Therefore, Alde-low EPCs can be considered promising candidates for wound treatment (13).

Autologous transplantation of EPCs is considered a prospective approach for therapeutic revascularization and chronic wound (14). It is reported that autoimmune diseases and its drug-based therapy, such as GC, account for 20% of chronic wound cases (15). Therefore, the patients who have been receiving GC treatment are one of the major targets for EPC therapy. However, similar to other cell sources, the introduction of autologous EPCs transplantation into clinical treatment is still being met with difficulty due to the negative influence of patients' medical backgrounds on the outcome of the treatment (16, 17). Ensuring the EPCs' potential is crucial for achieving the best outcome of EPC-based therapy. A previous study suggested that chronic GC treatment reduces the number of circulating EPCs in the patients (16). However, no report has yet clarified the influences of GCs on the wound healing ability of EPCs. Thus, whether or not the outcomes of EPC-based therapy are worsened in patients who have been chronically treated by GCs remains unclear.

In the present study we examined whether or not GCs interfere with the wound healing ability of Alde-low EPCs. We found that GCs downregulated the expression of CXCR4 in a transplantation flap mouse model, which impaired the migration and wound healing ability of EPCs. Treatment with PGE2 upregulated the EP4 receptor and activated the PI3K/AKT signaling which were involved in rescuing the detrimental effects of GC on the CXCR4 expression of EPCs. In addition, similar detrimental effects of GCs on the PGE2/CXCR4 pathway were noted in EPCs under hypoxic conditions. Of note, under hypoxic conditions, independent with the PEG2 pathway, the HIF2α pathway was also involved in the GC-impaired CXCR4 expression in EPCs. Taken together, these findings highlight the negative effects of GCs on the EPC functions, suggesting that autologous EPC therapy for patients receiving GC treatment be considered carefully.

MATERIALS AND METHODS

Isolation of Umbilical Cord Blood-Derived EPCs

All experiments involving human subjects were performed in accordance with the Guidelines for Medical and Health Research Involving Human Subjects, Ministry of Education, Culture, Sports, Science and Technology, Japan and the permission of the Institutional Ethics Review Committee of the University of Tsukuba. Human EPCs were isolated from umbilical cord blood (UCB), as previously described (13). Isolated EPCs were sorted based on the ALDH activity using ALDEFLUOR® system reagents (StemCell Technologies, Vancouver, Canada). The EPCs with the low ALDH activity (Alde-low EPCs) were used for further experiments in the present study. Alde-low EPCs were cultured with maintenance medium (Iscove's modified Dulbecco medium-IMDM, Invitrogen, Carlsbad, CA, USA)/10% FBS/5 ng/mL bFGF (PeproTech, NJ, USA), and 0.1%

(v/v) penicillin-streptomycin (100 U/mL penicillin, 0.1 mg/mL streptomycin; Invitrogen) and incubated at 37°C in 5% CO_2.

A Fluorescence Activated Cell Sorting (FACS) Analysis of EPCs

EPCs were harvested with trypsin, counted the cell number and a number of 2×10^5 cells were reconstituted in 100 μl 2% FBS-containing PBS. For staining purpose, cells were incubated with the desired antibodies for 30 min at 4°C.

The following antibodies were utilized with the volume of 5μl as the recommendation of the manufacturer: PE-labeled anti-CD105 (323206, BioLegend), PE-labeled anti-CD73 (550257, BD Pharmingen), PE-labeled anti-CD31 (303106, BioLegend), allophycocyanine (APC)–labeled anti-CD45 (555485, BD Biosciences, San Jose, CA), and FITC-labeled anti-CD34 (555821, BD Biosciences). Purified-anti-VEGFR-2 were used as previously reported (13). APC-labeled anti-IgG1 (555751, BD Biosciences), PE-labeled anti-IgG1 (555749, BD Biosciences), FITC-labeled anti-IgG1 (555748, BD Biosciences) were used as the isotype controls. After staining, cells were washed by 2% FBS-containing PBS, centrifuged at 1,800 RPM for 3 min at 4°C, and reconstituted in 300 μl 2% FBS-containing PBS. A flow cytometer (MoFlo XDP; Beckman Coulter, Pasadena, CA, USA) collected 10,000 events for each group, and the isotype control IGg was used as the negative control.

In order to quantify CXCR4 expression in EPCs, the cells were stained with 5μl of PE-labeled anti-CXCR4 (306506, BioLegend) and performed the FACS analysis with the similar above protocol. PE-labeled anti-IgG1 (555749, BD Biosciences) was used as the isotype controls. The threshold for CXCR4-positivity was quantified by means of fluorescent intensity value subtracted with isotype control value IgG of each group.

Cell Proliferation Assay

The proliferation of EPCs was evaluated by the growth curve and proliferation assay staining with Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan).

For growth curve, EPCs were seeded at a number of 4×10^3 cells/well in the 24 well-plate dishes and cultured at 37°C in 5% CO_2 for 9 days. The culture medium was changed every 3 days. The cells were washed with sterile PBS and treated with 0.05% trypsin/EDTA (Invitrogen) at 24-h intervals for 9 days to separate single cells. Dead cells were excluded using trypan-blue staining solution (35525-02; Nacalai Tesque, Kyoto, Japan), and the numbers of live cells in triplicate dishes were counted using a hemocytometer.

For proliferation assay staining with CCK-8, EPCs were seeded at a number of 2.5×10^4, 1.25×10^4, and 6.25×10^3 cells/well in 96-well microplate. Cells were incubated for 48 h and 10 μl of Cell Counting Kit-8 reagent was added to each well, then incubate for 2 h. The number of live cells were measured by colorimetric reading using a microplate reader (Varioskan, ThermoFisher Scientific, Massachusetts, USA) for 450 nm.

Annexin-V/7AAD Staining Assay

EPCs were trypsinized and centrifuged at 1,000 RPM for 3 min at 4°C. Cells were then resuspended in 100 μl of 2% FBS and stained with 5 μl of PE annexin V and 7-AAD (BD BioSciences (CA, USA), and then incubated at 4°C in the dark for 30 min. FBS (2%, 300 μl) was added, and the apoptotic cells were analyzed by flow cytometry.

In vitro Migration Assay

Scratch assay: An *in vitro* migration assay was performed as previously reported with minor modifications (18). EPCs with a number of 1.5×10^5 cells were seeded onto 4-well plates with maintenance medium until they reached confluency after 24 h. Scratch wounds ∼1 mm wide were created. After gentle washing of the detached cells with PBS, the growth medium was changed to 2% FBS-containing IMDM. The pictures of wound closure were taken every 6 h during 24-h post-scratching at 100× magnification (10× objective and 10× eyepiece) under a microscope (Olympus, Tokyo, Japan). The cell migration was calculated using the ImageJ software program (NIH, MD, USA). The wound closure distance was measured at the beginning (T0) and end of the experiment (Tx). The following formula was used to convert the migrated area to percentage: Percentage (%) of wound closure $= T_0 - T_X = [1 - X/T_0 \times 100]$.

Transwell Migration Assay

A volume of 600 μl SDF-1 (100 ng/ml) in IMDM medium was placed in the lower chamber of each well of 24 well-plates. Afterwards, a volume of 200 μl IMDM medium containing 3×10^4 EPCs added in the upper chamber of 8 μm pore sized of filter membrane. After 24 h of incubation, the lower side of filter was washed with PBS and fixed with 2% PFA. The number of migrated EPCs which migrate toward SDF-1 was determined by Hematoxylin & Eosin staining and counted under the observation using a microscope (Olympus) at 100× magnification.

Gene Expression Analyses

Total RNA was isolated from cultured EPCs using extraction reagent (Sepasol-RNA I Super G; Nacalai Tesque), and reverse transcription was performed with 1 μg of the total RNA using a reverse transcription polymerase chain reaction (RT-PCR) kit (Toyobo, Osaka, Japan). The expression of the target genes was analyzed using a GeneAmp PCR System (Life Technologies, CA, USA) with Thunderbird SYBR qPCR Mix (Toyobo). Experiments were carried out in triplicate, and the expression of the target genes was calculated using the $2^{-\Delta\Delta CT}$ method. β-Actin was used as an internal control. The primer sequences used for quantitative RT-PCR are shown in **Table 1**.

In vivo Wound Healing Model

C57BL/6 mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). All experiments were performed in compliance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. Each experiment was repeated triplicate. In order to compare the wound healing ability of untreated EPCs and GC-treated EPCs, total 15 male mice at 12th week of age were used in

TABLE 1 | The primer sets for the quantitative polymerase chain reactions.

Gene	Primer	Sequence
βactin	Forward	GTGCGTGACATTAAGGAGAAGCTGTGC
	Reverse	GTACTTGCGCTCAGGAGGAGCAATGAT
VEGF	Forward	AGATGAGCTTCCTACAGCACAAC
	Reverse	AGGACTTATACCGGGATTTCTTG
PDGF-AA	Forward	TAGGGAGTGAGGATTCTTTGGACACCA
	Reverse	CAAATGCTCCTCTAACCTCACCTGGAC
FGF	Forward	AGAGCGACCCTCACATCAAGCTACAAC
	Reverse	ATAGCTTTCTGCCCAGGTCCTGTTTTG
Ang-1	Forward	GCCTGATCTTACACGGTGCT
	Reverse	GGCCACAAGCATCAAACCAC
SDF-1	Forward	AGAGCCAACGTCAAGCATCT
	Reverse	CTTTAGCTTCGGGTCAATGC
CXCR4	Forward	CCTTATCCTGCCTGGTTATTGTC
	Reverse	AGGATGAGGATGACTGTGGTCT
CXCR7	Forward	AGAGCTCACAGTTGTTGCAAAGTGC
	Reverse	GGTTCAAGATGTAGCAGTGCGTGTC
CCL2	Forward	GAATCACCAGCAGCAAGTGT
	Reverse	GTTTGGGTTTGCTTGTCCAGG
TGF-β	Forward	AGAGCTCCGAGAAGCGGTACCTGAACCC
	Reverse	GTTGATGTCCACTTGCAGTGTGTTATCC
COX-2	Forward	GGCAGGAGGTCTTTGGTCT
	Reverse	AACTGCTCATCACCCCATTC
mPGES-1	Forward	GGAACGACATGGAGACCATGTAC
	Reverse	TCCAGGCGACAAAAGGGTTA
EP-4	Forward	CCTCAGCGACTTTCGGCG
	Reverse	ACGAATACTCGCACACGAG

each experiment and divided into three groups (5 mice/group): PBS, EPCs, GC-treated EPC. In order to examine the role of CXCR4 in the wound healing ability of EPCs, total nine male mice at 12th week of age were used in each experiment and divided into three groups (3 mice/group): untreated EPCs, GC-treated EPCs, and CXCR4 antibody-treated EPCs (AMD3100, Sigma-Aldrich). 100 nM of AMD3100 at 24-h treatment was used to block the CXCR4. The mice were anesthetized using avertin, and a skin incision (3 × 2 cm) was made to create an ischemia gradient as previously described (19). EPCs were treated with 100 nM GC for 24 h prior to surgery then labeled with PKH26 (Sigma-Aldrich, Missouri, USA) as the instruction of the manufacturer before transplantation. PBS and PKH26-labeled untreated EPCs were used as the controls. The EPCs were injected at a number of 5×10^5/mouse through the tail vein. Skin tissue samples were fixed overnight with 4% paraformaldehyde; then, washed with PBS, soaked in sucrose 10% for 2 h, and finally in sucrose 20% overnight. Then, the frozen blocks of samples were made by embedded the samples in the O.C.T compound (Sakura Finetek, Tokyo, Japan) and freeze in liquid nitrogen. After that the tissues were sectioned before immunostaining. For observation, the flap tissues were collected at two different time points: on the third day of transplantation to analyze the inflammatory cell recruitment and on the seventh day of transplantation to analyze the neovascularization. Images of the ischemic flaps were captured on the seventh day of transplantation, and the necrotic areas were quantified using the Image J software program (NIH).

Histological Analyses

The frozen flap tissue sections were mounted, stained with hematoxylin and eosin (Wako, Osaka, Japan) and observed under a microscope (Olympus, Tokyo Japan). The inflammatory cells recruited in the ischemic area were visualized by immunohistochemical staining with rat anti-mouse CD45 (553078, BD Pharmigen), rat anti-mouse Mac1 (rat anti-mouse CD11b 550282, BD Pharmigen). The neovascularization was analyzed by immunohistochemical staining with rat anti-mouse CD31 (553370, BD Pharmigen) as the instruction of the manufacturer. Briefly, sections were washed in PBS and then rinsed in 10% H_2O_2 in PBS before blocking with blocking buffer containing Phosphate-Buffered Saline with 0.5% Tween 20 (PBST) and normal rabbit serum at a dilution of 4:1 (v/v) for 1 h at room temperature. After that, the sections were incubated with appropriate antibody overnight. On the following day, the sections were washed with PBST before incubating in the secondary antibody (POD Conjugate Anti-rat, MK-201, Takara, Shiga, Japan) for 30 min at room temperature. The sections were then washed with PBS and covered with 0.05% 3,3′-Diaminobenzidine (DAB) solution (Sigma-Aldrich) in PBS with H_2O_2 for 3 min then stopped detection by water. Quantification of the number of positive cells was performed at 200× magnification (20× objective and 10× eyepiece) under a microscope (Olympus) by counting the brown dots in 10 fields.

Western Blotting

Cultured EPCs were harvested, counted and a number of 10^6 cells were suspended in low-salt buffer (10 mM HEPES, 10 mM KCL, 1 mM dithiothreitol, 1 mM EDTA, protease inhibitor cocktail (PIC), and 1% Nonidet P-40, Roche Diagnostics, Basel, Switzerland), and nuclear pellets were collected by centrifugation. The nuclear pellets were then suspended in high-salt buffer (20 mM HEPES, 400 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA, PIC), and the nuclear extract was obtained. The total protein concentration was measured by Bradford assay (Bio-rad, CA, USA) as the instruction of the manufacturer. 30μg protein of each nuclear extract samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (EMD Millipore, Darmstadt, Germany).

An immunoblotting analysis was performed. Briefly, the membranes were blocked with 5% Bovine Serum Albumin (Sigma-Aldrich, Missouri, USA) in Tris-Buffered Saline with 0.5% Tween 20 (TBST) for an hour at room temperature, then incubated incubation with the appropriate primary antibody overnight. Primary antibodies were diluted at the dilution of 1:1,000 (v/v) as the instruction of the manufacturer, including: Rabbit anti-human AKT (9272S, Cell Signaling Technology, Massachusetts, USA, phosphorylated AKT antibody (9271S, Cell Signaling Technology), rabbit anti-human HIF-2α antibody (NB100-122, Novus Biologicals, CO, USA), and goat anti-Lamin

B antibody (M-20, Santa Cruz Biotechnology, Inc., CA, USA. The membranes were washed with TBST buffer then incubated with secondary antibodies, including: Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (32460, ThermoFisher Scientific) and rabbit anti-goat IgG (31402, ThermoFisher Scientific) with the dilution of 1:10,000 (v/v) for an hour at room temperature. Then, the membranes were washed and the enhanced chemiluminescence (GE Healthcare, Illinois, USA) was used for detection by a luminescent image analyzer (ImageQuant LAS4000, GE Healthcare). The band densities of target proteins were analyzed by ImageJ software (NIH). The relative expressions of target proteins were normalized with the total-LaminB expression. For the phosphorylation analysis, after incubation with phosphorylated antibody and detected the phosphorylated protein, the membranes were striped with Restore Stripping Buffer (ThermoFisher Scientific) for 15 min at room temperature, then, continued to incubate with rabbit Akt antibody. The Akt signals were detected and the membranes were then stripped and re-probed with LaminB antibody.

Statistical Analyses

Data were statistically analyzed using Mann Whitney U test using the GraphPad Prism 5 software program (GraphPad Software, CA, USA) as appropriate. Data are presented as the mean ± standard deviation. $P < 0.05$ was considered as significant.

RESULTS

GC Treatment Impaired the Proliferation Ability of EPCs

A previous report stated that GCs induced apoptosis in lymphocytes, demonstrating the negative effects of GCs on cells (20). However, thus far, no report has described the effects of GCs on EPCs; therefore, in the present study, we examined the characteristics of EPCs, including the cell morphology, viability, surface markers, and proliferation, under treatment with 100 nM dexamethasone as a GC. We found that GC treatment exerted no effects on the adherence or visual appearance of EPCs (**Figure 1A**). In addition, Annexin-V/7AAD staining assay data showed that 24-h treatment of GC showed no apoptosis induction or any cytotoxicity in EPCs (**Figure 1B**).

To examine the effects of GC on the EPC surface markers, a FACS analysis was performed. The data showed that there were no significant changes in the self-surface markers of EPCs under GC treatment, with both control and treated groups expressing high levels of CD31, CD34, CD105, CD73, and VEGFR2 and not expressing CD45 hematopoietic marker at all (**Figure 1C**). Next, we investigated the effects of GC treatment on the proliferation ability of EPCs. The data showed that the proliferation ability of EPCs treated with GC was significantly impaired compared to the untreated EPCs (**Figures 1D,E**). Taken together, these data show that GC impaired the proliferation ability of EPCs.

GC Impaired the *in vitro* Migration Ability of EPCs via the Downregulation of CXCR4

Previous studies have described the negative effects of GCs on the wound healing process (3, 20). Recently, we also reported that GCs impair the wound healing function of mesenchymal stem cell by reducing the expression of SDF-1 (5). Therefore, in the present study, we thought that GCs might also affect the wound healing function of EPCs. To test this hypothesis, we evaluated the wound healing function of EPCs under GC treatment. First, because the EPC migration ability is critical for their biological properties, the effect of GC on the migration ability of EPCs was evaluated by a scratch assay as an *in vitro* wound healing model (21). As shown in **Figure 2A**, GC-treated EPCs showed a lower capability of wound closure than the control, indicating a reduced migration ability of EPCs on exposure to GC (**Figures 2A,B**).

Next, we assessed the expression profiles of the wound healing-related genes in EPCs. Numerous reports have shown that EPCs express multiple chemokines and their receptors related to mobilization (SDF-1, CXCR4, CXCR7), angiogenesis (Ang-1, TGFβ), immunomodulatory (CCL2), and growth factors (PDGF, VEGF, FGF) that play a critical role in wound healing (22, 23). To clarify how GC interferes with the wound healing capacity of EPCs, we examined the mRNA expression of those genes in GC-treated EPCs.

We observed a significantly impaired expression of CXCR4, which related to the mobility (24) and CCL2, which related to the inflammatory cell recruitment (25, 26) in GC-treated EPCs compared to that in control cells (**Figure 2C**). In addition, several genes that contribute to EPC migration, such as the extracellular matrix (ECM) protease MMP9 and homing and adherence factor VCAM 1, were also downregulated due to GC treatment (data not shown). Consistent with the mRNA expression data, a further FACS analysis showed a reduction in the CXCR4 surface protein level (**Figure 2D**).

It is reported that the SDF-1/CXCR4 cascade is responsible for the mobilization of EPCs (10). Therefore, we next performed the transwell assay to examine the effects of GC treatment on the migration ability of EPCs toward the signal of SDF-1. As expected, GC-treated EPCs showed the low migration ability toward SDF-1 compared to the untreated cells (**Figure 2E**). In addition, treatment EPCs with a CXCR4 antibody, AMD3100, also showed the similar impaired migration ability, suggesting the direct role of CXCR4 that regulates the mobility of EPCs (**Figure 2E**). Taken together, these results showed that GC impaired wound healing ability *in vitro* and the migration ability toward SDF-1 of EPCs by the downregulation of CXCR4 and CCL2.

GC Impaired the Wound Healing Ability of EPCs

We next examined the effects of GC on the wound healing ability of EPCs by a transplantation study using an *in vivo* mouse flap model. The wound healing functions of GC-treated EPCs were analyzed and compared to those in untreated cells. We found that the injection of untreated EPCs healed the wounds of mice with a reduction in the necrotic areas at 7 days after transplantation. In contrast, the wound healing ability of the GC-treated EPCs was significantly impaired, with some

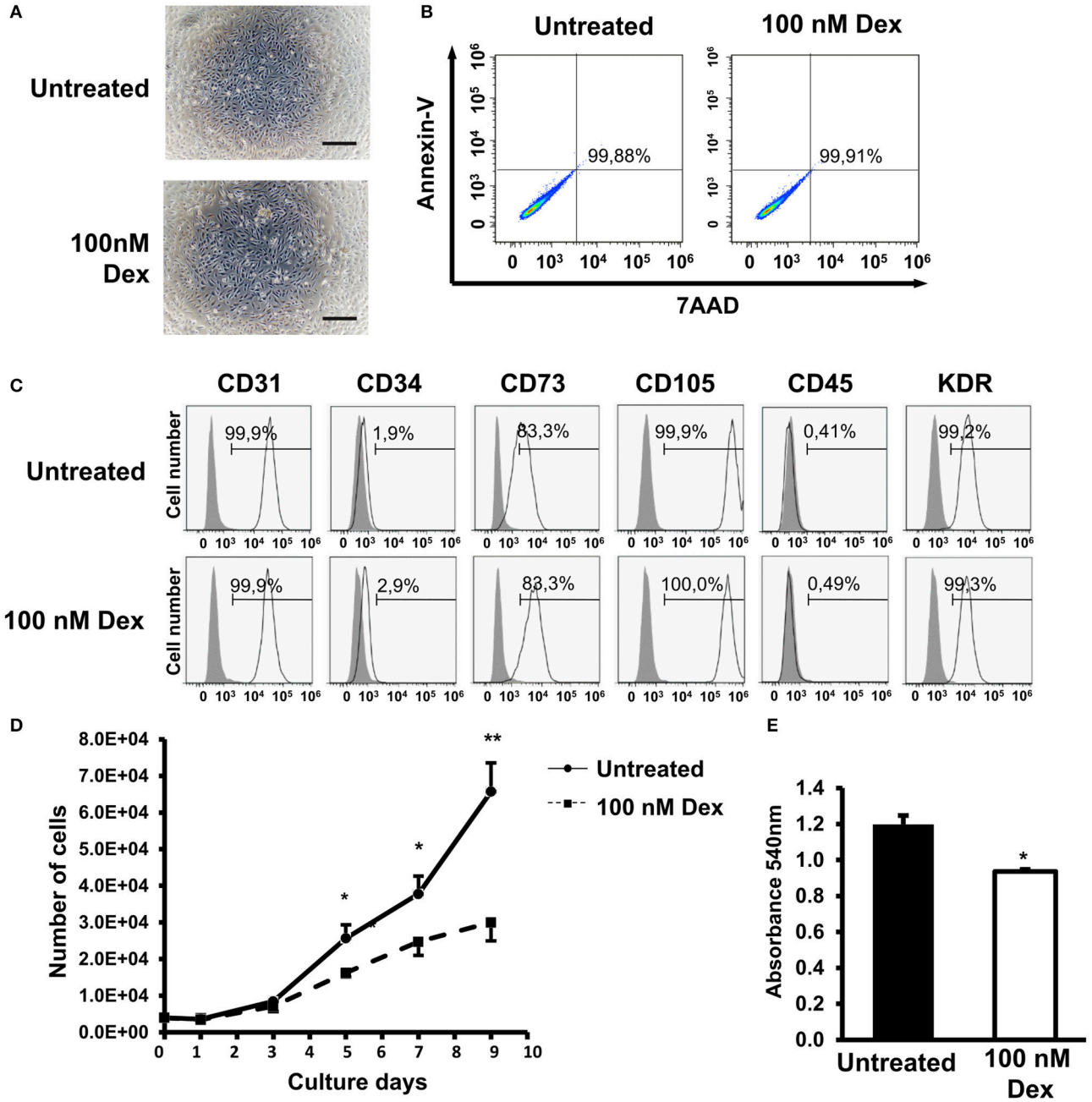

FIGURE 1 | Glucocorticoid (GC) treatment impaired the proliferation ability of EPCs. **(A)** Cell morphology of untreated and GC-treated EPCs from one representative experiment. **(B)** Apoptotic cells of untreated and GC-treated EPCs. Cells were stained by Annexin V staining, then analyzed by the FACS analysis. The histograms were from one representative experiment. **(C)** Cell surface markers of untreated and GC-treated EPCs analyzed by FACS analysis. The histograms were from one representative experiment. **(D)** The growth curve of untreated and GC-treated EPCs from one representative experiment. **(E)** The proliferation assay of untreated and GC-treated EPCs examined by Cell counting kit-8. The chart shows the mean data from all experiments. Dexamethasone (100 nM, 24-h treatment) was used as a GC. Untreated: untreated EPCs, 100 nM Dex: GC-treated EPCs. The data represent the mean ± SD. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. The scale bar indicates 100 μm. The experiment was repeated triplicate.

necrotic areas remaining, similar to control mice without any cell transplantation (**Figures 3A,B**).

Because the GC-treated EPCs showed the impaired expression of CCL2 which related to the inflammatory cell recruitment ability (25, 26), we performed a histological analysis of the wound healing process on the third day after transplantation and the data revealed that mice transplanted with untreated EPCs showed an increased recruitment of CD45- and Mac-1 positive

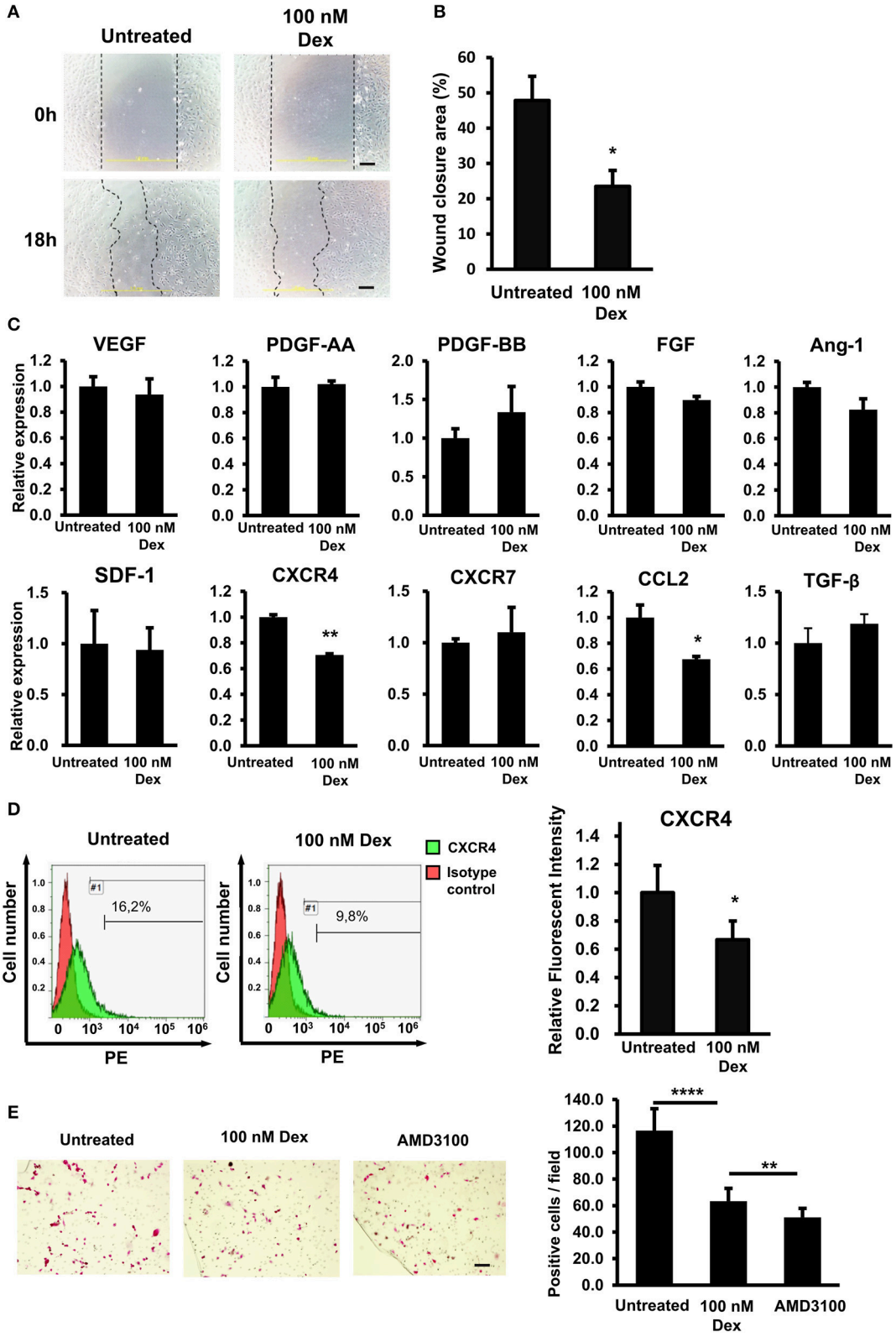

FIGURE 2 | GCs impaired the *in vitro* migration ability of EPCs via the downregulation of CXCR4. **(A)** The *in vitro* wound healing ability of untreated and GC-treated EPCs by a scratch assay. The images showed the wound closures from one representative experiment taken under a microscope at 100× magnification.

(Continued)

FIGURE 2 | (B) Quantification of the wound closure area in a scratch assay of untreated and GC-treated EPCs. The data were displayed as the means from all experiments. **(C)** The relative mRNA expression of wound healing genes in untreated and GC-treated EPCs. The chart shows the mean data from all experiments. **(D)** The relative protein expression of CXCR4 in untreated and GC-treated EPCs identified by a FACS analysis. The FACS histograms were from one representative experiment. The chart shows the quantification of relative expression of CXCR4 protein. The data were displayed as the means from all experiments. **(E)** The transwell assay of untreated, GC-treated EPCs, and CXCR4 antibody-treated EPCs toward SDF-1. AMD3100 (100 nM, 24-h treatment) was used as the CXCR4 antibody. The images were the migrated EPCs from one representative experiment taken under a microscope at ×100 magnification. The chart shows the quantification of number of migrated EPCs. The data were displayed as the means calculated from all experiments. Dexamethasone (100 nM, 24-h treatment) was used as a GC. Untreated: untreated EPCs, 100 nM: GC-treated EPCs. The data represent the mean ± SD. $n = 3$, $^{****}P < 0.0001$, $^{**}P < 0.01$, $^{*}P < 0.05$. The scale bar indicates 100 μm. The experiment was repeated triplicate.

cells to the wound sites compared to mice transplanted with GC-treated EPCs (**Figures 3C,D**). In addition, on the seventh day after transplantation, mice injected with untreated EPCs showed a greater number of CD31-positive vascular endothelial cells than mice injected with GC-treated EPCs (**Figures 3C,D**). In addition, to examine the effects of GC treatment on the homing and migration ability of EPCs to the wound sites, we quantified the PKH-labeled EPCs in the wound regions on the first day of transplantation. We found that there were fewer PKH-positive cells at the wound sites of mice transplanted with GC-treated EPCs than in those injected with untreated EPCs (**Figures 3E,F**).

We previously reported the impaired wound healing ability of EPCs with high activity of ALDH (Alde-high EPCs) due to the low expression of CXCR4 compared to those with low activity of ALDH (Alde-low EPCs) (19). In order to clarify the direct role of CXCR4 in the wound healing ability of EPCs, we blocked the activity of CXCR4 by its antibody, AMD3100, and compared the necrotic area in the transplantation study. As expected, similar to the effects of GC, blocking CXCR4 in EPCs showed the impaired wound healing function in the flap mice (**Figure 3G**), suggesting CXCR4 is responsible for the wound healing ability of EPCs.

Taken together, these data demonstrated that GCs impaired the wound healing functions of EPCs.

GC-Impaired Prostaglandin E2 Production Was Involved in the Downregulation of CXCR4 in EPCs

Previous studies have shown that GCs are a strong inhibitor of the arachidonic acid cascade, resulting in less production of prostaglandin E2 (PGE2) in macrophages, vascular smooth muscle cells, and MSCs (27–30). In addition, other studies have suggested that the expression of CXCR4 is elevated in the presence of PGE2 (31). Therefore, we next investigated whether or not GC impaired the production of PGE2 to cause the downregulation of CXCR4 expression in EPCs. Cyclooxygenase (COX) and prostaglandin dehydrogenase (mPGES) are reported to be key enzymes in the catabolism of PGE2 (32–34). We found that GC treatment impaired the expression of COX-2 and mPGES-1 in EPCs, implying the negative effects of GCs on the PGE2 production of these cells (**Figure 4A**).

Next, in order to clarify the involvement of PGE2 in the downregulation of the CXCR4 expression in GC-treated EPCs, we examined whether or not treatment with PGE2 could reverse the expression of CXCR4 of these cells. The data showed that treatment of PGE2 rescued the GC-impaired mRNA and protein expression of CXCR4 in EPCs (**Figures 4B,C**). Furthermore, consistent with the upregulation of CXCR4 in the presence of PGE2, the migratory capacity of GC-treated EPCs was also recovered by PGE2 treatment (**Figure 4D**). Taken together, these results suggested that GC downregulated the expression of CXCR4 in EPCs by the impairment of PGE2 synthesis via COX-2 and mPGES-1.

EP4/AKT Signaling Was Involved in the GC-Downregulated PGE2 in EPCs

A previous reports showed that PGE2 exerted its activity by interaction with a G-protein-coupled receptor family (GPCRs) consisting of EP1, EP2, EP3, and EP4 subtypes, resulting in the different signal transduction and regulation of the expression of numerous target genes (35). To identify which PGE2 receptors are affected by GC, we analyzed the gene expression of these subtypes in EPCs in the presence of GC. We found that, among these four receptors, GC treatment only impaired the expression of EP4 in EPCs. In addition, treatment with PGE2 reversed the negative effects of GC on the EP4 expression, suggesting that EP4 is the PGE2 receptor related to the effects of GC on EPCs (**Figure 5A**).

The EP2 and EP4 receptors which mediate the increased cAMP concentrations had been thought to have similar effects in some biological process; however, recently, their distinct roles were reported (36, 37). It might be because of the selective expression of either of them in cells and the selective actions on the different signaling pathways (37). It is reported that EP4 but not EP2 couples to PI3K which regulates the migration of dendritic cells in the mouse suggesting the role of EP4/PI3K/AKT in the regulation of migration ability of the cells (37). Therefore, we next examined the role of PI3K/AKT pathways in the GC-impaired CXCR4 expression in EPCs. As expected, we found the impaired phosphorylation of AKT in EPCs in the presence of GC; this effect was rescued by the adding of PGE2 which showed by the upregulation of pAKT (**Figure 5B**). In order to clarify the role of PGE2/PI3K/AKT pathway in the CXCR4 regulation of GC-treated EPCs, we analyzed the expression of CXCR4 in the present of a PI3K inhibitor. The effect of PI3K inhibitor on the phosphorylation of AKT was confirmed (**Figure 5C**). The data showed that treatment with PI3K inhibitor abolished the rescued effects of PGE2 on the GC-treated EPCs in which CXCR4 expression was impaired (**Figure 5D**) indicating that PGE2 reversed the GC-impaired CXCR4 expression in EPCs via the activation of PI3K/AKT pathway.

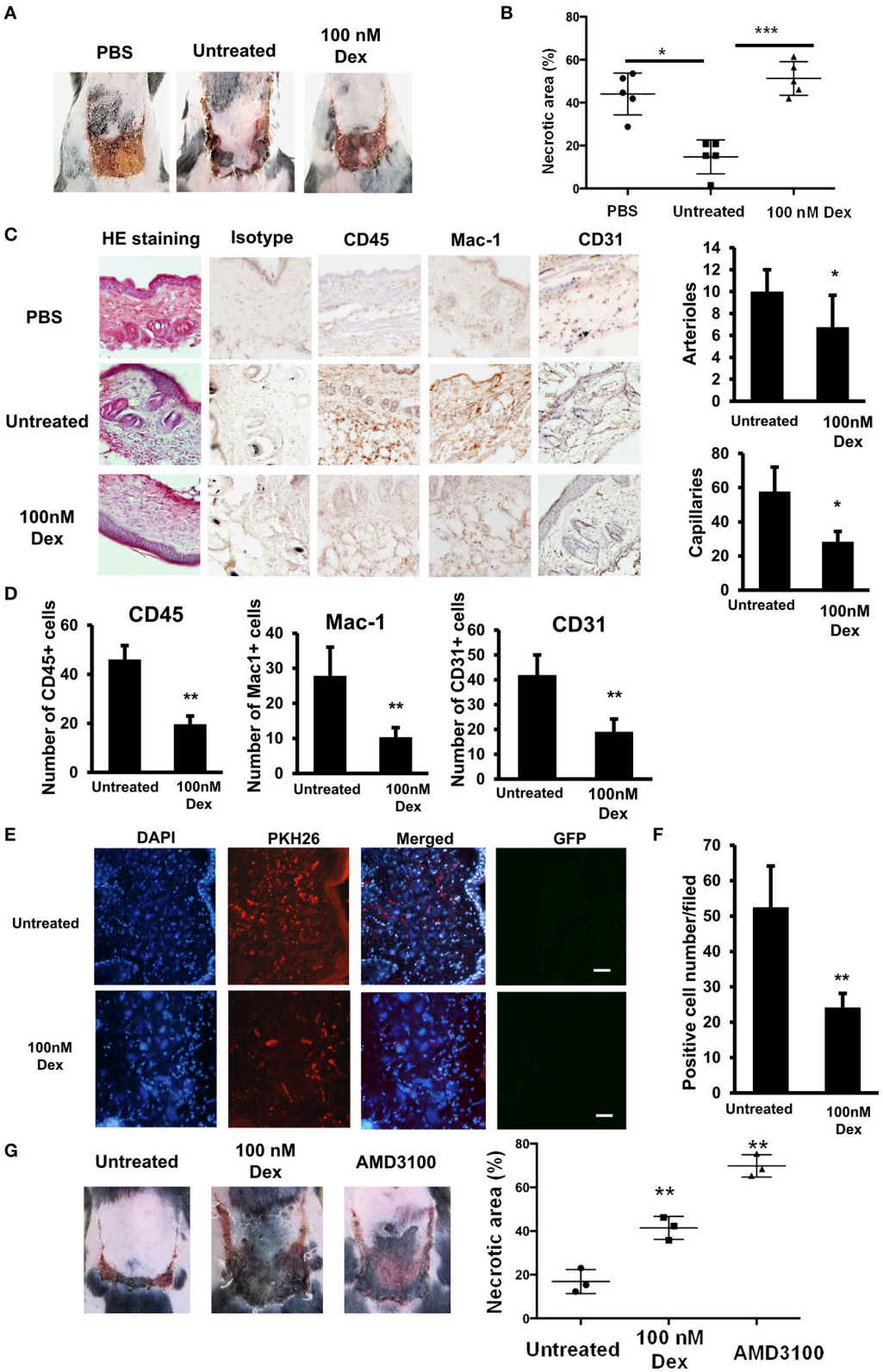

FIGURE 3 | GCs impaired the wound healing ability of EPCs. **(A)** Wound healing ability of untreated and GC-treated EPCs in flap mouse model. **(B)** The necrotic area of the flap tissues injected with untreated and GC-treated EPCs. **(C)** Immunohistochemistry of CD45-, MAC-1- (at day 3 post-transplantation), and CD31-positive

(Continued)

FIGURE 3 | cells (at day 7 post-transplantation) in flap tissues injected with untreated and GC-treated EPCs observing under a microscope at 200× magnification. **(D)** Quantification of CD45-, MAC-1- (at day 3 post-transplantation), and CD31-positive cell numbers (at day 7 post-transplantation) in flap tissues injected with untreated and GC-treated EPCs observing under a microscope at 200× magnification. **(E)** PKH-positive EPCs in flap tissues injected with untreated and GC-treated EPCs observing under a microscope at 200× magnification. **(F)** Quantification of PKH-positive EPCs in flap tissues injected with untreated and GC-treated EPCs observing under a microscope at 200× magnification. From **A–F**, the data were calculated from five individual mice from one representative experiment. The data represent the mean ± SD. $n = 5$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. **(G)** Wound healing ability of untreated and CXCR4 antibody-treated EPCs in flap mouse model. AMD3100 (100 nM, 24-h treatment) was used as a CXCR4 antibody. The data were calculated from three individual mice from one representative experiment. The data represent the mean ± SD. $n = 3$, $^{**}P < 0.01$. Dexamethasone (100 nM, 24-h treatment) was used as a GC. Untreated: untreated EPCs, 100 nM: GC-treated EPCs. The scale bar indicates 100 μm. The experiment was repeated triplicate.

Taken together, these results demonstrated that GC impaired the EP4/AKT signaling which was involved in the downregulation of CXCR4 expression of EPCs.

GC Downregulated the Expression of CXCR4 via the Independent Impairment of the HIF2α and PGE2 Pathways

We previously reported the HIF2α-dependent upregulation of CXCR4 in Alde-low EPCs under hypoxic conditions, which promotes the migration and wound healing function of the cells (13, 19). To examine whether or not GC causes similar negative effects that impair the CXCR4 expression in EPCs under hypoxic conditions, we next analyzed untreated and GC-treated EPCs under hypoxic conditions. As expected, in the control EPCs without treatment of GC, we observed the upregulation of CXCR4 at both the mRNA and protein levels under hypoxic conditions (**Figures 6A,B**). However, in the GC-treated EPCs, the upregulation of CXCR4 under hypoxic conditions was abrogated (**Figures 6A,B**). Interestingly, this detrimental effect of GC on the CXCR4 expression was rescued by treatment with PGE2 (**Figures 6C,D**), indicating the similar effects of GC on EPCs under both normoxic and hypoxic conditions of downregulating the expression of CXCR4 via the impairment of the PGE2 pathway.

Our previous report described the functions of the HIF2α pathway, which plays a key role as an upregulator of the CXCR4 expression in EPCs under hypoxic conditions (19). In order to examine whether or not the HIF2α pathway is involved in the GC-impaired reduction of the CXCR4 expression under hypoxic conditions, we next examined the effects of GC on the expression of HIF2α. Interestingly, we found that GC also impaired the expression of HIF2α under hypoxic conditions (**Figure 6E**).

COX2/PGE2 was reported to upregulate the expression of HIF2α under hypoxic conditions in hepatocellular carcinoma cells (38). Therefore, we thought that PGE2 might also upregulate the expression of HIF2α in EPCs under hypoxic conditions. In order to test this hypothesis, untreated EPCs were cultured in the presence of PGE2 under hypoxic conditions, and the expression of HIF2α was examined. Unexpectedly, we found that PGE2 showed no effect on the expression of HIF2α (**Figure 6F**). This suggested that the HIF2α and PGE2 pathways independently regulated the expression of CXCR4 in EPCs under hypoxic conditions.

Taken together, these results indicated that GC exerted similar effects on the CXCR4 expression in EPCs under both normoxic and hypoxic conditions. However, under hypoxic conditions, the GC-impaired CXCR4 expression in EPCs was caused independently by the downregulation of the HIF2α or PGE2 pathway.

DISCUSSION

In the present study, we showed that GC downregulated the expression of CXCR4, thus abrogating the wound healing ability of EPCs. GC-treated EPCs showed a poor migration ability and dysfunction in the recruitment of inflammatory cells and neovascularization. Of note, the production of PGE2 was associated with the detrimental effects of GCs on EPCs. GCs impaired the expression of COX2 and mPGES1, which are the main enzymes of PGE2 synthesis. Treatment with PGE2 upregulated the expression of EP4 receptors and activated the PI3K/AKT signaling pathways, thereby reversing the impaired effects of GCs on the expression of CXCR4 in EPCs. Importantly, our data demonstrated that, GCs similarly impaired the expression of CXCR4 under hypoxic conditions as under normoxic conditions, as proven by the impairment of PGE2 and another pathway related to HIF2α. Of note, PGE2 and HIF2α acted independently in the regulation of CXCR4 in EPCs under hypoxic conditions (**Figure 7**, proposed model).

Although no significant influence on the EPCs was observed under microscopic observation, GC treatment decreased the proliferation ability of EPCs. GCs are known to exert anti-proliferative activities against various cells, including neural cells, osteoblasts, osteosarcoma cells, hepatoma cells, and lung, ovarian, and prostate cancer cells (39–41). It has been reported that GC inhibits cell proliferation by arresting the cell cycle via several molecular mechanisms, including the upregulation of cyclin-dependent kinase inhibitors (CKIs), such as p21, that inactivate the cyclinD/Ckd4 complex, leading to G1/G0 arrest (42, 43), or affects other regulators, such as cyclinD1 and c-myc, which induce arrest in the G1 phase (44, 45). In addition, although in our present study, GC treatment showed no apoptosis induction in EPCs after 24-h treatment, a further time-course study is necessary to examine the effects of GC to induce EPCs apoptosis at later time points.

Additionally to the impaired-proliferation, GC treatment downregulated the expression of wound healing genes in EPCs, including CCL2 and CXCR4. CCL2 is the proinflammatory chemokine which is responsible for the inflammatory cell recruitment (25). It is reported that CCL2 promotes wound healing in diabetic mice by inducing macrophage (26). The downregulation of CCL2 in GC-treated EPCs might be involved

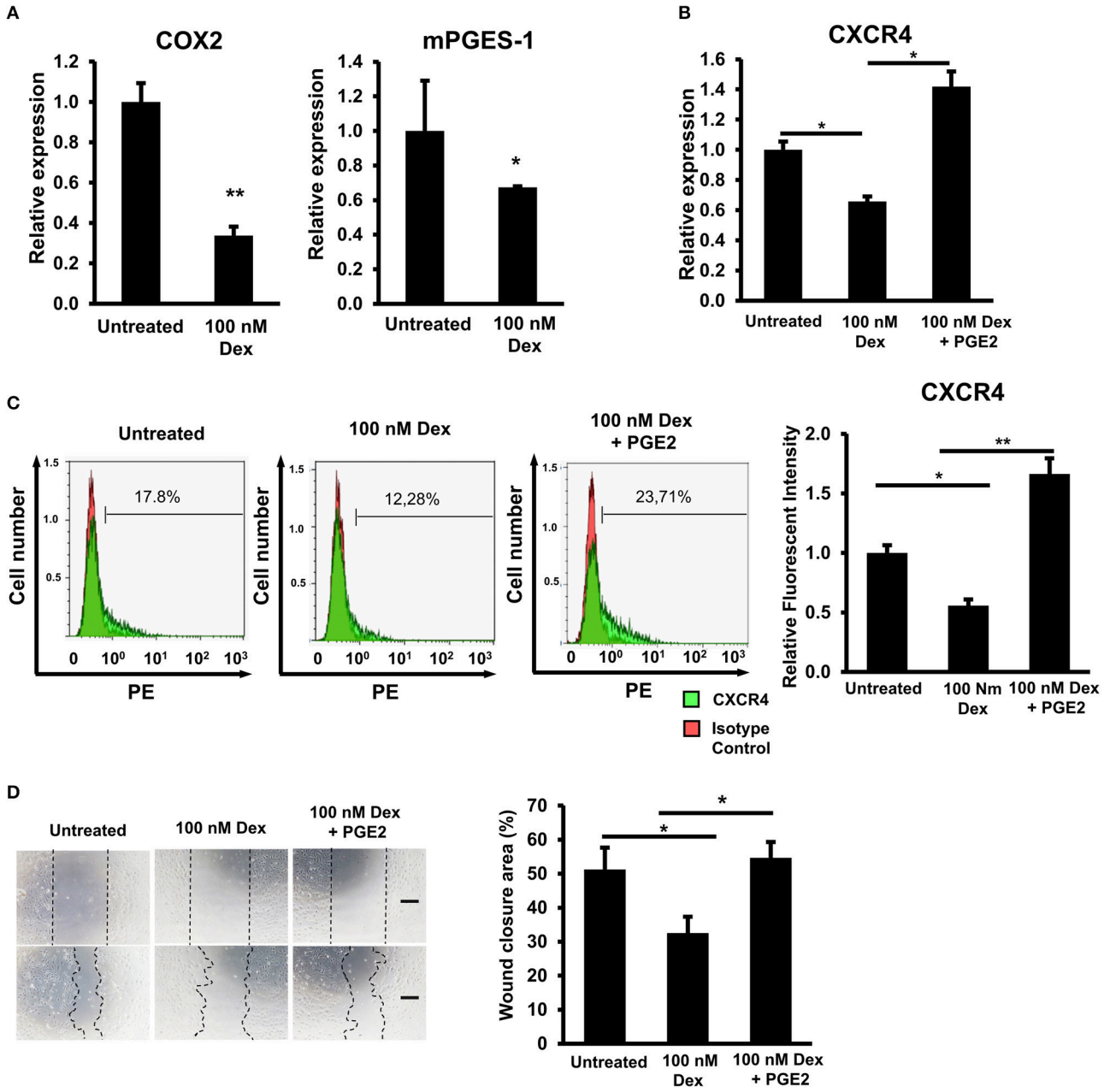

FIGURE 4 | GC-impaired prostaglandin E2 production was involved in the downregulation of CXCR4 in EPCs. **(A)** The relative mRNA expression of COX2 and mPEGS1 in untreated and GC-treated EPCs. **(B)** The relative mRNA expression of CXCR4 in untreated, GC-treated EPCs, and GC-treated EPCs in the presence of PGE2. **(C)** The relative protein expression of CXCR4 in untreated, GC-treated EPCs, and GC-treated EPCs in the presence of PGE2 by a FACS analysis. **(D)** An *in vitro* scratch assay of untreated, GC-treated EPCs, and GC-treated EPCs in the presence of PGE2 observing under a microscope at 100× magnification. Dexamethasone (100 nM, 24-h treatment) was used as a GC. Untreated: untreated EPCs, 100 nM: GC-treated EPCs, 100 nM + PGE2: GC-treated EPCs in the presence of PGE2. The data represent the mean ± SD. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. The scale bar indicates 100 μm. The experiment was repeated triplicate. The charts showed the overall mean data from all experiments.

in the low recruitment of CD45 and Mac1-possitive cells to the wound sites on the third day after transplantation of GC-treated EPCs in the mouse model. The SDF-1/CXCR4 cascade is a major cascade underlying the mobilization of EPCs (46). We previously reported on the GC-impaired wound healing function of MSCs by the suppression of SDF-1 (5). In the present study, although no significant effects on the expression of SDF-1 were noted, GC still adversely affected the wound healing function of EPCs via interference with the regulation of CXCR4. CXCR4 is a G-protein coupled receptor that is highly expressed on EPCs

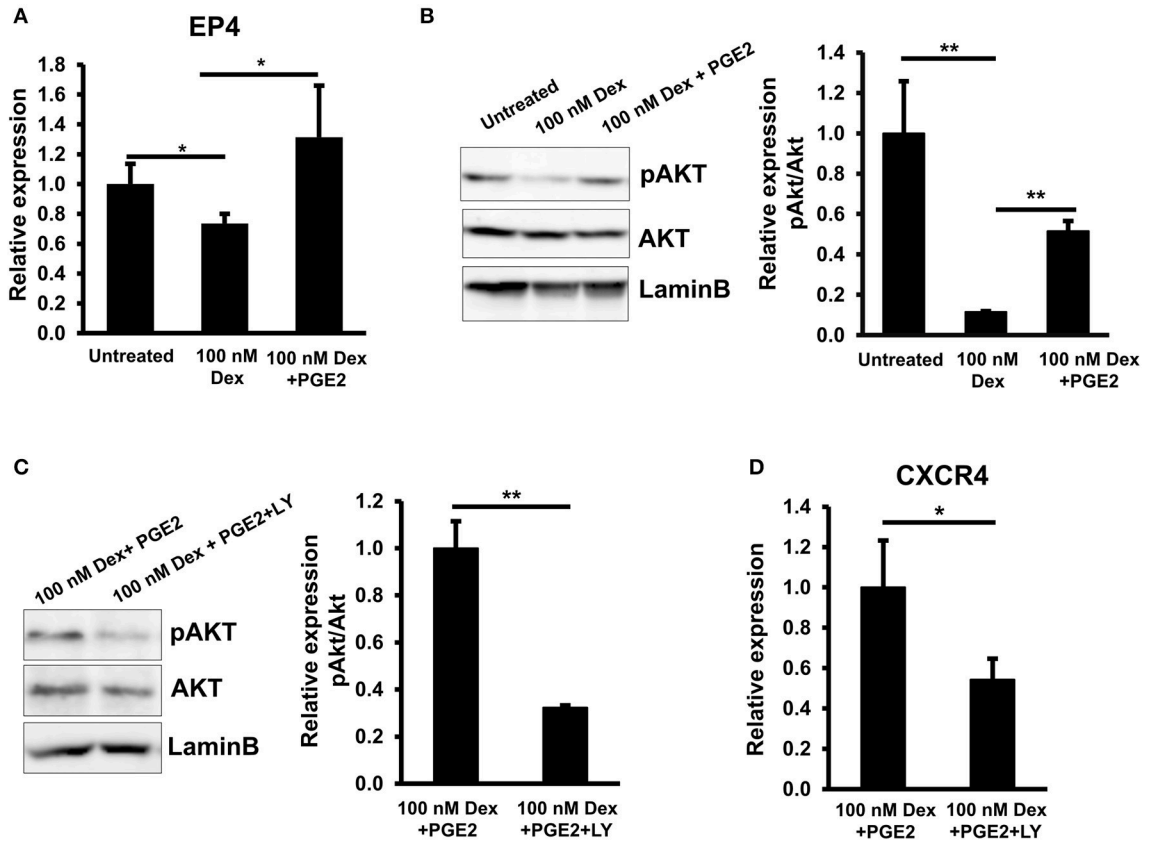

FIGURE 5 | GCs downregulated CXCR4 via the impaired PGE2/EP4/AKT signaling in EPCs. **(A)** The relative mRNA expression of EP4 in untreated, GC-treated EPCs, and GC-treated EPCs in the presence of PGE2. **(B)** The relative protein expression of pAKT/AKT in untreated, GC-treated EPCs, and GC-treated EPCs in the presence of PGE2. **(C)** The inhibition of PI3K/AKT pathway in GC-treated EPCs in the presence of PGE2. LY294002 was used as a PI3K inhibitor. **(D)** The relative mRNA expression of CXCR4 in GC-treated EPCs in the presence of PGE2 and PI3K inhibitor. Dexamethasone (100 nM, 24-h treatment) was used as a GC. Untreated: untreated EPCs, 100 nM: GC-treated EPCs, 100 nM + PGE2: GC-treated EPCs in the presence of PGE2, 100 nM + PGE2 + LY: GC-treated EPCs in the presence of PGE2 and PI3K inhibitor. LY: LY294002. The data represent the mean ± SD. $n = 3$, $^{**}P < 0.01$, $^{*}P < 0.05$. The experiment was repeated triplicate. The charts showed the overall mean data from all experiments.

(47). In injured tissues, the surrounding cells secrete SDF-1, which recruits the CXCR4-expressing cells to the wound sites and play their functions in wound healing (48). A study in coronary artery disease patients showed that the dysregulation of CXCR4 signaling reduced the migratory capacity of EPCs in these patients compared to healthy subjects (49). In our study, we found that, under GC treatment, the downregulation of CXCR4, together with the contribution of impaired proliferation, led to a reduced *in vitro* wound healing ability of EPCs in the migration scratch assay. These impaired effects might be involved in the low homing ability of EPCs to the injured tissues in the mouse flap model. These findings suggested the need for further studies on the CXCR4 regulatory effects and functions of EPCs derived from patients receiving GC therapy.

The present findings raised questions about how GCs reduce the CXCR4 expression in EPCs. PGE2 has been proven to induce the expression of CXCR4 in several cells, such as myeloid-derived suppressor cells and microvascular endothelial cells (31, 50). In addition, the inhibitory effects of GCs on prostaglandin synthesis have been well-demonstrated in various cell types and tissues by the mediation of multiple pathways (51, 52). Therefore, we hypothesized that GCs impaired the expression of CXCR4 in EPCs through activity against prostaglandin synthesis. Indeed, our data demonstrated that GCs interfered with the production of PGE2 by the impairment of two enzymes: COX2 and mPGES1. Of note, treatment with PGE2 rescued the expression of CXCR4 in the GC-treated EPCs, which confirmed our hypothesis.

PGE2 is a potent upstream mediator of several genes through interaction with its receptors (EPs), including EP1, EP2, EP3, and EP4, thereby recruiting the transcription factors and modulating the target gene expression (37). Several reports have described the role of PGE2-EPs signaling in the SDF-1-CXCR4 chemokine system (53, 54). EP3 or EP4 knockout suppressed SDF-1 and CXCR4-positive stromal cells in mice (53). PGE2 promotes the homing ability of CD34-positive cells through EP2 and EP4 (54). However, thus far, how GC affects the PGE2 receptors and its effects on the regulation of CXCR4 in EPCs have been unclear. We found that GC impaired the expression of EP4 but not EP1,

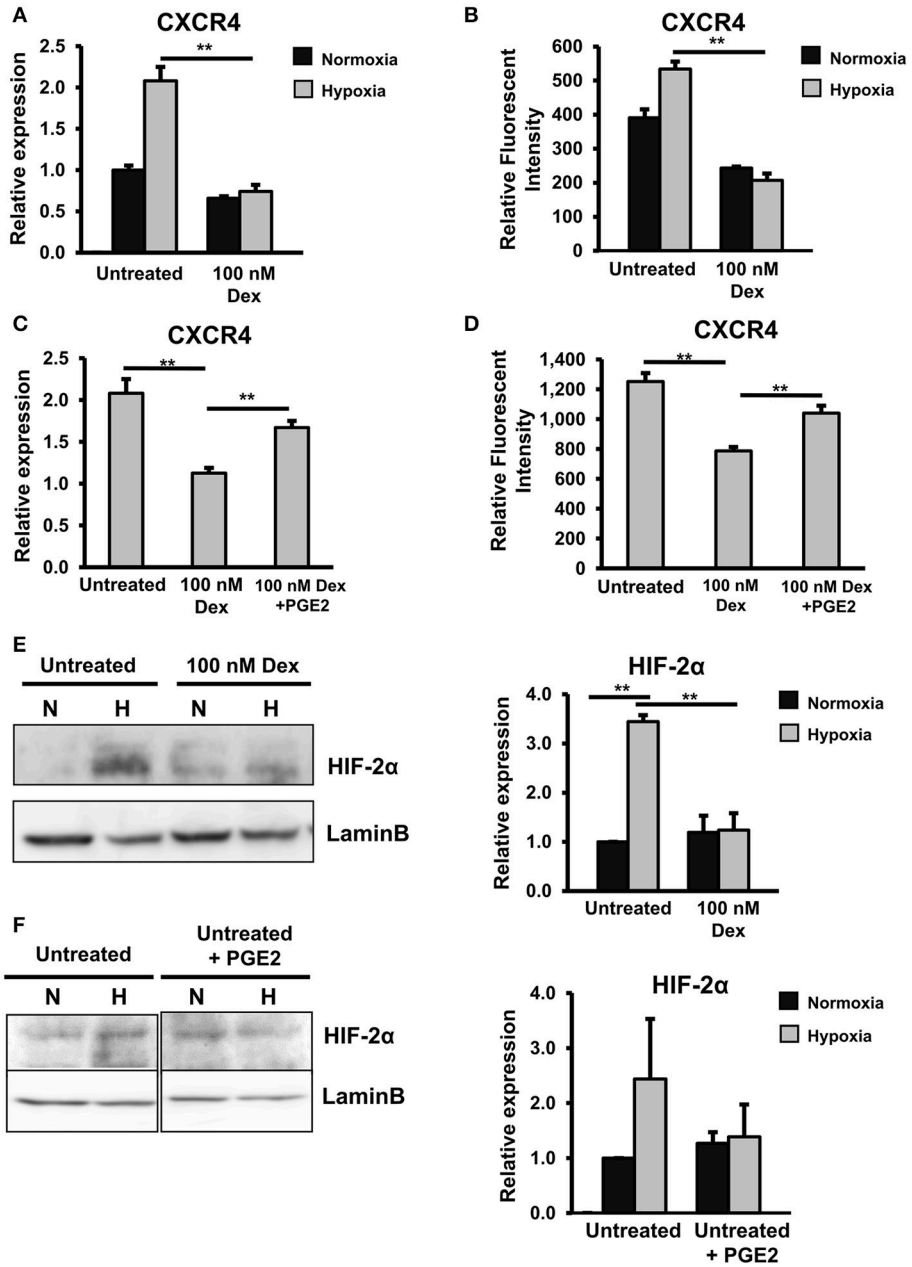

FIGURE 6 | GCs downregulated the expression of CXCR4 via the independent impairment of the HIF2α and PGE2 pathways. **(A)** The relative mRNA expression of CXCR4 in untreated and GC-treated EPCs under normoxic and hypoxic conditions. **(B)** The relative protein expression of CXCR4 in untreated and GC-treated EPCs under normoxic and hypoxic conditions. **(C)** The relative mRNA expression of CXCR4 in untreated EPCs, GC-treated EPCs, and GC-treated EPCs in the presence of PGE2 under hypoxic conditions. **(D)** The relative protein expression of CXCR4 in untreated EPCs, GC-treated EPCs, and GC-treated EPCs in the presence of PGE2 under hypoxic conditions. **(E)** The expression of HIF2α protein in untreated and GC-treated EPCs under normoxic and hypoxic conditions. **(F)** The expression of HIF2α protein in untreated EPCs in the presence of PGE2 under normoxic and hypoxic conditions. Dexamethasone (100 nM, 24-h treatment) was used as a GC. Untreated: untreated EPCs, 100 nM: GC-treated EPCs, 100 nM + PGE2: GC-treated EPCs in the presence of PGE2, Untreated + PGE2: untreated EPCs in the presence of PGE2. The data represent the mean ± SD. $n = 3$, $^{**}P < 0.01$. The experiment was repeated triplicate. The charts showed the overall mean data from all experiments.

EP2, or EP3 in EPCs which suggested the involvement of EP4 receptor in this pathway. In addition, previous reports showed that only EP4 regulates the migration of numerous cells (55–57). For instance, EP4 regulates the migration of dendritic cells in mice via selective action on PI3K (55). In addition EP4 is also involved in breast cancer cell migration during tumor invasion (56) and enhances the migration of rat smooth muscle cells (57).

Hypoxia plays a crucial role in modulating the functions of many types of cells via the activation of HIF (58). Hypoxic preconditioning promoted the survival, differentiation, and

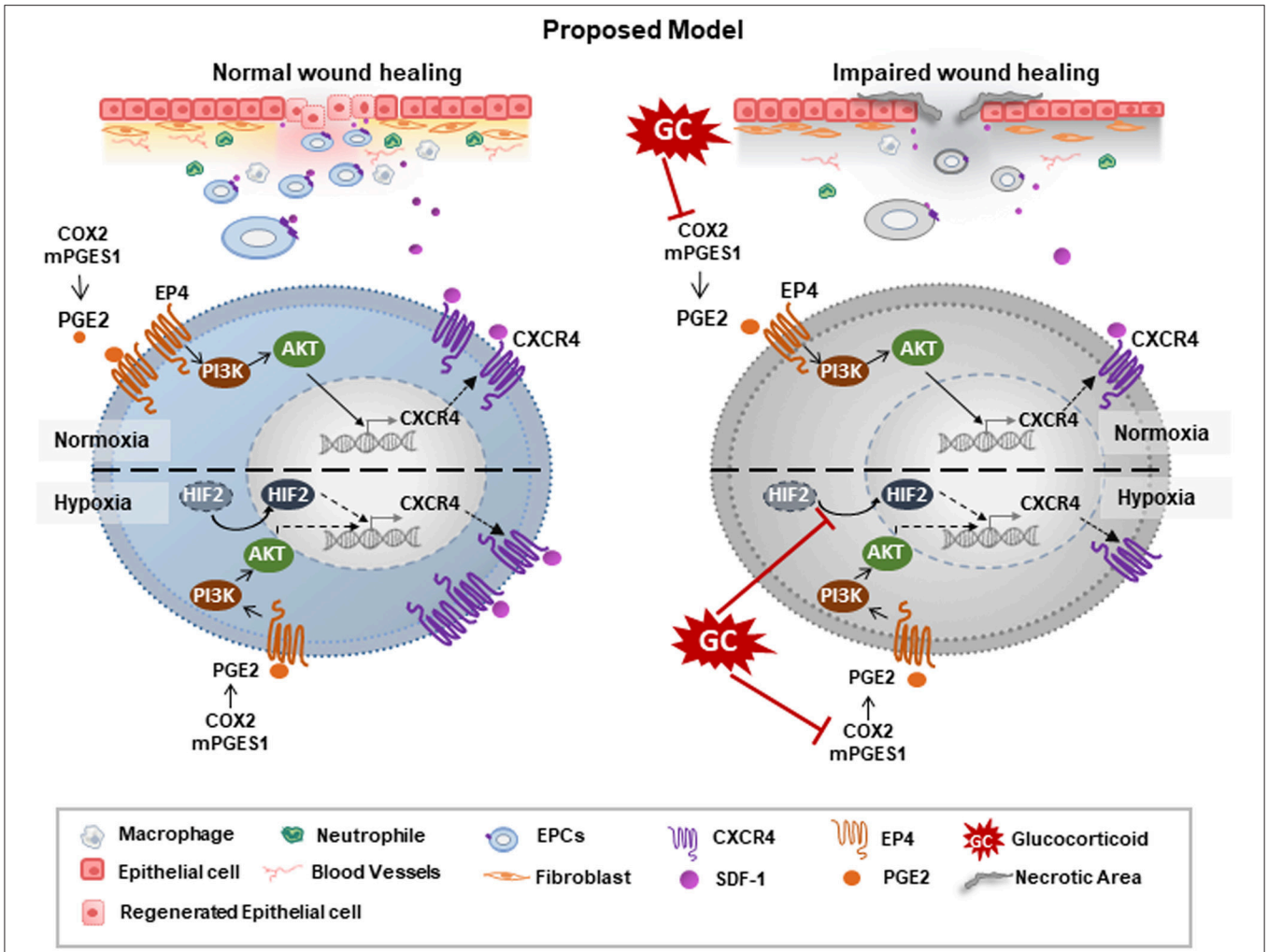

FIGURE 7 | Proposed model: GCs impaired the wound healing functions of EPCs via the impairment of CXCR4 regulated pathways. GCs downregulated the expression of CXCR4 under both normoxic and hypoxic conditions, thereby reducing the migration and wound healing ability of EPCs. Under normoxic condition, GCs impaired the expression of COX2/mPEGS1, thereby suppressing the PGE2/EP4/AKT pathway, which downregulated the CXCR4 expression. Under normoxic conditions, in addition to the PEG2 pathway, GCs independently impaired the HIF2α pathway, consequently decreasing the expression of CXCR4.

function of EPCs for the preservation of the left ventricle in acute myocardial ischemia mice (59). In addition, hypoxic treatment upregulated the expression of CXCR4 in EPCs, thereby enhancing the migration of the cells (60). We therefore expected that hypoxic treatment might reverse the negative effects of GCs on the impaired expression of CXCR4. However, our data showed that GC reduced the expression of CXCR4 in EPCs under not only normoxic but also hypoxic conditions, although this impairment was able to be rescued by PGE2. We previously showed that the CXCR4 expression is directly regulated by HIF2α under hypoxic conditions in Alde-low EPCs (19). In our current study, we found that the expression of HIF2α was also downregulated by GC treatment under hypoxic conditions, highlighting another CXCR4-regulated pathway impaired by GC. Previous report described the role of the COX2/PGE2 pathway in the upregulation of HIF2α in carcinoma cells (38); however, in our present study, PGE2 exerted no significant effects on the expression of HIF2α in EPCs, suggesting the cell-specific regulation of HIF2α would exist. In addition, our data indicated the different mechanisms underlying the regulation of CXCR4 under normoxic and hypoxic conditions. Under hypoxic conditions, PGE2 and HIF2α operate as independent pathways that are impaired by GC, consequently suppressing the expression of CXCR4 in EPCs.

Under trauma conditions, peripheral tissues—through myofibroblasts, epithelial cells, and keratinocytes—show an increased production of EPC-mobilizing factors, like VEGF, G-CSF, bFGF, PDGF, and most importantly SDF1, as a potent chemoattractant of EPCs (61). MSCs are considered to contribute to the vascular niche development by providing growth factors (62). SDF-1 production by resident MSCs might therefore encourage the homing ability of EPCs to ischemic sites. A previous report found that the level of SDF-1 protein in the serum of GC-treated patients was significantly decreased

compared with untreated patients (63). Because the biologic effects of chemokines are mediated by their corresponding receptors, the interplay between SDF-1 and CXCR4 may provide another way to regulate the distribution of circulating EPCs (64). Similar to our previous finding that GCs reduced SDF-1 production in MSCs (5), the present study showed that GCs also reduced the CXCR4 expression in EPCs. These two discoveries thoroughly show why GC-treated patients have a reduced number of circulating EPCs and a consequently impaired angiogenic ability that leads to dreadful outcomes, like avascular necrotic femoral head (ANFH).

Synthetic GCs have been developed to help treat many different conditions, such as autoimmune disorders, allergies and asthma, cancer, and surgery (24). Despite the multi-functions, GCs causes various side effects including chronic wound (65). It is reported that patients who receive GC treatment for 30 days prior to wounding or operation have a 2-fold increase in wound infection, three times increase in wound dehiscence, and four times increased mortality compared to those who not get the GC treatment (66). In addition, rheumatoid arthritis patients who receive long term GC treatment and surgery have the high risk of delayed wound healing (67). Therefore, it can be implied that the patients with GC-induced chronic wound have become the target of EPC therapy which helps to accelerate wound healing. Our study indicated the negative influences of GC on the wound healing ability of EPCs by the impairment of PGE2/CXCR4, which suggested a strategy to improve the function of EPCs. As autologous cell sources are preferred, the intervention of PGE2 or CXCR4 to improve wound healing ability of GC-treated patients-derived EPCs might be useful before the application to clinical settings.

Previous studies showed that glucocorticoid treatment impairs the corneal neovascularization in mice and rabbit which implied the possibility of abnormal vascularization process, including the dysfunction of EPCs and ECs (68, 69). In addition, EPCs from mice with aldosterone treatment showed the impaired differentiation ability to ECs and migration ability. These studies suggested the *in vivo* negative effects of GCs on EPCs functions (70). However, up to now, the influences of GCs on wound healing ability of EPCs have not yet reported. Therefore, our present study focused on the effects of *in vitro* treated GC on EPCs to provide an idea of how GCs influence EPCs wound healing functions. Further studies related to *in vivo* effects of GCs on EPCs using GC-induced mouse model and GC-treated patients derived EPCs are required to clarify the regulatory ability of GCs on EPCs.

In summary, our study demonstrated that GCs suppress the migration ability and wound healing function of EPCs by the downregulation of CXCR4 under both normoxic and hypoxic conditions. Under normoxic conditions, the impairment of prostaglandin synthases COX2 and mPEGS1 and the prostaglandin receptor EP4 are involved in the detrimental effects on GCs on EPCs. Treatment with PGE2 upregulated the expression of EP4 and consequently activated the PI3K/AKT pathway, which might be involved in rescuing the GC-impaired CXCR4 expression in EPCs. Under hypoxic conditions, in addition to impairment of the PGE2 pathway, GCs exerted similarly detrimental effects on the HIF2α pathway that independently downregulated the expression of CXCR4 in EPCs. Further studies should be performed to carefully assess wound healing functions of EPCs derived from patients who have been receiving long-term treatment with GCs.

AUTHOR CONTRIBUTIONS

EC, TK, and VK contributed to the study concept, design and access to all the data, data analysis and interpretation, and writing of the manuscript. EC, VK, and OO contributed to the writing and editing of the manuscript. KM, KT, and HH provided samples, technical and laboratory support for the research. TY contributed to the study concept, design, and technical support. OO contributed to the study concept and design, editing of the manuscript and final approval.

ACKNOWLEDGMENTS

We thank the Japanese Ministry of Education, Culture, Sports, Science & Technology (MEXT) for their support.

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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.

frontiers in Medicine

REVIEW
published: 16 October 2018
doi: 10.3389/fmed.2018.00273

The Vasoreparative Potential of Endothelial Colony Forming Cells: A Journey Through Pre-clinical Studies

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For over a decade various cell populations have been investigated for their vasoreparative potential. Cells with the capacity to promote blood vessel regeneration are commonly known as endothelial progenitor cells (EPCs); although such a definition is currently considered too simple for the complexity of cell populations involved in the reparative angiogenic process. A subset of EPCs called endothelial colony forming cells (ECFCs) have emerged as a suitable candidate for cytotherapy, primarily due to their clonogenic progenitor characteristics, unequivocal endothelial phenotype, and inherent ability to promote vasculogenesis. ECFCs can be readily isolated from human peripheral and cord blood, expanded *ex vivo* and used to revascularize ischemic tissues. These cells have demonstrated efficacy in several *in vivo* preclinical models such as the ischemic heart, retina, brain, limb, lung and kidney. This review will summarize the current pre-clinical evidence for ECFC cytotherapy and discuss their potential for clinical application.

Keywords: endothelial progenitor cells (EPC), angiogenesis, cell therapy, endothelial colony forming cells (ECFCs), ischemia, vascular repair

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *29 June 2018*
Accepted: *05 September 2018*
Published: *16 October 2018*

Citation:
O'Neill CL, McLoughlin KJ, Chambers SEJ, Guduric-Fuchs J, Stitt AW and Medina RJ (2018) The Vasoreparative Potential of Endothelial Colony Forming Cells: A Journey Through Pre-clinical Studies. Front. Med. 5:273. doi: 10.3389/fmed.2018.00273

THE POTENTIAL OF VASCULAR REGENERATION AS A THERAPEUTIC GOAL

Vasodegeneration and the ensuing tissue ischemia remains a significant challenge for healthcare systems worldwide. Diseases such as ischemic heart disease, stroke, peripheral artery disease, and ischemic retinopathies are complex to treat because there are diverse underpinning causes of non-perfusion and each tissue exhibits a distinct response to hypoxia. Current available treatments aim to remove damaged tissue, widen obstructed blood vessels or replace blocked vasculature with bypass surgery. A potential new approach would be to regenerate the compromised vasculature using so-called "therapeutic angiogenesis". While delivery of pro-angiogenic peptides have often provided scope for achieving re-perfusion, the use of cell therapy products have gained favor since they can offer sustained delivery of a multitude of angiogenic factors and/or provide direct replacement of damaged vascular cells (1). Although several cell-types such as mesenchymal stem cells (MSCs) (2), embryonic stem cells (ESCs) (3), and induced pluripotent stem cells (iPSCs) (4) have been tested pre-clinically for their therapeutic potential, endothelial progenitor cells (EPCs) have emerged as a population of cells with promising tissue regenerative properties.

EPCs have been the subject of considerable controversy due to their ambiguous definition (5) and this term actually encompasses a range of very different cell types (6). Cells named as myeloid angiogenic cells (MACs), circulating angiogenic cells (CACs), and early EPCs, all of which are hematopoietic cells, have been shown to promote vascular repair through paracrine mechanisms.

Recently, leading experts in the field published a consensus statement on EPC nomenclature to standardize terminology (7). Endothelial progenitors are thus defined as cells with an unequivocal endothelial phenotype, self-renewal potential and a capacity for *de novo*, *in vivo* blood vessel formation. It is now widely accepted that endothelial colony forming cells (ECFCs) comply with this definition and are considered the "*bona fide* endothelial progenitor" with emerging therapeutic potential (7).

WHAT ARE ECFCS?

Endothelial colony forming cells (ECFCs) are sometimes referred to in the literature as late Endothelial Progenitor cells (due to their later appearance in culture), blood outgrowth endothelial cells, or outgrowth endothelial cells. ECFCs are isolated *in vitro* from the cultured mononuclear fraction of peripheral blood or umbilical cord blood, grown under endothelial conditions. They appear in culture as cobblestone shaped colonies within weeks of mononuclear cell plating and have significant proliferative potential (**Figure 1**) (8). It has been demonstrated that ECFCs can also be derived from human induced pluripotent stem cells by sorting for markers Neuropilin-1 and CD31 (9). In addition to cord blood, ECFCs have also been successfully isolated from fat tissue (10), placenta (11), and lungs (12); these findings suggest that ECFCs originate from tissue resident vascular progenitors. Recent reports pinpoint specific endothelial subsets within the vasculature and these may constitute "vascular stem cells" with homeostatic reparative roles. These vascular stem cells are identified by the expression of CD201, the protein C receptor (PROCR) EPCR, a type 1 transmembrane receptor which is known to be highly expressed on vascular endothelial stem cells (VESCs). PROCR+ selection facilitates their isolation and enriches for highly clonogenic cells with bipotent differentiation capacity into endothelial cells and pericytes (13). CD157, also known as bone marrow stromal antigen 1 has also been identified as a marker of tissue resident VESCs, it is expressed in endothelial cells of large vessels and CD157+ cells possess significant vascular regenerative potential (14).

THE HALLMARKS OF ECFCS

ECFCs typically exhibit high clonogenic capacity. Indeed, these cells can yield a hierarchy of different sized colonies with umbilical cord blood giving rise to the highest frequency of largest colonies that have high proliferative potential (HPP) when compared to adult peripheral blood (15). ECFCs are characterized by the expression of endothelial markers CD31, CD146, VEGFR2, vWF, and VE-cadherin. ECFCs also express CD34, although the frequency of this marker is variable and can diminish as the cells are expanded *in vitro*. Importantly, ECFCs are negative for hematopoietic markers CD14 and CD45. An essential property of ECFCs is their ability to form either a *de novo* vascular network *in vitro* while *in vivo* these cells integrate seamlessly with the host vasculature (**Figure 1**).

PRE-CLINICAL APPLICATION OF ECFCS

The Ischemic Retina

Ischemic retinopathies such as retinal vein occlusion, diabetic retinopathy, and retinopathy of prematurity are common causes of visual impairment and are characterized by vasodegeneration (16). Pre-clinical evidence shows that ECFC cell therapy may be a potential treatment strategy for such ischemic retinopathies (17). The retina differs from other organs, as it has a certain degree of immune privilege and so provides a unique environment to examine the effects of human ECFCs. When these cells were injected into murine models of retinal ischemia, they promoted vascular repair, decreased the avascular area, enhanced the normovascular area, and importantly, decreased pathological neovascular tuft formation. Furthermore, human ECFCs could be found directly integrating and forming new vessels within the host murine vasculature (8). The same effects were seen when ECFCs derived from induced pluripotent stem cells were used (9). In addition, beneficial effects of ECFCs may be enhanced using agents that alter growth factor signaling pathways. For example, AAV2.COMP-Ang1 was shown to enhance the therapeutic benefit of intravitreally delivered ECFCs by promoting their integration into the murine vasculature (18). Although there is a lot more work needed before translation of ECFCs into therapy for the ischemic retina, we have recently evaluated the effect of ECFCs in the mouse oxygen-induced retinopathy model, by examining dose, delivery route, and immunogenicity. Human ECFCs delivered to the murine ischemic retina demonstrated a vasoreparative effect both by intravitreal and intracarotid delivery. Importantly, cells conferred therapeutic benefit by promoting vascular repair. In addition, if ECFCs were delivered into healthy adult mice, they were completely cleared from the retina within 3 days (17). It has also been reported that ECFCs promote vascular repair in the ischemic retina through release of paracrine factors. A subset of ECFCs was found to be effective in rescuing retinal degeneration, this subset was found to express high levels of CD44, the hyaluronic acid receptor (19). Taken together, these data provide convincing scientific evidence to support ECFCs as a potential cell therapy for ischemic retinopathies.

The Ischemic Brain

Ischemic stroke is a common worldwide cause of mortality. Fewer than 10% of patients experiencing ischemic stroke are suitable for thrombolysis treatment which can effectively restore some cerebral blood flow (20), therefore there is a need for therapies that induce vascular repair and more effectively restore blood flow for greater number of patients. ECFCs are emerging as a promising new treatment option for ischemic stroke and their therapeutic potential in the rodent brain has been demonstrated. For example, GFP-labeled ECFCs were tracked for cell engraftment in a photothrombotic ischemic stroke mouse model. Cells were delivered via the left cardiac ventricle 24 h after stroke. Bioluminescence signals were highest in the brain on day 1 and decreased steadily until day 14. GFP-positive ECFCs were found at the infarct border demonstrating a successful homing response to regions of tissue hypoxia. Importantly,

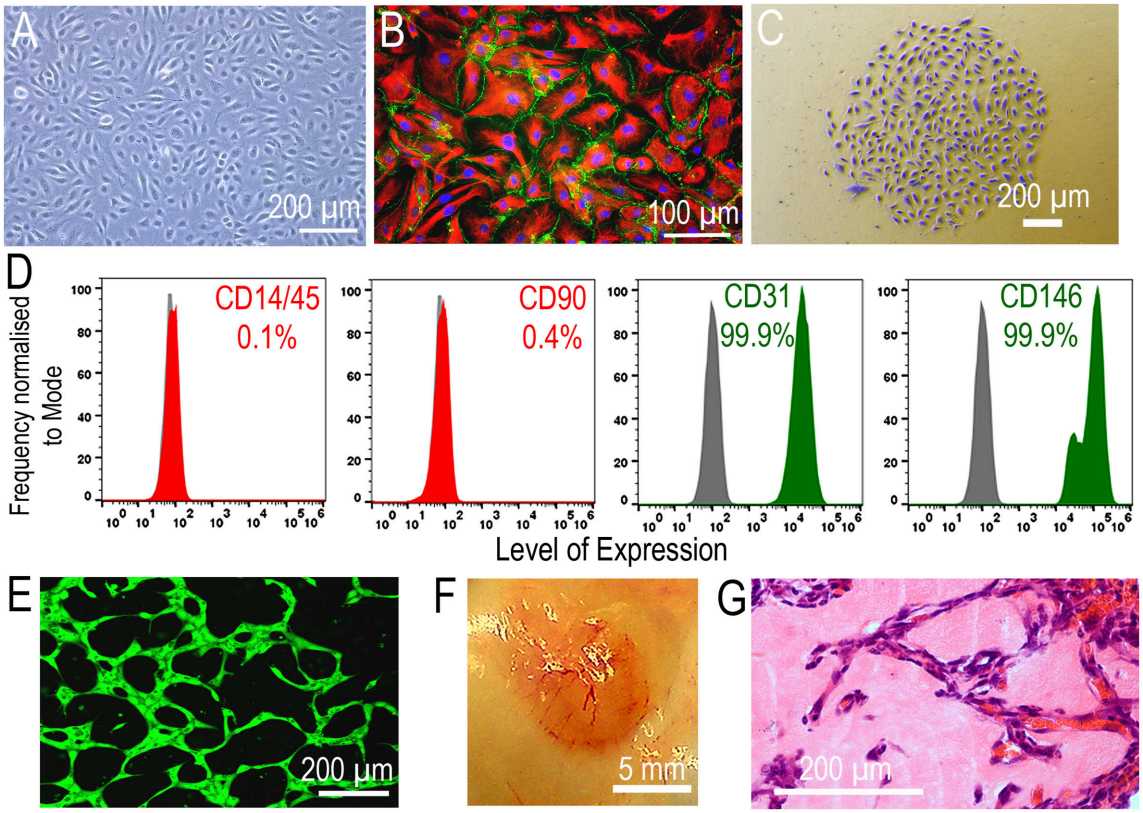

FIGURE 1 | Properties of ECFCs. **(A)** ECFC cobblestone monolayer morphology (Scale bar 200 μm). **(B)** A tight monolayer of ECFCs with adherens junctions (β catenin = green, Vimentin = red, scale bar 100 μm). **(C)** A colony derived from a single cell demonstrating clonogenic potential of ECFCs (crystal violet stained, scale bar 200 μm). **(D)** ECFCs express endothelial markers CD31 & CD146 and are negative for hematopoietic markers CD14/45 and stromal marker CD90. **(E)** ECFCs have tubulogenic capacity *in vitro* (scale bar 200 μm) **(F,G)**. ECFCs form perfused vessels *in vivo* in the Matrigel plug assay (Scale bar, 5 mm and 200 μm respectively).

ECFC therapy led to improved angiogenesis, neurogenesis, decreased neuronal apoptosis, and ultimately led to restoration of brain function (21). In addition, in a rat model of transient focal cerebral ischemia and middle cerebral artery occlusion (MCAO), ECFCs primed with erythropoietin (EPO) were shown to have enhanced efficacy for reversing stroke injury (22, 23). In another study using the same model, labeled ECFCs administered intravenously 24 h after MCAO were seen to specifically home to the ischemic hemisphere and settled in the injured area. ECFCs transplantation stimulated an increase in capillary density at the site of injury. Although in this study, ECFCs were not detected integrating within the vasculature, they stimulated an increase in proangiogenic growth factor expression at the ischemic site, which was also associated with a reduction in the number of apoptotic cells (24).

ECFCs have also been examined for their potential to repair vascular damage in pre-clinical models of traumatic brain injury (TBI) in rodents. TBI, created by lateral fluid percussion injury was used to assess the effects of cord blood-derived ECFCs. Cells were intravenously infused 1 h after injury. ECFCs successfully homed to the injured site, were detected in the injured brain after 24 h and were effective in promoting neovascularization and improving neurological function (25). A further follow-up study demonstrated that infusion of ECFCs can repair blood brain barrier tight junction functionality (26). In addition, in a rat model of cerebral aneurysm, ECFC transfusion was shown to inhibit inflammatory signaling, protect smooth muscle cells from apoptosis and promote vascular regeneration (27). Taken together, these studies highlight the potential therapeutic effects of ECFCs for vascular repair in the brain.

Peripheral Artery Disease

Peripheral artery disease (PAD) can lead to ischemic injury and amputation. Prognosis is poor and current treatments for PAD patients are limited (50); therefore there is a pressing need for new strategies to enhance angiogenesis and collateral arterial growth. The potential for ECFC treatment in PAD has been demonstrated in several preclinical studies using the murine hindlimb ischemia model. Firstly, ECFCs were shown to increase perfusion by rapidly relocating to the ischemic hind limb within 6 h after injection. In addition, there was enhanced benefit when a combination of ECFCs and mesenchymal stem cells (MSCs) were used. Further analysis showed that the reparative effects of ECFC therapy were due to direct vascular incorporation (28). Although it has also been suggested that ECFCs can function as paracrine mediators, modulating MSC engraftment

TABLE 1 | Summary of Pre-clinical studies using ECFCs in various disease models.

	Retina	Brain	Limb	Heart	Skin	Lung	Kidney
Pre-clinical model	Mouse oxygen induced retinopathy	Mouse ischemic stroke Rat transient focal cerebral ischemia Rat middle cerebral artery occlusion	Mouse hindlimb ischemia	Pig myocardial infarction Mouse myocardial infarction	Mouse tissue injury Mouse full thickness dermal wound	Rat oxygen induced bronchopulmonary dysplasia	Mouse bilateral kidney I/R injury rat I/R injury
ECFC source	Human cord blood iPS-derived	Human cord blood	Human cord blood iPS-derived Human white adipose tissue	Pig peripheral blood Mouse bone marrow Human cord blood	Human cord blood	Human cord blood	Human cord blood
Delivery	intra-vitreal Intra-carotid	Intra-arterial Intra-venous	Intra-muscular Retro-orbital	Intra-arterial Intra-myocardial	Intra-jugular Embedded in scaffold & grafted onto wound site	Intra-jugular	Intra-jugular
Results	Promote vasoreperfusion Decrease avascular area & pathological neovascular tufts	Improve neurological function & blood brain barrier integrity Improve angiogenesis & neurogenesis Decrease apoptosis & inhibit inflammatory signaling	Increase perfusion & vessel density Improve limb salvage Recruit myeloid cells	Increase blood vessel density Improve left ventricular function & myocardial remodeling Decrease myocardial fibrosis, infarct size, apoptosis & oxidative stress Anti-inflammatory	Induce wound vascularization Contribute to functional vessels Reduced levels of hypoxia Enhanced matrix organization Accelerate re-epithelialization	Improve lung compliance & architecture of alveoli Preserve lung vascularity Prevents pulmonary hypertension	Attenuate I/R-induced renal injury & renal superoxide formation Prevent tubule necrosis Reduce macrophage infiltration Decrease apoptosis
Integration into host vasculature	Direct integration and formation of new blood vessels detected 72 h post-delivery	Not detected to integrate within the vasculature	Direct incorporation into vasculature detected 20 days post delivery	Engraft into host myocardium Incorporate into vessels at border zone detected 7 weeks post delivery	Integrate into host vasculature & form functional hybrid vessels detected 10 days post delivery Scaffolds embedded with ECFCs	Low engraftment in recipient lung vasculature 14 days post delivery	Barely detectable in any tissue 24 h post delivery
Paracrine Function	Promote vascular repair through release of paracrine factors	Increased expression of pro-angiogenic growth factors	Function as paracrine mediators by modulating msc engraftment	Induce secretion of angiogenic cytokines at ischemic site	Secreted factors enhance collagen matrix organization, promote migration & proliferation of keratinocytes	ECFC-CM showed similar therapeutic effects to ECFCs	Secrete exosomes as primary mediators of anti-apoptotic effect
References	(8, 9), (17–19)	(21–27)	(9), (28–35)	(36–39)	(40–44)	(45, 46)	(47–49)

via PDGF-BB/PDGFRb signaling (29). Similar beneficial effects were reported in a later study in which ECFCs combined with mesenchymal progenitor cells enhanced blood flow recovery of the ischemic limb. Interestingly, analysis showed that improved blood flow was in part due to the recruitment of host myeloid cells with, presumably, concomitant release of pro-angiogenic growth factors (30). Another study combining ECFCs and MSCs, via retro-orbital delivery, demonstrated the homing ability of the cells and increased vessel density via an endoglin-dependent mechanism (31). Modification or pre-treatment of ECFCs has been shown to enhance their effects. For example, ECFC-induced functional recovery and limb salvage were markedly improved by Fucoidan pre-treatment, which protected the cells from replicative cellular senescence (32). Overexpression of integrin B1 was also reported to augment ECFC vasoreparative potential as it improved homing of the cells to the ischemic tissue, leading to improved perfusion in the ischemic limbs (33). Similarly, adiponectin pre-treatment has been demonstrated to increase ECFCs neovascularization capacity in a hind limb model in streptozotocin-induced hyperglycemic nu/nu mice (34). ARA290, an agonist of Erythropoietin has also been shown to enhance ECFC function by increasing the homing of these cells to the ischemic limb (35). In addition, ECFCs derived from human induced pluripotent stem cells (iPSCs) have also been shown to contribute to hind limb vascular repair. In a model of hindlimb femoral vessel removal in nude mice, hiPSC ECFCs were shown to improve blood perfusion and limb salvage as well as cord blood-derived ECFCs (9). This result is important as it means that it may be possible to generate patient specific hiPSC-derived ECFCs for autologous treatment of vascular disease.

The Ischemic Myocardium

Ischemic heart disease is a common cause of mortality worldwide. Cell therapy to regenerate the ischemic heart is a rapidly emerging concept. Most clinical trials have used bone marrow mononucleated cells (MNCs). Although bone marrow MNCs have demonstrated some therapeutic efficacy for the ischemic myocardium (51), they remain a very heterogeneous population of cells and therefore it is difficult to decipher the individual contributions of cell populations responsible for the repair. In addition, bone marrow MNCs from patients with ischemic heart disease have been shown to have a reduced neovascularization capacity (52). ECFCs remain to be tested clinically in the ischemic myocardium. The first pre-clinical studies were performed using CD34+ cells with presumed endothelial precursor capacity. These cells increased vessel density within the infarct area and improved left ventricular function although whether these heterogeneous cells were acting as paracrine reservoirs or endothelial "building blocks" remains unknown (36). Further pre-clinical studies have shown that ECFC therapy is beneficial; cells participate in the vascular repair process and promote vascular recovery of the ischemic heart. In an acute myocardial infarction model in pigs, infusion of autologous ECFC-like cells improved myocardial remodeling; decreased infarct size, increased vessel density and ECFCs were seen to incorporate within vessels at the border zone (37). Myocardial injection of ECFCs expressing AKT/HO-1 into a murine myocardial infarction model demonstrated an increase in blood vessel density, a decrease in apoptotic cells at the infarct site, reduction in oxidative stress and pro inflammatory molecule TNFα as well as an improvement in ejection fraction (38). More recently, the transplantation of ECFCs pre-treated with Genistein was shown to increase cellular proliferation at the ischemic sites, enhance neovascularization, decrease myocardial fibrosis, and improve cardiac function (39).

Wound Healing

The inability to efficiently repair wounds is a common feature of patients suffering with vascular disease. Due to their angiogenic potential, ECFCs have been examined for their capacity to aid vascular repair in several wound injury models. In a murine model where tissue injury was induced by dye laser, digital intravital microscopy revealed that human umbilical cord blood-derived ECFCs delivered via infusion through the jugular vein migrate to sites of injury and promote endothelial regeneration (40). Interestingly, this study found that recruitment of ECFCs was dependent on the presence of neutrophils at the site of injury via the binding of P-selectin glycoprotein ligand-1 (PSGL-1) (40). In a murine model of full thickness dermal wound in athymic nude mice, unlike HUVECs, treatment with ECFCs was shown to induce wound vascularization by direct integration with host vasculature, demonstrated by blood filled vasculature. Furthermore, cells were detectable up until day 10, wounds that received ECFCs showed reduced levels of hypoxia, enhanced matrix organization and accelerated epithelial coverage. Interestingly, the border of the ECFC treated wounds contained smooth muscle cells likely mobilized by the secretion of PIGF and PDGF-BB by the ECFCs. This study also demonstrated ECFCs pro-angiogenic potential by paracrine factors: ECFCs expressed higher levels of pro-angiogenic growth factors such as VEGF, PIGF, and Ang-1 compared to HUVECs; ECFC-conditioned medium (CM) significantly improved collagen matrix organization from human dermal fibroblast sheets; and ECFC-CM boosted keratinocyte migration and proliferation across the wound via secretion of KGF and HGF (41). ECFCs also have the potential to be embedded within scaffolds or skin substitutes (42) where they can vascularize the scaffolds alone or in combination with accessory cells, and following subcutaneous transplantation, can anastomose within the host vasculature enabling perfusion (43). Cord blood-derived ECFCs seeded in a RGD-g-PLLA biosynthetic scaffold (designed to promote survival and retention of the cells at the wound site) enhanced dermal wound neovascularization and labeled ECFCs were seen to be retained in the wound up to a week after transplantation (44).

The Lungs

The lungs are highly vascularized organs. Recent research indicates that lung microvascular endothelium is a rich source of resident ECFCs, which contribute to normal vascular homeostasis (12, 14). Studies investigating the effects of ECFCs in the lung have shown beneficial effects. For example, in a rat injury model of oxygen-induced bronchopulmonary dysplasia, a lung disease of prematurity, human cord blood-derived ECFCs were administered through the jugular vein. ECFCs significantly improved lung compliance and the architecture of the alveoli.

It also showed that ECFC therapy improved lung angiogenesis and prevented pulmonary hypertension in hyperoxia-exposed newborn mice. Treatment with ECFC-CM showed similar therapeutic effects in such models of bronchopulmonary dysplasia (45, 46). Such studies highlight that ECFCs may provide vasoreparative effects through paracrine mechanisms, in addition to direct vascular engraftment.

The Kidney

Ischemia reperfusion injury is the main cause of acute kidney injury (AKI) which results in endothelial cell loss and apoptosis, leading to reduction in peritubular capillary blood flow (53). ECFCs may facilitate the development of new treatments for AKI. The administration of ECFCs in a mouse model of ischemic AKI was shown to reduce tubular injury, renal apoptosis, and infiltration of macrophages and attenuate increases in plasma creatinine levels. Because there was little evidence of ECFCs remaining within the murine kidneys, their protective effects were primarily attributed to the release of exosomes, as injecting ECFC-derived exosomes alone protected against the multiple parameters of kidney injury (47). A follow-up study showed that specifically the microRNA miR-486-5p, present at high amounts within the ECFC exosomes, accounted for the therapeutic effects of ECFCs (48). In agreement with this, other studies have also shown that ECFCs secrete soluble factors to preserve microvascular function. Conditioned media from human cord blood-derived ECFCs offered protection in a rat model of ischemia reperfusion injury. Interestingly, ECFC-CM significantly reduced ICAM-1 expression and decreased the number of differentiated lymphocytes recruited to the kidney after renal ischemia (49). These studies suggest that ECFCs and their CM may provide a therapeutic option for the treatment of AKI.

FUTURE PERSPECTIVES

The studies discussed in this review (**Table 1**), support the case for ECFCs as a potential novel endothelial cell therapy that promotes vascular repair in many different vascular beds albeit tissue specific endothelial heterogeneity. It is possible that ECFCs, as progenitors, have required plasticity to adopt organ specific endothelial characteristics. A sole mechanism of action (MOA) for ECFC beneficial effects remains unclear. In some of these studies, ECFCs facilitate vascular repair by directly integrating within the host vasculature, while in others vascular engraftment of these cells is low or completely absent and their therapeutic benefit can be explained by the paracrine release of proangiogenic growth factors. More research is needed to fully elucidate mechanisms of action, but these are likely to depend on the experimental model used. Demonstration of long-term engraftment in pre-clinical models is challenging considering ECFCs are human cells delivered into immunocompetent mice. Other factors that need to be considered for each target disease are cell dosage, delivery route, and timing of ECFC administration (1).

To induce maximal therapeutic efficacy, it may be worthwhile considering co-transplantation of ECFCs with accessory cells such as MSCs (28, 30), smooth muscle cells (SMCs) (54), adipose tissue-derived stem cells (43) or myeloid angiogenic cells (55). Several studies have shown this to be an effective approach as it may promote long-term neovascularization by directly supporting and stabilizing ECFC-derived neovessels and also by providing proangiogenic factors to aid the regenerative process.

The majority of studies described in this review have been performed using a xeno-allogenic approach, testing human cells in mouse and rat models. However, in order to translate this work toward the clinical setting; it might be necessary to collect data from larger animal models, which may also allow an autologous approach. ECFCs have successfully been isolated from rabbits (56), dogs (57), pigs (58), sheep (59), horses (60), and monkeys (61) and there is some evidence for autologous ECFC therapy to be efficacious in pigs (37). Despite these results, when considering the use of ECFCs as a potential cytotherapy for human disease, it is likely that initial ECFC therapy may be allogeneic due to the fact that ECFCs cannot always be obtained efficiently from adult peripheral blood and those isolated from patients may be dysfunctional and therefore not optimal for cell transplantation. Another consideration is that ECFCs in human blood are very rare, and numbers are reduced with disease (62), thus it may not be possible to isolate them from every patient. Furthermore, isolation, characterization and *ex-vivo* expansion of autologous ECFCs requires 4–6 weeks which is a limiting factor for diseases with a narrow therapeutic window. Therefore, we consider ECFC allogeneic therapy as the most feasible and practical strategy in which ECFCs will be HLA typed to match patients and will be stored in cell banks as an "off-the-shelf" cell therapy product.

There is quite a high failure rate in successful translation of regenerative medicine therapies to clinical use. This is mainly due to potential cell products not passing the regulatory requirements, clinical efficacy standards, and the high manufacturing cost (63). Successful translation in the EPC field has been mainly impaired due to the use of heterogeneous populations of non-endothelial cells such as BM-MNCs, which have led to conflicting results and discourage pursue of further clinical trials (64). High cell population heterogeneity and low purity characterized the first generation of cell therapies, but next generation cell therapies require a highly pure and well-defined cell therapy product for consistency. In the EPC field, a recently published consensus on EPC nomenclature recommended accurate cell definitions and meaningful nomenclature (7).

This review shows pre-clinical evidence suggesting that ECFCs have therapeutic value for ischemic diseases. Advances in cell therapy manufacture technologies in combination with a first-in-man clinical trial are needed to facilitate the translational pathway for ECFCs into patients. Importantly, both cells GMP manufacture and clinical trial design have to align with regulatory frameworks for advanced therapy medicinal products.

Finally, before ECFCs are used in the clinical setting, protocols for their isolation, culture, and expansion must strictly adhere to GMP standard operating procedures. GMP guidelines may vary from country to country (65). In addition, xeno-free culture conditions are preferable. Replacing the use of animal products

required for ECFC culture, such as fetal bovine serum and rat Collagen I are being investigated by many groups. Strategies to overcome these issues include the use of human platelet lysate (66, 67) and the manufacture of GMP synthetic basement membrane substrates (68). In addition, the use of automated cell culture facilities will enable scalable and standardized methods to create a reliable and reproducible cell therapy product.

In conclusion, harnessing the reparative angiogenic capacity of ECFCs may provide an exciting new regenerative therapy for vascular disease, however there are still challenges to overcome, and more research is warranted before these can be used in the clinic.

AUTHOR CONTRIBUTIONS

CO and RM: conception and design; CO, RM, and AS: manuscript writing; KM, SC, and JG-F: final approval of manuscript; CO, RM, KM, and SC: figure and table design.

FUNDING

This work was supported by the Juvenile Diabetes Research Foundation, the Medical Research Council, the Leverhulme Trust, the National Eye Research Centre, Northern Ireland Health and Social Care R&D Division, The Sir Jules Thorn Trust and Fight for Sight.

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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.

frontiers in Medicine

REVIEW
published: 23 October 2018
doi: 10.3389/fmed.2018.00295

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

OPEN ACCESS

Edited by:
Reinhold J. Medina, Queen's University Belfast, United Kingdom

Reviewed by:
Hisamichi Naito, Osaka University, Japan
Roger Preston, Royal College of Surgeons in Ireland, Ireland

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *17 July 2018*
Accepted: *28 September 2018*
Published: *23 October 2018*

Citation:
Paschalaki KE and Randi AM (2018) Recent Advances in Endothelial Colony Forming Cells Toward Their Use in Clinical Translation. Front. Med. 5:295. doi: 10.3389/fmed.2018.00295

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 heterogenous 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*$^{+}$ hematopoietic progenitors (29). Initial studies in patients with Philadelphia-negative myeloproliferative neoplasms identified the Janus kinase 2 V617F (JAK2^{V617F}) mutation only in hematopoietic cells and not in ECFC (30). However, subsequent studies revealed a subgroup of patients with the JAK2^{V617F} 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^{V617F} 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^{V617F}-mutated ECFC showed increased adhesion to normal mononuclear cells (31). Further studies are required to determine whether JAK2^{V617F} 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

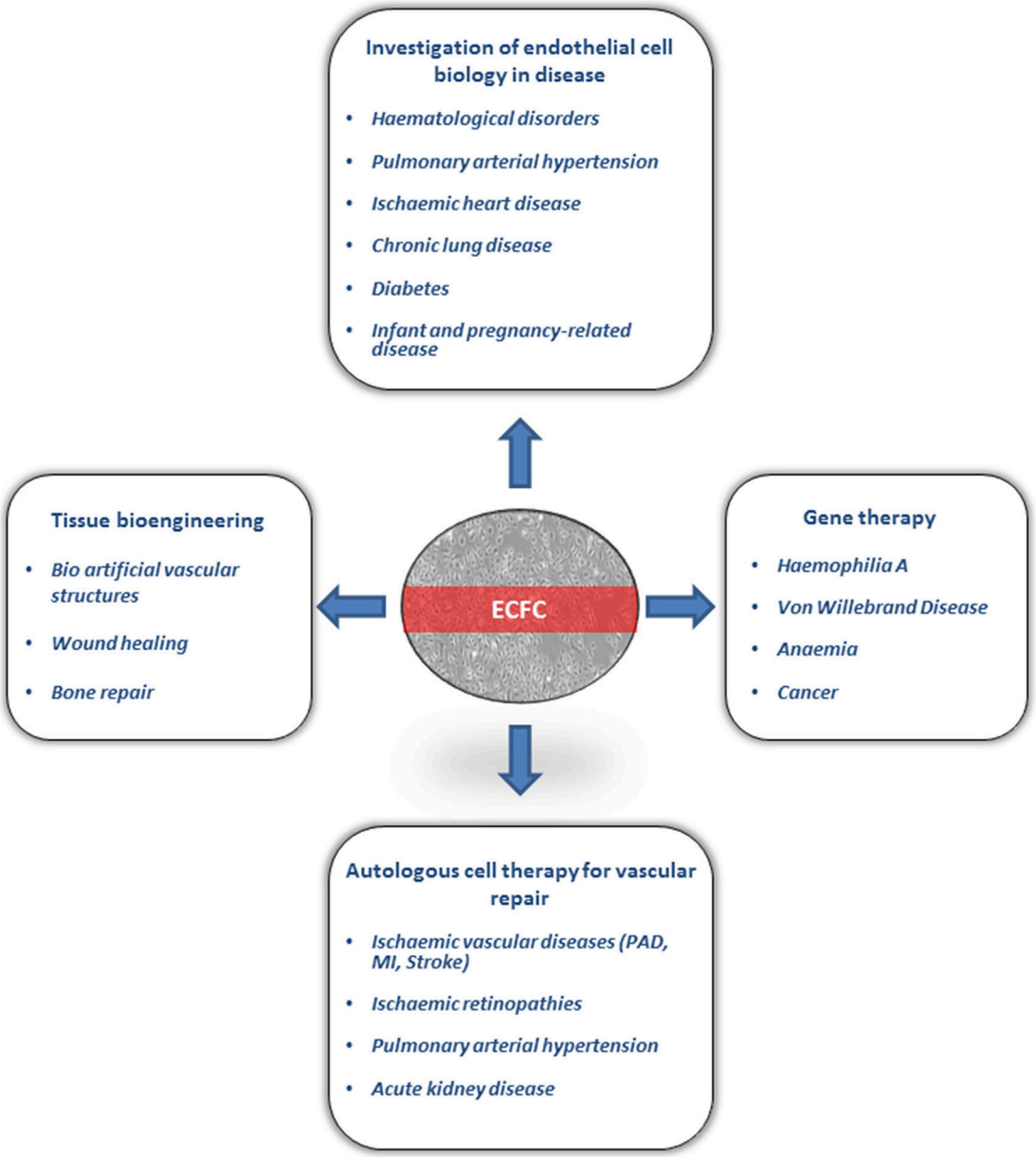

FIGURE 1 | Clinical applicaions of Endothelial Colony Forming Cells (ECFC). ECFC have been used for investigation of endothelial cell biology in several diseases. ECFC are currently under investigation in preclinical models for autologous cell therapy, gene therapy and tissue bioengineering. PAD, peripheral arterial disease; MI, myocardial infraction.

higher risk group showed increased RelA activation in response to stimulation with interleukin-1β/tumor necrosis factor α (TNF-α) (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-β 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-β 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-κB, followed by stabilization of hypoxia-inducible factor-1α 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 α show an abnormal increased release of interferon γ 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 pro-angiogenic 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-α 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.

FUNDING

KP is financially supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. AMR's research is partly supported by the BHF Centre for Vascular Regeneration (Rm/30158).

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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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BRIEF RESEARCH REPORT
published: 27 November 2018
doi: 10.3389/fmed.2018.00331

Osteoprotegerin Induces CD34$^+$ Differentiation in Endothelial Progenitor Cells

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Endothelial progenitor cells (EPCs) are the main hypothetical cells that could give rise to vessels and in particular one subtype isolated from peripheral or cord bloods: endothelial colony forming cells (ECFCs). These ECFCs are clonogenic precursors committed to endothelial lineage and have robust vasculogenic properties. However, their low number and poor expansion properties when isolated from human adult bloods, currently limit their use as an autologous cell therapy product. We previously reported that osteoprotegerin (OPG), a well-characterized regulator of bone metabolism, contributes to ischemic tissue revascularization, tumor growth *in vivo,* and potentiates ECFCs proangiogenic properties through the secretion of SDF-1. The current study investigated the role of OPG in ECFCs differentiation and expansion from cord blood CD34$^+$ cells. OPG increased the number of ECFCs after endothelial differentiation of CD34$^+$ cells, enhancing the time of EPCs colonies initial appearance and the growth kinetic of endothelial cell progeny. OPG-exposed ECFCs expressed higher levels of CD34$^+$ compared to control ECFCs. In conclusion, our findings provide novel insights into OPG in regulation of CD34$^+$ progenitor cells. These results give new opportunities for *ex vivo* expansion of human ECFCs using OPG as a cell culture component for future ECFC product manufacture according to GMP.

Keywords: osteoprotegerin, endothelial progenitor cells, endothelial-colony forming cells, CD34$^+$ cells, proliferation

OPEN ACCESS

Edited by:
Ondrej Viklicky, Institute for Clinical and Experimental Medicine (IKEM), Czechia

Reviewed by:
Marian Klinger, Opole University, Poland
Tomomi Toubai, Yamagata University, Japan

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *13 July 2018*
Accepted: *12 November 2018*
Published: *27 November 2018*

Citation:
Boisson-Vidal C, Benslimane-Ahmim Z, Lokajczyk A, Heymann D and Smadja DM (2018) Osteoprotegerin Induces CD34$^+$ Differentiation in Endothelial Progenitor Cells. Front. Med. 5:331. doi: 10.3389/fmed.2018.00331

INTRODUCTION

Endothelial progenitor cells (EPCs) are the main hypothetical cells that could give rise to vessels and in particular a specific subgroup of circulating EPCs isolated from cord and adult peripheral blood: Endothelial colony forming cells (ECFCs) (1). These highly proliferative non-hematopoietic phenotype ECFCs are precursors committed to endothelial lineage. They are clonogenic and have robust vasculogenic properties. The absence of any specific markers in contrast to mature cells limited the ability to identify them in early 2000. We now have convincing data showing a clear difference between EPCs with mature cells (2–5). Their low number and poor expansion properties when isolated from human blood or bone marrow currently limit their use as an autologous cell therapy product (6). Strategies to improve ECFCs therapeutic potential are needed and one of the challenges is to understand the main effectors allowing an endothelial differentiation from immature cells and/or circulating progenitors.

Osteoprotegerin (OPG), a soluble member of the tumor-necrosis-factor family, is a well-characterized regulator of bone metabolism, which acts by blocking osteoclast maturation. It plays key roles in regulating numerous other physiological and pathological processes especially in vascular system (7). OPG is constitutively secreted by endothelial cells and their progenitors, as well as by smooth muscle cells, megakaryocytes, and platelets (8, 9). It promotes growth and tubule formation of mature endothelial cells (10). We recently described an increase in ECFCs vasculogenic properties *in vitro* and *in vivo* (11, 12). These beneficial effects have been attributed at least in part to SDF-1/CXCR-4 and heparan proteoglycan pathways (13). OPG has also been described to induce hematopoietic stem cells expansion (14) and to mediate cardioprotection by protecting the cells from reactive oxygen species-induced cell death (15). Because commitment of stem/progenitor cells into ECFCs is a main limitation to their clinical use, we hypothesized that OPG might regulate biological ECFCs maturation and investigated the role for OPG in ECFCs differentiation from cord blood $CD34^+$ cells.

MATERIALS AND METHODS

Buffy Coat Cell Preparation and Culture of Umbilical Cord Blood Endothelial Cells

The study was approved both by the relevant ethics committee (Hôpital Saint Louis, Paris, France) and the French Ministry of Higher Education, Training and Scientific Research (AC-2008-376).

Mononuclear cells were isolated from human cord blood by density-gradient centrifugation on Pancoll and $CD34^+$ mononuclear cells by magnetic-bead separation according to the manufacturer's instructions (Miltenyi Biotec, France). ECFCs were obtained and cultured with or without OPG (25 ng/mL) as previously described (5, 11). Colonies were identified by their characteristic morphology then by immunostaining for von Willebrand factor and double-positivity for DiI-AcLDL uptake and BS-1 lectin binding.

Cell Proliferation Potential

ECFCs appeared as small compact cell clusters about 10 to 20 days after plating. Cells from each colony were replated on 12-well dishes then on 6-well dishes and finally in a T25 flask. Subsequently, confluent cells were replated in T25 flasks every 3–4 days until day 50. The trypan blue exclusion test was used to determine cell counts at each passage. These counts served to plot a growth kinetic curve and calculate the population doubling time (PDT) and cumulative population-doubling level (CPDL) as previously described (16).

Immunophenotyping of Endothelial Cells

On culture days 25 and 40, ECFCs cultured with and without OPG were detached using accutase then incubated with primary or isotype control antibody and analyzed by fluorescence-activated cell sorting using a FACSCalibur cytometer (Becton Dickinson, France). We used directly conjugated primary murine monoclonal antibodies specific for the following surface antigens: CD34, CD144, CD105 (Beckman Coulter, Cylex, France); CD133 (Miltenyi Biotec, France); CD31, CD73, CD61/51, VEGF-R2 (BD Pharmingen, France); CD146 (Santa Cruz Biotechnology, France); CD54, CD106 (AbCys, France), CD45, and CD115 (Immunotech, France). Corresponding isotype stains were used as negative controls. Data plotting was performed using CellQuest software (BD Biosciences, France).

Statistical Analysis

Differences between groups were assessed using Student's paired *t*-test, with the statistical software package GraphPad Prism, version 5 (GraphPad Software, USA). $P \leq 0.05$ were considered statistically significant.

RESULTS

$CD34^+$ Cells Cultured With OPG Exhibits Increased Clonogenic Capacity and Proliferative Potential

We firstly examined the possible involvement of OPG in cord blood $CD34^+$ commitment to ECFCs. We harvested $CD34^+$ cells from umbilical cord bloods and observed ECFCs formation in the presence or absence of 25 ng/ml of OPG added to the culture medium EGM2 from the first day of culture. We obtained colonies in both groups that displayed the same cell phenotype with a cobblestone morphology (**Figure 1A**). As shown in **Figure 1B**, OPG increased significantly the number of colonies (2.9 ± 0.6 vs. 1.4 ± 0.5 colonies per equivalent cord blood volume for OPG and control conditions, respectively, $p = 0.0084$) and decreased the timing of colony emergence (**Figure 1C**, $p = 0.0008$). The EPCs-derived colonies emerged 4 days earlier than in control culture medium. These observations suggested that OPG stimulated ECFCs formation.

We then compared the proliferative kinetics of ECFCs cultivated in the presence or absence of OPG from the very first day of $CD34^+$ culture. As shown in **Figure 1D**, the growth curves indicate greater proliferative potential of ECFCs exposed to OPG compared to controls conditions: after a 6-day latency period, the number of cells in the OPG group showed a rapid increase to 9×10^6 on day 30 followed by a slower increase. Control ECFCs also proliferated rapidly but less actively, so that 37 days were needed to obtain 9×10^6 cells. The presence of OPG in the culture medium induces a 1.8-fold decrease in the average PDT and a 1.7-fold increase in the CPDL of ECFCs the first 15 days of culture (**Figure 1E**, $p < 0.0001$).

OPG Modulates CD34 Surface Expression by ECFCs

To investigate OPG influence on ECFCs phenotype, we used flow cytometry to monitor the expression of selected markers on days 25 and 40 in the OPG and control groups. Mature endothelial cells markers (vWF, P-selectin) were highly expressed

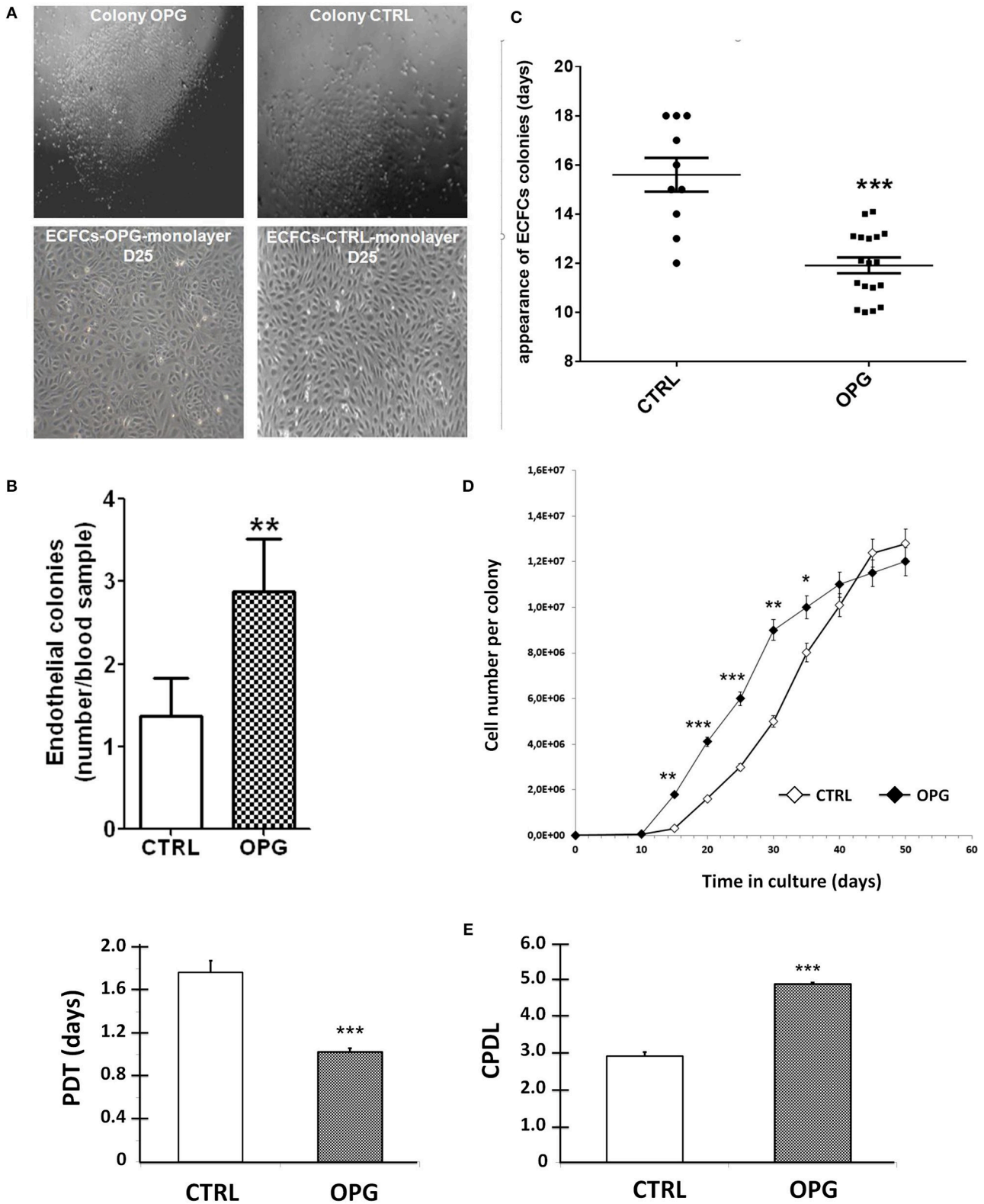

FIGURE 1 | Osteoprotegerin (OPG) increases the colony-forming capacity and proliferative potential of EPC-derived endothelial colony-forming cells (ECFCs). Mononuclear cells isolated from cord blood were cultured in EGM2 with (OPG) or without (CTRL) 25 ng/ml of OPG. **(A)** Representative photomicrographs (10 × magnification) of endothelial progenitors-derived ECFCs from the OPG-exposed and control groups after 14- and 25-day of the endothelial cell progeny derived from

(Continued)

FIGURE 1 | the CD34$^+$ EPC colonies grown to confluence. **(B)** Number of colonies per equivalent cord blood volume. The number of colonies formed was identified by phase-contrast microscopy. Results represent the mean ± SEM ECFCs of 8 independent experiments. **(C)** Time of initial ECFCs appearance after culture initiation from equivalent volumes of cord blood. Results represent the mean ± SEM number of days before initial ECFCs appearance of 10 independent experiments for CTRL conditions and 18 independent experiments for OPG conditions. **(D)** Growth kinetics of the endothelial cell progeny derived from cord EPC colonies. $n = 10$, cells were enumerated at each passage. **(E)** Population doubling time (PDT) and Cumulative Population-Doubling Level (CPDL) of EPCs-derived ECFCs during 15 days of culture. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ by Student paired *t*-test.

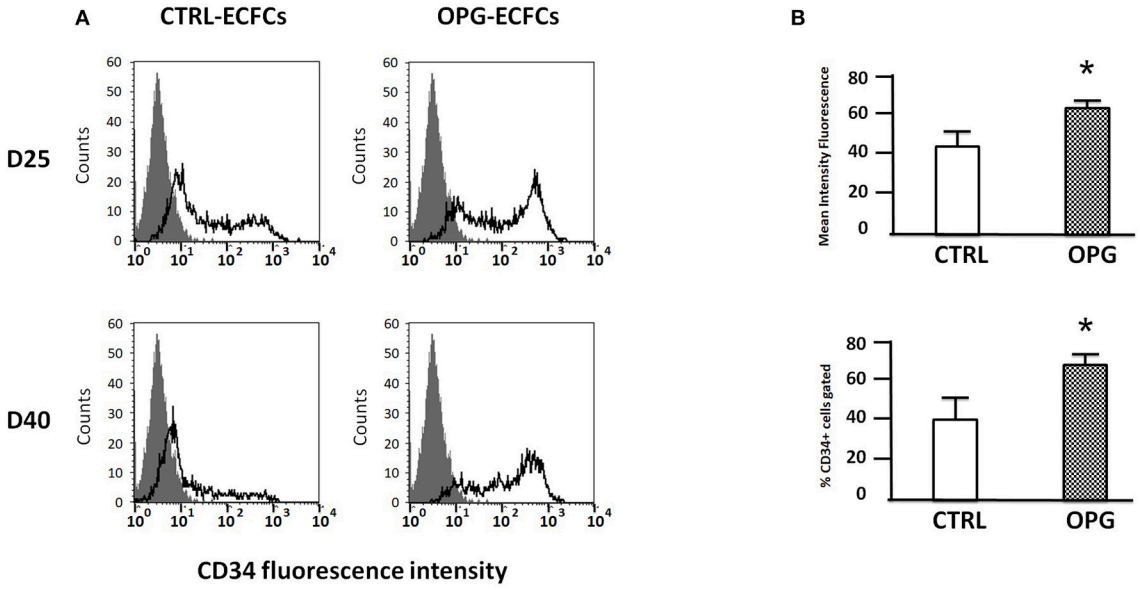

FIGURE 2 | Comparative immunophenotyping of osteoprotegerin (OPG)-exposed and control endothelial colony-forming cells (ECFCs) after 25 and 40 days of culture. **(A)** Representative examples of CD34 expression profiles 25 and 40 days following the seeding. The isotopic control is in gray and the specific antibody in black. **(B)** Mean fluorescence intensity of OPG-exposed and control ECFCs CD34$^+$ marker analyzed by flow cytometry. MFI ≥ 2 were considered positive (MFI ± SD, $n = 4$ for CTRL and $n = 5$ for OPG conditions, $^*P < 0.05$ by Student paired *t*-test).

(data not shown). Immunophenotyping revealed that ECFCs cultivated under both conditions express the endothelial cell-surface antigens CD31, CD105, CD144, CD146, and VEGR-R2 at approximatively the same level. None of the groups expresses any significant amount of hematopoietic cell surface antigen CD45, CD14, and CD115. However, we found on day 25 that OPG-exposed ECFCs expressed higher levels of CD34$^+$ compared to control ECFCs (**Figures 2A,B**, $p = 0.04$). The percentage of CD34-positive cells was increased 1.6-fold for OPG-exposed ECFCs compared to control cells (**Figure 2B** $p = 0.04$). By day 40, the CD34 expression decreased in both groups but remained slightly higher in the OPG group (data not shown).

DISCUSSION

Our present study demonstrates the promoting effect of OPG on CD34$^+$ differentiation in ECFCs which have then stronger capacity to proliferate. ECFCs cultured in the presence of OPG strongly expressed CD34$^+$ and are devoid of hematopoietic markers (CD45, CD14) conforming that they are not originated from hematopoietic stem cell pool. This suggests that OPG could contribute to endothelial commitment and give rise to expanded CD34$^+$-ECFCs with better vasculogenic properties. ECFCs exposed to OPG may display a higher proliferative ability allowing a faster generation of sufficient number of cells for clinical applications.

Current protocols for isolation and expansion of ECFCs relies on the presence of serum and various growth factors to promote endothelial cells proliferation and differentiation (16–20). Previous studies have shown that long term expansion reduces their proliferative and angiogenic potency (16, 21–24). We demonstrated here that exogenous OPG is able to increase endothelial differentiation from CD34$^+$ stem cells and to give them enhanced proliferative potential. This further highlights the importance of OPG in progenitors related biology.

Few data describe the pathways that allow the endothelial differentiation of stem/progenitor cells (25). Several methods developed in the purpose of improving the isolation and long term expansion of ECFCs while preserving their angiogenic potential have shown that human platelet lysate (PL) are suitable serum substitutes (1, 20, 26, 27). This could be related at least in part to the presence of OPG contained in megakaryocytes and alpha-granules of platelets (8, 9). Our results could explain the efficacy of PL to yield twice more colonies per ml blood

compared to the conventional isolation medium with fetal bovine serum (27).

Regarding CD34+ Cells Survival and Expansion

Numerous factors have been described to promote ECFCs proliferation and maturation in culture (TGF-beta, VEGF, BMP4, etc.) (6). We can explain the observed enhanced proliferative properties of ECFCs primed with OPG through different mechanisms. (i) OPG could increase directly or indirectly the proliferation of ECFCs. We previously observed that OPG increase SDF-1 expression and secretion in ECFCs (11). However, SDF-1 does not have any effect of ECFCs proliferation (28). Furthermore, OPG does not have any effect on RNA expression nor secretion of VEGF, the main effector known in ECFCs proliferation and maturation [data not shown, (29)]. OPG could induce the secretion of specific growth factor(s) that could be involved in proliferation. OPG has an additive effect to VEGF, angiopoietin-1, TGFβ-1, and IL8 in PL, and may further support efficient outgrowth of ECFCs (30, 31). (ii) Effects of OPG on ECFCs proliferation could result from pro-apoptotic genes down-regulation already described (13). OPG protects HUVECs and ECFCs from apoptosis induced by growth factor deprivation and preserves their viability by stimulating mTOR and Akt cascades (8, 13). We previously demonstrated that OPG drastically reduced caspase-3/7 activities and maintained ECFCs viability after 48 h of treatment. This was supported by an observed down-regulation of pro-apoptotic genes (Hyou1, Taldo1 (transaldolase), crk (adapter molecule crk), and Sh3glb1 (endotphilin-B1) in OPG-stimulated ECFCs that reflects the capacity of OPG to protect cells from apoptosis and to promote their survival.

ECFCs Priming of OPG Induces Overexpression of CD34+

ECFCs originate from the blood-derived mononuclear progenitor cells fraction expressing CD34 but during their *in vitro* endothelial expansion part of the cells loses CD34 upon differentiation. The function of this transmembrane cell surface glycoprotein remains obscure, though CD34 has been shown to be a ligand for L-selectin allowing leucocytes adhesion to the endothelium at sites of inflammation (15, 16). Our finding that OPG increases CD34 could reflect a mobilization of a more immature state of ECFCs. Previous study on CD34 in ECFCs suggests that CD34 expression is not related to different ECFCs subpopulations but is a reflect of cell plasticity and the result of angiogenic stimuli interfering with the endothelial tube formation (32). The CD34 expression is known to be inducible and down-regulated, in HUVECs, by angiogenic growth factors (32, 33). Thus, upregulation of CD34 observed in OPG-exposed ECFCs could be both attributed to progenitor subtype expansion but also to an increased ECFCs vasculogenic potential.

Our data obtained on cord blood-derived ECFCs need now to be confirmed with adult peripheral blood mobilized or not with G-CSF. This validation will allow us to propose OPG as a cell culture adjuvant for ECFCs isolation in GMP culture condition. Moreover, this potential link between OPG and endothelial differentiation could also be tested on stem cell at the origin of endothelial lineage. We recently described that very small embryonic-like stem cells (VSELs) were able to differentiate in endothelial lineage (34, 35). making these cells a potential cell source for cell therapy. VSELs exhibit several features of pluripotent stem cells and do not form teratomas after transplantation into deficient mice. As a prerequisite step to cell therapy trials, as an expansion model have just been described by Pr Ratajczak's group, OPG could be now used in new differentiation ongoing protocol.

In conclusion, this study provides further evidence that OPG plays a functional role in ECFCs commitment and expansion In addition to its action on angiogenesis, our work confirms its important contribution to the angiogenic/vasculogenic process. OPG secretion from vascular cells and platelets may be involved in blood-derived ECFCs maturation, proliferation, and their ability to form new blood vessels. It may provide new opportunities for optimization of *ex vivo* expansion and maintenance of human CD34+ progenitors such as ECFCs or VSELs in appropriate media free from feeder-layer cells, and may have value for their application in regenerative medicine. A better understanding of this OPG/CD34 pathway in vasculogenesis could lead to new strategies for *ex vivo* ECFCs expansion.

ETHICS STATEMENT

The study was approved both by the relevant ethics committee (Hôpital Saint Louis, Paris, France) and the French Ministry of Higher Education, Training and Scientific Research (AC-2008-376). The protocol complied with the Declaration of Helsinki. Umbilical cord blood were collected after normal full-term deliveries with the written informed consent of the mother, and used within 24 h.

AUTHOR CONTRIBUTIONS

CB-V and ZB-A designed the *in vitro* experiments and performed data analysis. ZB-A and AL conducted the experiments, interpreted, and analyzed data. ZB-A and CB-V wrote the manuscript. DH and DS performed critical revising of the intellectual content. CB-V and DH financially supported the experiments. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We are grateful to the consent mothers for their commitment and are indebted to the nursing services of Hôpital Bicêtre for providing umbilical cord blood samples. ZB-A received grants from the *Groupe d'Etude et de Recherches sur l'Hémostase* (GEHT). The CNRS pays the salary of CB-V.

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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.

frontiers in Medicine

MINI REVIEW
published: 18 December 2018
doi: 10.3389/fmed.2018.00354

Recent Advances in Endothelial Progenitor Cells Toward Their Use in Clinical Translation

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Since the discovery of Endothelial Progenitor Cells (EPC) by Asahara and colleagues in 1997, an increasing number of preclinical studies have shown that EPC based therapy is feasible, safe, and efficacious in multiple disease states. Subsequently, this has led to several, mainly early phase, clinical trials demonstrating the feasibility and safety profile of EPC therapy, with the suggestion of efficacy in several conditions including ischemic heart disease, pulmonary arterial hypertension and decompensated liver cirrhosis. Despite the use of the common term "EPC," the characteristics, manufacturing methods and subset of the cell type used in these studies often vary significantly, rendering clinical translation challenging. It has recently been acknowledged that the true EPC is the endothelial colony forming cells (ECFC). The objective of this review was to summarize and critically appraise the registered and published clinical studies using the term "EPC," which encompasses a heterogeneous cell population, as a therapeutic agent. Furthermore, the preclinical data using ECFC from the PubMed and Web of Science databases were searched and analyzed. We noted that despite the promising effect of ECFC on vascular regeneration, no clinical study has stemmed from these preclinical studies. We showed that there is a lack of information registered on www.clinicaltrials.gov for EPC clinical trials, specifically on cell culture methods. We also highlighted the importance of a detailed definition of the cell type used in EPC clinical trials to facilitate comparisons between trials and better understanding of the potential clinical benefit of EPC based therapy. We concluded our review by discussing the potential and limitations of EPC based therapy in clinical settings.

Keywords: endothelial colony forming cells (ECFCs), Endothelial progenitor cell (EPC), clinical translation, clinical trial, preclinical study

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *11 September 2018*
Accepted: *03 December 2018*
Published: *18 December 2018*

Citation:
Keighron C, Lyons CJ, Creane M, O'Brien T and Liew A (2018) Recent Advances in Endothelial Progenitor Cells Toward Their Use in Clinical Translation. Front. Med. 5:354. doi: 10.3389/fmed.2018.00354

INTRODUCTION

The term "putative endothelial cell progenitors" was pioneered by Asahara et al in their seminal publication in Science over two decades ago (1). They showed that this cell population can be successfully isolated from peripheral blood derived mononuclear cells of healthy volunteers, utilizing magnetic bead positive selection of two cell surface antigens, CD34, and Flk1. They also demonstrated that EPCs could home specifically to areas of ischemia. This forms the basis of vasculogenesis, whereby new blood vessels are formed by EPCs or angioblasts, which home,

differentiate, proliferate, and incorporate into resident mature vessels in response to various stimuli such as ischemia (2). Prior to the discovery of this cell population, the principal mechanism of vascularization after an ischemic event was thought to be due to the process of angiogenesis, whereby new vessels are formed by direct migration, differentiation, and proliferation of the existing mature endothelial cells (3). Furthermore, the discovery of this novel EPC concept has overturned the previous dogma which suggested that vasculogenesis could only occur during embryogenesis. In fact, both vasculogenesis and angiogenesis may potentially have a synergistic role in postnatal revascularization.

The new paradigm shift in the understanding of vascular regeneration has led to multiple publications using the term "EPC." Specifically, this term has been widely utilized in many studies as a surrogate biomarker to assess the risk of cardiovascular disease in human subjects (4–6) and as a potential novel therapeutic agent for vascular regeneration (7, 8). The initial nomenclature of EPC encompasses a heterogeneous cell population, including early EPC [or circulating angiogenic cells (CAC), myeloid angiogenic cells (MACs), pro-angiogenic haematopoietic cells (PACs)] and late EPC [or outgrowth endothelial cells, endothelial colony forming cells (ECFC)] (9–12).

Early EPCs typically are cultured on fibronectin coated plates and appear early in culture (4–7 days). They are defined as spindled shaped cells with low proliferative potential, with cells only surviving up to 4 weeks in culture (13, 14). Functionally, early EPCs neither form a colony in culture nor vessels *in vitro* in 2D Matrigel assays. They also do not integrate into pre-existing vessels. Despite this, they have been found to have pro-angiogenic paracrine capabilities, demonstrated by their ability to increase the number of tubules formed by mature endothelial cells (13). Medina et al. showed that early EPCs possessed an expression profile more similar to monocytes than endothelial cells, with cells expressing haematopoietic markers (RUNX1,WAS,LYN) as well as inflammatory markers (TLRs,CD14,HLAs) (12). Subsequently, Medina et al. showed that early EPCs were of myeloid origin as opposed to endothelial in origin (10). In contrast, late EPCs are cultured on collagen coated plates and tend to appear later in culture (usually after 1 week) as colonies with well circumscribed monolayers of cells pertaining a cobblestone morphology. Late EPCs behave functionally like endothelial cells with the ability to form vessels in 2D Matrigel angiogenesis assays. They also have an expression profile similar to mature endothelial cells ($CD34^+$,VE-Cad^+,vWF^+) but have a much higher proliferation rate, and survival, with cells surviving up to 12 weeks. These cells secrete less angiogenic factors compared to early EPCs (9, 10, 12, 14). A more in depth comparison of these cell types can be found in a review by Hirschi et al. (15).

A recent Consensus Statement on Nomenclature of endothelial progenitors has discouraged the current liberal use of the term "EPCs," and recommends the term "ECFC" instead. They have also proposed a more precise characterization of ECFCs based on a pre-defined cellular phenotype and function (10). This is a crucial step in defining the term "EPC" which will lead to the harmonization and standardization of the cell type used in clinical studies, thus allowing comparisons to be made across different studies.

The objective of this review was to search the current literature and critically appraise the current use of EPCs as therapeutic agents by summarizing: (a) clinical trials using EPCs as a therapeutic agent currently registered in www.clinicaltrials.gov; (b) published clinical trials using EPCs as a therapeutic agent; and (c) the efficacy of ECFCs in preclinical and clinical studies. We also highlight the potential and limitations of EPC based therapy in clinical settings.

MATERIALS AND METHODS

Three separate literature searches were conducted to generate the data presented here (**Appendices 1–3**). "EPC" was used as the first search term and this was carried out using the www.clinicaltrials.gov database, focusing specifically on interventional therapies only. The second literature search focused on clinical trials in humans using the term "EPCs" as a therapeutic intervention using the PubMed and Web of Science databases published within the last 10 years. The third literature search (PubMed and Web of Science databases) was performed using the synonymous names of ECFCs, "Endothelial Colony Forming Cells or Outgrowth Endothelial Cells or Blood Outgrowth Endothelial Cells or Endothelial Outgrowth Cells or Late Endothelial Progenitor Cells or Late Outgrowth Endothelial Progenitor Cells," as defined by Medina et al. (10) as the search term. Results were limited to primary interventional studies carried out within the last 10 years.

RESULTS

Clinical Trials Using EPCs As Therapeutic Agent, Registered Under www.clinicaltrials.gov Registry

A total of 341 clinical trials were found when the term "EPC" was searched using the www.clinicaltrials.gov registry (22/09/05 to 12/04/17). Many of the registered trials related to the assessment of EPCs across various clinical states and were non-interventional in nature. There are 26 clinical trials which utilized EPCs as a therapeutic agent currently registered in www.clinicaltrials.gov (**Table 1**) with a total of 1,148 participants expected to be enrolled into the trials. Approximately two thirds of these registered clinical trials involved patients with ischemic conditions such as peripheral artery disease ($n = 8$), coronary artery disease ($n = 7$) and ischemic stroke ($n = 2$). The remaining conditions registered were pulmonary arterial hypertension ($n = 4$), liver cirrhosis ($n = 2$), lymphodema ($n = 1$), erectile dysfunction ($n = 1$), and traumatic bone defects ($n = 1$) (**Table 1**).

The majority of the 26 registered clinical trials specified the source for EPC in the registry ($n = 19$), such as bone marrow derived ($n = 9$) or peripheral blood derived ($n = 10$), but only 10 trials specified the cell surface markers used for EPC definition in the registry, including $CD133^+$($n = 7$), $CD34^+$($n = 2$), and $CD14^-$($n = 1$). The status of half of these trials were labeled as "complete" ($n = 12$). Two trials

TABLE 1 | Clinical trials using EPCs as therapeutic agent, registered under www.clinicaltrials.gov registry (22nd Sept 2005–12th Apr 2017).

NCT	Condition	Phase	n	Cell type	Cell surface marker(s)	Cell culture condition	Status	Sponsor(s)	Published results
NCT00936819	Acute Myocardial Infarction	II	100	PB-EPCs or eNOS-EPCs	CD31, CD34, CD14, CXCR4, VEGFR2	2-3 days culture in fibronectin coated plates	Recruiting	Ottawa Hospital Research Institute	NR
NCT01049867	Coronary Artery Disease	I/II	10	BM-EPCs	$CD133^+$	NR	Unknown	Hospital y Clinica OCA, S.A. de C.V.	NR
NCT00384514	Coronary Artery Disease	II	24	PB-EPCs	NR	NR	Unknown	TheraVitae Ltd	NR
NCT00289822	Coronary Artery Disease	II	75	PB-EPCs	NR	0 or 3 days culture	Terminated	Johann Wolfgang Goethe University Hospital	De Rosa et al. (16)
NCT00629096	Dilated Cardiomyopathy	II	27	BM-MNCs	NR	NR	Completed	Fundación Pública Andaluza Progreso y Salud	NR
NCT00221182	Heart Disease	I/II	1	PB-EPCs	$CD34^+$	NR	Terminated	Foundation for Biomedical Research and Innovation	NR
NCT00694642	Refractory Angina	I/II	28	EPCs	$CD133^+$	N/A	Completed	Pilar Jimenez Quevedo	Jimenez-Quevedo et al. (17)
NCT02605707	Chronic Ischemic Stroke	I/II	30	EPCs	NR	NR	Unknown	Southern Medical University, China	NR
NCT01468064	Ischemic Stroke	I/II	20	BM-EPCs	NR	NR	Unknown	Southern Medical University, China	NR
NCT01595776	Critical Limb Ischemia	I/II	8	PB-EPCs	$CD133^+$	NR	Completed	IRCCS Policlinico S. Mattteo	Arici et al. (18)
NCT02454231	Critical Limb Ischemia	II/III	45	PB-EPCs	$CD14^+$, $CD34^+$	NR	Completed	University of Florence	NR
NCT00523731	Critical Limb Ischemia	I	6	PB-EPCs	NR	Cells cultured in X-vivo 15 serum free medium supplemented with autologous human serum, VEGF and heparin for 5 days	Completed	TheraVitae Ltd	Mutirangura et al. (19)
NCT02287974	Critical Limb Ischemia	I/II	20	BM-EPCs	$CD133^+$	NR	Active	Andalusian Initiative for Advanced Therapies - Fundación Pública Andaluza Progreso y Salud	NR
NCT02474381	Diabetic Foot	NR	60	EPCs	$CD133^+$	NR	Unknown	Shanghai 10th People's Hospital	Zhang et al. (20)
NCT00221143	No Option Critical Limb Ischemia	I/II	15	PB-EPCs	$CD34^+$	NR	Completed	Translational Research Informatics Centre, Kobe, Hyogo, Japan	Kawamoto et al. (21), Kinoshita et al. (22)

(Continued)

TABLE 1 | Continued

NCT	Condition	Phase	n	Cell type	Cell surface marker(s)	Cell culture condition	Status	Sponsor(s)	Published results
NCT02915796	Peripheral Arterial Disease	I	345	PB-EPCs	$CD133^+$	Mononuclear cells isolated, re-suspended in RPMI-1640 and then injected into the ischemic area	Recruiting	Shanghai 10th People's Hospital	Huang et al. (23)
NCT00306085	Peripheral Atherosclerosis	I	20	BM-Cells	$CD34^+$	Cells sorted by the MarrowXpress system and immediately transplanted	Unknown	University of Naples	Malone et al. (24), Cobellis et al. (25)
NCT00641836	Idiopathic PAH	NR	98	PB-EPCs	VE-Cad^+, KDR^+, $CD34^+$, $AC133^+$	Cultured on fibronectin coated flasks in Medium 199 for 5 days	Completed	Zhejiang University	NR
NCT00257413	Idiopathic PAH	NR	31	PB-EPCs	VE-Cad^+, KDR^+, $CD34^+$, $AC133^+$	Cultured on fibronectin coated flasks in Medium 199 for 5 days	Completed	Zhejiang University	Wang et al. (26)
NCT00372346	Idiopathic PAH	NR	40	PB-EPCs	vWF^+, $CD31^+$, $CD34^+$	Cultured on fibronectin coated flasks in EGM-2 for 10-14 days	Unknown	Zhejiang University	NR
NCT00469027	PAH	I	7	PB-eNOS-EPCs	$CD14^+$, $CD31^+$	Isolated and cultured on fibronectin coated flasks for 7-12 days	Completed	Northern Therapeutics	Granton et al. (27)
NCT01333228	Liver Cirrhosis	I/II	14	BM-EPCs	CD31, CD34, CD14, VEGFR-2, VEGFR-1, CD133, CD90, CD117, vWF, CXCR1, CD45, ID1	BM-EPCs isolated via Ficoll gradient, cultured on fibronectin coated plates in endothelial complete medium for 4 days	Completed	Clinica Universidad de Navarra + Universidad de Navarra	D'Avola et al. (28)
NCT03109236	Liver Cirrhosis	III	66	BM-EPCs	$CD133^+$	NR	Recruiting	National University Hospital, Singapore	NR
NCT01112189	Lymphedema	I/II	20	BM-EPCs	NR	NR	Completed	Hospital Universitario Dr. Jose E. Gonzalez	Maldonado et al. (29)
NCT01089387	Post Prostatectomy Erectile Dysfunction	I/II	18	BM-MNCs	NR	NR	Completed	Institut National de la Santé Et de la Recherche Médicale, France	NR
NCT03103295	Traumatic Bone Defects	I/II	20	PB-EPCs	NR	Cultured in EGM	Active	A.A. Partners, LLC	Vasyliev et al. (30)

BM, Bone Marrow; eNOS, Endothelial Nitric Oxide Synthase; EPCs, Endothelial Progenitor Cells; MNC, Mononuclear Cells; N/A, Not Applicable; NR, Not Reported; PAH, Pulmonary Arterial Hypertension; PB, Peripheral Blood.

were labeled as "terminated." Slow recruitment was noted for one of these two trials (NCT00221182). The other trial was subsequently published (NCT00289822) (16). The remaining trials were labeled as "active" ($n = 2$), "recruiting" ($n = 3$), and "unknown" ($n = 7$).

The results of this search showed that the term "EPC" has been widely used to encompass a heterogeneous population of cells. In addition, these results also highlight the paucity of information registered within the www.clinicaltrials.gov for these clinical trials, thereby preventing direct comparison with other trials. A detailed definition of the cell type used in clinical trials is warranted, to facilitate better understanding of the potential clinical benefit of EPC based therapy.

Published Clinical Trials Using EPCs As the Therapeutic Agent

Five relevant published clinical trials using EPCs were found following the PubMed and Web of Science database searches (01/05/2008 to 01/05/2018). These clinical trials involved various disease states including peripheral arterial disease (31, 32), coronary artery disease (33), pulmonary hypertension (34), and liver cirrhosis (28) (**Supplementary Table 1**). Despite using the term "EPC" as a therapeutic agent, the cell types used in all the five published trials differ, specifically, in their methods of cell isolation and culture, as well as the cell surface markers used for phenotypic characterization. Furthermore, the cell dose, and the route of administration varies between trials.

Lara-Hernandez et al. showed that intramuscular administration of EPCs into the ischemic limbs of 28 patients with no-option critical limb ischemia was safe and feasible (31). They used 50 mls of G-CSF mobilized blood and then selected for $CD34^+$ and $CD133^+$ cells. This treatment resulted in a significant reduction in the pain score of no option CLI patients with increased tissue perfusion and no adverse effects noted after a follow-up of 14 months (31). Tanaka et al. also isolated EPCs from G-CSF mobilized blood, but selected for $CD34^+$ EPCs prior to intramuscular administration into non-healing diabetic feet. They demonstrated the safety of EPC therapy, no serious adverse events noted, coupled with increased vascular perfusion and complete wound closure after ∼18 weeks (32).

Zhu et al. demonstrated the safety and feasibility of EPCs by injecting thymosin β1 pre-treated EPCs, into patients with ST segment elevated myocardial infarction, with some efficacy data showing improved exercise capacity and left ventricular function after 6 month follow-up (33). The EPCs used by Zhu et al. were cultured on fibronectin and phenotypically characterized as VE-$Cadherin^+$, KDR^+, $CD34^+$, and $CD133^+$ (33) cells. Similarly, Zhu et al demonstrated the safety and feasibility of intravenously administered $CD34^+$, $CD133^+$, and KDR^+ EPCs into children with idiopathic pulmonary arterial hypertension (IPAH). Although safety was their primary endpoint, significant increases in exercise capacity and pulmonary hemodynamics were also noted (34).

D'Avola et al. showed that a single administration of EPCs (maximum of 100×10^6 cells) via the hepatic artery in 12 patients with decompensated liver cirrhosis is safe and feasible (28). No treatment-related severe adverse events were seen for up to 1 year of follow up. Extensive characterization of EPCs was performed following 7 days of *ex vivo* culture of fibronectin adherent mononuclear cells (from 50-100 mls of bone marrow aspirate) using extensive cell surface markers (CD31, CD34, CD14, VEGFR2, VEGFR1, CD133, CD90, CD117, vWF, CXCR4, ID1, and CD45) and functionality assessment with DiL-acetylated-LDL cholesterol uptake and lectin binding capacity as well as demonstration of tube-structure formation over Matrigel matrix (28).

While some of the clinical data from **Table 1** was published, several of these papers were not included in this section for overall analysis for the following reasons: one paper did not use the term EPC but instead used the term "angiogenic precursor cells" (19), five papers included cells identified only using a single cell surface marker [e.g., $CD133^+$ (17, 18, 20) or $CD34^+$ (21, 29)], and one did not specifically define the cell type (24). It was concluded that these papers could not be classified according to our search criteria as being interventional EPC studies, and thus excluded. Two papers were published beyond the date range specified in our search (23, 26). Finally, two other papers from **Table 1** met our classification criteria did not appear in our literature search (27, 30). Granton et al. used $7–50 \times 10^6$ peripheral blood eNOS modified EPCs for pulmonary arterial hypertension and showed an improvement in pulmonary resistance after treatment (27), whereas Vasyliev et al. used $20–60 \times 10^6$ autologous peripheral blood derived EPCs in a scaffold of allogeneic bone and fibrin gel for bone fracture repair and demonstrated an improvement in bone regeneration (30). Of note, neither study had a control group to compare against.

Overall, all trials were single arm trials, with four of the five published trials being early phase clinical trials and demonstrated safety and feasibility of EPC therapy. Only Zhu et al. conducted a randomized controlled trial (33).

Efficacy Data Using ECFCs

In the previous sections, we examined human interventional studies using "EPCs" as a therapeutic but had noted that the field is moving toward a cell type that is considered to be the "true EPC." Thus, we examined the published data on ECFCs, the novel and "bone-fide" EPC, using the PubMed and Web of Science databases. From our search using all the relevant search terms, only 39 out of 1,316 papers retrieved used hECFCs as an interventional therapy in preclinical models (01/05/2008 to 01/05/2018) (**Table 2**). The majority of the published articles assessed the potential therapeutic role of ECFC in ischemic disease models including hindlimb ischemia ($n = 20$), cerebral ischemia ($n = 4$), retinal ischemia ($n = 2$), myocardial ischemia ($n = 2$), ischemic acute kidney injury ($n = 1$) and ischemic reperfusion injury ($n = 1$). However, ECFCs were also being investigated for other conditions such as islet graft retention ($n = 3$), vascular injury ($n = 2$), pulmonary arterial hypertension ($n = 1$), bronchopulmonary dysplasia ($n = 1$) and traumatic brain injury ($n = 2$). In these 39 papers, hECFCs were predominantly derived from the umbilical cord ($n = 31$), followed by

peripheral blood ($n = 6$) (**Table 2**). However, despite a robust preclinical evidence to suggest the efficacy of ECFCs in several conditions, this cell type has yet to be tested in human clinical trials.

Medina et al. highlighted the importance of ECFC identification with more than one surface marker (10). Despite the use of the term "ECFC," four preclinical studies did not confirm the identity of ECFCs using surface expression markers, with eight studies utilizing only one surface expression marker. Of the papers which used multiple markers, the most commonly used surface markers were: $CD34^+$ ($n = 11$), $CD31^+$ ($n = 16$), $VEGFR2/KDR^+$ ($n = 13$), and $CD45^-$ ($n = 14$).

DISCUSSION

The results of our literature search showed that the term "EPC" has been widely used to encompass a heterogeneous population of cells in both the published and registered clinical studies, thereby, rendering direct comparison amongst studies impossible. Our findings also highlight the paucity of information registered within www.clinicaltrials.gov for these clinical trials, specifically, the cell phenotype that is being tested. Approximately half of the EPC clinical trials (11 out of 26 trials) did not define the culture conditions used for their therapeutic product, and a majority of the trials (17 out of 26 trials) did not specify the cell surface markers used to characterize the cell therapy product used in their trials. Whilst the results from many of the ongoing clinical trials are yet to be published/released, only 12 out of 26 trials have reached the completion phase.

From the published EPC human clinical trials, there is a marked inconsistency in terms of the culture conditions and characterization of EPCs with D'Avola et al. and Vasyliev et al using multiple different cell surface markers to identify the EPCs they used (28, 30), Lara-Hernandez et al. and Granton et al using two cell surface markers (27, 31), Tanaka et al. only utilizing one cell surface marker (32), and Zhu et al. and Zhu et al. did not mention the marker used for defining the EPCs in their studies (33, 34). This lack of standardization makes it difficult to compare the results of different clinical trials and will in the future hinder the translation of these therapies into medical practice. A similar lack of consistency is again observed with the methods to culture EPCs with some reports administering EPCs without prior culture (31, 32), some culturing on fibronectin before re-injection into the patient (27, 28, 33, 34), and Vaysilev et al. culturing cells on uncoated flasks with EGM-2 medium (30). Despite the positive results from the above papers, EPC therapy is still limited by the immunogenicity of allogeneic EPCs, along with its poor definition, isolation and expansion standardization, as outlined above.

From the above data it is clear that a detailed definition of the cell type used in clinical trials is warranted, to facilitate better understanding of the potential clinical benefit of EPC based therapy. Consistent with the recommended nomenclature by Medina et al. the term "ECFC," a more accurate description of a specific cell type within the EPC population can be carried out, allowing the standardization of cell definition for potential therapeutic use (10).

In fact, currently, there are an increasing number of preclinical studies which have demonstrated the efficacy of ECFCs in various disease models (36, 40, 56, 59, 61, 65, 68, 73). These studies predominantly focus on ischemic conditions such as limb ischemia, cerebral ischemia, myocardial ischemia, ischemic reperfusion injury, and ischemic kidney injury. The likely reason for this is beyond the scope of this review article but it may be related to the angiogenic effect of these cells, which facilitate the revascularization in these ischemic states; however these cells may have other unexplained therapeutic effects, which may not be related to its angiogenic properties. However, similar to EPC studies, there is a lack of standardization of cell surface markers and culture protocols when producing ECFCs for therapeutic intervention between the different studies. This lack of standardization, while addressed by Medina et al. (10), has not yet been fully adopted by the field of vascular regeneration.

The ability to successfully isolate ECFCs is crucial prior to its consideration for clinical use. We and others have previously published methods for ECFC isolation (74–78). However, the yield of ECFCs varies depending on the method used (9). Most methods used collagen as the matrix molecule for cell seeding, rather than fibronectin, suggesting that the former molecule is a better cell selection method (74–78). Direct comparison between collagen and fibronectin by Colombo et al. showed the contrasting impact of the type of matrix molecule used for cell seeding on the pharmacodynamics of ECFC colonies. Seeding cells on fibronectin, as compared with collagen, resulted in earlier appearance of ECFC colonies. In contrast, ECFC colonies cultured on collagen demonstrated a better cell proliferation and lifespan, which might be IL-6 and IL-8 dependent (79). Interestingly, the immunophenotype and the ability for *in vitro* tubule formation remains similar despite the type of matrix molecule used for cell seeding (79).

Tasev et al. have further refined the ECFC isolation method with better cell expansion rates using platelet lysate supplemented culture medium, for large scale propagation for potential clinical use (80). Hofmann et al. have also described an easily applicable method for isolating ECFCs directly using adult human blood to generate more than 100 million functional ECFCs (77). They collected 5 ml of peripheral blood from patients and plated directly into a T75 flask discarding supernatants at various time points to remove any blood cells that were not ECFCs. Their method used human platelet lysate, thereby making it a xeno-free protocol, and they were able to consistently isolate and expand ECFCs up to 30 population doublings (81). Moreover, using the culture method outlined in their paper, ECFCs can be cryopreserved without resulting in genomic instability or changes in cell phenotype and function (81).

Siegel et al. have successfully produced an ECFC product by leukapheresis of peripheral blood in accordance with Good Manufacturing Products (GMP) standard (82). Their isolation method can produce approximately 1.44×10^8 ECFC per

TABLE 2 | Preclinical work using ECFCs as a therapeutic agent.

Condition	Cell source	Cell number	Cell surface marker(s)	Administration method	Recipient	n	Outcome	References
Hind Limb Ischemia	hUC-ECFCs or EPO Primed hUC-ECFCs	1×10^5	$CD131^+$, $EPOR^+$	IV	Athymic Nude Mice	5–7	Primed ECFCs have improved graft efficiency, improved cell survival and improved angiogenic potential	Bennis et al. (35)
Hind Limb Ischemia	hUC-ECFCs	1×10^5	$CD34^+$, $CD31^+$, $Tie\text{-}2^+$, KDR^+, $Flt\text{-}1^+$, $CD144^+$, $CD14^-$, $CD45^-$	IV	Athymic Nude Mice	10	Improved residual muscle blood flow and increased collateral vessel formation	Sarlon et al. (36)
Hind Limb Ischemia	hUC-ECFCs	1×10^6	CD34, CD146, CD45, KDR	IM injection to 3 sites (In 10 mice VEGF was blocked)	C57BL/6 N mice	50	ECFC treated mice showed significantly better outcomes in recovery quality and length	Flex et al. (37)
Hind Limb Ischemia	hUC-ECFCs	1×10^5	$CD34^+$, $CD31^+$, $Tie\text{-}2^+$, KDR^+, $Flt\text{-}1^+$, $CD144^+$, $CD14^-$, $CD45^-$	IM injection to ischemic area	Athymic nude mice	6	Improved blood flow	Mena et al. (38)
Hind Limb Ischemia	hUC-ECFCs	1×10^6	NR	Injection into three sites (20 μl/each site) of the gracilis muscle in the medial thigh three times/ week	Male C57BL/6J mice	8	Improved blood flow	Kim et al. (39)
Hind Limb Ischemia	hUC-ECFCs	1×10^5 cells dissolved in 500 μl of PBS	CD34+	IV	NOD/Shi-scid, IL-2Rγnull mice	15	Improved blood flow	Goto et al. (40)
Hind Limb Ischemia	hUC-ECFCs	(i) CAC-CM (50 μl) (ii) ECFC-CM (50 μl) (iii) ECFC (2×10^5 cells/50 μl), (iv) CAC (10^6 cells/50 μl), (v) a mix containing CAC-CM (25 μl) and ECFC suspension (10^5cells/25 μl), or (vi) a mix containing CAC suspension (5×10^5cells/25 μl) and ECFC-CM (25 μl)	$CD31^+$, $CD144^+$, KDR^+, $VEGF^+$, $Flk\text{-}1^+$, $CD14^-$, $CD45^-$	Matrigel implantation into ischemic site	C57BL/6 N mice	3	Endothelial cell retention and vascular maturation	Odent Grigorescu et al. (41)
Hind Limb Ischemia	hUC-ECFCs	5×10^5 cells (IM) or 1×10^6 cells (IV)	$CD34^+$, vWF^+, $CD133^+$, KDR^+, $CD31^+$, $c\text{-}kit^+$, $CXCR4^+$, $CD144^+$, $eNOS^+$, $p\text{-}eNOS^+$, $VEGFR2^+$	IM or IV injection	Balb/C Nude Mice	5	Significantly enhanced blood perfusion, capillary density, proliferation and angiogenic cytokine secretion	Lee et al. (42)
Hind Limb Ischemia	hUC/PB-ECFCs & hMSCs	NR	$CD31^+$, KDR^+, $CD34^+$	IV	Nude Mice	8–10	Enhanced neovascularization	Schwarz et al. (43)
Hind Limb Ischemia	hUC-ECFC	1×10^5	$CD34^+$	IV	Type 2 diabetic C56BL/6 J male athymic Nude mice	6	Increased blood flow recovery and vascular density, with reduced inflammation	Mena et al. (44)

(Continued)

TABLE 2 | Continued

Condition	Cell source	Cell number	Cell surface marker(s)	Administration method	Recipient	n	Outcome	References
Hind Limb Ischemia	Egfl7 repressed hUC-ECFCs	1×10^5	$CD31^+$, $CD34^+$, $CD144^+$, $CD133^-$, $CD45^-$, $CD90^-$	IV	Athymic nude mice	14	Improved revascularisation	D'Audigier et al. (45)
Hind Limb Ischemia	hUC-ECFCs treated with epigenetic drugs (GSK-343 and panobinostat)	5×10^5	$CD31^+$, $CD34^+$, $CD45^-$	IM	NOD/SCID and athymic nude CD1 female mice	7–8	Increased vasculogenesis	Fraineau et al. (46)
Hind Limb Ischemia	hBM-MSC conditioned medium + hUC-ECFCs	1×10^5	$CD34^+$, $CD144^+$, $CD146^+$, KDR^+, $CD45^-$, $CD14^-$	IV	NMRI-nude mice	6	Increased blood perfusion	Poitevin et al. (47)
Hind Limb Ischemia	Trichostatin A treated hUC-ECFCs	5×10^5	$CD34^+$, $CD31^+$, $CD105^+$, $CD144^+$, $VEGFR2^+$, vWF^+, $CD45^-$, $CD14^-$	IM	Athymic nude CD1 female mice	3–4	Enhanced vascular repair capacity	Palii et al. (48)
Hind Limb Ischemia	hUC-ECFCs	1×10^6	$CD31^+$, Flk^+, vWF^+, $eNOS^+$, phospho-$eNOS^+$	IV	Male C57BL/6J or BALB/c-nu/nu mice	8	Improved neovascularization and limb salvage	Heo et al. (49)
Hind Limb Ischemia	α6 knockdown hUC-ECFCs	1×10^5	$CD31^+$, $CD34^+$, $CD144^+$, $CD146^+$, $CD45^-$, $CD14^-$	IV	Male athymic nuce Foxn-1 mice	5	No ECFC integration or neovascularization	Bouvard et al. (50)
Hind Limb Ischemia	BMP2 or BMP4 treated hUC-ECFCs + hPB-ECFC	NR	$VEGFR2^+$, $CD31^+$, $CD34^+$, $CD45^-$, $CD14^-$	IV	Nude mice	NR	Increased therapeutic potential of ECFCs exposed to BMP	Smadja et al. (51)
Hind Limb Ischemia	PB-ECFCs derived from white European and south Asian males	3×10^5	$CD31^+$, $CD144^+$, $CD146^+$, $CD309^+$, $CD45^-$, $CD14^-$	IV	Male immunodefic-ient CD1 nude mice	5	Superior recovery in ECFCs from white European compared to those from South Asian men	Cubbon et al. (52)
Hind Limb Ischemia	Non-diabetic controls (young + age matched) + type 2 diabetic hPB-ECFCs treated with globular adiponectin	5×10^5	$CD34^+$, $CD31^+$, $VEGFR2^+$	IV	Diabetic female athymic NMRI nu/nu mice	4–8	Increased and prolonged neovascularization in adiponectin treated diabetic ECFCs compared to untreated diabetic ECFCs	Leight et al. (53)
Hind Limb Ischemia	ECFCs + hBM-MSCs	NR (1:1 ratio)	NR	Retro-orbital injection	Athymic male nude mice	6–7	Significantly higher vessel perfusion in ECFC only group, and significantly higher density and foot perfusion after co-transplantation	Rossi et al. (54)
Ischemic retina	hUC-ECFCs	1×10^3, 1×10^4, 1×10^5	$CD31^+$, $CD105^+$, $CD14^-$, and $CD45^-$	Intravitreal Delivery	P13 mice	1–8	Low dose cohort showed best improvement	Reid et al. (8)

(Continued)

TABLE 2 | Continued

Condition	Cell source	Cell number	Cell surface marker(s)	Administration method	Recipient	n	Outcome	References
Ischemic retina	Low passage and late passage hPB-ECFCs + hUC-ECFCs	NR	VEGFR2$^+$, Caveolin 1$^+$,CD45$^-$, CD14$^-$, CD31$^+$, CD105$^+$, CD146$^+$, CD34$^+$	Intravitreal injection	C57BL/6 mice	6	Late passage ECFCs had impaired vasoreparative properties	Medina et al. (55)
Ischemic Myocardium	OECs	5×10^6	CD45$^-$, CD133$^+$	Intramyocardial injection	Rabbits	8	Improved cardiac function	Tan et al. (56)
Myocardial Infarction	hUC-ECFCs	5×10^6	CD31$^+$, CD34$^+$, CD105$^+$, CD144$^+$, CD146$^=$, KDR, Tie-2$^+$, CD45$^-$	Intramyocardial injection	Male Sprague-Dawley rats	5	Increased angiogenesis and improved cardiac function	Kim et al. (57)
Ischemia Reperfusion Injury	hUC-ECFC + MPCs	2×10^6 (2:3)	CD31$^+$	Intracoronary injection	Nude Rats	3–17	Higher LV dimensions, higher heart weight to tibia length ratio. Improved cardiac function	Kang et al. (58)
Ischemic AKI	hUC-ECFCs	1×10^6	CD31$^+$, VEGFR2$^+$, CD45$^-$, CD14$^-$, CD133$^-$	IV	Male NOD-SCID (NOD.CB17-*Prkdc*^{scid}/J)	5-7	ECFCs protect against ischemic AKI damage	Burger et al. (59)
Vascular Injury	MSC derived ECFCs	5×10^5	CD133$^+$ CD34$^+$ KDR$^+$, vWF$^+$, CD31$^-$, CD45$^-$	IV through tail vein	Male nude mice	120	Accelerated re-endotheliazation and inhibits neointimal hyperplasia	Wang et al. (60)
Vascular Injury	hPB-ECFCs pretreated with recombinant BMP4	5×10^5	CD31$^+$, KDR$^+$, Tie-2$^+$	IV	Male NRMInu/nu athymic nude mice	5	Accelerated endothelial repair capacity	Xia et al. (61)
Traumatic Brain injury	hUC-ECFCs	3×10^5	CD34$^+$, KDR$^+$, vWF$^+$, VE-Cad$^+$, UEA-1$^+$	Intra-cerebroventricular Infusion	Balb/C Nude Mice	36	Reduced Evans blue extravasation, reduced brain water content. Increased microvascular density. Improved neurological function	Huang et al. (62)
Traumatic Brain injury	hUC-ECFC	1×10^6	CD31$^+$, vWF$^+$, VE-Cad$^+$	IV	Balb/C Nude Mice	21	Improved rate of neurologic disability. Increased microvessel density and proangiogenic growth factors SDF-1 + VEGF	Zhang et al. (63)
Cerebral Ischemia	hUC-ECFCs	4×10^6	CD146$^+$	IV via tail vein	Adult male Sprague–Dawley rats	33	Erythropoietin primed ECFCs showed best improvement	Garrigue et al. (64)
Transient Focal Cerebral Ischemia	hUC-ECFCs + EPO	5×10^6	NR	IV	Sprague-Dawley Rats	24	Completely restored neurological function	Pellegrini et al. (65)
Stroke (Middle Cerebral Artery Occlusion)	hUC derived ECFCs	4×10^6	CD54$^+$, CD31$^+$, CD146$^+$, CD34$^+$, CD144$^+$, KDR$^+$ CD45$^-$, CD14$^-$, CD133$^-$	IV via femoral vein	Adult male Sprague-Dawley rats	4–37	Improved functionality	Moubarik et al. (66)
Ischemic Stroke	hUC-ECFCs	1×10^6	CD31$^+$, CD34$^+$, VEGFR2$^+$, CD133$^-$	Injected into the left ventricle	Male BALB/c-nu mice	NR	Functional recovery, improved angiogenesis + neurogenesis with reduced apoptosis	Ding et al. (67)

(Continued)

TABLE 2 | Continued

Condition	Cell source	Cell number	Cell surface marker(s)	Administration method	Recipient	n	Outcome	References
Islet Graft Retention	hPB-ECFC	5×10^5	NR	Infra-Kidney Transplantation	Hypergylcaemic NOD-SCID Mice	6-9	Improved β-cell survival and graft-vessel and β-cell volume	Coppens et al. (68)
Islet Graft Retention	hUC-ECFCs	6×10^5	$CD31^+$, $VE\text{-}Cad^+$, $CD105^+$, vWF^+, KDR^+	Infra-Kidney Islet Transplantation	Balb/C Nude Mice	6-7	Absence of blood inflammatory reaction	Kim et al. (69)
Islet Graft Retention	hUC-ECFCs	NR	$VE\text{-}Cad^+$, KDR^+, $Flt\text{-}1^+$, $eNOS^+$, vWF^+, $CD31^+$	Intraportal Islet Transplantation	Diabetic Balb/C Nude Mice	23	Improved rate of neurologic disability. Increased microvessel density and proangiogenic growth factors SDF-1 + VEGF	Jung et al. (70)
PAH	hPB-ECFCs and hPB-EPCs	1.5×10^6	$CD31^+$, KDR^+, $CD14^-$, $CD34^+$	IV	Male nude rats	4–22	ECFCs had poor retention and no efficacy. EPCs resulted in right ventricular hypertrophy and increased right ventricular systolic pressure	Ormiston et al. (71)
BD	hUC-ECFCs	1×10^5 into mice, 2.5×10^5 into rats	$CD31^+$, $CD105^+$, $CD144^+$, $CD146^+$, $CD14^-$, $CD45^-$	IV	Rag–/– mice and RNU nude rats	5	No adverse effects with improvements in lung structure, exercise capacity and pulmonary hypertension	Alphonse et al. (72)

AKI, Acute Kidney Injury; BD, Bronchopulmonary Dysplasia; BM, Bone marrow; BOEC, Blood Outgrowth Endothelial Cells; CACs, Circulating Angiogenic Cells; CM, Conditioned Media; IM, Intramuscular; IV, Intravenous; KDR, Kinase Insert Domain Receptor; LV, Left Ventricular; MPCs, Mesenchymal Progenitor Cells; m/r/pECFCs, Murine/Rabbit/Porcine Endothelial Colony Forming Cells; MSCs, Mesenchymal Stem Cells; NR= Note reported; OECs, Outgrowth Endothelial Cells; PAEC, Pulmonary Arterial Endothelial cells; PAH, Pulmonary Arterial Hypertension; PB, Peripheral Blood; PMVEC, Pulmonary microvascular endothelial cells; SDF-1, Stromal Cell-Derived Factor-1; UC, Umbilical Cord; vWF, VonWillebrands Factor; VE-Cad, Vascular Endothelial Cadherin; VEGFR2, Vascular Endothelial Growth Factor Receptor 2.

white blood cell, following leukaparesis of up to 6.8 liters of peripheral blood. These ECFCs showed a significant Dil-AcLDL uptake and showed $CD29^+$, $CD31^+$, $CD34^+$, $CD44^+$, $CD105^+$, $CD117^+$, $CD133^+$, $CD144^+$, $CD146^+$, and $VEGFR2^+$ expression. Furthermore, they showed that their ECFCs could reach up to twelve cumulative population doublings. More importantly, these ECFCs showed no evidence of telomerase activity, as well as capable of *in vitro* tubule formation and secretion of epidermal growth factor, HGF, VEGF-A, platelet derived growth factor-B, IL-8, and monocyte chemoattractant protein-1 (82).

The major limitations of ECFC therapy are the long culture times to generate a therapeutic dose, due to its low frequency in peripheral and cord blood, and that it can only be administered in an autologous fashion due to its inherent immunogenicity. Furthermore, despite the potential for ECFC cryopreservation for future use, the intrinsic function of autologous ECFC may be impaired due to the underlying diseased state such as diabetes mellitus, with Jarajapu et al. reporting to be able to isolate ECFC from three in every ten diabetic patients, compared to eight out of nine in non-diabetic controls (83). However, ECFCs can be genetically modified to augment their function *in vivo* which may facilitate correction of disease-induced cell dysfunction. Examples of genetic modification include β1 integrin overexpression to improve blood perfusion in CLI (40), erythropoietin overexpression to promote erythropoiesis (84), or GSK-3β inhibition which improves the angiogenic capabilities of ECFCs (85).

In addition to genetic modification, ECFCs can be combined with other cell types to improve a specific aspect of ECFC therapy, such as combining them with mesenchymal stem cells (MSCs) or mesenchymal progenitor cells to reduce the immunogenic effect of allogeneic ECFCs and to increase cell survival post transplantation (86). This is due to the anti-inflammatory effects of mesenchymal stem cells. Reports have also noted that MSCs can differentiate into pericyte like cells which act to stabilize the vasculature formed (86–90). Based on the results of the above studies, next generation vascular cell therapies will likely consist of genetically modified ECFCs or ECFC combination.

CONCLUSIONS

To date, EPC based therapy has been shown to be feasible and safe with suggestion of efficacy. However, it is important

to note that only one trial included a control arm. While EPCs have been previously the favored cell type utilized for vascular therapeutics, they consist of a heterogeneous population of cells which will produce challenges in terms of GMP compliant cell manufacturing and definition of a cell product. "ECFCs," as defined by Medina et al. have several advantages over EPCs as a therapeutic product, as outlined above, including being a more defined cell type with enhanced proliferation, and possessing the ability to form new vessels, while also integrating into pre-existing vasculature. The use of ECFCs facilitates the harmonization and standardization of the cell type used in clinical studies, allowing direct comparison between studies (10). To translate ECFCs into routine clinical practice, issues surrounding their immunogenicity will need to be overcome, along with the issues regarding the standardization of markers used to identify them. To date, there have been no clinical trials using this cell type.

AUTHOR CONTRIBUTIONS

CK, TO, and AL: conception and design of the study. CK, CL, MC, and AL: analysis and interpretation of data. CK, CL, and AL: drafting of the manuscript. All authors revised the manuscript critically for important intellectual content and final approval for the submission of the manuscript.

ACKNOWLEDGMENTS

This work was supported by the Science Foundation Ireland (SFI) Investigator Programme (15/IA/3136).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2018.00354/full#supplementary-material

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Conflict of Interest Statement: TB is a founder, director and equity holder in Orbsen Therapeutics Ltd. TB and AL are both authors on a patent entitled 'Osteopontin for the prediction and treatment of cardiovascular diseases' (US Patent Number: US8323968B2).

The remaining 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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 20 December 2018
doi: 10.3389/fmed.2018.00356

Hypoxia Impairs Initial Outgrowth of Endothelial Colony Forming Cells and Reduces Their Proliferative and Sprouting Potential

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Vascular homeostasis and regeneration in ischemic tissue relies on intrinsic competence of the tissue to rapidly recruit endothelial cells for vascularization. The mononuclear cell (MNC) fraction of blood contains circulating progenitors committed to endothelial lineage. These progenitors give rise to endothelial colony-forming cells (ECFCs) that actively participate in neovascularization of ischemic tissue. To evaluate if the initial clonal outgrowth of ECFCs from cord (CB) and peripheral blood (PB) was stimulated by hypoxic conditions, MNCs obtained from CB and PB were subjected to 20 and 1% O_2 cell culture conditions. Clonal outgrowth was followed during a 30 day incubation period. Hypoxia impaired the initial outgrowth of ECFC colonies from CB and also reduced their number that were developing from PB MNCs. Three days of oxygenation (20% O_2) prior to hypoxia could overcome the initial CB-ECFC outgrowth. Once proliferating and subcultured the CB-ECFCs growth was only modestly affected by hypoxia; proliferation of PB-ECFCs was reduced to a similar extent (18–30% reduction). Early passages of subcultured CB- and PB-ECFCs contained only viable cells and few if any senescent cells. Tube formation by subcultured PB-ECFCs was also markedly inhibited by continuous exposure to 1% O_2. Gene expression profiles point to regulation of the cell cycle and metabolism as major altered gene clusters. Finally we discuss our counterintuitive observations in the context of the important role that hypoxia has in promoting neovascularization.

Keywords: ECFCs, hypoxia, colony growth, angiogenesis, tissue repair, proliferation

OPEN ACCESS

Edited by:
Reinhold J. Medina, Queen's University Belfast, United Kingdom

Reviewed by:
Chad L. Barber, California Lutheran University, United States
Kazuhide Hayakawa, Massachusetts General Hospital, United States

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *18 June 2018*
Accepted: *06 December 2018*
Published: *20 December 2018*

Citation:
Tasev D, Dekker-Vroling L, van Wijhe M, Broxterman HJ, Koolwijk P and van Hinsbergh VWM (2018) Hypoxia Impairs Initial Outgrowth of Endothelial Colony Forming Cells and Reduces Their Proliferative and Sprouting Potential. Front. Med. 5:356. doi: 10.3389/fmed.2018.00356

INTRODUCTION

The majority of wounds heal through physiological tissue repair. But if tissue repair fails, tissue engineering or regenerative medicine and transplantation is necessary (1). One of the problems during regenerative medicine is that oxygen diffusion is limited in a cellular tissue-engineered scaffold (2), resulting in reduced (hypoxia) or lack of oxygen (anoxia) within the deeper regions of the scaffold and finally cell death (2, 3). Therefore, it is important either to prevascularize tissue-engineered scaffolds by generating a blood vessel network in a scaffold *in vitro* or to create a scaffold with an environment (matrix composition, incorporation of blood vessel-generating cells and growth factors) that facilitates rapid angiogenesis when implanted in the body (4–7).

The primary vector of angiogenesis is the endothelial cell. However, in many disease conditions or after implantation of an engineered graft the ability of the endothelium to generate new vessels proceeds too slowly to overcome tissue hypoxia and subsequent cell death. As initially shown by Asahara et al. (8), within the blood the mononuclear cell (MNC) fraction expressing CD34 contains a subset of circulating progenitors committed to endothelial lineage, which proliferate at a high rate and contribute to an accelerated assembly of a new vascular network. Subsequent studies showed that the cells originally identified as endothelial progenitor cells harbored various cell types, in particular myeloid cells that acquired endothelial marker properties and endothelial colony-forming cells (ECFCs), that actively participate in neovascularization (9–13). ECFCs—also called blood-originated endothelial cells (BOECs)—exhibit high proliferative and colony-forming ability, do belong to the endothelial cell lineage and not to the hematopoietic cell lineage, and possess robust *in vitro* and *in vivo* neovascularization ability including participation in the lining of new vessels (9, 14).

Low oxygen tension in ischemic tissues determinates the fate and proliferation of progenitor or stem cells (15–17). On the one hand, hypoxia can limit growth in stem cell niches (18, 19). On the other hand, a hypoxic environment can enhance recruitment of circulating angiogenesis promoting cells, e.g., via the chemokine SDF-1 (20, 21). One may anticipate that ECFCs proliferation is also increased in hypoxic conditions, as there is a need for cells to enable expansion of the new vascular bed. However, a number of studies demonstrated that the proliferation of ECFCs was markedly inhibited by hypoxia (22–25), although some controversy exists (26, 27). Hypoxia also reduced ECFC migration as well as tubule formation into matrigel (22–25), although Decaris et al., (23) reported a difference in effect between acute and chronic hypoxia. The effect of hypoxia was mimicked by the α-ketoglutarate homolog dimethyl-oxo-glutarate (DMOG) supporting a role for HIF stabilization (24). However, the role of HIF has been debated. When the HIF-1α, one of the hypoxia-inducible factor α-subunits in endothelial cells, was overexpressed in CB-ECFCs, Kütscher et al. (28) observed improved proliferation, reduced apoptosis and increased sprouting. In contrast, recently, He et al. (21) reported that continued hypoxia reduced the proliferation of peripheral blood (PB) ECFCs by HIF-1α-mediated signaling. This differs from microvascular endothelial cells in which sprouting is enhanced by HIF-1α, while HIF-2α facilitates stabilization of vascular structures (29, 30).

In this study we summarize our findings on the effects on hypoxia on ECFCs using a custom designed hypoxia work station, which allows handing of the cells over longer periods in a defined oxygen atmosphere (30). Initially, we investigated the clonal outgrowth of ECFCs from human cord- and peripheral blood under hypoxic conditions. Subsequently, we evaluated the effect of various oxygen concentrations on the proliferation of CB- and PB-ECFCs that were cultured in the presence of platelet lysate, which improved serial propagation of ECFCs (31). Finally, we determined the effect of hypoxia on tubule formation in a fibrin matrix and compared its effect on gene expression in basal and tubule formation-stimulating conditions.

MATERIALS AND METHODS

Isolation of CB and PB-ECFCs Under Hypoxia and Normoxia

The study was executed in accordance with the Declaration of Helsinki and was approved by the University Human Subjects Committee of the VU University Medical Center. Written informed consent was obtained from all donors in accordance with the institutional guidelines. CB-ECFCs and PB-ECFCs were isolated as previously described with minor modifications (32). Namely, after isolation of MNCs by Ficoll-Paque density gradient centrifugation, the CB- and PB-derived MNCs were re-suspended in complete EBM-2 (Lonza, Walkersville, MD, United States) supplemented with 10% FBS, 0.1% penicillin-streptomycin, 2 mM L-glutamine, and EGM-2 SingleQuotes (without hydrocortisone and gentamycin/ amphotericin-B). MNCs were divided in two equal inoculation parts and seeded in a density of at least 2.5×10^6 cells per cm^2 onto 0.1% gelatin (Sigma) pre-coated 6- or 48-well plates. One culture was placed at 20% O_2/5% CO_2 and the second inoculum at 1% O_2/5% CO_2 atmosphere.

For optimal evaluation of hypoxic conditions cells were cultured inside a custom-designed hypoxia workstation (T.C.P.S., Rotselaar, Belgium) as previously described (30).

After 3 days, the medium was renewed for the first time, followed by daily renewals during the first week; from day 7 until the end of primary culture the medium was changed every other day.

The renewal medium that was used for the CB- and PB-MNCs cell cultures at 1% O_2 was pre-incubated as a thin layer (2 ml/10 cm^2) in empty culture dishes in the hypoxia-chamber for at least 2 h in order to allow the medium to become fully hypoxic. Renewal of cell culture medium was performed within the hypoxia-chamber preventing the cells being exposed to a normoxic environment. The outgrowth of primary ECFC colonies was monitored daily and counted in the appropriate oxygen environment by phase contrast microscopy on the basis of their characteristic endothelial cobblestone morphology (**Figure 1**).

Endothelial phenotype of the ECFCs obtained from primary cultures at 20 and 1% O_2 was confirmed using immunofluorescence and flow cytometry as previously described (31). Flow cytometry showed that both CB-ECFCs and PB-ECFCs were positive for CD31, CD105, CD146, VEGFR2 (CD309), and negative for CD14, CD45, and CD133 (33, 34). Expression of CD34 was highest in dense cultures (34). In addition, the CB- and PB-ECFCs stained positive for VE-cadherin, von Willebrand factor and Acetyl-LDL uptake.

To determine the minimal exposure time to 20% O_2 needed for overcoming the lack of colony outgrowth in 1% O_2, CB-MNCs of three different donors were isolated and plated in individual 6-well plates which were transferred after 24, 48, 72, or 96 h (24, 48, 72, and 96h) from 20% O_2 to 1% O_2. Cells cultured only in 20% O_2 or 1% O_2 served as controls (T0). ECFC colonies were quantified when the colonies had become visible in the culture in 20% O_2 (maximal evaluation period of 4 weeks). All

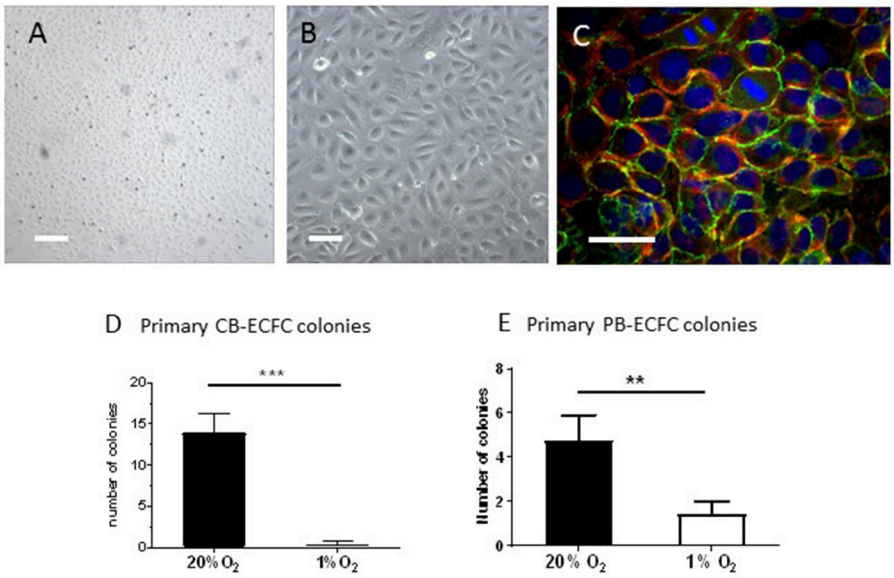


FIGURE 1 | Inhibition of clonal outgrowth of ECFCs from cord and peripheral blood MNCs by hypoxia**. (A,B)** Primary colony of CB-ECFCs isolated at 1% of oxygen. Bars are 500 μm **(A)** and 100 μm **(B)**, respectively. **(C)** Staining of VE-cadherin (green), f-actin (red), and nuclei (DAPI) of a primary colony of CB-ECFCs. Note two dividing cells in the middle top part. Bar = 100 μm **(D)** Enumeration of outgrowth colonies from umbilical cord blood-derived MNCs at 20 and 1% O_2 expressed as average number ± SEM ($n = 14$) of counted colonies per donor. Statistical significance was determined by Wilcoxon matched-pairs signed rank test; ***$p < 0.005$. **(E)** Enumeration of outgrowth colonies from peripheral blood-derived MNCs at 20 and 1% O_2 expressed as average number ± SEM ($n = 9$) of counted colonies per donor. Statistical significance was determined by Wilcoxon matched-pairs signed rank test; **$p < 0.01$.

experiments were performed with passage 2 (proliferation assay) to passage 5 (RNAseq experiments) CB-ECFCs or PB-ECFCs.

Western Blot Analysis for HIF-1α and HIF-2α

Subcultured CB-ECFCs were seeded on 5 cm^2 dishes coated with 0.1% gelatin. The dishes were placed in 1% O_2 for various time periods (0, 3, 6, 24, 48, and 96 h). The CB-ECFCs were washed with PBS and were lysed with 100 μl Laemmli sample buffer (including β-mercaptoethanol, Biochemical, 1:20). The samples were heated for 5 min at 96°C and briefly centrifuged before loading. Equal volumes of samples were loaded for separation of HIF-1α and HIF-2α via 6% SDS-PAGE using a semi-dry transfer system (Biorad, Veenendaal, Netherlands). Rabbit polyclonal antibody HIF-1α (1:250, Cayman chemical, Ann Arbor, MI, United States) and rabbit polyclonal antibody HIF-2α (1:250, Novus Biologicals, Edinburgh, United Kingdom) and β-actin (Sigma-Aldrich, St Louis, MO, United States) were used as primary antibodies. Horse-radish peroxidase coupled anti-rabbit was used as a secondary antibody (1:250, DakoCytomation, Neverlee, Belgium).

Transfection of CB-MNCs With dssiRNA HIF-1α and HIF-2α

Freshly isolated CB-MNCs were transfected with dssiRNA HIF-1α and HIF-2α (Qiagen, Venlo, Netherlands) using 'Human monocyte nucleofactor kit' (Lonza, VPA-1007). Briefly, for each condition an equal amount of CB-MNCs was centrifuged, and 100 μl nucleofactor was added to the cell pellet. From both dssiRNA HIF-1α and HIF- 2α, 1 μg was added to the cell suspension, and transferred to the cuvette and placed in the electroporation system (Amaxa, Lonza Verviers) according to the manufacturer. Transfected CB-MNCs were resuspended in complete EGM medium, transferred to 0.1% gelatin coated wells, and further cultured under 1% O_2 according the CB-ECFC culture protocol. Mock transfected cells (only electroporation step, no dssiRNA transfection) served as a control in 20 and 1% O_2. Further details are given in Nauta et al. (30).

Assessment of Proliferative Capacity of CB- and PB-ECFCs at 20 and 1% O_2 Conditions

The effect of oxygen tension on proliferative capacity of subcultured CB- and PB-ECFCs was assessed at 20, 5, 2, and 1% O_2 cell culture conditions. Early passages (p2–p3) of CB- and PB-ECFCs obtained from the primary colonies at 20% O_2 cultures ($n = 3$ individual donors) were seeded at density of 500 cells/cm^2 on 0.1% gelatin coated cell culture vehicles in complete EGM-2 and placed at 20, 5, 2, and 1% O_2 incubators, respectively. Medium change of cultures placed at the corresponding O_2 hypoxic chambers was performed as described in the previous section of material and methods.

Proliferation of subcultured ECFCs was calculated from cell counts. To that end, cells were incubated for indicated periods at different oxygen tensions. Subsequently they were washed, fixated with glutaraldehyde and their nuclei were stained by

crystal violet. For each donor and time point/condition triplicate 10 cm^2 wells were evaluated. From each stained well two pictures were taken at fixed positions (covering 60% of the plate surface), and the nuclei were counted using Image J software. Viability was determined by trypan blue exclusion after enzymatic detachment of unfixed cells (31). β-Galactosidase activity was assayed as previously indicated (31).

Tube-Formation in Fibrin Matrix

Assessment of sprouting ability of PB-ECFCs expanded in PL-EGM was performed at 20% O_2 and 1% O_2 seeding 20,000 cells on 3D human fibrin matrices prepared as previously described (30). Following overnight incubation in M199 supplemented with 10% inactivated human serum and 10% new-born calf serum, tube formation was induced by stimulating the cells with the combination of 10 ng/ml TNF-α and 25 ng/ml $VEGF_{165}$ and refreshed after 2 days. All growth factors were purchased from ReliaTech GmbH, Wolfenbuttel, Germany. After 96 h stimulation, the cells were fixed with 2% paraformaldehyde/HBSS and quantification of the length of formed tube-like structures was performed using Optimas image analysis software as previously described (35). The tube formation ability of PB-ECFCs was determined in triplicate wells for each of 7 donors.

RNA Isolation and Genome-Wide RNA-Sequencing

To investigate the transcriptomic response of established PB-ECFCs under hypoxia, cells from 6 female and 6 male donors were grown at 20% O_2 and exposed to 1% O_2 for 24 h in EBM-2 media (SingleQuots omitted) supplemented with 5% platelet lysate prepared as previously described (31). The cell lysates were collected in 350 μL per 20 cm^2 cells of solution containing RLT buffer (Qiagen) + 10 μL/mL β-mercaptanol. Mechanical disruption was accomplished using 1mL syringes and 21G needles, and the cell lysates were stored at −20°C overnight and transferred to −80°C the next day. Total RNA was isolated using RNeasyMinElute Cleanup Kit (Qiagen, Netherlands) and the RNA quality was tested with a Nanodrop 1,000 spectrophotometer. An RNA pool for deep sequencing of 20% O_2 condition was prepared by mixing 2.5 μg of RNA of each donor. The same procedure was repeated for preparing the pool of 1% O_2 RNA samples. The genome-wide RNA-sequencing was performed using the Illumina system accordingly to procedures described previously (36).

Deep-Sequencing mRNA Analysis

Statistical analysis of genome-wide RNA-sequencing data of the biological response of PB-ECFCs during exposure to 1% O_2 for 24 h was performed using significance analysis of microarrays (SAM) (36). The genes were defined as significantly changed by a q-value of 0.05 and an N-fold change >1.5 or <0.66. Only genes that complied to this double criteria were further analyzed using the online tools WEB-based GEne SeT AnaLysis Toolkit Webgestalt (37) and Gene Set Enrichment Analysis (GSEA, Broad Institute, United States). For visualization of protein-protein interactions STRING10 analysis was used (38).

Statistical Analysis

Data on initial outgrowth of colonies from cord- or peripheral blood MNC are given as Mean ± SD. Data on subcultured ECFCs are expressed as means ± SEM. At least four independent experiments, with ECFCs isolated from different donors, were performed for all analyses, unless otherwise indicated. Single comparisons were made with Student's *t* tests for normally distributed data or the Wilcoxon matched-pairs signed rank test for data not normally distributed. Comparisons between multiple groups were performed using one- or two-way ANOVA with Bonferroni *post hoc* test. Significance was defined as a $p < 0.05$.

RESULTS

Hypoxia Impairs the Initial Outgrowth of ECFCs From Cord Blood—Derived Mononuclear Cell Fraction

The development of colonies of ECFCs from the MNC fraction of blood takes 10–14 days before they become visible and start to expand rapidly (also referred to as late-outgrowth EPCs). To investigate whether hypoxia stimulates the initial outgrowth of ECFCs from progenitor cells that reside within the MNC fractions of cord- and peripheral blood, CB-MNC inoculates of 14 individual donors were each incubated at 1% or 20% O_2 (both with 5% CO_2) for up to 30 days with regular renewal of the culture medium. The cultures were monitored daily for presence of colonies. ECFC colonies became detectable after 8–14 days and subsequently monitored and counted three times a week till the colonies started to merge. If no or only a few colonies were observed, the incubation was continued up to 30 days (to check for late coming colonies). The initial ECFC colonies were visible as densely packed cobblestone cell monolayers, which display VE-cadherin and cortical F-actin staining and multiple cell divisions (**Figures 1A–C**). There was no differences in morphology or differences in immunohistochemical characterization between colonies obtained at 20% or 1% of oxygen (the last one shown in **Figures 1A–C**).

At 20% O_2, on average 13.9 (± 8.5) colonies were obtained from cultures of CB-MNCs (**Figure 1D**; individual data in **Supplemental Table 1**). However, under 1% O_2 conditions 11 out of these 14 CB-MNCs generated no CB-ECFC colony outgrowth at all, while CB-ECFC outgrowth was limited to 2 colonies per isolation in the remaining 3 cultures. This failing in initiation of outgrowth resulted in a significant reduction ($p < 0.001$) of the mean number of CB-ECFCs colonies per isolation from 13.9 ± 8.5 (20% O_2) to 0.4 ± 0.9 (1% O_2) (**Figure 1D** and **Supplemental Table 1**).

Initial Clonal Outgrowth of PB-ECFCs in Hypoxia

The initial outgrowth from PB-MNCs of nine individual adult donors also displayed a reduction in the number of ECFCs colonies developed in hypoxic PB-MNCs cultures as compared to their counterparts exposed to 20% oxygen levels. However, this effect was less extreme than in CB-ECFCs. Initiation of

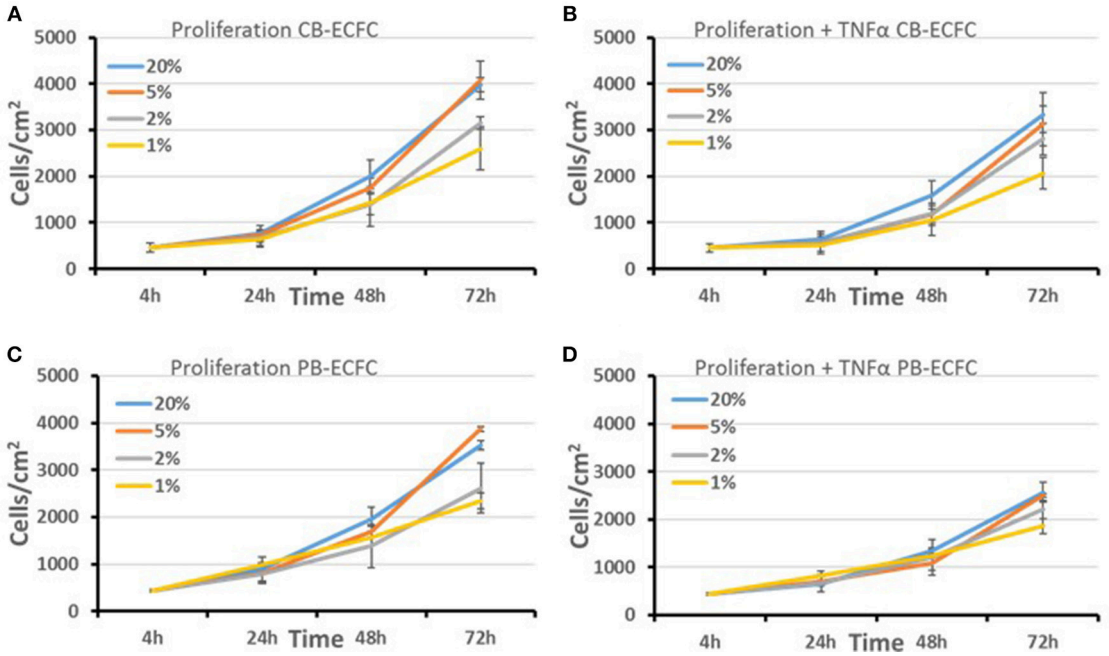


FIGURE 2 | Proliferation of subcultured CB and PB-ECFCs at various oxygen concentrations. The effect of oxygen concentration (20, 5, 2, and 1%) in the absence **(A**,**C)** or presence of 10 ng/ml TNFα **(B**,**D)** on the proliferation rate of subcultured CB-ECFCs, expressed as mean number of cells/cm^2 of 3 CB-ECFC and 3 PB-ECFC isolations of different donors ± SEM, is shown.

outgrowth was observed in 6 out of 9 (= 66% of total isolations) resulting in a significant ($p < 0.05$) reduction of the mean number of ECFCs colonies per isolation from 4.7 ± 3.4 (20% O_2) to 1.4 ± 1.7 (1% O_2) (**Figure 1E** and **Supplemental Table 1**). At the end of the colony isolation and outgrowth period of 30 days three times more colonies were counted in normoxic than in hypoxic cultures. However, those colonies that developed at 1% O_2 also expanded rapidly, close to those that developed at 20% O_2. Although PB-ECFCs are less sensitive to the outgrowth arrest observed with CB-ECFCs, our data indicate that hypoxia by itself is not suitable to speed up the clonal outgrowth of initial ECFC colonies.

Effect of Oxygen Atmosphere on the Proliferation of Subcultured ECFCs

To evaluate whether a decrease in cell division rate could explain the lack of initial colony outgrowth we cultured already established CB-ECFCs and PB-ECFCs at low density in various oxygen atmospheres and assayed their proliferation during their logarithmic growth phase (**Figures 2A,C**). For each of these conditions the oxygen atmosphere (1, 2, 5, and 20%) was maintained during all manipulations, including microscopic inspection of cells and renewal of medium, which was pre-balanced at the appropriate O_2 atmosphere (30). From the obtained proliferation data we calculated the number of divisions and cell duplication time. As a colony of 32 cells is easily detectable during initial outgrowth assay and the division time of CB-ECFCs is 1.18- to 1.30-fold faster for cells in 20% O_2 than in 1% O_2, a reduced proliferation can (partly) contribute to but does not explain the lack or reduction of initial colony formation by CB-ECFCs and PB-ECFCs, respectively. Furthermore, the proliferation rates of CB- and PB-ECFCs in 20 and 1% O_2 are comparable and do not explain the fully impaired initial colony outgrowth in CB-ECFCs. Since inflammation is an important factor during angiogenesis and thereby also involved in the (out) growth of ECFCs, we evaluated the effect of the inflammatory mediator TNFα on proliferation of subcultured ECFCs. TNFα reduced the proliferation rate of ECFCs independent of the oxygen concentrations (compare **Figures 2A,B**, and **Figures 2C,D**, respectively).

Prior Exposure to Oxygen Overcomes Hypoxia-Induced Impairment of Initial CB-ECFC Outgrowth

We hypothesized that the precursors of PB-ECFCs in the MNC fraction have been circulating in the blood, while those of CB-ECFCs may be released recently from the umbilical cord and less exposed to the oxygenated blood. Therefore, we evaluated whether temporal exposure of freshly isolated CB-MNCs to 20% oxygen contributed to the initiation of the process of ECFC colony formation by CB cells. To that end, freshly isolated CB-MNC fractions of three donors were seeded and exposed for varying periods (0–24–48–72–96 h) to ambient oxygen (20% O_2) before transferred to 1% O_2 atmosphere for an additional hypoxic culture up to 4 weeks. Colony formation was monitored during this 4 weeks of culturing and compared to colony generation while exposed continuously to 20% oxygen. **Figure 3**

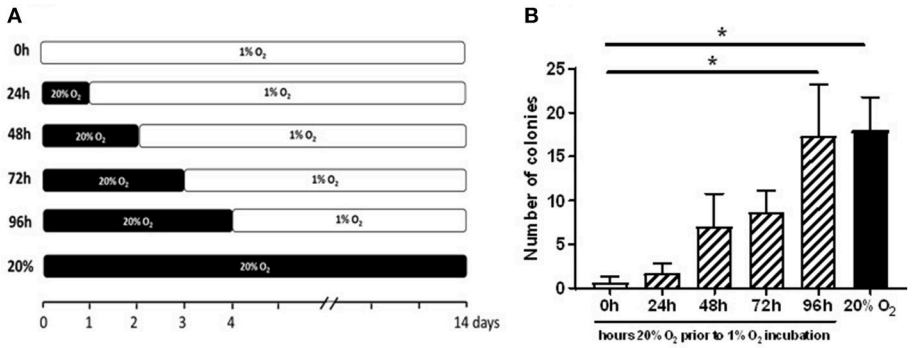


FIGURE 3 | Prior exposure to 20% O_2 restores the induction of ECFC colony formation in 1% O_2. The graph depicts enumeration of ECFC colony outgrowth from umbilical cord blood-derived MNCs. **(A)** The freshly isolated CB-MNC fractions of three donors were seeded and exposed for varying periods (0–96 h) to ambient oxygen (20% O_2) and subsequently transferred to 1% O_2 atmosphere for additional culture **(B)** ECFC colony outgrowth from umbilical cord blood-derived MNCs obtained from three different donors after 24, 48, 72, or 96 h exposure to 20% O_2. Cells cultured only in 20% O_2 (20%), or 1% O_2 (0 h) served as controls. ECFC colonies were quantified when the colonies had become visible in the culture in 20% O_2 and expressed as average number ± SEM ($n = 3$) of counted colonies. Statistical significance was determined by a One-way ANOVA with Bonferroni *post-hoc* test; $^*p < 0.05$.

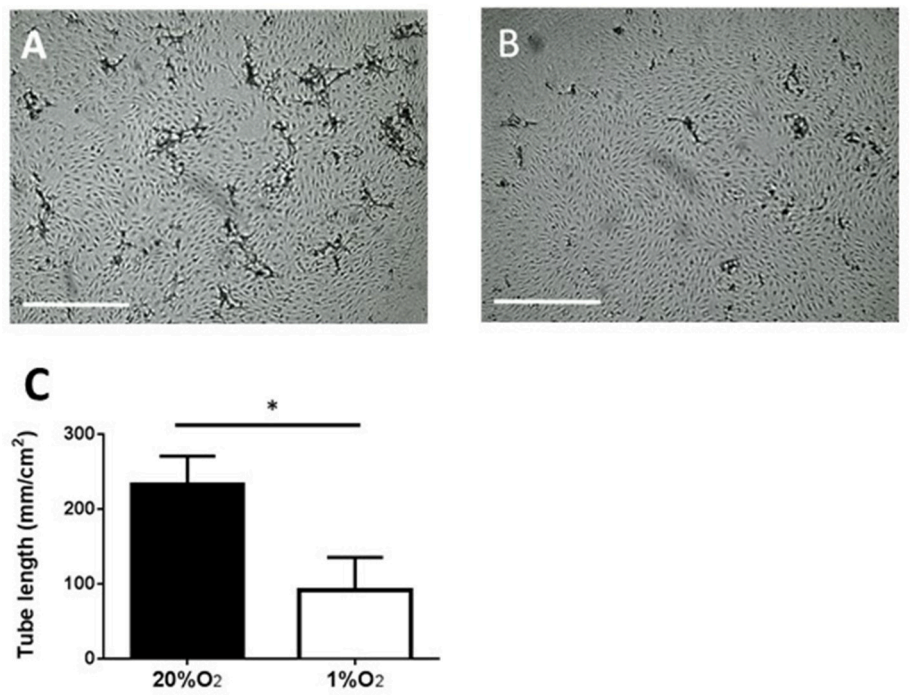
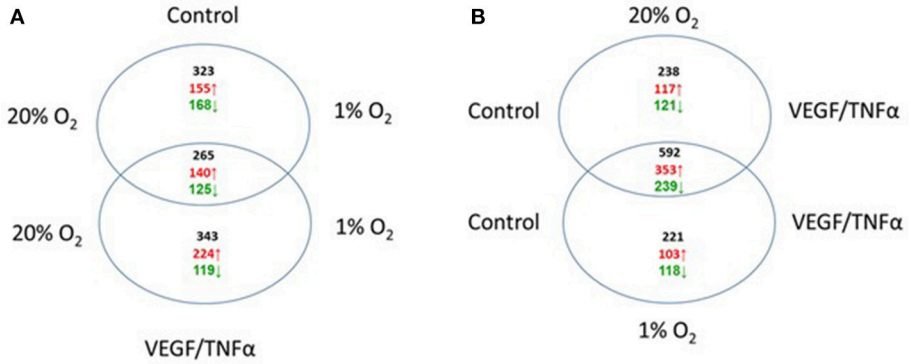


FIGURE 4 | Inhibition of tube-forming capacity of PB-ECFCs when cultured at 1% O_2. PB-ECFCs obtained from different donors were serially expanded in medium supplemented with PL at 20 or 1% of oxygen for 7 days. The PB-ECFCs were then seeded on 3D fibrin matrices and the sprouting ability of cells in fibrin matrices was then assessed after stimulation with the combination of 10 ng/ml TNFα and 25 ng/ml VEGF at 20% O_2 (phase contrast picture **A**) or 1% O_2 (phase contrast picture **B**) for a period of 96 h (bar = 1,000 μm). Results represent the mean ± SEM ($n = 7$) of the length of tube-like structures of the donors **(C)** Statistical significance between two oxygen concentration conditions was determined by unpaired *t*-test; $^*p < 0.05$).

shows that subsequent to 2–3 days of 20% O_2 pre-exposure, but not shorter, initial outgrowth of ECFC colonies occurred in hypoxia. This reached statistical significance after 4 days (mean of 18 colonies in 20% of oxygen and mean of 17 colonies after 4 days of priming at 20% O_2 and subsequent 1% O_2 incubation). The subsequent expansion of these primed colonies proceeded fast, suggesting that the prior exposure to 20% O_2 largely overcame the growth arrest during the initial incubation period.

In agreement with previous findings (24) and similar to in other types of endothelial cells, exposure of CB-ECFCs to hypoxia induced both HIF-1α and HIF-2α (**Supplemental Image 1**). Transient deletion of the combination of HIF-1α and HIF-2α residues by siRNA, which lasted for at least 72h (30), did



C 20% vs C1%		Up	Down	Total
	p<0.05	295	293	588
	P<0.01	161	150	311
	q<0.05	83	47	130

VT 20% vs VT 1%		Up	Down	Total
	p<0.05	364	244	608
	P<0.01	168	106	274
	q<0.05	54	17	71

C 20% vs VT 20%		Up	Down	Total
	p<0.05	470	360	830
	P<0.01	248	143	391
	q<0.05	88	31	119

C 1% vs VT 1%		Up	Down	Total
	p<0.05	456	357	813
	P<0.01	249	146	395
	q<0.05	90	50	140

FIGURE 5 | Venn diagram of genes upregulated of downregulated in control **(A)** and VEGF/TNFα-stimulated **(B)** PB-ECFCs.

also overcome the hypoxia-induced outgrowth arrest of ECFC colonies, similar to 20% O_2 exposure (**Supplemental Image 2**, **Supplemental Table 1** independent experiments, however each with low number of colonies).

Endothelial Tube Formation by PB-ECFCs at 1 and 20% Oxygen

Subsequently, we focused on PB-ECFCs, as their autologous nature makes them the preferential ECFC type in tissue engineering applications. For an angiogenic response endothelial cells and ECFCs not only require proliferation, but also have to form tubular structures (sprouts) that invade into the hypoxic tissue. Exposure of monolayers of PB-ECFCs on top of a 3D fibrin matrix to the combination of VEGF/TNFα indeed induced endothelial tubule invasion in the fibrin matrix when cultured in the presence of 20% O_2. However, similar as earlier observed with human microvascular endothelial cells (30), the continuous exposure to 1% hypoxia during sprout formation significantly inhibited (59% reduction compared to PB-ECFCs at 20% O_2, $p < 0.05$) tubule outgrowth from the PB-ECFC monolayer (**Figure 4**). At 20% O_2, tubule formation in fibrin fully depends on the availability of u-PA and uPAR, which indicates that migration/invasion plays a pivotal role (33, 34). On the other hand, the effect of hypoxia on proliferation of subcultured PB-ECFCs was limited–also after TNFα-stimulation (**Figure 2D**), and may contribute only in a limited way to the reduction of tube formation. These effects reflect earlier findings in human microvascular endothelial cells (30).

Gene Array Analysis of Hypoxic Response of Subcultured PB-ECFCs

While HIF-1α enhances endothelial sprouting, HIF-2α has been suggested to stabilize endothelial tubules and limits endothelial sprouting (29). We recently identified by siRNA screen four HIF-2α-regulated genes that inhibited endothelial sprouting during prolonged hypoxia: ARRDC3, MME, PPARG, and RALGPS2 (36). To evaluate whether these genes were also regulated during the response of PB-ECFCs to hypoxia, we analyzed gene expression of a pool of 10 subcultured PB-ECFCs (from 5 male and 5 female donors) that were exposed for 24 h to 20% O_2 or 1% O_2, both in control and in VEGF/TNFα-stimulated conditions as tubule formation was induced by the simultaneous exposure to VEGF-A and TNFα. **Figure 5** summarizes the differential gene expression at the $p < 0.05$, $p < 0.01$, and $q < 0.05$ levels as revealed by genome-wide RNA-sequencing. It shows that in control and VEGF/TNFα-stimulated PB-ECFCs hypoxia altered the expression of 588 and 608 genes, respectively ($p < 0.05$). From these gene populations making up together 931 genes only 265 overlapped (140 up- and 125 down-regulated) (**Figure 5**). When the effects of VEGF/TNFα exposure was compared in ECFCs exposed to 1 or 20% O_2, the overlap accounted 592 out of a total of 1041 genes (**Figure 5**). The top 25 up and down regulated genes ($q < 0.05$) are shown in **Tables 1A-D**, and all the genes ($p < 0.05$) are listed in **Supplemental Data Sheets 1**, **2**. Pathway analysis using the online tools Gene Set Enrichment Analysis (GSEA) and the WEB-based GEne SeT AnaLysis Toolkit (Webgestalt) revealed that the upregulated genes by hypoxia showed an enrichment in several metabolic pathways (see **Figure 6B**) including amino acid metabolisms, glycolysis/gluconeogenesis, and carbon-, fructose- and mannose metabolism. The downregulated genes were categorized mainly in cell cycle (**Figure 6B**), p53 signaling pathway and cytokine-cytokine receptor interactions (**Table 2**). The major regulated pathways were enriched in both control and VT-treated PB-ECFCs. These data comply with anticipated

TABLE 1A | Top ($q < 0.05$) 25 Up regulated genes by hypoxia (non-stimulated PB-ECFCs).

Gene symbol	*n*-fold	Name
PIK3R6	107,9	phosphoinositide-3-kinase, regulatory subunit 6
MIR210HG	22,48	MIR210 host gene (non-protein coding)
ANGPTL4	17,06	angiopoietin-like 4
GDF6	16,87	growth differentiation factor 6
SYTL2	16,32	synaptotagmin-like 2
PROM1	15,77	prominin 1
VEGFA	12,62	vascular endothelial growth factor A
ENO2	10,35	enolase 2 (gamma, neuronal)
SLC2A1	10,02	solute carrier family 2 (facilitated glucose transporter), member 1
INHBA	10	inhibin, beta A
DNAH8	9,74	dynein, axonemal, heavy chain 8
ADM2	9,19	adrenomedullin 2
FBLN2	8,85	fibulin 2
STC2	8,82	stanniocalcin 2
ELN	8,4	Elastin
GALNTL2	7,58	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2
PODN	7,11	Podocan
FER1L4	6,35	fer-1-like 4 (C. elegans) pseudogene
ALDOC	6,13	aldolase C, fructose-bisphosphate
AK4	6	adenylate kinase 4
NPTX1	5,92	neuronal pentraxin I
SDC2	5,38	syndecan 2
SMAD7	5,37	SMAD family member 7
TGFBI	5,29	transforming growth factor, beta-induced, 68 kDa
ALDH1L2	5,28	aldehyde dehydrogenase 1 family, member L2

TABLE 1B | Top ($q < 0.05$) 25 Up regulated genes by hypoxia (VT-stimulated PB-ECFCs).

Gene symbol	*n*-fold	Name
EGLN3	61,41	egl nine homolog 3 (C. elegans)
NUPR1	13,83	nuclear protein, transcriptional regulator, 1
FABP3	12,52	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
PPARG	9,30	peroxisome proliferator-activated receptor gamma
TGFBI	9,22	transforming growth factor, beta-induced, 68kDa
DNER	7,56	delta/notch-like EGF repeat containing
SLC8A3	7,23	solute carrier family 8 (sodium/calcium exchanger), member 3
SYTL2	6,43	synaptotagmin-like 2
HIF3A	6,38	hypoxia inducible factor 3, alpha subunit
ANGPTL4	6,16	angiopoietin-like 4
COL25A1	5,05	collagen, type XXV, alpha 1
PHGDH	4,95	phosphoglycerate dehydrogenase
NDRG1	4,81	N-myc downstream regulated 1
ENO2	4,65	enolase 2 (gamma, neuronal)
ADM2	4,65	adrenomedullin 2
CSPG5	4,48	chondroitin sulfate proteoglycan 5 (neuroglycan C)
JDP2	4,39	Jun dimerization protein 2
GPNMB	4,38	glycoprotein (transmembrane) nmb
KLF4	4,28	Kruppel-like factor 4 (gut)
VEGFA	3,99	vascular endothelial growth factor A
NPTX1	3,94	neuronal pentraxin I
SLC2A1	3,88	solute carrier family 2 (facilitated glucose transporter), member 1
MEGF6	3,83	multiple EGF-like-domains 6
MN1	3,82	meningioma (disrupted in balanced translocation) 1
COL8A2	3,72	collagen, type VIII, alpha 2

roles for cell division and metabolic control in the behavior of subcultured ECFCs in hypoxia.

Next, we looked at individual genes that were significantly altered by hypoxia and that were suggested earlier to contribute to endothelial sprouting. VEGF-A (as a positive regulator of angiogenesis) and ARRDC3 (negative regulator) were both significantly increased ($p < 0.05$; $q < 0.05$) in control (12.6- and 4.7-fold, respectively) and VEGF/TNFα-exposed cells (4.0- and 2.7-fold, respectively). MME and PPARG increased 2.2- and 1.9-fold in hypoxic control ECFCs ($p < 0.05$, q ns), while their mRNAs were increased by 3.2- and 9.3-fold in VEGF/TNFα-exposed ECFCs. No changes were observed in the expression of RALGPS-2. While the increase in VEGF-A may have little additional effect on cells that were already stimulated by VEGF/TNFα, changes in VEGFRs probably do. VEGFR2 decreased consistently in hypoxia-treated cells, i.e., by 50% and 51% in control and VEGF/TNFα-treated cells, respectively ($p <$ 0.05, q ns). The 40% and 12% increases in VEGFR1 were not significant, but—together with the drop in VEGFR2 - are likely to decrease ECFC sprouting and/or proliferation. In control and VEGF/TNFα-exposed cells, respectively, a 20% and 16% drop in PlGF mRNA [a negative regulator as pointed out by Hookham et al. (24)] remained statistically non-significant. in VEGF/TNFα-exposed ECFCs. No changes were observed in the expression of RALGPS-2. ANGPL4, which was recently recognized as a Wnt signaling antagonist (39), HIF3A and EGLN3 (PDH-3), were strongly upregulated, particularly in VEGF/TNFα exposed ECFCs (all p and $q < 0.05$).

DISCUSSION

Data presented in this study indicate that hypoxia does not stimulate, but impairs the initial outgrowth of ECFC colonies from cord and peripheral blood. This inhibition is stronger in CB-ECFCs than in PB-ECFCs. In CB-ECFCs hypoxia causes an initial outgrowth arrest that could largely be overcome by a 3–4

TABLE 1C | Top ($q < 0.05$) 25 Down regulated genes by hypoxia (non-stimulated PB-ECFCs).

Gene symbol	*n*-fold	Name
PRND	−22,49	prion protein 2 (dublet)
APLNR	−18,14	apelin receptor
GJA4	−15,17	gap junction protein, alpha 4, 37 kDa
INHBB	−6,37	inhibin, beta B
AQP1	−5,00	aquaporin 1 (Colton blood group)
RPS17	−4,88	ribosomal protein S17
RPS17L	−4,83	ribosomal protein S17-like
CDC20	−4,28	cell division cycle 20 homolog (S. cerevisiae)
LYVE1	−4,10	lymphatic vessel endothelial hyaluronan receptor 1
NUF2	−4,02	NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)
HMMR	−3,74	hyaluronan-mediated motility receptor (RHAMM)
NQO1	−3,70	NAD(P)H dehydrogenase, quinone 1
UCP2	−3,63	uncoupling protein 2 (mitochondrial, proton carrier)
CKAP2L	−3,62	cytoskeleton associated protein 2-like
BUB1B	−3,54	budding uninhibited by benzimidazoles 1 homolog beta (yeast)
PAK6	−3,54	p21 protein (Cdc42/Rac)-activated kinase 6
PLK1	−3,52	polo-like kinase 1
ANLN	−3,28	anillin, actin binding protein
KIF4A	−3,28	kinesin family member 4A
SHCBP1	−3,23	SHC SH2-domain binding protein 1
BIRC5	−3,21	baculoviral IAP repeat containing 5
TPX2	−3,16	TPX2, microtubule-associated, homolog (Xenopus laevis)
SPAG5	−3,11	sperm associated antigen 5
KIFC1	−3,09	kinesin family member C1
CDCA8	−3,09	cell division cycle associated 8

TABLE 1D | Top ($q < 0.05$) 17 down regulated genes by hypoxia (VT-stimulated PB-ECFCs).

Gene symbol	*n*-fold	Name
CCL8	−23,88	chemokine (C-C motif) ligand 8
C3	−5,96	complement component 3
IL1B	−4,88	interleukin 1, beta
KYNU	−4,51	kynureninase
EFNB2	−3,95	ephrin-B2
PLK1	−3,85	polo-like kinase 1
ADAMTS1	−3,79	ADAM metallopeptidase with thrombospondin type 1 motif, 1
DLGAP5	−3,02	discs, large (Drosophila) homolog-associated protein 5
CD200	−3,02	CD200 molecule
CHST1	−2,95	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1
BUB1B	−2,66	budding uninhibited by benzimidazoles 1 homolog beta (yeast)
PAK6	−2,66	p21 protein (Cdc42/Rac)-activated kinase 6
TNFRSF11B	−2,66	tumor necrosis factor receptor superfamily, member 11b
TOP2A	−2,65	topoisomerase (DNA) II alpha 170 kDa
BMX	−2,61	BMX non-receptor tyrosine kinase
RRM2	−2,56	ribonucleotide reductase M2
MKI67	−2,44	antigen identified by monoclonal antibody Ki-67

days pre-incubation in ambient air (20% O_2) before the explanted MNCs were transferred to the hypoxic atmosphere. Furthermore, the proliferation rate of subcultured CB- and PB-ECFCs was comparable at 5 and 20% oxygen, while it progressively dropped after exposure of the cells at 2% and 1% O_2, independent of a proliferation reducing effect of TNFα. The ability of PB-ECFCs to form VEGF/TNFα-induced tubules in a 3D- fibrin matrix was inhibited by hypoxia. These effects were accompanied by a marked change in the expression of genes, including cell cycle, metabolism controlling, and angiogenesis controlling genes.

Initial Colony Formation in Hypoxia

In agreement with most of these studies on CB-ECFCs, hypoxia reduced proliferation. A reduced proliferation will prolong the time period before initial colonies from explanted freshly isolated CB-MNC become visible. However, the 18–30% reduction in proliferation rate that we found with subcultured CB-ECFCs is not sufficient to explain the absence of any ECFC clone after a 30 days evaluation period as seen in 11 out of 14 cultures. Other possibilities may regard a differentiation arrest of the progenitor cells that develop into ECFC colonies or the loss of a positive interaction (or induction of a negative interaction) between hypoxic accessory MNCs and the endothelial progenitors. In preliminary experiments we could rule out the accumulation of soluble inhibitory factors as we did not find an effect of conditioned media taken from the hypoxic CB-MNC on the clonal outgrowth of subcultured CB-ECFCs. Furthermore, as 4 days pre-incubation in 20% O_2 or transient deletion of HIF-1α and HIF-2α was sufficient to overcome the lack of colony formation it is likely that a differentiation step is needed to "awake" the rapid proliferation of CB-ECFCs. As monocyte/macrophages also express HIF-1α and HIF-2α (40), we cannot discriminate yet whether such differentiation step is within the ECFC progenitors themselves or within the accompanying mononuclear monocytes.

Several lines of research pointed to an important effect of oxygen tension on cell differentiation (41, 42). Hypoxia reversibly arrested stem cells in an undifferentiated state (42), while low oxygen tensions have also been used to maintain the pluripotency of different progenitors (43). Therefore, it is not excluded that hypoxia by maintaining a quiescence phenotype of putative endothelial progenitor cells suppresses their endothelial differentiation toward ECFCs especially if the microenvironment is devoid of pro-neovascularization clues. It is plausible that in ischemic tissues—once the pro-angiogenic environment is established—growth factors that are essential for EC differentiation such as VEFG, FGF, or HGF in conjunction with pro-angiogenic myeloid cells such as circulating angiogenic cells would eventually overcome the hypoxia-induced inhibition of differentiation of EPCs toward EC phenotype.

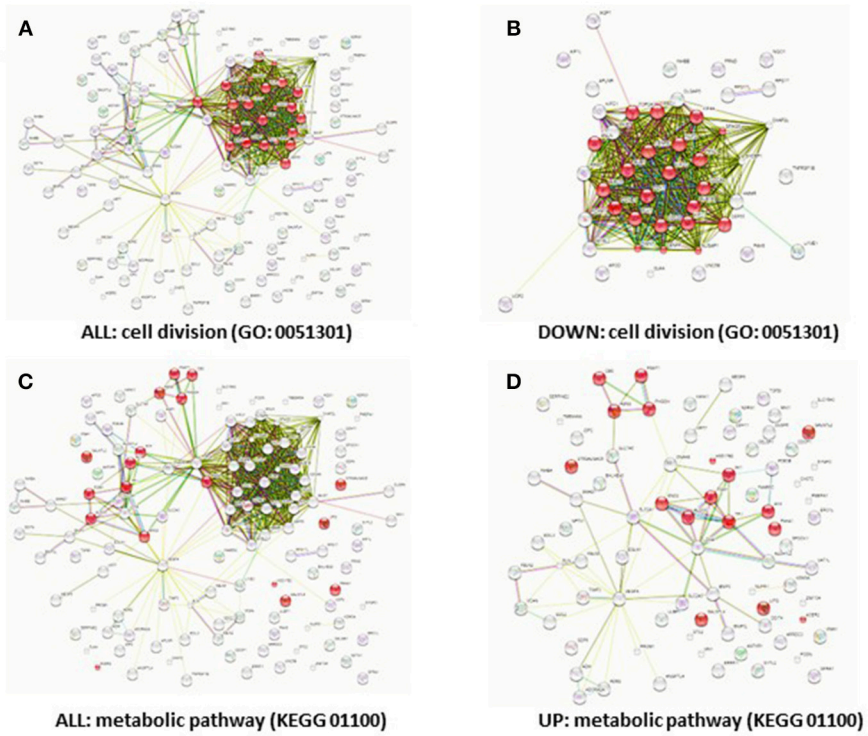


FIGURE 6 | Downregulation of cell cycle genes and upregulation of metabolic genes by hypoxia. Predicted protein-protein interactions show different protein clusters. Genes that were significantly differentially regulated (FDR < 5%) in hypoxia **(A,C)**, down-regulated **(B)** or upregulated in hypoxia **(D)** were clustered based on protein-protein interactions. The nodes represent the proteins and a shared function of the proteins are shown as interconnecting lines. The genes were clustered based on the GO (0051301) cell division pathway **(A,B)** or the KEGG (01100) metabolic pathway; genes involved in these pathways are indicated in red.

The initial outgrowth of PB-ECFCs was also inhibited by hypoxia, but to a much lesser extent than that of CB-ECFCs. The difference in the ability of CB-MNCs and PB-MNCs to generate primary clones under normoxia and hypoxia suggests that beside oxygen tension other factors play an important role in the initial outgrowth of ECFCs from MNCs. The microenvironment which imprint stem and progenitor cells during fetal and post-natal life can accounted for by observed differences between CB-MNCs and PB-MNCs in their ability to generate ECFCs colonies. Furthermore, to the best of our knowledge, there is no information about the circulation time of CB- and PB-endothelial progenitors. Hence, we cannot exclude that PB-endothelial progenitors have been exposed to the oxygenated milieu of the blood for a longer period than their CB counterparts. Once isolated and cultured, ECFCs obtained from umbilical cord blood differ from PB-ECFCs with respect to proliferation (43) gene expression (44–46), and *in vivo* vessel formation (46, 47).

Hypoxia and the Propagation of Subcultured ECFCs

Hypoxia not only inhibited the initial outgrowth of ECFCs from CB- and PB-MNCs but also has an impact to the clonal and proliferative ability of subcultured cells. Interestingly, the CB-ECFCs displayed a small reduced proliferation at 1% O_2 which is in agreement with previously reports (23, 24), while similar data have also been reported very recently on PB-ECFCs (25). The whole genome sequencing data also showed a change in metabolic pathways and in cell cycle genes during exposure to hypoxia comparable with that of human foreskin MVECs (36). While the metabolic adaptation helps the cell to overcome the limitation of energy supply, the suppression of cell cycle genes likely contributes to the hypoxia-induced reduction of ECFC proliferation. Hypoxia-driven HIF-1α activation with subsequent proliferation cell cycle arrest in G1/S phase and induction of apoptosis has been reported as a mechanism that restricts the growth of EC obtained from post-natal tissues (48). Whatever is the transcriptomic background of observed hypoxia-induced cell proliferation in CB- and PB-ECFCs, future investigation is warranted.

During neovascularization, ECFC proliferation matches the growth of the vascular tree. Our finding that hypoxia reduces ECFC proliferation is contra-intuitive suggesting that other factors that play a crucial role during *in vivo* neovascularization and are accountable for proper *in vivo* multiplication of EC are absent in our *in vitro* hypoxic assay. Indeed, rapid neovascularization reported in animal studies of addition of VEGF to ECFCs (49) as well as co-implantation of ECFCs with myeloid cells (50) or MSCs (51) pinpoint to the necessity to include these clues during *in vitro* assaying in order to unravel the true behavior of ECFCs under hypoxia.

TABLE 2 | Hypoxia pathway analysis.

Non-stimulated PB-ECFCs		VT-stimulated PB-ECFCs	
Pathway name	**Statistics**	**Pathway name**	**Statistics**
Metabolic pathways	$p = 3.25e{-}08$	Metabolic pathways	$p = 9.94e{-}05$
Cell cycle	$p = 1.78e{-}05$	Cell cycle	$p = 0.0027$
Glycolysis / Gluconeogenesis	$p = 1.78e{-}05$	Glycolysis / Gluconeogenesis	$p = 0.0088$
Renal cell carcinoma	$p = 0.0003$	Renal cell carcinoma	$p = 2.92e{-}06$
Glycine, serine and threonine metabolism	$p = 0.0003$	Glycine, serine and threonine metabolism	$p = 0.0030$
TGF-beta signaling pathway	$p = 0.0003$	TGF-beta signaling pathway	$p = 0.0112$
p53 signaling pathway	$p = 0.0031$	p53 signaling pathway	$p = 0.0088$
Purine metabolism	$p = 0.0037$	Purine metabolism	$p = 0.0052$
Pentose phosphate pathway	$p = 0.0068$		
Mucin type O-Glycan biosynthesis	$p = 0.0077$		
Cysteine and methionine metabolism	$p = 0.0093$		
Fructose and mannose metabolism	$p = 0.0093$		
Cytokine-cytokine receptor interaction	$p = 0.0140$	Cytokine-cytokine receptor interaction	$p = 4.74e{-}05$
mTOR signaling pathway	$p = 0.0167$		
Adipocytokine signaling pathway	$p = 0.0247$	Adipocytokine signaling pathway	$p = 0.0088$
Pathways in cancer	$p = 0.0247$	Pathways in cancer	$p = 0.0007$
ECM-receptor interaction	$p = 0.0338$		
Ribosome	$p = 0.0371$	Ribosome	$p = 0.0064$
		PPAR signaling pathway	$p = 4.74e{-}05$
		Axon guidance	$p = 0.0219$
		Focal adhesion	$p = 0.0450$

The KEGG pathways involved in angiogenesis and metabolism with a $p < 0.05$ are show. Red is upregulated and green is downregulated.

Endothelial Tube Formation by PB-ECFCs

Hypoxia not only reduced proliferation, but also inhibited endothelial tube formation by PB-ECFCs into a fibrin matrix. This is in line with earlier observations on CB-ECFCs (22, 24). The observed inhibition of tube formation in hypoxia was highly similar to the inhibition observed in hypoxic human microvascular EC, which was largely corrected after inhibition of HIF-2α (29, 30). Hookham et al. (24) found that Placental growth factor (PlGF) was a major player in the inhibition of tube formation by hypoxic CB-ECFCs. How PlGF exerts its effect is not yet clear. As it binds to VEGFR-1 and NRP-1 (52), it may on the one hand prevent quenching of VEGF-A by VEGFR-1 (53), and at the other hand withdraw NRP-1 from assisting cis-oriented VEGFR-2 endocytosis which is required for VEGFR-2 signaling (54). Only the latter would contribute to inhibition of tubule formation. In our experimental conditions tubule formation was fully inhibited by anti-u-PA antibodies (33) or si-uPAR (34), which suggest that migration/invasion plays a dominant role in tube formation in the fibrin matrix. A comparable mechanism has been observed in tubule formation by human microvascular endothelial cells, a process that depended on migration/invasion independent of proliferation (35).

The suppression of ECFC sprouting and proliferation by hypoxia seems contra-intuitive, but it may reflects the monolayer stabilizing properties of endothelial cells induced by HIF-2α. This response normally balances the sprouting-inducing effect of HIF-1α (29). In an hypoxic environment the surrounding non-endothelial tissue cells mainly support the HIF-1α part of this balance. However, the suggestion of a HIF-2α-suppressed ECFC sprouting contrasts to a very recent study of He et al. (25), who demonstrated by specific inhibition that inhibition of HIF-1α, but not HIF-2α, could overcome the hypoxia-induced inhibition of angiogenesis. This conclusion, which is opposite to studies on microvascular endothelial cells (29, 30), needs further evaluation and underpinning.

Altogether, there is firm consensus that hypoxia suppresses proliferation and sprouting of CB- and PB-ECFCs *in vitro*, but how the variety of suggested mediators participate and interact requires further elucidation. Notwithstanding, the crucial question raised by our findings is "why is there poor outgrowth of circulating ECFCs in hypoxia, while expansion of endothelial cells would be essential for vessel repair or new vessel forming abilities in hypoxic conditions?" One cannot exclude that this reflects the fact that ECFCs were studied in isolation, while the presence of many hypoxic tissue cells may shift the balance more into pro-angiogenic direction. Alternatively, one may anticipate that the highly proliferation of ECFCs can in particular contribute to new vessels with improved tissue circulation at the interface of the hypoxic tissue and circulating blood. If non-perfused vessel-like structures would be made in the center of a chronic hypoxic environment, it would take much energy without improvement of blood and oxygen supply. However, under these conditions, alternative

sources of "endothelial precursor cells" e.g., the quiescent resident endothelial cells in vessels (55) or endothelial cells derived from erythro-myeloid progenitors (56) might be involved. Getting more insight in all these alternatives is needed to improve the use of endothelial precursor cells and ECFCs in regenerative medicine.

ETHICS STATEMENT

The collection of cord and peripheral blood was approved and conducted according to the guidelines by the Medical Ethics Committee of the VU University medical center in Amsterdam, Netherlands.

AUTHOR CONTRIBUTIONS

DT prepared, performed and evaluated experiments (on PB-ECFCs) and made initial draft of the manuscript. LD-V prepared, performed and evaluated experiments (on CB-ECFCs) and made initial draft of the manuscript. MvW performed experiments. HB was involved in supervising and evaluating experiments, corrected the manuscript. PK planned and supervised experiments, evaluated and assembled data, corrected the manuscript. VvH planned and supervised, final coordination of the manuscript.

FUNDING

This work was supported by Grant 1.6 of the Netherlands Institute for Regenerative Medicine (NIRM).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2018.00356/full#supplementary-material

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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.

frontiers in Medicine

MINI REVIEW
published: 30 January 2019
doi: 10.3389/fmed.2019.00010

Endoglin as an Adhesion Molecule in Mature and Progenitor Endothelial Cells: A Function Beyond TGF-β

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Endoglin (ENG) is a transmembrane glycoprotein expressed on endothelial cells that functions as a co-receptor for several ligands of the transforming growth factor beta (TGF-β) family. ENG is also a recognized marker of angiogenesis and mutations in the endoglin gene are responsible for Hereditary Hemorrhagic Telangiectasia (HHT) type 1, a vascular disease characterized by defective angiogenesis, arteriovenous malformations, telangiectasia, and epistaxis. In addition to its involvement in the TGF-β family signaling pathways, several lines of evidence suggest that the extracellular domain of ENG has a role in integrin-mediated cell adhesion via its RGD motif. Indeed, we have described a role for endothelial ENG in leukocyte trafficking and extravasation *via* its binding to leukocyte integrins. We have also found that ENG is involved in vasculogenic properties of endothelial progenitor cells known as endothelial colony forming cells (ECFCs). Moreover, the binding of endothelial ENG to platelet integrins regulate the resistance to shear during platelet-endothelium interactions under inflammatory conditions. Because of the need for more effective treatments in HHT and the involvement of ENG in angiogenesis, current studies are aimed at identifying novel biological functions of ENG which could serve as a therapeutic target. This review focuses on the interaction between ENG and integrins with the aim to better understand the role of this protein in blood vessel formation driven by progenitor and mature endothelial cells.

Keywords: endoglin, endothelial progenitors, EPC, ECFCs, HHT1, integrins, TGF-β

OPEN ACCESS

Edited by:
Peter S. Steyger, Oregon Health and Science University, United States

Reviewed by:
Eleni Papakonstantinou, Aristotle University of Thessaloniki, Greece
Valerie Kouskoff, University of Manchester, United Kingdom
Michelle Letarte, Hospital for Sick Children, Canada

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *27 June 2018*
Accepted: *14 January 2019*
Published: *30 January 2019*

Citation:
Rossi E, Bernabeu C and Smadja DM (2019) Endoglin as an Adhesion Molecule in Mature and Progenitor Endothelial Cells: A Function Beyond TGF-β. Front. Med. 6:10. doi: 10.3389/fmed.2019.00010

ENDOGLIN STRUCTURE AND FUNCTION

Endoglin (ENG; also known as CD105) is a type I transmembrane glycoprotein predominantly expressed in endothelial cells (ECs) (1, 2) and endothelial colony-forming cells (ECFCs) (3). It is known as an essential co-receptor for the transforming growth factor β (TGF-β) family, playing an important role in angiogenesis. At the cell surface, ENG associates with TGF-β type I receptors, including the ECs-specific ALK1 (activin receptor-like kinase 1) and the ubiquitous ALK5, as well as to a TGF-β type II receptor. In this receptor complex, ENG and ALK1 are able to bind to bone morphogenetic protein 9 (BMP9; also known as growth differentiation factor 2 [GDF2]), a member of the TGF-β family, with much higher affinity than to TGF-β1 and mediate its proliferation signal (4–7).

Upon ligand binding, the TGF-β signaling receptor complex phosphorylates members of the Smad family of transcription factors which, in turn, undergo nuclear translocation to regulate gene expression (**Figure 1A**).

In addition to the membrane bound form of endoglin, a circulating form of the ENG ectodomain, was found to be released upon a C-terminal cleavage of ENG by matrix metalloproteinase 14 (MMP-14) (8, 9). Increased levels of circulating endoglin have been detected at early stages of preeclampsia (5, 10) in hypertensive or diabetic patients (11) and in certain cancer patients (12), suggesting its role as a predictive biomarker in these pathologies. Interestingly, a detailed characterization of plasma from women with preeclampsia has revealed that circulating endoglin can be complexed within exosomes, rather than as individual soluble endoglin (13).

A pioneer article on human ENG described that this glycoprotein is present on ECs as a dimer (two subunits of ∼90-kDa), each displaying the tripeptide arginine-glycine-aspartic acid (RGD) in the extracellular region of the protein (1). The RGD motif and homologous sequences in mouse and pig are recognition motifs for cell surface integrins present in extracellular matrix proteins such as fibronectin, tenascin, thrombospondin, vitronectin, von Willebrand factor as well as fibrinogen and prothrombin (14–16). Integrin-mediated adhesion is involved in hemostasis, thrombosis, and inflammation, processes in which the endothelium plays a critical role. Therefore, the presence of the RGD motif within a hydrophilic environment (1) and its accessibility in the 3D structure of ENG (7), clearly suggest the involvement of ENG in integrin binding, as postulated in early studies (17, 18). In fact, endothelial endoglin binds to leukocyte integrins, allowing leukocyte extravasation (19) suggesting a possible novel function for ENG independent of TGF-β signaling (**Figures 1B,C**). Several lines of evidence support the role of ENG in leukocyte trafficking: (i) ENG expression is markedly up regulated in endothelial cells of inflamed tissues with an associated inflammatory cell infiltrate (19); (ii) ENG is up-regulated in the post-ischemic kidney and ENG-haploinsufficient mice are protected from renal ischemia-reperfusion injury, due to a reduction of cellular inflammatory responses (20); (iii) Arterial, venous, and capillary endothelia in lymphoid organs are highly reactive with anti-ENG antibodies and a marked staining pattern is observed in high endothelial venules (21); and (iv) Although ENG is present throughout the vascular endothelium, its expression, as compared to veins or arteries, is stronger in capillaries, where most leukocyte infiltration to organs occurs.

In addition to leukocytes, other cell types present in the circulatory system have also revealed an integrin-mediated adhesion activity toward endothelial endoglin (22–24) (**Figures 1D,E**). Thus, endoglin plays a role in integrin-mediated adhesion of vascular mural cells to endothelium (23). Also, a new role for endoglin in αIIbβ3 integrin-mediated adhesion of platelets to the endothelium, conferring resistance of adherent platelets to detachment has been described (24) (**Figure 1E**).

VASCULAR PATHOPHYSIOLOGY OF ENDOGLIN IN HEREDITARY HEMORRAGIC TELANGECTASIA (HHT)

Hereditary Hemorrhagic Telangiectasia (HHT) is an autosomal dominant disease with a prevalence of one case per 5,000–8,000 individuals (25, 26). HHT patients develop muco-cutaneous lesions known as telangiectasia in the nose, mouth, and gastrointestinal tract, as well as larger AVMs in major organs such as the lung, liver, and brain (25). The telangiectasia are comprised of fragile vessels that are susceptible to rupture and hemorrhage, which means that HHT patients can suffer recurrent anemia following frequent and severe bleeding episodes. Mutations in the endoglin gene (*ENG*) are responsible for HHT type 1 (HHT1) (27), while mutations in activing receptor-like kinase (*ACVRL1* alias *ALK1*) are responsible for HHT2 (2, 27). Taken together, HHT1 and HHT2 account for more than 80% of all HHT cases, whereas a small number of patients show mutations in SMAD4 (*MADH4*) or in BMP9 gene (*GDF2*), which are responsible for less common HHT variants such as a combined syndrome with juvenile polyposis (JP-HHT) and HHT5, respectively (28, 29). Remarkably, all of these genes code for proteins involved in the TGF-β family signaling pathways (30). Nowadays, the most widely accepted hypotheses for AVMs formation in HHT1 and HHT2 involves a "first hit" (gene haploinsufficiency) based on the loss of one functional allele $Eng^{+/-}$ (HHT1) or $ALK1^{+/-}$ (HHT2), which causes a 50% reduction in protein expression, followed by a "second hit" based on an angiogenic trigger such as inflammation, hypoxia or vascular injury (31). Together, these events may induce a deficient endothelial function of ENG that could result in HHT vascular lesions. The relevant role of endoglin in the pathophysiology of the vascular system is illustrated by the phenotype of different HHT1 animal models (32). While endoglin heterozygous mice are viable and reproduce the vascular phenotype of HHT patients, total loss of ENG expression leads in mice to cardiovascular defects and embryonic death by mid-gestation (33–35). A conditional knock out mouse model for HHT1 has revealed that the arteriovenous malformations (AVMs), induced upon ENG loss, appear to be the result of delayed vascular remodeling and inappropriate ECs proliferation responses (36). In ENG null mice, vasculogenesis does not seem affected, suggesting that ENG is not required for initial differentiation of ECs or formation of a primitive vascular plexus. The yolk sac of $Eng^{-/-}$ embryos has enlarged fragile vessels, while the embryo proper shows cardiac cushion defects and delayed maturation of major vessels (34). The reason why the total loss of ENG expression leads to cardiovascular defects and embryonic death is not completely understood. Some authors have postulated that the primary defect is in the heart (37), while others suggest that the loss of smooth muscle cells (SMCs) coverage of the endothelium could play a decisive role in the lethality of $Eng^{-/-}$ animals (34).

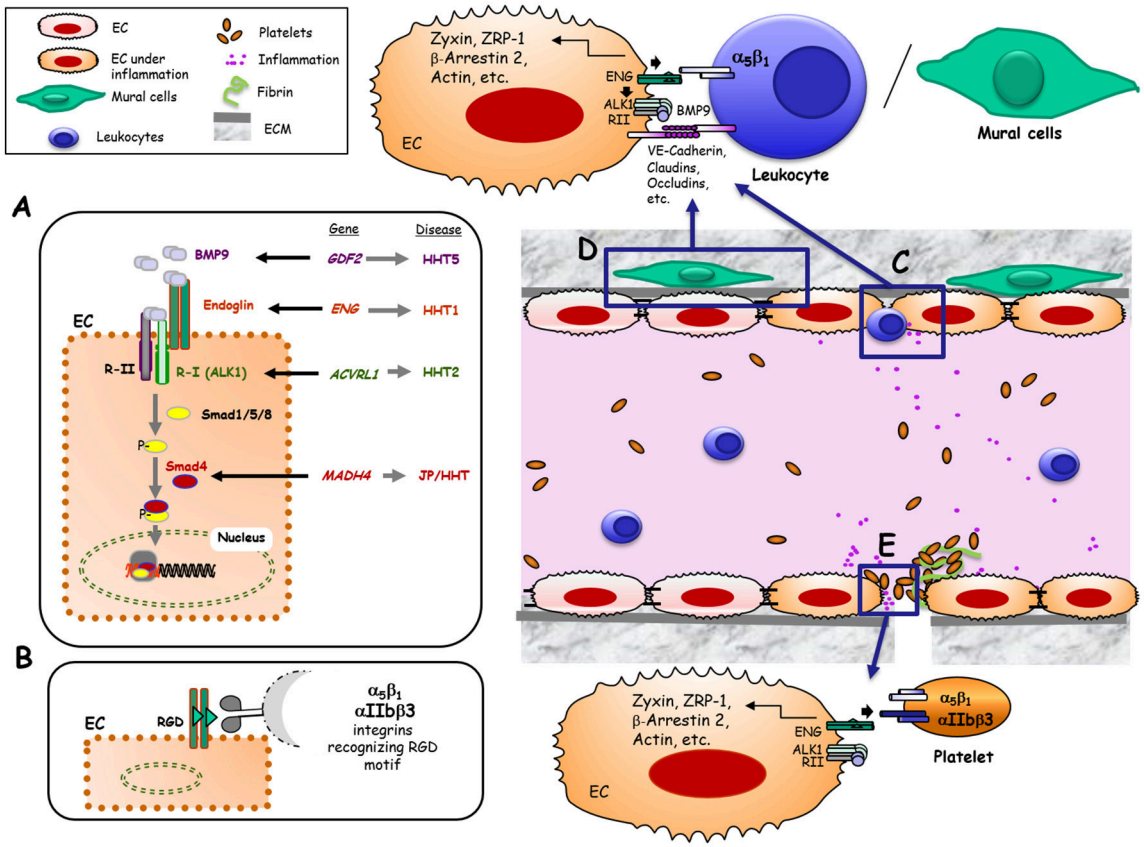

FIGURE 1 | Hypothetical model of Endoglin and its role as TGF-β co-receptor and adhesion molecule in different physiological contexts. **(A)** Canonical pathway that implicates Eng as a co-receptor of TGF-β in ECs. Bone morphogenetic protein 9 (BMP9), and other members of the TGF-β family, bind to an EC receptor complex composed by the type I (R-I) receptor named ALK1 and the type II (R-II) receptor, both exhibiting serine/threonine kinase activity, as well as the auxiliary receptor endoglin. Upon ligand binding, the R-II transphosphorylates ALK1, which subsequently propagates the signal by phosphorylating the receptor-regulated Smad (R-Smad) family of proteins Smad1/5/8. Phosphorylated R-Smads form heteromeric complexes with Smad4, translocating into the nucleus to regulate the transcriptional activity of different target genes. BMP9, Endoglin, ALK1 and Smad4 proteins are encoded by GDF2, ENG, ACVRL1 and MADH4 genes, whose pathogenic mutations give rise to HHT5, HHT1, HHT2, and JPHT, respectively (30). **(B)** Endothelial endoglin as an adhesion molecule involving its RGD sequence when binding to cell surface integrins from leukocytes, VMCs or platelets. **(C)** Leukocyte transmigration through the vessel endothelium. Under inflammatory conditions, different soluble factors are released leading to leukocyte adhesion and transmigration through ECs. This process is mediated, at least, by the interaction between leukocyte integrin α5β1 and endothelial endoglin involving the specific recognition of the RGD motif in Eng (22). **(D)** Adhesion between endothelial cells and vascular mural cells (VMCs). This intercellular association involves, at least, the interaction between integrin α5β1 in VMCs and endothelial endoglin via the specific recognition of the RGD motif in Eng (23). **(E)** Platelet-dependent hemostasis. Endothelial endoglin via its RGD binds to platelet integrins αIIbβ3 and α5β1, contributing to stabilize platelets adhesion to endothelium (24).

ENDOGLIN INTERACTING PARTNERS IN THE VASCULATURE

Previous studies reported that endothelial ENG is associated with several proteins critically involved in endothelial cell adhesion, proliferation, migration, angiogenesis, and vascular permeability, including integrins (38), zyxin (39), zyxin-related protein 1 (ZRP-1) (40), VEGF receptor type 2 (VEGFR-2) (41), beta-arrestin 2 (42), and vascular endothelial-cadherin (VE-cadherin) (43). It has been shown that VE-cadherin interacts with several components of the TGF-β receptor complex, including ENG, ALK1, and the TGF-β type II receptor TGFBR2 in adherents junctions. Of note, ENG, TGFBR2, and ALK1 also interact with p21-activated kinase (PAK-1), GTP-binding proteins, and Ras-related C3 botulinum toxin substrate 2 (Rac-2), involved in endothelial barrier maintenance, cytoskeletal remodeling and cell migration (44). Using *in vitro* ECs lines derived from ENG homozygous null, heterozygous, and control mouse embryos, Jerkic and Letarte have reported that ENG-deficient mouse embryonic ECs are hyper-permeable and unresponsive to stimulation by VEGF and TGF-β1, factors that regulate vascular permeability (45). Interestingly, VE-cadherin is barely detected in ENG-deficient ECs, a finding that could explain the EC junction destabilization and impairment of TGF-β signaling. Furthermore, permeability alterations in $Eng^{+/-}$ mice are in line with findings in patients with Hereditary Hemorrhagic Telangiectasia type 1 (HHT1). Thus, ECs from HHT1 patients show a disorganized actin cytoskeleton prone to

cell breaking with changes in shear stress and blood pressure (46). This reorganization of actin filaments in HHT1 might lead to vessel hemorrhages and eventual disappearance of the capillary network, as reported in this disorder. In line with the pathogenic hypothesis of an endothelial dysfunction in HHT1, the involvement of ENG in integrin-mediated trans-endothelial leukocyte trafficking was proposed (31). Accordingly, following inflammation triggering, the extracellular region of ENG binds to leukocyte integrin α5β1 and promotes leukocyte transmigration (31). In contrast, a different experimental approach showed an increased gut inflammation and myeloid infiltration in colitis $Eng^{+/-}$ vs. control mice (47). Overall, this novel role of ENG may contribute to a better understanding of the inflammation process and the high incidence of infections in HHT patients (48). Because pericytes embrace the endothelium to regulate the blood retinal barrier, the role of endothelial endoglin in this context has also been analyzed. Thus, vascular permeability studies carried out *in vivo* showed that retinas of $Eng^{+/-}$ heterozygous mice displayed higher retinal permeability than that of $Eng^{+/+}$ mice, suggesting a destabilization of the endothelial barrier function due to ENG haploinsufficiency (23). The importance of the interaction between ECs and vascular mural cells is also underlined by studies showing that thalidomide stabilizes the vasculature in a mouse model of HHT1 (49). Indeed, pericyte recruitment and stabilization appears as a therapeutic strategy to reduce the severity of epistaxis in HHT (50). Thus, ENG, directly, or as a component of the TGF-β receptor complex may regulate ECs integrity, whereas its absence may result in vascular hyper-permeability, thus contributing to the fetal lethality associated with the homozygous null genotype (39, 40).

Although this review focuses on the role of endoglin in ECs, it is worth mentioning that endoglin is also expressed at lower levels in other cell types, some of which present in the vasculature as vascular SMCs, activated monocytes or mesenchymal cells (18, 51–53). However, a review on the endoglin role in these cell types deserves an independent study.

ENDOGLIN AND ENDOTHELIAL COLONIES FORMING CELLS (ECFCS)

The formation of blood vessels from mesoderm is driven by the recruitment of undifferentiated cells which can differentiate into endothelial lineage. In the adult, populations of bone marrow-derived endothelial progenitor cells (EPCs) are mobilized into the circulation by stimuli such as estrogen and VEGF (54). Interestingly, human EPCs could derive from very small embryonic like stem cells (55) that can also give rise to hematopoietic cells (56). Two populations of EPCs have been differentially characterized from circulating endothelial progenitors: (i) early EPCs that exhibit a hematopoietic profile with a genomic fingerprint similar to monocytes; and (ii) ECFCs, also known as blood outgrowth endothelial cells (BOECs), which are considered late EPCs with an endothelial-like genomic profile (57). ECFCs display an intrinsic tube forming capacity *in vitro* and *in vivo* and they are involved in blood vessel formation and vascular repair (58, 59). For example, upon vascular injury, tissue perfusion of blood flow involves not only angiogenic sprouting of ECs from nearby intact vessels, but also the recruitment circulating ECFCs allowing the formation of new blood vessels (60). Acting at the interface between blood and tissues, ECFCs have a remarkable ability for migration and proliferation, being key cells in angiogenesis and vascular remodeling. It is therefore not surprising that perturbations in these critical ECFCs functions contribute to several vascular pathologies. Accordingly, ECFCs have opened a new area for treatment of cardiovascular diseases using cell therapy, advancing in the knowledge of vessel reconstruction ability in postnatal life. In this line, much effort has been recently devoted to identify novel molecular targets within ECFCs able to enhance their angiogenic potential. ECFCs are positive for endoglin (CD105), VE-cadherin (CD144), CD31, VEGFR2 (KDR), EGFL7, and CD146, and negative for CD45 and CD14 (58, 59). Among these, ENG is emerging as an interesting therapeutic target based on its involvement in angiogenesis and vascular remodeling (46, 61).

Many strategies of therapeutic revascularization, based on the administration of growth factors or stem/progenitor cells from diverse sources including EPCs, have been proposed and are currently being tested in patients with peripheral arterial disease or cardiac diseases (62). Some pretreatment of ECFCs with erythropoietin (63), fucoidan (64), soluble CD146 (65), tumor necrosis factor-α (TNF-α) (66), or platelet lysates (67, 68) have also been proposed to improve the therapeutic efficacy of *in vivo* administered ECFCs. Interestingly, integrins represent a major molecular determinant of EPCs function. More specifically, integrin α4β1 is a key regulator of EPCs retention and/or mobilization from the bone marrow, while integrins α5β1, α6β1, αvβ3, and αvβ5 are involved in EPCs homing, invasion, differentiation and paracrine factor production (69). The involvement of ECFCs in angiogenesis has been shown to be essential for ischemic disease recovery (70, 71). Furthermore, the differentiation of mesenchymal stem cells (MSCs) into mural cells appears to be stimulated by their co-culture with ECFCs, whereas overexpression of the Notch ligand Jagged1 in ECs further enhanced the differentiation of MSC into pericytes (72). This cooperation prompted some authors to postulate an improvement of ECFCs efficiency by co-injecting ECFCs and SMC progenitors (73). Therefore, a combined treatment of ECFCs with mesenchymal stem/progenitor cells (MSCs/MPCs) appears to operate synergistically, enhancing neovascularization compared to either individual cell population (74, 75). Recently, we proposed a role for ENG in the ECFCs-MSCs interplay involved in the revascularization process (61). Indeed, ENG silencing in ECFCs markedly inhibited ECFCs adhesion to MSCs *in vitro*, without affecting MSCs differentiation into perivascular cells or other mesenchymal lineages. Mesenchymal stem cells (MSCs) increase muscle recovery of ECFC in HLI model and we found that ENG-silenced ECFCs co-injected with MSCs in mice abolish beneficial effects of MSCs leading to decreased vessel density and foot perfusion upon ischemia. Our results suggest ENG involvement in the crosstalk between ECFCs and MSCs, leading to vessel formation and stabilization (51) (**Figures 2A,B**).

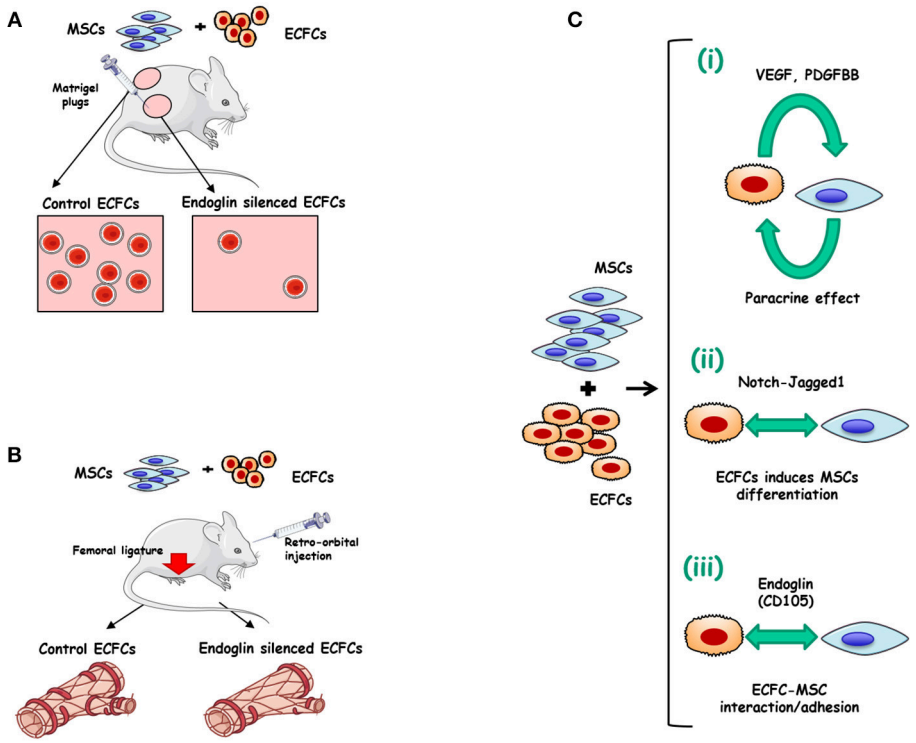

FIGURE 2 | Endoglin in ECFCs. **(A)** Role of endoglin matrigel vascularization *in vivo*. Matrigel plugs were mixed with either ECFCs treated with control siRNA plus mesenchymal stem cells (MSCs) (left) or with ECFCs treated with endoglin specific siRNA plus MSCs (right). The Matrigel mixture was injected into nude mice and the number of vessels was analyzed after 1 week. Control plugs display a marked vascularization with functional vessels (presence of erythrocytes). Plugs with endoglin-silenced ECFCs display less vascularization than controls, suggesting an important role for this protein in cell adhesion (23). **(B)** MSCs combined with ECFCs, accelerate muscle recovery in a mouse model of hind limb ischemia, through an endoglin-dependent mechanism. After femoral ligature and retro-orbital injection of ECFC+MSC Doppler analysis shows a revascularization induced by co-injection of ECFCs plus MSCs (left). This synergistic effect is abolished when endoglin is previously silenced in ECFCs (right) (61). **(C)** Mechanisms involved in the synergy between ECFCs and MSCs. (i) Mutual paracrine effect between ECFCs and MSCs involving growth factors like VEGF and PDGFBB (76, 77). (ii) ECFCs-induced differentiation of MSCs into perivascular cells via Notch-Jagged1 (72) (iii) Adhesion between ECFCs and MSCs involving endoglin (61).

As postulated in **Figure 2C**, three steps may be involved during the formation of stable vessels by ECFC and MSC and/or perivascular cells: (i) Mutual paracrine effects by VEGF and/or PDGF-BB (76, 77). (ii) ECFCs-driven perivascular maturation of MSC and/or SMC progenitors via the notch-dependent pathway; and (iii) Adhesion between ECFCs and perivascular cells in an ENG-dependent manner. The relevance of endothelial ENG adhesive properties in mature vessel formation in mice (61) is in agreement with a previous report on the active role of human ENG in the adhesion between mature ECs and vascular mural cells (23).

Relevance of ENG in ECFC has been demonstrated also in ECFCs isolated directly from HHT1 patients. Indeed, these ECFCs have disorganized and depolymerized actin fibers and impaired tube formation, as compared to healthy ECFCs (46). The molecular basis for the abnormal behavior of HHT1 ECFCs appears to be mediated, at least in part, by the regulatory role of endoglin and we can speculate *via* its interaction with zyxin family members involved in the actin cytoskeletal organization as it was proposed for endothelial cells (39, 40). Thus, the ENG cytoplasmic domain binds to zyxin and ZRP1, which concentrate at focal adhesions within actin polymerization points. Consequently, the decrease of ENG levels in HHT1 ECFCs could be related to the cytoskeleton alteration.

CONCLUSION

Blood vessel formation and remodeling are essential processes in the maintenance of tissue homeostasis and function, and therefore, their alteration causes a variety of pathologic conditions. ENG involvement in the function of mature and progenitor endothelial cells is a hot topic not completely understood and developed yet. Recent findings underline the importance of ENG not only as a co-receptor for several ligands of the TGF-β family, but also as a molecule involved in cell adhesion, which can regulate ECFCs behavior in the context of angiogenic processes. It is recognized that in mature ECs, ENG plays a key role in angiogenesis and blood vessel homeostasis, becoming a potential therapeutic target for pro- and anti-angiogenic approaches in the treatment of diseases such as HHT, cancer, preeclampsia,

diabetes complications or post-ischemic disease. While ENG expression is relatively low in quiescent ECs, it is upregulated in the active endothelium involved in angiogenesis and vessel remodeling and permeability. In these processes, endothelial ENG regulates ECs proliferation, migration, and actin cytoskeletal organization, leukocyte extravasation, and interaction with mural cells. In addition, a role for ENG in platelet-endothelium interaction, including the resistance of adherent platelets to shear under inflammatory conditions, was also recently proposed, opening new perspectives on ENG functions.

This review highlights the emerging roles of endothelial ENG as a cell adhesion molecule in mature and progenitor endothelial cells interacting with vascular mural cells, leukocytes, and platelets, independently or in parallel to its TGF-β co-receptor role.

While evidence-based therapeutics are coming into play in the traditionally empirical base for endoglin related protein derivatives, future studies of ENG in endothelial progenitor cells may pave the way for a better understanding of its function in the vascular system and for the development and understanding of the use of these immature cells as a cell therapy product.

AUTHOR CONTRIBUTIONS

ER wrote the manuscript. CB provided helpful suggestions and contributed to writing the manuscript. DS wrote the manuscript and provided funding support.

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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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 11 March 2019
doi: 10.3389/fmed.2019.00033

Selection of a Real-Time PCR Housekeeping Gene Panel in Human Endothelial Colony Forming Cells for Cellular Senescence Studies

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Endothelial Colony Forming Cells (ECFCs) represent a subset of endothelial progenitors with well-documented vasoreparative capacity. However, cellular senescence, which occurs due to aging, diabetes, smoking, or tissue inflammation, renders these cells dysfunctional. Therefore, there is growing interest in studying expression of senescence markers in ECFCs. RT-qPCR is the most commonly used technique to quantify gene expression and the proper choice of reference genes used for data normalization is critical for accurate quantification. It has been reported that the expression of commonly used housekeeping genes is often unstable in senescence. To identify the most suitable reference genes for ECFC senescence studies, we analyzed a microarray dataset, which compared the gene expression between proliferating and senescent ECFCs. In addition to replicative senescence, the data included X-ray-induced and Etoposide-induced senescence. We used the geNorm algorithm to identify the most stable genes across all studied conditions. Gene Ontology analysis found that the most stable genes belonged to the KEGG category of Genetic Information Processing. The optimal combination of housekeeping genes for ECFC senescence was found to include four ribosomal protein genes; RPL13, RPL31, RPL37, and RPL30. The RT-qPCR validation confirmed that normalization with our novel panel was more sensitive in identifying senescence markers compared to commonly used genes such as ACTB, UBC, and GAPDH.

Keywords: housekeeping gene, ECFCs, senescence, RT-qPCR, computational biology

OPEN ACCESS

Edited by:
Enrico Capobianco, University of Miami, United States

Reviewed by:
Petra Hruba, Institute for Clinical and Experimental Medicine (IKEM), Czechia
Nicholas Ekow Thomford, University of Cape Coast, Ghana

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *12 November 2018*
Accepted: *04 February 2019*
Published: *11 March 2019*

Citation:
McLoughlin KJ, Pedrini E, MacMahon M, Guduric-Fuchs J and Medina RJ (2019) Selection of a Real-Time PCR Housekeeping Gene Panel in Human Endothelial Colony Forming Cells for Cellular Senescence Studies. Front. Med. 6:33. doi: 10.3389/fmed.2019.00033

1. INTRODUCTION

Endothelial progenitor cells (EPCs) are defined as cells circulating in blood with the capacity to differentiate into endothelial cells and contribute to new blood vessel formation. There has been controversy and confusion in the literature when studying EPCs. We and others have proposed nomenclature guidelines for precise terminology (1). A subtype of EPC which is clearly defined by their immunophenotype and functional properties are endothelial colony forming cells (ECFCs). ECFCs exhibit both progenitor and endothelial characteristics and have been considered the *bona fide* EPC (2). There is agreement that ECFCs have remarkable vasoreparative potential and consequently represent an ideal candidate for cell therapy (3–5). ECFCs are isolated as small clusters of cells and therefore, require *in vitro* cell number amplification from tens to millions to meet numbers needed for a cell therapy. Our data demonstrated that this is feasible, and we can expand ECFC numbers from 100,000

to 2.5 billion cells in 14 days. Although ECFCs have significant proliferative potential, they have a Hayflick limit and undergo replicative senescence (6). In order to characterize the senescence program in ECFCs at the gene expression level, there is a requirement to optimize and validate reference genes. While microarray and RNA-seq are high-throughput technologies that allow genome-wide assessment of transcriptomes, reverse transcriptase real time quantitative polymerase chain reaction (RT-qPCR) remains the most frequently used methodology for small scale studies of gene expression. RT-qPCR is extremely sensitive, has a broad dynamic range, is fast and highly reproducible (7). Despite these advantages, RT-qPCR accuracy is highly dependent on the choice of reference genes (8). These internal controls, also known as housekeeping genes, are used as the normalization factor and therefore its expression ideally should not be affected by experimental treatments. Poor choice of housekeeping genes has a major impact on results and could lead to generation of misleading information. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines have indicated the need for validation of the housekeeping gene choice to ensure stable expression across the experimental settings (9). The general assumption that "classical" housekeeping genes are appropriate is not justified because it has been demonstrated that the expression GAPDH and β-actin change under certain *in vitro* experimental conditions, as well as with respect to source material. For example, Glare and colleagues found that β-actin and GAPDH expression is reduced in both broncoalveolar lavage fluid cells and endobronchial biopsy tissue in asthmatics compared to healthy controls (10, 11). In particular, several studies have highlighted that the expression of common housekeeping genes is often unstable in aging and cellular senescence (12–14). Therefore, considering that aging is associated with major metabolic and structural changes in cell phenotypes, it is critical to identify the most stable gene normalizers for senescence studies. Here, we established and validated a panel of housekeeping genes for cellular senescence studies using ECFCs.

2. MATERIALS AND METHODS

2.1. ECFC Isolation and Characterization

ECFCs were obtained from human umbilical cord blood with appropriate maternal consent and under ethical approval in accordance with the Declaration of Helsinki. The mononuclear cell fraction was isolated by density gradient fractionation. Umbilical cord blood was diluted using Alsever's solution (Sigma-Aldrich) and carefully layered on Histopaque-1077 (Sigma-Aldrich) in a 1:1 ratio. The fraction at the interphase between the Histopaque and the plasma was carefully removed using a transfer pipette and resuspended in EGM-2 (Lonza Ltd.) supplemented with 20% fetal bovine serum (Hyclone), and plated in 24-well NUNC tissue culture plates precoated with rat tail collagen type I (BD Biosciences). ECFCs were characterized by immunophenotyping for CD31, CD105, CD14, and CD45 (eBioscience) using an Acoustic Focusing Cytometer (Attune NxT, Life Technologies) following already established methodology (5). For all experiments, ECFCs at passage 9 were used.

2.2. Induction of Senescence

Both replicative and stress-induced senescent ECFCs were generated for this study. Stress-induced senescence was induced by treating ECFCs with 1 μM Etoposide (Sigma) for 4 days. Media was replaced with fresh EGM-2 supplemented with 10% fetal calf serum (Gibco) and ECFCs were cultured for an additional 4 days to establish senescence, before RNA was extracted using Maxwell RSC automated RNA extraction system (Promega). For X-ray induced senescence cells were treated with 10 Gy and cultured for 5 days to allow establishment of senescence. For replicative-induced senescence, cells were allowed to grow until their Hayflick limit was reached. Quiescence was induced via contact inhibition by allowing cells to reach and maintain 100% confluency for 5 days before RNA was extracted using Maxwell RSC automated RNA extraction system (Promega).

2.3. Microarray

Total RNA was extracted using miRNeasy Mini RNA extraction kit (Qiagen). Total RNA from each sample was quantified by the NanoDrop ND-1000 and RNA integrity was assessed by Agilent 2100 Bioanalyser. The microarray hybridization was performed by Arraystar (Rockwille, US) following established protocols. Microarray data was deposited in GEO with accession number GSE125792.

2.4. Quantitative Real Time PCR

RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription kit (ThermoFisher). Primer sequences shown in **Table S1** were designed using Primer BLAST and experimentally validated; only primers matching the MIQE guidelines (**Table S2**) were used (9). RT-qPCR was performed on Roche LightCycler 480 with the Power SYBR-green PCR Master Mix (ThermoFisher), following the manufacturer instructions. Amplification reactions were done in three technical replicates. Following a polymerase activation step at 95°C for 15 min, 40 cycles, including denaturation at 94°C for 10 s; annealing at 58°C for 10 s, and amplification at 72°C for 10 s were performed. Fluorescent signals generated during PCR amplification were monitored and analyzed with LightCycler 480 software (Roche). The expression level was estimated using 35 cycles as limit of detection (LOD). For both microarray data and RT-qPCR data, 3 biological replicates were used; however, different biological replicates were used between techniques (i.e., microarray used 3 different biological replicates to those used for RT-qPCR). Therefore, independent samples were used for RT-qPCR validation because this provides higher confidence to generalize results as 6 different biological replicates tested using two techniques yielded similar results.

2.5. GeNorm Implementation

Stability of gene expression was assessed using the selectHK() function form the NormqPCR package (15). The source code applied is presented below:

```
# library(BiocInstaller)
# biocLite("NormqPCR")
library(NormqPCR)
input_file <- "input.csv"
df_test <- read.csv(paste0("~/folder/",
input_file))

# identify the numeric varible
# df_test<-df_test[c(1:100,nrow(df_test)),]
id_test <- unlist(lapply(df_test,is.numeric))
mat2 <- as.matrix(df_test[-nrow(df_test),
id_test])
str(mat2)
raw2 <- new("qPCRBatch", exprs = mat2,
 featureCategory =
            as.data.frame(array("OK",
             dim=dim(mat2))))
sampleNames(raw2) <- colnames(df_test)[-1]
featureNames(raw2) <- as.character
(df_test[,1][-nrow(df_test)])
head(exprs(raw2))
t <- Sys.time()
out <- selectHKs(raw2, method = "geNorm",
               Symbols = featureNames(raw2),
                minNrHK = 2, log = T)
d<-Sys.time()-t
# print the time enlapsed
d
saveRDS(object = out,file = paste0("~/out_",
input_file,".rds"))
```

2.6. Statistical Analysis

Data handling and statistical analysis was done using R (16). Unless otherwise specified, the alpha value for statistically significant was set to 5%. The tests were performed using the default parameters and in a two-sided manner. Where appropriate, *p*-values were corrected for multiple testing. To calculate the confidence interval of the correlation coefficient, the cor.test() function from the "stats" package (16) has been used.

3. RESULTS

3.1. Characterizing Gene Stability From the ECFC Transcriptome in a Cellular Senescence Investigation Using geNorm

To identify ECFC genes with the most stable expression during cellular senescence, we analyzed a microarray dataset of non-senescent and senescent ECFCs. Senescence programs investigated were replicative, X-ray induced, and Etoposide-induced. Since microarray and RT-qPCR are two different techniques in terms of dynamic range and limit of detection (17), and to avoid the selection of candidate housekeeping genes that were difficult to detect within our RT-qPCR experimental setup, we decided to filter the dataset focusing on genes with high expression. After normalization, the microarray dataset provided values for average expression of 18,667 probes (**Figure 1A**). We screened for the level of expression by RT-qPCR along the overall distribution defined by the microarray. Based on these results (**Figure 1A** bottom panel), we decided to use the top 9,000 probes for further analysis. This threshold was applied as it was both a combination of the halfway point of the microarray data and the point at which transcripts could be unequivocally assessed *in vitro* using RT-qPCR. We applied the geNorm algorithm using the normalized expression level of the probes in the microarray. GeNorm is an algorithm developed by Vandesompele and colleagues which can be used to assess the level of variation in gene expression between different experimental conditions. It works by taking the standard deviation of the genes between experimental conditions and comparing it to the other genes within the dataset. The least stable gene is then excluded and the process is repeated, until two genes remain at the end. Full details can be found in Vandesompele et al. (18). It is important to highlight that the geNorm algorithm provides a stability score, which is inversely proportional to the common idea of stability; therefore, a lower value depicts higher stability. GeNorm indicated that 8616 probes had stability values lower than 1; and 4,444 transcripts had stability values lower than 0.5 (**Figure 1B**). Furthermore, we confirmed results by visualizing the expression of some genes at different stability ranking levels. As expected, by moving down the ranking, both inter- and intra- variability of the gene expression increased (**Figure 1B** bottom panel). Interestingly, we noticed a general trend in the dataset; if a gene is more highly expressed, then it seems more likely to be ranked as stable as shown by the dotted red line (**Figure 1C**). This is also highlighted by the MA plot showing that genes with the higher expression (above 15 variance stabilizing transformation VST units) are the most stable (**Figure 1D**). When choosing and designing a panel of housekeeping genes, the most stable transcripts from different biologically relevant categories (defined by GO term) were identified from microarray analysis using the geNorm algorithm and validated with PCR. Transcripts with middle and low stability were also sampled at random in order to establish variances in expression based on differing stability values. In summary, we applied the well-established geNorm algorithm to a subset of ECFC senescence microarray dataset to screen for stable targets.

3.2. Most Stable Genes Belong to Genetic Information Processing

Starting from the geNorm-based results, we used the top 1000 genes, ranked by stability among all senescence samples, to perform GO analysis (19, 20), using Cellular Component domain. We found that GOs for cytosolic large and small ribosomal subunits were among the most enriched cellular components in terms of stability (**Figure 2A**). To further characterize the identity of the most stable genes, we also performed enrichment analysis using KEGGs terms (21, 22). We found that mRNAs coding for proteins involved in genetic information processing, which include ribosomal protein genes, were among the most stable ECFC genes in the dataset used (**Figure 2B**).

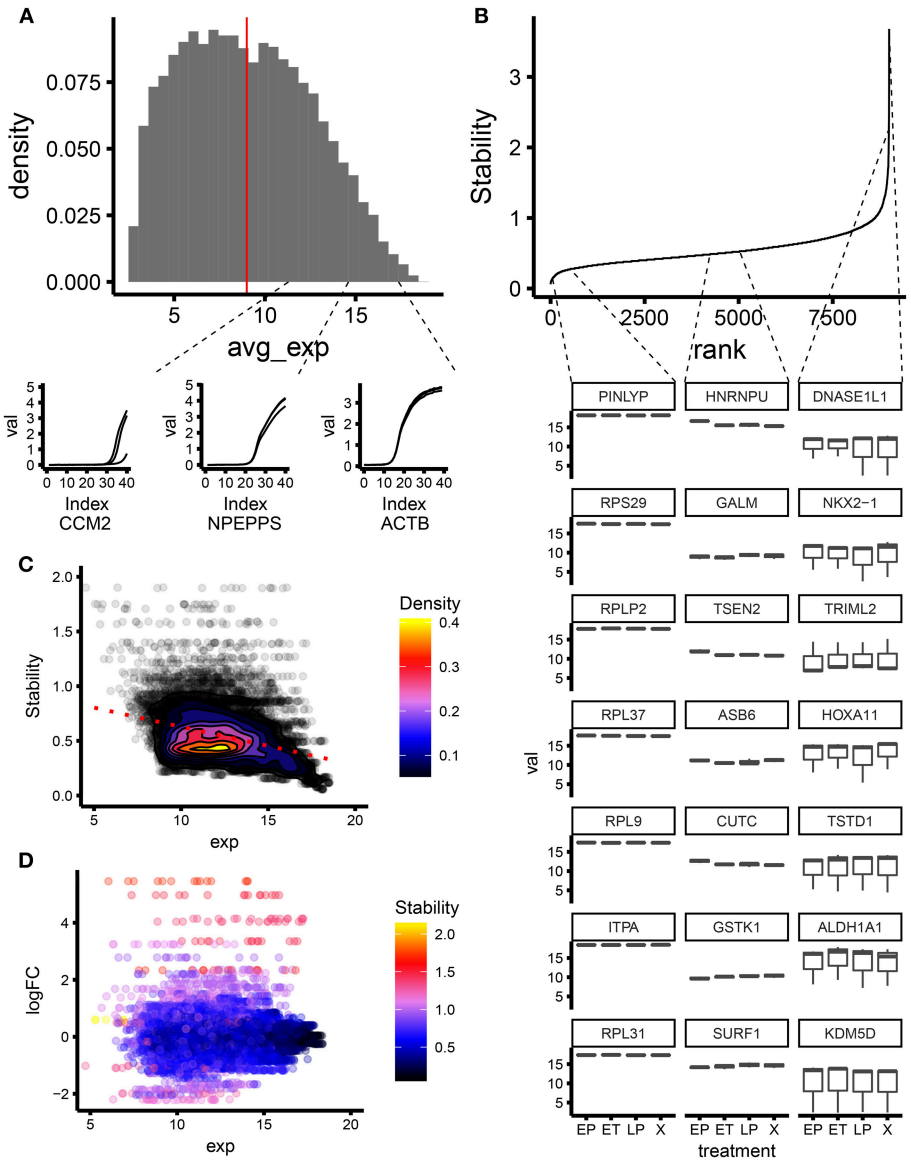

FIGURE 1 | Screening for stable genes using geNorm on microarray data for an investigation of ECFC senescence. **(A)** Distribution of normalized gene expression from microarray data. The red line defines the arbitrary threshold used to filter the dataset. In the lower panel, the qPCR amplification plots for some genes along the distribution are shown. Genes with expression level close to the threshold are close to the LOD of our experimental setup. **(B)** Stability vs. ranking obtained from geNorm. In the lower panel, the distributions of expression levels (from the microarray) for some genes along the ranking are shown. Genes with lower rank have higher within and between treatment variability in gene expression. **(C)** Scatter plot of stability vs. expression (from microarray). The dotted line shows the trend in the dataset; genes with a higher expression level seem to have a lower stability score i.e., more stable. **(D)** MA plot showing level of expression vs. expression fold change. GeNorm stability score depicted using a colored scale.

3.3. Four Ribosomal Protein Genes RPL13, RPL31, RPL37, and RPL30 Provide the Optimal Combination of Housekeeping Genes to Normalize Gene Expression in ECFC Senescence Studies

To refine our ECFC housekeeping gene selection and establish the ideal number of housekeeping genes to be used in a panel, we designed and successfully validated RT-qPCR primers for 28 target genes (**Table S1**), including targets from geNorm screening of microarray data plus "classical" housekeeping genes i.e., ACTB, GAPDH, and UBC. Those were further tested using different RNA samples including non-senescent, quiescent, and senescent ECFCs. RT-qPCR results were analyzed with geNorm and ranked according to their stability (**Figure 3A**). As expected, this independent experiment confirmed ribosomal genes as the most stable and validated RPL13, RPL31, RPL37, RPL30, and RPS6 as the top 5 most stable genes among

A

GO.ID	Term	Annotated	Significant	Expected	Fisher
GO:0022625	cytosolic large ribosomal subunit	52	30	5.88	5.90E-16
GO:0070062	extracellular exosome	1550	265	175.19	1.30E-14
GO:0005925	focal adhesion	288	72	32.55	2.60E-11
GO:0022627	cytosolic small ribosomal subunit	38	19	4.29	3.70E-09
GO:0031012	extracellular matrix	224	42	25.32	2.30E-08
GO:0043209	myelin sheath	122	31	13.79	1.40E-05
GO:0005747	mitochondrial respiratory chain complex ...	42	15	4.75	2.90E-05
GO:0005829	cytosol	2967	410	335.35	0.00014
GO:0000276	mitochondrial proton-transporting ATP sy...	10	6	1.13	0.00029
GO:0016020	membrane	3976	475	449.39	0.00076

B

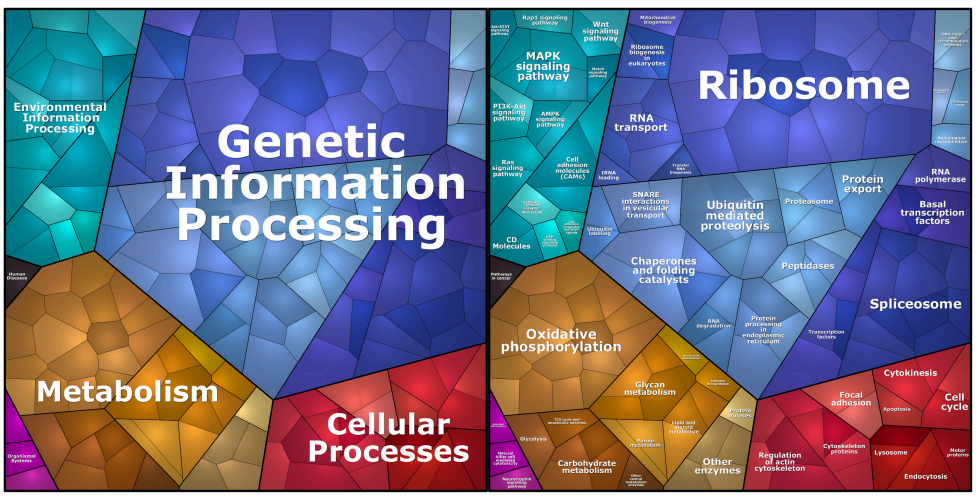

FIGURE 2 | Gene Ontology analysis for most stable genes. **(A)** GO enrichment analysis. The table shows the top 10 GO terms for the Cellular Component domain, using the top 1,000 most stable genes in the dataset. Ribosomal proteins seem to be the most enriched component within the most stable genes. **(B)** Proteomap of the most stable genes. The terms shown in the Voroni plot are based on the KEGG Pathways gene classification. Each gene is shown by a polygon and functionally related proteins are arranged in common regions.

the 28 candidates. In addition, since MIQE (9) guidelines recommend to use at least two housekeeping genes for accurate normalization of gene expression in qPCR experiments; we used the geNorm algorithm to calculate the variability provided by the normalization factor using different number of housekeeping genes. Interestingly using our data and housekeeping candidates, we found that, as expected, the higher the number of genes used, the lower the variability; but from our 28 candidates, we reached a maximum low variability with 17 genes; as using more showed increased variability. In addition, the pairwise variation using the top two housekeeping genes was already below the suggested threshold of 0.15. Nevertheless, results from geNorm variability highlighted that there was an evident improvement in the pairwise variation using 4 genes in the definition of a normalization factor; however, there was a minimal improvement when using 5 instead of 4 genes (**Figure 3B**). This is also supported by comparing the correlation of the NFs using 3 vs. 4 genes and 4 vs. 5 genes **Figure S1**).

3.4. The Normalization Factor From Our Housekeeping Panel Is Different to the Normalization Factor Using Classical Housekeeping Genes

Although we followed stringent criteria to select optimal housekeeping genes for ECFC senescence studies, it is difficult to define the "real normalizer"; therefore, we decided to compare our set of candidate housekeeping genes with a set of "classical" housekeeping genes including ACTB, UBC, and GAPDH in terms of their normalization factor. The objective was to test if our set of housekeeping genes provides a normalization factor which is not correlated with the one obtained by using the classical housekeeping genes (therefore different). Correlation analysis demonstrated that the normalization factor using our set of selected housekeeping genes is not significantly correlated with any normalization factor provided by other classical approaches (ACTB alone, GAPDH alone, UBC alone and ACTB GAPDH and UBC altogether) (**Figure 4A**). Consequently, considering that

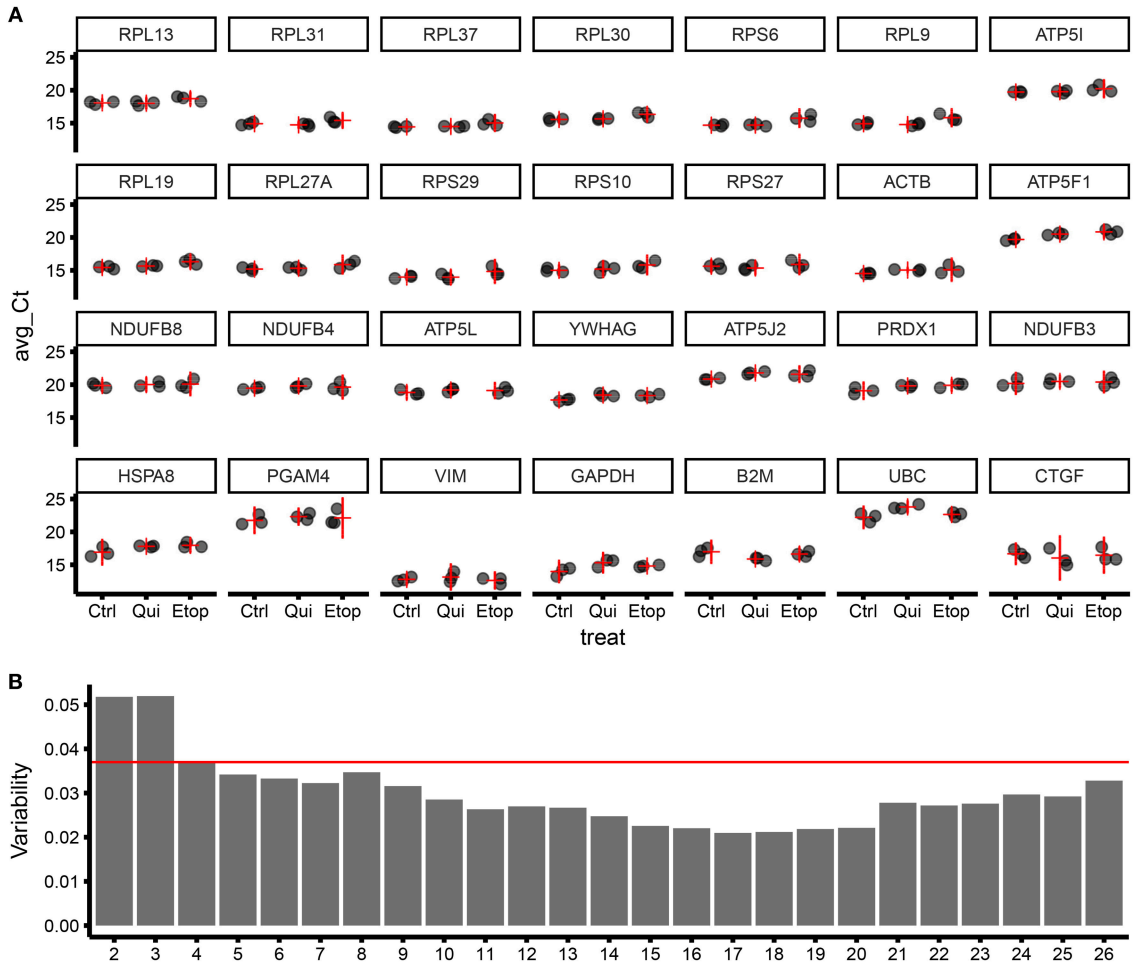

FIGURE 3 | Validation of gene stability from geNorm results using RT-qPCR for 28 candidate housekeeping genes.**(A)** Level of expression (qPCR) for different genes ordered by stability (starting from the most stable; by row; top to bottom), expressed as Ct of three biological replicates (for each biological replicate, three technical replicates were produced). The horizontal red line is the estimate of the average expression for the studied gene in the specific treatment group. The vertical red line is the 95% confidence interval of the estimate of the average expression. The variability within and between treatment increase moving from top-left to bottom-right. Ctrl, control ECFCs; Qui, quiescent ECFCs; Etop, Etoposide-induced senescent ECFCs. **(B)** Bar plot showing sample variability by the number of genes used for calculating the normalization factor (NF).

our set of genes has been proven to be more stable compared to the classical ones, we can conclude that our normalization factor is more reliable than the one provided by classical housekeeping genes alone and combined. This accuracy is further underscored by different results found within our experimental setup using ECFC and senescence/quiescence *in vitro* models. The expression of UBC was significantly downregulated in quiescent ECFCs when using our housekeeping gene panel (**Figure 4B**). Similarly, we found significant upregulation of UBC gene expression in senescent ECFCs, confirming UBC as an unsuitable housekeeping gene for senescence and quiescence studies in ECFCs. This is in agreement with previously published data from another group investigating cell size and gene expression; which reported similar changes in UBC expression in both A549 cells and primary human fibroblasts (23). In addition, we evaluated gene expression of IGFBP7 and IL6, which are components of the senescent associated secretory phenotype (SASP) and were expected to be upregulated. While both, our new and the classical housekeeping panels identified a significant upregulation of IGFBP7, only our new housekeeping panel was able to detect a significant upregulation of IL6 (**Figure 4C**). Taken together, we were able to validate our set of new housekeeping genes as highly accurate for ECFC senescence studies.

4. DISCUSSION

We have shown a workflow for the identification of housekeeping genes for RT-qPCR using an unbiased approach starting from a transcriptomic dataset. Considering the growing number of -omic dataset freely available online, we demonstrate that relying on classically reported housekeeping gene is not always accurate. Moreover, with this approach, it is possible to identify candidates for specific cell types and biological processes that are not yet validated for RT-qPCR experiments. Here, we have used such

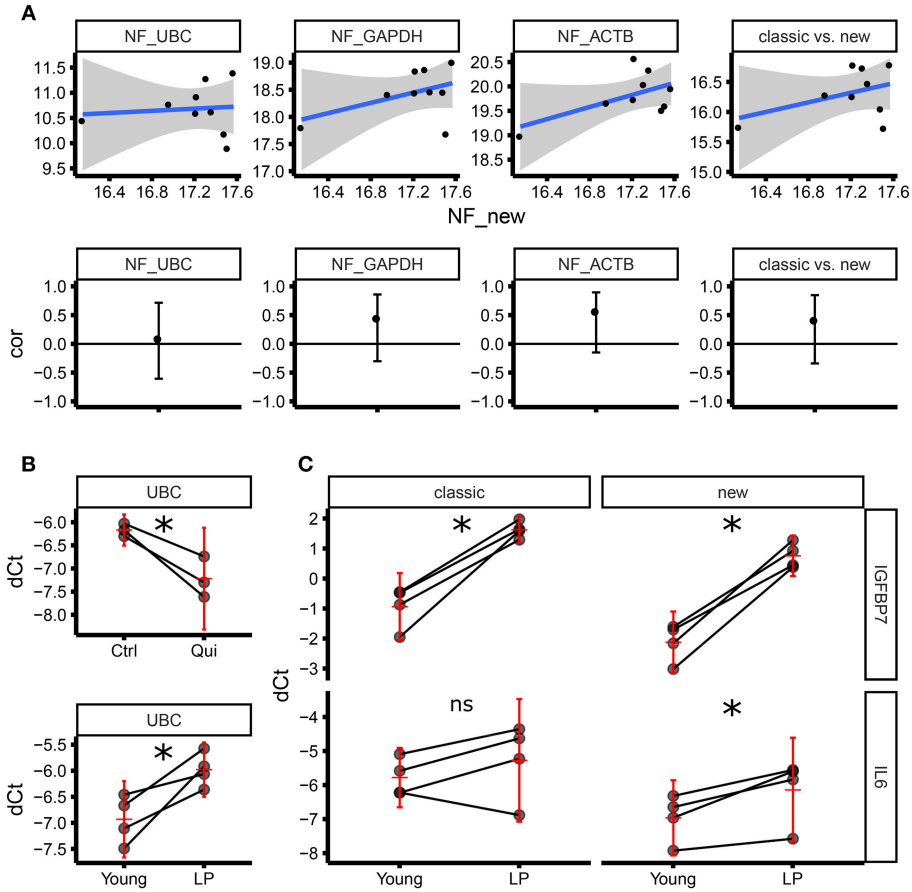

FIGURE 4 | Normalization factor using "classic" housekeeping genes does not correlate with our new geNorm-based housekeeping panel.**(A)** The upper panel shows the scatter plots comparing normalization factors (NFs) from single genes (UBC, GAPDH, ACTB) or the geometric average of the expression of the three (classic) vs. the new NF based on the genes identified by this study (RPL13, RPL31, RPL37, RPL30). The lower panel shows the estimate of the Pearson correlation r (the black dot) and its 95% confidence interval (vertical black line) for each of the scatter plots above. All the confidence intervals include zero (horizontal black line), therefore we can conclude there is not enough evidence of a correlation between any of the classical NFs and the new NF. **(B)** Level of expression (qPCR) for UBC as dCt using the new NF. The upper panel shows the expression in three biological replicates (three independent ECFC clones) in control vs. quiescence (matched samples). The lower panel shows the expression in four biological replicates (four independent ECFC clones) in young vs. late passage (LP) (matched samples). The horizontal red line corresponds to the estimate of the average expression in the treatment group. The vertical red line is the 95% confidence interval of the estimate of the average expression. **(C)** Level of expression (qPCR) for IGFBP7 (upper panel) and IL6 (lower panel) as dCt, using either the new NF (right side) or the classical NF (left side) in four biological replicates (matched samples). IGFBP7 is significantly upregulated (as expected comparing LP vs young cells) using both the classical NF and the new NF. IL6 is significantly upregulated (as expected comparing LP vs young cells) only using the new NF. The horizontal red line corresponds to the estimate of the average expression in the treatment group. The vertical red line is the 95% confidence interval of the estimate of the average expression. *p <0.05; ns, not significant.

analytical workflow to identify the optimal housekeeping panel for studies investigating ECFCs and cellular senescence. The use of a non-stable reference gene leads to incorrect results. As established by MIQE guidelines (9), reference gene validation must be carried out for every experiment and every type of sample, whether treated or not. In this paper, we propose a panel of the most stable genes to be used in ECFCs using different means of inducing senescence. This study has identified and validated the genes RPL13, RPL31, RPL37, and RPL30 as the most stable in ECFCs for senescence studies. Here, we report a significant decrease in expression of the classical housekeeping gene UBC under quiescence, coupled with a significant increase under senescence, when using the novel panel. This is in agreement with previous work showing that UBC expression is reduced in fibroblasts under quiescence (23) further strengthening the argument that housekeeping genes must be optimized for each experimental condition and cell type to ensure correct interpretation of results. The optimal number of housekeeping reference genes needed for analysis of ECFCs in senescence studies was established. Although MIQE guidelines published in 2009 recommend using at least two housekeeping genes, a systematic review found that the average number of housekeeping genes used across 130 studies between 2010 and 2015 was 1.2 (13). This highlights that while MIQE guidelines are widely accepted, there is a major lack of compliance. In terms of recommendations for future studies, we feel that the

use of at least three housekeeping controls is a relatively modest request to ensure that RT-qPCR data investigating senescence mechanisms in ECFCs is accurately analyzed. The mechanistic and organelle-based locations of candidate housekeeping genes was characterized in detail. We found that the majority of identified stable genes were located within the KEGG category genetic information processing, which include also genes coding for ribosomal proteins. This information highlights why a similar workflow as the outlined in this report, should be used to identify future candidates when experimental parameters or cell types change. For example, our housekeeping panel, although validated for senescence studies, will not be ideal for investigations into pathologies focussing on ribosomal proteins or genes. We want to emphasize that there is no perfect "universal" housekeeping gene control for every condition; for any experimental setup, it is essential to first identify the most effective controls. There is little available information in the literature about housekeeping genes for ECFCs; however, a recent publication identified a selection of optimal housekeeping genes for differentiating ECFCs from umbilical cord blood mononuclear cells on scaffold delivery systems (24). This investigation yielded a panel of genes different to ours (YWHAZ, GAPDH, UBC) although the experimental conditions did not investigate senescence and only utilized seven common candidate genes. To our knowledge, this is the first investigation which has used microarray data as a starting point for identifying reference gene candidates in ECFCs. Microarrays have been used before for identifying a shortlist of novel candidate genes in grapevine exposed to water and heat stress (25). In this study, a method was established for cleaning raw microarray data by excluding genes below the threshold of detection for qPCR and those genes which would be unsuitable due to high variation (measured by observing the standard deviation and excluding those of which the standard deviation was unacceptable). This improves the screening process in two ways: Firstly, it allows the identification of robust candidates by excluding those which are unsuitable. Secondly, it reduces the computational burden required to run the algorithm for identifying genes. This is a particular disadvantage of geNorm, where the algorithm follows a polynomial time kinetic (**Figure S2**). This study will contribute to the current field by providing an in-depth methodology for identifying potential housekeeping candidates for ECFCs from microarray data. Although the authors chose senescence as the focus for the analytical pipeline, the methodology is transferable to other cell types, pathologies, and experimental settings. Other cell types, such as human umblical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs), have already undergone rigorous housekeeping gene selection from a broad range of sources (26, 27) including microarrays. As such, it is important that ECFCs are held to the same rigorous standards. As ECFCs are a promising candidate for cellular therapy applications, it is paramount to determine the mechanisms which may disrupt their function in pathological conditions. For example, it is important to study ECFCs in the context of hypoxia, high glucose, inflammation, oxidative stress, among others. Since senescence of endothelial progenitors is a major component driving vascular aging, this study decided to focus on housekeeping genes for ECFCs when evaluating their senescence programme. Results shown here have practical applications for future studies investigating ECFC senescence. This study also recommends screening transcriptomic changes as a valid strategy to identify precise reference controls in any future ECFC studies.

AUTHOR CONTRIBUTIONS

KM and EP contributed equally to this manuscript. KM and EP carried out the experiments. JG-F generated samples for the microarray. RM, KM, and EP wrote the manuscript with support from JG-F. MM provided technical advice. RM conceived the original idea.

FUNDING

This work was supported by the Leverhulme Trust RPG-2015-357, the Juvenile Diabetes Research Foundation 5-CDA-2014-225-A-N (JDRF), the National Eye Research Centre SAC 016, and the Medical Research Council (MRC).

ACKNOWLEDGMENTS

We thank the Belfast Health and Social Care Trust, in particular, Dr. Inez Cooke and Katie Corry for the constant support and assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2019.00033/full#supplementary-material

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