Potentials of Endothelial Colony-Forming Cells: Applications in Hemostasis and Thrombosis Disorders, from Unveiling Disease Pathophysiology to Cell Therapy

Das Potential von endothelialen koloniebildenden Zellen: Anwendungen bei hämostatischen und thrombotischen Störungen, von der Aufklärung der Pathophysiologie von Krankheiten bis zur Zelltherapie

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Abstract

Endothelial colony-forming cells (ECFCs) are endothelial progenitor cells circulating in a limited number in peripheral blood. They can give rise to mature endothelial cells (ECs) and, with intrinsically high proliferative potency, contribute to forming new blood vessels and restoring the damaged endothelium in vivo. ECFCs can be isolated from peripheral blood or umbilical cord and cultured to generate large amounts of autologous ECs in vitro. Upon differentiation in culture, ECFCs are excellent surrogates for mature ECs showing the same phenotypic, genotypic, and functional features. In the last two decades, the ECFCs from various vascular disease patients have been widely used to study the diseases' pathophysiology ex vivo and develop cell-based therapeutic approaches, including vascular regenerative therapy, tissue engineering, and gene therapy. In the current review, we will provide an updated overview of past studies, which have used ECFCs to elucidate the molecular mechanisms underlying the pathogenesis of hemostatic disorders in basic research. Additionally, we summarize preceding studies demonstrating the utility of ECFCs as cellular tools for diagnostic or therapeutic clinical applications in thrombosis and hemostasis.

Keywords

- endothelial cells
- endothelial colony-forming cells
- ex vivo model
- von Willebrand disease
- thrombosis

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Zusammenfassung

Endotheliale koloniebildene Zellen (endothelial colony forming cells, ECFCs) sind endotheliale Vorläuferzellen, die in begrenzter Zahl im peripheren Blut zirkulieren. Sie unterstützen das Wachstum adulter Endothelzellen (ECs), haben ein hohes intrinsisches Proliferationspotential und tragen zur Neubildung von Blutgefäßen und Reparatur von Geweben in vivo bei. ECFCs können aus peripherem oder Nabelschnurblut isoliert werden und können in vitro in großen Mengen als autologe ECs kultiviert werden. Nach Differenzierung in Kultur liefern ECFCs einen exzellenten Ersatz für adulte Endothelzellen, da sie dieselben phenotypischen, genotypischen, und funktionellen Eigenschaften besitzen. In den letzten zwanzig Jahren wurden ECFCs von Patienten mit verschiedenen vaskulären Erkrankungen ex vivo verwendet, um die Pathophysiologie von Krankheiten zu studieren und Zell-basierte therapeutische Ansätze wie vaskuläre regenerative Therapie, tissue engineering, oder Gentherapie zu entwickeln. In diesem Review geben wir eine Übersicht über vergangene Studien, in denen ECFCs zur Aufklärung molekularer Mechanismen, die der Pathogenese hämostaseologischer Erkrankungen unterliegen, in der Grundlagenforschung verwendet wurden. Außerdem zeigen wir eine Zusammenfassung vorangehender Studien, die den Nutzen von ECFCs als zelluläres Werkzeug für Diagnose oder Therapie im klinischen Feld von Thrombose und Hämostase demonstrieren.

Schlüsselwörter

- Endothelzellen
- endotheliale koloniebildene Zellen
- ex vivo Modell
- von Willebrand Syndrom
- Thrombose

Introduction

Endothelial colony-forming cells (ECFCs) represent a rare population of endothelial progenitor cells (EPCs) circulating in the blood flow at a frequency of approximately 1 per 10^7 peripheral mononuclear cells (MNCs), and they are considered to be the only true endothelial cell precursors.[1] ECFCs can promote mature endothelial cell growth and, with an intrinsic clonal proliferative potency, contribute to forming new blood vessels and reconstructing the injured endothelium in vivo.[2,3] They can be isolated from peripheral blood or an umbilical cord and cultured/expanded in vitro (►Fig. 1). They are highly proliferative, and their growth in culture is robust, yielding up to 10^{19} cells.[4,5] Upon differentiation in culture, ECFCs highly resemble vascular endothelial cells phenotypically, genetically, and functionally. They display a cobblestone-shaped endothelial cell morphology, and they express typical endothelial cell markers CD31, VE-cadherin (CD144), CD146, vascular endothelial growth factor receptor 2 (VEGFR-2), and von Willebrand factor (VWF) (►Fig. 1). Besides, ECFCs express stem cell marker CD34, while they lack hematopoietic surface antigens CD14, CD45, and CD115 (►Table 1).[3,6–8] They also demonstrate the ability to ingest acetylated low-density lipoprotein,[6] form tube-like structures, and assemble into bidimensional capillary-like networks in Matrigel scaffolds.[9–12] Moreover, the whole-transcriptome profiling confirmed that cultured ECFCs resemble the endothelial lineage.[1,13] Interestingly, even epigenetic alterations associated with diseases in endothelial cells are preserved, making ECFCs excellent surrogates for mature endothelial cells and, therefore, a suitable cellular model for vascular diseases.[14] While nowadays, ECFCs are well-characterized, they have been previously confused with other EPC subtypes in scientific literature and have been named using several terms, including late outgrowth endothelial cells,[15–17] blood outgrowth endothelial cells,[18–22] or EPCs.[6,10,11,13]

In the late-1990s, Asahara et al and Lin et al established the technique enabling thriving endothelial cell culture from human peripheral blood, which was a vastly exciting innovation due to their potential usage for various clinical applications.[23,24] From that time on, numerous laboratories conducted research on cultured ECFCs, including cell therapy for vascular diseases (for the treatment of diabetes, ischemic disorders, etc.), tissue engineering (vascularization of bioengineered tissue constructs), gene therapy, liquid biopsy, and understanding of the molecular pathogenesis of endothelial dysfunction in various vascular disorders.[10,17,18,25,26]

In the current review, we will first briefly describe recommended methodologies for the isolation and culture of ECFCs and the corresponding limitations. We will also provide an overview of past studies using ECFCs to understand the pathophysiology of hemostatic disorders better. Furthermore, we summarize preceding studies using ECFCs as a potential therapeutic instrument in the clinical field of hemostasis and thrombosis (►Fig. 2).

Standardized Methodologies and Limitations of ECFCs Cultures

Methodologies and Guidelines for Isolation and Culture of ECFCs

Following the widespread use of ECFCs for various medical applications, different approaches for isolation and cultivation of ECFCs have been developed. We have summarized examples of variations in these approaches in ►Table 2.[1,6,20,23,24,27–32]

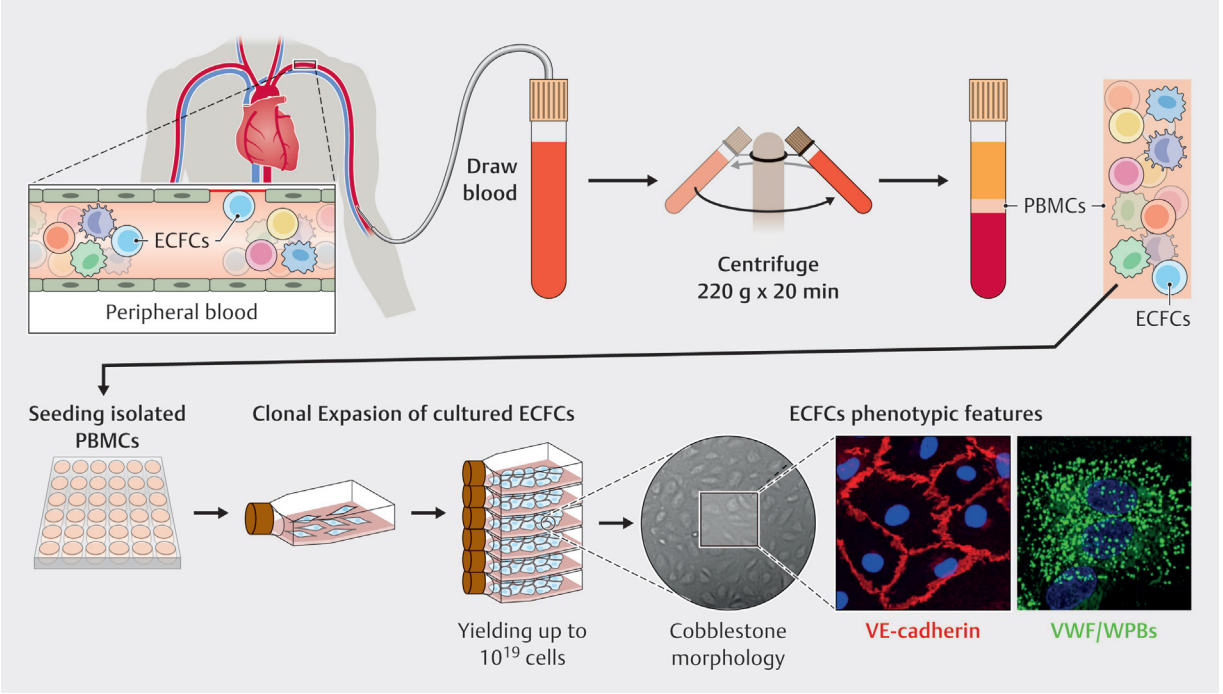

Fig. 1 Schematic of origin, isolation, culture, and characterization of ECFCs. ECFCs are endothelial progenitor cells circulating in a limited number in peripheral blood. They can give rise to mature endothelial cells and contribute to forming new blood vessels and constructing the injured endothelium in vivo. ECFCs can be cultured by isolating PBMCs from peripheral blood and seeding them on suitable extracellular matrix protein (e.g., collagen) in an endothelial-specific medium. ECFCs are typically identified by their cobblestone-like morphology and expression of endothelial cell-specific markers, such as VWF and VE-cadherin. ECFCs, endothelial colony-forming cells; PBMCs, peripheral blood mononuclear cells; VWF, von Willebrand factor.

Table 1 Characterization of the cell surface antigen expression and Ac-LDL uptake by ECFCs

Antigen	Lin et al[25]	Ingram et al[6]	Ingram et al[8]	Yoder et al[3]	de Boer et al[4]
CD144 (VE-cadherin)	+	+	+	99%	+
CD146 (MCAM)	nd	+	+	92%	+
CD31 (PECAM-1)	+	+	+	92%	+
VWF	+	+	+	97%	nd
CD105	nd	nd	+	97%	nd
CD141 (thrombomodulin)	+	+	+	nd	nd
CD309 (KDR/VEGFR-2)	nd	nd	nd	67%	+
CD51/56 (αvβ3 integrin)	nd	nd	nd	nd	+
CD34	+	+	+	nd	+
Ac-LDL	+	nd	nd	100%	nd
CD14 (monocyte marker)	–	–	–	–	–
CD45 (leukocyte marker)	nd	–	–	–	–
CD115	nd	nd	nd	–	nd
CD133 (hematopoietic stem cell marker)	nd	–	nd	nd	–

Abbreviations: –, indicated as not expressed or negative; +, indicated as expressed or positive; Ac-LDL, acetylated low-density lipoprotein; KDR, kinase insert domain receptor; MCAM, melanoma cell adhesion molecule; nd, no data; PECAM-1, platelet endothelial cell adhesion molecule; VEGFR-2, vascular endothelial growth factor receptor 2; VWF, von Willebrand factor.
Source: Yoder et al[3] reported the approximate percentage (%) of cell populations that expressed specific surface antigens.

The Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (ISTH) gave recommendations and guidelines for standardized isolation, quantification, and evaluation of ECFCs.[5] To isolate MNCs (known as peripheral blood mononuclear cells [PBMCs]), blood collection into either EDTA-, sodium citrate, or heparin-containing tubes is proposed. The MNCs can be plated on various extracellular matrix types such as fibronectin- or collagen

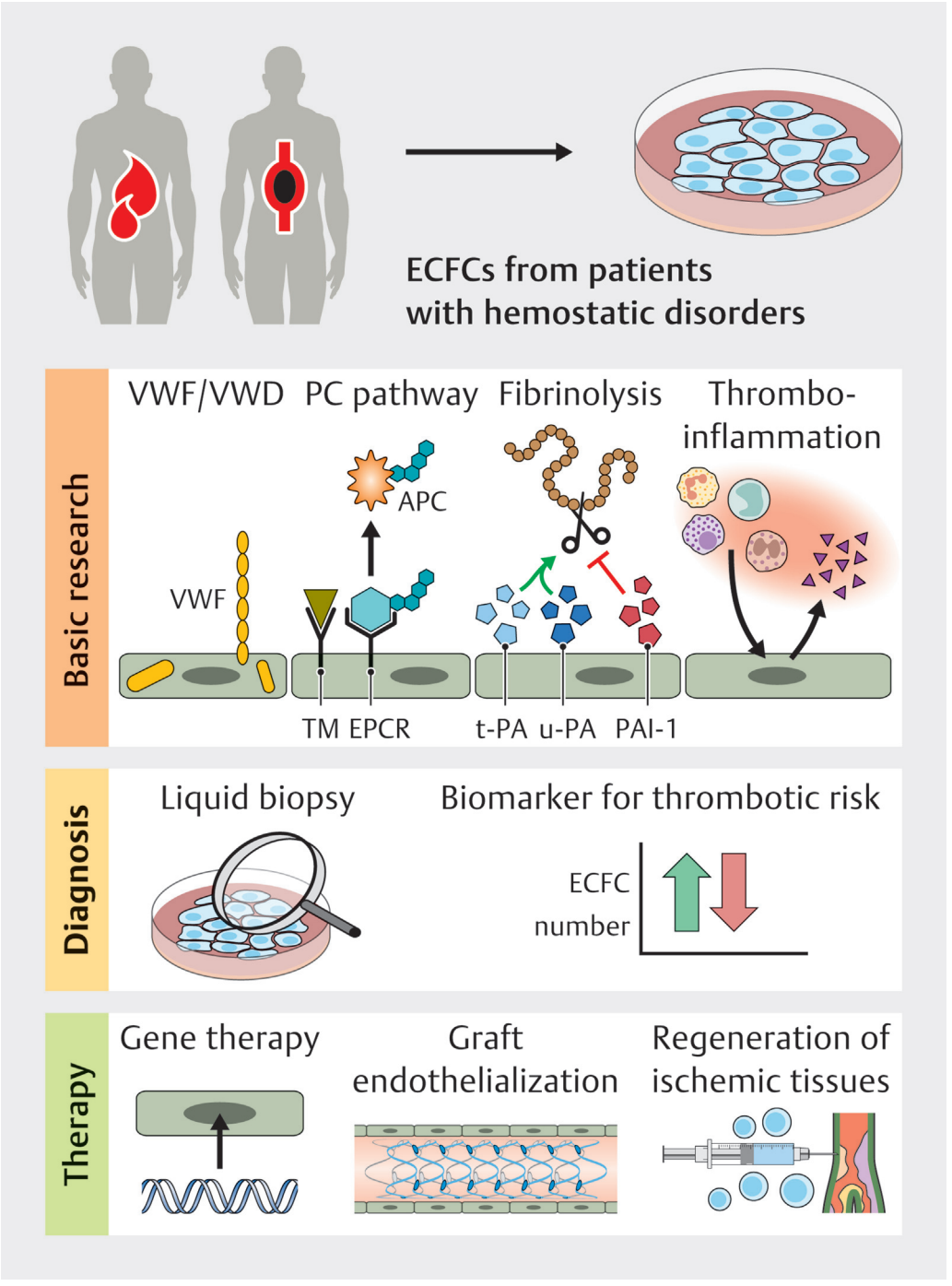

Fig. 2 Applications of patient-derived ECFCs in basic research, diagnosis, or therapy of hemostatic disorders. APC, activated protein C; ECFCs, endothelial colony-forming cells; EPCR, endothelial protein C receptor; PAI-1, plasminogen activator inhibitor-1; PC, protein C; TM, thrombomodulin; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator; VWD, von Willebrand disease; VWF, von Willebrand factor.

1-coated wells, and a commercially specialized endothelial growth medium (EGM2) available supplemented with 5 to 10% fetal bovine serum is used for cultivation. The first ECFC colonies in culture emerge after 7 to 21 days of plating. Therefore, it is recommended that they be identified as well-circumscribed colonies with a cobblestone appearance and more than 50 adherent cells. Furthermore, the following information should be documented and reported: (1) the number of colonies per seeded 10^7 MNCs, (2) failure of ECFC isolation defined as "zero colonies" (the report of zero colonies might give information on potential disease mechanisms), (3) both passage number and population doubling time, (4) number of days in culture from the day of the MNC isolation. Immunophenotyping using flow cytometry and microscopy immunofluorescence staining is used to characterize and validate the isolated ECFCs.

Table 2 A list of examples of methods for isolation and cultivation of ECFCs using different strategies for isolation of PBMCs, material for coating cell culture plates, and type of medium for endothelial cell growth

Literature	PBMCs isolation strategy	Coating material	Medium
Asahara et al[23]	Magnetic beads (CD34-positive mononuclear blood cells)	Collagen (enrichment process) /and fibronectin	M199 medium FBS: 5% (vol/vol)
Lin et al[24]	Histopaque-1077/CEC enrichment with P1H12-conjugated beads	Collagen type I (enrichment process)/and fibronectin	EGM-2 medium (Clonetics)
Ingram et al[6]	Histopaque 1077 (ICN)	Collagen type 1 rat tail (BD Biosciences)	EBM-2 medium (Cambrex) FBS: 10% (vol/vol) Penicillin/streptomycin (Invitrogen): 2% (vol/vol) Amphotericin B (Invitrogen): 0.25 µg/mL
Martin-Ramirez et al[27]	Ficoll-Paque Plus (GE Healthcare)	Collagen type 1 rat tail (Becton Dickinson)	EGM-2 BulletKit (Lonza, cat. no. CC-3162) FBS: 18% (vol/vol)
Ormiston et al[28]	Ficoll-Paque Plus	Type 1 collagen rat tail (BD Biosciences)	EGM-2MV Bullet Kit (Lonza cat. no. CC-3202) FBS: 10% (vol/vol)
Lin et al[29]	Ficoll-Paque Plus or microfluidic devices with antihuman CD34 antibody	1% gelatin-coated plates	EGM-2 BulletKit (except for hydrocortisone), (Lonza, cat. no. CC-3162) FBS: 20% (vol/vol) Glutamine–penicillin–streptomycin: (Invitrogen)
Holnthoner et al[30]	Lymphocyte separation medium (LSM1077, PAA)	Collagen I/III (only for seeding PBMCs), Fibronectin (Sigma)	EGM-2 BulletKit (Lonza, cat. no. CC-3162) FBS: 5% (vol/vol)
Sakimoto et al[31]	Lymphoprep (STEMCELL Technologies)	Rat collagen type 1 (Corning) /Animal component-free cell attachment substrate (STEMCELL Technologies)	EGM-2 BulletKit (Lonza, cat. no. CC-3162), FBS: 10% (vol/vol); or EC-Cult-XF Culture Kit (STEMCELL Technologies, cat. no. 08000)[a]
Selvam et al[20]	Vacutainer cell preparation tubes (BD Biosciences)	Collagen	EGM-2 BulletKit (Lonza, cat. no. CC-3162) FBS: 10% (vol/vol) Antibiotics: 1% (vol/vol)
Kutikhin et al[1]	Histopaque density media 1077 (Sigma)	Collagenase type I (Gibco)	EGM-2MV (CC-3202, Lonza) FBS: 5% (vol/vol)
Poyatos et al[32]	Ficoll-Paque Plus (GE Healthcare)	Type 1 collagen rat tail (BD Biosciences)	ECM-2 medium (ScienceCell Research Laboratories) FBS: 20% (vol/vol)

Abbreviations: ECFCs, endothelial colony-forming cells; FBS, fetal bovine serum; hFGF-B, human fibroblast growth factor; IGF, insulin-like growth factor; PBMCs, peripheral blood mononuclear cell; VEGF, vascular endothelial growth factor.
Notes: EGM-2 BulletKit (Lonza, cat. no. CC-3162) contains hEGF, hydrocortisone, GA-1000 (gentamicin, amphotericin-B), FBS (2%), VEGF, hFGF-B, R3-IGF-1, ascorbic acid and heparin. EGM-2MV Bullet Kit (Lonza cat. no. CC-3202) contains hEGF, hydrocortisone, GA-1000 (gentamicin, amphotericin-B), FBS (5%), VEGF, hFGF-B, R3-IGF-1 (insulin-like growth factor), and ascorbic acid. ECM-2 medium (ScienceCell Research Laboratories) contains essential and nonessential amino acids, vitamins, organic and inorganic compounds, hormones, growth factors, trace minerals, and FBS (5%). Additional FBS is added to the endothelial culture medium to achieve a final FBS concentration of 5 to 20%.
[a]EC-Cult-XF Culture Kit (STEMCELL Technologies, cat. no. 08000) is a xeno-free medium.

Consensus between laboratories following the isolation, documentation, and characterization recommendations will yield ECFCs as a robust and valid ex vivo model for comparable study outcomes.

Issues and Limitations

Using ECFCs as patient-derived cellular material for hematological research, diagnosis, and therapy has many advantages. However, there are issues and limitations, which are addressed in the following section.

Unclear origin: The origin of ECFCs is the subject of scientific debate since previous findings, with conflicting results, have found evidence that circulating ECFCs arise from both bone marrow and alternative vascular niche origin.[16,24,33] The poor characterization of the cell's stemness represents an explicit limitation for the potential use as a

human cell therapy source. Therefore, more studies addressing the question of ECFC origin are needed.

Low cell number and lack of isolation success: In peripheral blood, ECFCs appear at a very low number of approximately 1 per 10^7 MNCs, and numbers are highly variable between individuals.[5] For some patient groups (e.g., in coronary artery disease[34,35] or pulmonary arterial hypertension patients),[5] this low number is even further reduced, making autologous treatment difficult. In some individuals, ECFCs cannot be successfully isolated, likely due to a very low number in peripheral blood. Although alternative approaches, such as VEGF gene transfer, have been applied to augment circulating EPCs,[36] the low number of circulating ECFCs represents a burden with the need to overcome for autologous cell therapy of some patients.

Heterogeneity between donors: Differences in proliferation and clonal behavior within ECFCs from different donors have been described. Ingram et al identified a hierarchy of ECFCs based on their clonogenic and proliferative potential, dividing them into high proliferative potential colony-forming cells and low proliferative potential colony-forming cells.[6] Ferreras et al also divided ECFCs into progenitor-like CD34− and mature CD34+ subtypes, while they also observed colonies with mixed CD34 +/− phenotype.[15] Similar results were found by de Boer and colleagues, who described three groups of ECFCs according to differences in morphologic appearance, endothelial marker expression, proliferation rate, and VWF secretion.[4] Furthermore, Ferratge et al found high interindividual heterogeneity in proliferation associated with the enhanced angiogenic potential of ECFCs.[37] While it is no major issue for diagnostic and therapeutic approaches, this heterogeneity might affect the results of disease studies comparing ECFCs from different individuals.

Applications of ECFCs to Understand Pathogenesis Mechanisms of Bleeding and Thrombotic Disorders

Here, we review previous studies using patient-derived ECFCs as biological tools to better understand pathogenesis mechanisms and the impact of gene variations causing bleeding (von Willebrand disease [VWD]) and thrombotic disorders (including disorders of the protein C pathway, systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), sickle cell disease, COVID-19, and disorders of the fibrinolytic system).

Bleeding Disorder: VWD

VWD is the most prevalent hereditary bleeding disorder in humans, which is caused due to quantitative or qualitative defects in the VWF. VWD is classified as type 1 (due to VWF partial deficiency), type 2 (caused by dysfunctional VWF), and type 3 (due to the complete absence of VWF in plasma).[38,39] The VWF is a multimeric plasma glycoprotein essential for normal hemostasis by mediating platelet adhesion to injured vascular subendothelium and stabilizing coagulant factor VIII (FVIII).[40] VWF is predominantly produced in endothelial cells and stored in endothelial-specialized secretory organelles called Weibel-Palade bodies (WPBs) before its secretion into plasma. In addition to VWF, the main component of the WPBs, several angiogeneses and preinflammatory molecules, such as angiopoietin-2 (Ang-2), P-selectin, interleukin-6, etc., are stored in these organelles.[41] The cultured ECFCs have demonstrated the production of VWF and the presence of WPBs, making them a feasible true-to-nature cell model to study VWF and VWD. In the past decade, several studies have used ECFCs to understand molecular and cellular mechanisms underlying the pathogenesis of VWD and to investigate the consequence of VWF gene (*VWF*) variations on VWF biosynthesis and its intracellular processing.[21,22] Additionally, the cultured ECFCs were analyzed to evaluate the effect of VWF variants on endothelial's phenotype and function, such as cell proliferation, migration, and angiogenesis in patients with VWD. These studies using ECFCs isolated from VWD patients have discovered that VWF plays a role in regulating angiogenesis.[20,42,43]

Pathogenesis of VWD and effect of mutations on VWF processing: Our group and others have used VWD patients-derived ECFCs to elucidate the underlying cause of VWF deficiency in patients with ambiguous genotypes, as well as to assess the consequence of the VWF variants with unknown significance (including exonic synonymous variants, deep intronic variants besides exonic substitutions changing amino acid sequences). In these studies, VWF mRNA and secreted VWF protein from patient ECFCs were quantitatively and qualitatively analyzed, and trafficking of VWF inside ECFCs (retention of VWF in endoplasmic-reticulum and formation of WPBs) was inspected. The data obtained from these studies led to the discovery the novel pathogenesis mechanisms leading to VWF deficiencies in VWD patients and clarifying the effect of VWF variants on intracellular processing of VWF, including multimerization, storage/formation of WPBs, and secretion.[22,44–51] The use of the patient-derived ECFCs emphasized the significance of the exonic synonymous and deep intronic variants on VWF splicing and their contribution to the pathogenesis of VWD.[44–47,51] Furthermore, we inspected the gene expression profile and trafficking of the inflammatory/angiogenesis proteins Ang2 and P-selectin, co-stored with VWF in WPBs, in the patient- and healthy control-derived ECFCs. Subsequently, we demonstrated alterations in intracellular trafficking of Ang2 and P-selectin and gene expression profiles of patient-derived ECFCs, even in distinct patterns, highlighting the implication of ECFCs as a valuable source in VWD research.[45,46] Correspondingly, Schillemans and colleagues reported alternative trafficking and secretion of several WPBs proteins (Ang-2, IL-6, and IL-8) after generating VWF-knockout ECFCs using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) gene-editing method. With this study, they also showed that CRISPR/Cas9 strategy is a robust method to create VWF-deficient/or-defective ECFCs that can be used for investigating pathophysiological mechanisms of VWD.[52]

In another interesting study, using type 3 VWD patients-derived ECFCs, Bowman et al revealed a differential effect of a duplicate variant (c.8419_8422dupTCCC) on VWF expression

between platelets and endothelial cells.[48] Initially, the patients displayed discrepant platelet versus plasma VWF levels. Subsequently, they assessed the level of VWF content in platelets and ECFCs isolated from the patients and the healthy controls. Consequently, they detected VWF in platelet lysates of the patients (>10× higher than plasma VWF) compared with significantly reduced VWF levels in lysate and supernatant of the patient ECFCs. They suggested that this is likely due to the inability of VWF expressed in patient ECFCs to form the multimers required for normal WPB packaging and release, whereas the platelet α-granules may be less stringent in this regard.[48]

Furthermore, in an inventive study, Kloosterman et al used the patient-derived ECFCs to understand the pathogenesis of type 1 VWD. They applied whole-transcriptome messenger RNA and microRNA-sequencing to generate comprehensive transcriptome profiles of basal and stimulated VWF release in both type 1 VWD- and control-derived ECFCs. Consequently, they revealed distinct transcriptional differences between Type 1 VWD and control ECFCs during basal and stimulated VWF release. They suggested that dysregulation of particular miRNA–mRNA axes may result in Type 1 VWD pathogenesis. They further demonstrated that these differences are more notable during stimulated release, which challenges current guidelines that consider only baseline VWF levels for the diagnosis of Type 1 VWD.[53]

Pathophysiology of angiodysplasia in VWD: Apart from the crucial role of VWF in hemostasis, there is evidence that VWF is also involved in angiogenesis and blood vessel formation. VWF modulates angiogenesis through either the intracellular pathway (owing to its intriguing property to derive the biogenesis of WPBs) or extracellular pathways (direct interaction of VWF with the endothelial surface receptors of integrin αvβ3, and modulating VEGFR-2 signaling). It is also well-recognized that patients with VWD have an increased incidence of angiodysplasia and vascular malformation leading to gastrointestinal (GI) tract bleeding.[20] As noted earlier, ECFCs were previously utilized to investigate the impact of VWF mutations on angiogenic features of endothelial cells in VWD, though the number of these studies is limited. To date, three studies on the pathophysiological mechanisms of angiodysplasia in VWD, using ex vivo ECFCs from VWD patients, have been published by Dr. Jeroen Eikenboom's group in the Netherlands, Dr. Anna Randi's group in England, and Dr. Paula Denise James's group in Canada.[20,42,43] These studies have generated ECFCs from type 1, 2A, 2B, and 3 VWD patients, and subsequently, they have determined the angiogenic characteristics of expanded isolated ECs ex vivo. In general, they have found variability in the angiogenic characteristics of ECFCs from different VWD subtypes and even within a single VWD type. Frequent features of ECFCs from VWD patients included increased Ang2 secretion into cells medium, abnormal cell proliferation, and migration. However, an impairment in the directionality of migration, an increased migratory velocity, and tube formation were observed in some VWD ECFCs (mostly in type 3) compared with healthy ECFCs.[20,42]

Thrombotic Disorders

Venous thrombosis is caused by both genetic and environmental factors. While thrombotic risk assessment is classically focused on the evaluation of circulating coagulation factors and inhibitors, endothelial properties and responses are equally contributing to the development or prevention of thrombosis. This article gives an overview of studies on thrombotic disorders, in which ECFCs have been applied aiming for clarification of the endothelial-driven pathogenesis.

Disorders of the protein C pathway: The endothelial protein C pathway serves as an anticoagulant system where activated protein C (APC), the key enzyme, together with its cofactor protein S (PS), downregulates thrombin formation through proteolytic inactivation of FVa and FVIIa. The activation of PC involves binding the proenzyme to the endothelial protein C receptor (EPCR) and the binding of thrombin to thrombomodulin (TM), which provides the activating complex. Therefore, disorders of the protein C pathway such as PC or PS deficiency and the FVL mutation hindering the inactivation of FVa by APC increase the thrombotic risk. Our group and others have shown EPCR and TM expression and, accordingly, APC formation on ECFCs.[54,55] These findings suggest potential anticoagulant properties of the cells and approve ECFCs as a suitable ex vivo model for individualized protein C pathway assessment. Confirming in vivo data, we demonstrated increased APC formation in FVL carriers compared with non-FVL carriers using ECFCs and autologous plasma in an ex vivo model.[55] ECFC levels, proliferation, migration, and tubulogenesis in FVL carriers and PC-deficient patients with Budd-Chiari syndrome were shown to be lower compared with patients without the hereditary thrombotic risk factor.[56] These data indicate a potential connection between the protein C pathway and ECFC function modulating the thrombotic risk.

SLE and APS: Inflammatory signaling via cytokines or complement factors is both mediated from and received by vascular endothelial cells. Hence, imbalances can modulate endothelial function and thrombotic risk. SLE is an autoimmune disorder associated with endothelial dysfunction and thrombophilia. In addition to endothelial damage caused by autoantibodies, endothelial repair is severely compromised in SLE patients resulting in premature vascular damage.[57,58] Several studies found lower levels of circulating EPCs and impaired ECFC proliferation and angiogenesis in patients compared with controls.[59–62] Denny et al found an increased interferon-α (IFN-α) production in ECFC from SLE patients.[60] They also showed the cytotoxic effect of IFN-α on the cells and, thereby, the inhibitory effect on angiogenesis. IFN-α was confirmed being the main culprit, and other inflammatory pathways were identified by further studies using ECFCs from SLE patients.[63–65] While symptoms and autoantibodies in SLE are more diverse, APS, which can be associated with SLE, is specifically characterized by venous and/or arterial thrombosis and pregnancy morbidity in the presence of pathogenic antiphospholipid antibodies. Regarding endothelial progenitor status in the pathogenesis of APS, Gresele et al compared ECFC levels between 20 APS cases and

matched controls and did not find a significant difference.[66] As mentioned earlier, significantly decreased levels of ECFCs in SLE patients were found, which, however, did not correlate with the presence and/or levels of different antiphospholipid antibodies.[60] Although they repeatedly reported no significant difference regarding the levels in peripheral blood, Grenn et al found ECFCs from APS patients to be impaired in the ability of mature endothelial cell differentiation compared with healthy controls.[67] These results were supported by the reduced number of ECFCs growing from control PBMCs cultured with APS sera. While IgG depletion of the sera did not rescue and purified APS and IgG did not interfere with ECFC differentiation, elevated IFN-α levels were found in APS sera, and APS PBMCs showed upregulated IFN-responsive genes. Finally, an IFN receptor-neutralizing antibody was shown to rescue ECFC differentiation exposed to APS sera, which strongly indicates the influence of IFN-α on ECFC differentiation in APS patients. Using ECFCs as a model for inflammation and angiogenesis of SLE and APS patients, new insights into disease pathology were gained, giving new directions for research and therapy, and decreasing the thrombotic risk.

Sickle cell disease and stroke: Sickle cell disease is an inherited disorder resulting in abnormal hemoglobin that polymerizes in hypoxic conditions leading to a sickle-like shape of red blood cells with increased endothelial adhesion. Various complex mechanisms like hypercoagulability and abnormal endothelial function contribute considerably to disease pathology.[68] Using ECFCs, ischemic stroke risk and endothelial cell biology of sickle cell anemia (SCA) patients were studied. Chang Milbauer et al showed upregulated inflammatory signaling in at-risk subjects compared with subjects considered not at risk for ischemic stroke.[69] Another laboratory aimed to establish ECFCs as a study model for endothelial function, including adhesive and inflammatory properties of SCA patients. They found patient ECFCs to be stronger in red blood cell adhesion and higher in proinflammatory signaling and IL-8 production, compared with ECFCs from healthy individuals.[70] Ito et al performed gene expression profiling of SCA patients with and without stroke. As in previous studies, they confirmed the upregulation of genes related to inflammatory pathways.[71] In addition, they found upregulated genes associated with coagulation and angiogenesis and downregulation of genes associated with apoptosis and cell adhesion. Most significantly, matrix metalloproteinase 1, an enzyme that is crucially involved in angiogenesis, was 200-fold upregulated, supporting the association between sickle cell stroke and angiogenesis-related pathways. Overall, these studies using ECFCs from sickle cell disease patients gained more insights into the endothelial-related pathophysiology of the disease. Nevertheless, further studies are needed to elucidate endothelial mechanisms leading to increased sickle stroke risk.

COVID-19: Although COVID-19, caused by severe acute respiratory syndrome coronavirus-2, is mainly associated with respiratory symptoms, complications are also related to vascular endothelial damage and severe thrombotic events. About 30% of COVID-19 patients in intensive care units show venous thromboembolism (VTE),[72,73] and pulmonary embolism or VTE is the estimated cause of death in ~20% of deceased patients.[74] As indicators for vascular regenerative capacity and endothelial function, ECFCs from COVID-19 patients were studied. Alvarado-Moreno et al analyzed the frequency, function, and morphology of ECFCs from recovered male patients approximately 4 weeks after the onset of symptoms.[75] They found an increased number of ECFC colonies which appeared in a shorter period in samples from patients compared with controls. However, ECFC colonies from patients showed morphological alterations and lower proliferative capacity indicating endothelial dysfunction and reduced vascular repair. Confirming these results, Poyatos et al reported an increased number of ECFC colonies associated with a shorter time of ECFC appearance in COVID-19 patients 3 months after the infection.[32] In addition, they found a correlation between high ECFC colony numbers with high hemoglobin levels ($\geq$ 14.5 g/dL) and with low PaO_2 levels ($<$ 80 mm Hg), both indicating hypoxic conditions. Whether increased ECFC levels in post-COVID-19 patients indicate a protective or counteractive response is unknown so far and deserves further study.

Disorders of the fibrinolytic system: While acquired disorders of the fibrinolytic system due to inflammation mostly result in increased fibrinolytic activity and a bleeding tendency, congenital abnormalities are associated with the development of thrombosis. This includes abnormalities of endothelial cells, which express both major plasminogen activators, tissue plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) and the plasminogen activator inhibitor-1 (PAI-1), and are therefore crucial players in fibrinolytic and thrombotic regulation.

High expression levels of u-PA and its receptor were measured on ECFCs indicating a potential role in plasminogen activation and fibrin degradation.[76,77] In addition, Smadja et al showed t-PA and PAI-1 expression and demonstrated the interaction of ECFCs with thrombin in fibrin clots.[54] Bacha et al studied the capacity of ECFC-derived endothelial microparticles to modulate plasmin generation in idiopathic pulmonary fibrosis (IPF) patients and found higher plasminogen activation compared with healthy control microparticles.[78] Overall, these data demonstrate the contribution of ECFCs on fibrinolytic regulation and suggest ECFC cultures as an ex vivo model for endothelial disorders affecting the fibrinolytic pathway. Conversely, it has been shown that players of the fibrinolytic pathway affect endothelial angiogenesis in vitro and in mouse models.[79,80] These results were confirmed using ECFCs as an angiogenesis model. Lacroix et al showed plasminogen activation at the surface of endothelial microparticles and their dose-dependent effect on ECFC tube formation.[81] Holnthoner et al established a coculture system of ECFCs and adipose-derived stem cells from the same individual and showed the ability of vessel formation in a fibrin matrix.[30] The group later demonstrated the inhibitory effect of fibrinolysis inhibition on ECFC-mediated vascularization using the same system.[82]

However, mechanisms and disorders of the fibrinolytic pathway and their impact on inflammation, angiogenesis,

and thrombogenesis on an individual level remain poorly understood and deserve further studies.

Potential Use of ECFCs for Diagnostic Purposes

Abnormal ECFC levels and function have been shown in various vascular diseases. Therefore, ECFCs are proposed as potential biomarkers or liquid biopsies to investigate endothelial dysfunction in patients.

ECFCs as Liquid Biopsies for Bleeding or Thrombotic Disorders

Liquid biopsies are valuable sources for diagnosis and molecular profiling of cellular pathophysiology. Representing an individual's endothelial properties, ECFCs can be considered as a liquid biopsy of in situ endothelium. Several studies demonstrated the suitability of ECFCs as liquid biopsies for pulmonary diseases. For chronic obstructive pulmonary disease patients, reduced proliferation, adhesion, and migration capacities of ECFCs were demonstrated.[83,84] Using galactosidase staining, annexin-V staining, and p16 expression measurement of ECFCs, increased endothelial senescent and apoptotic states were shown for IPF patients.[85]

Patient-derived ECFCs were utilized to investigate the pathogenesis of the acquired VWD in patients with aortic stenosis (AS), a progressive valvular heart disease. The AS is caused by a narrowing heart valve, which causes structural changes in VWF and loss of large VWF multimers due to the generation of high shear force, resulting in acquired VWD.[14] Patients with AS can experience complications, including GI bleeding from angiodysplasia lesions. Selvam et al investigated AS patients-derived ECFCs ex vivo. Subsequently, they demonstrated that ECFCs from patients with AS were more proliferative than controls, along with increased retention of Ang-2 in patient-derived ECFCs. They suggested that this can be due to the loss of large VWF multimers and its consequent effect on angiogenesis through the extracellular pathway, which modulates VEGFR-2 signaling. The authors concluded that patient-derived ECFCs have implications for the clinical management of AS patients by monitoring them before and after surgery.[14]

In addition, the potential use of ECFCs as liquid biopsies has been proposed for thrombotic disorders.[86] As mentioned earlier, Chang Milbauer et al showed upregulated inflammatory signaling of sickle cell disease patients with increased thrombotic risk compared with subjects considered not at risk for ischemic stroke.[69] Comparing VTE patients and controls, Alvaredo-Moreno et al found differences in cytokine profiles, increased reactive oxygen species, and abnormalities in the mitochondrial membrane.[87] Therefore, gene expression profiling or culture supernatant evaluations of ECFCs from thrombotic patients might be applied as a liquid biopsy to assess an individual's thrombotic risk.

Circulating ECFCs as Biomarkers for Thrombotic Risk

Rosti et al found a correlation between elevated levels of cultured ECFCs and the occurrence of splanchnic vein thrombosis in patients with primary myelofibrosis.[88] They proposed increased ECFC frequency as a biological hallmark for a higher risk of thrombosis in these patients. Alvarado-Moreno et al studied ECFC frequency in culture and function in patients with a history of recurrent, unprovoked thrombosis. They found higher ECFC numbers and less time of appearance in VTE patients compared with controls, thereby confirming previous results.[87] In a multicenter randomized control trial, circulating EPC levels were quantified by flow cytometry in 193 VTE patients.[89] In patients with recurrent VTE (12%), significantly lower levels of EPCs were measured compared with patients without recurrent VTE. As an additional benefit, EPC levels as a biomarker were able to identify high VTE recurrence risk in anticoagulated patients, while D-dimers, most commonly used as a biomarker for VTE risk, were not indicative in these patients.[89]

Although the connection between the development of thrombosis and ECFC function remains poorly understood, these results suggest that numbers of circulating EPCs and ECFC outgrowth colonies might be used as hallmarks for VTE risk evaluation for improved treatment regimens.

ECFCs for Potential Therapeutic Applications

Due to the cells' vessel-regenerative function, ECFCs have been widely proposed for therapeutic application in conditions causing vessel and tissue damage. In animal models aiming for tissue regeneration and repair, ECFCs have been variously applied, including systemic or tissue-targeted bolus injection, in vitro pre-vascularization of tissue-engineered constructs or vascular grafts, or delivery of gene products.[90] Since the endothelium contributes to the development of many hemostatic disorders, it has been considered as a target for therapeutic application of bleeding and thrombotic disorders.

ECFCs for Gene and Cell Therapy for Bleeding Disorders

Genomic stability in culture and ease of genetic manipulation, besides obtaining them in a minimally invasive way, made ECFCs an attractive cell target for gene therapy to potentially treat bleeding disorders, including VWD and hemophilia A.[91] In an attempt for phenotypic correction of VWD type 3, De Meyer et al showed successful and highly efficient lentiviral vector (encoding full-length human VWF) transduction of canine ECFCs, which led to the expression of functional VWF.[26] Furthermore, in another study, de Jong and colleagues proved that ECFCs are also feasible cell targets for inhibiting the production of mutant VWF by allele-specific small-interfering RNAs (siRNAs) as a therapeutic strategy to improve VWD phenotypes. The transfection of isolated ECFCs from a type 2 VWD patient (exhibiting a defect in multimerization due to mutation VWF p.Cys1190Tyr) with the allele-specific siRNA demonstrated improvement of the laboratory phenotype, including the improvement in VWF multimers and VWF-binding activities.[92]

Stable and safe FVIII expression was shown in murine and canine hemophilia A models after transplantation of genetically modified ECFCs ex vivo alone or combined with other

cell types in multiple studies.[18,93–95] Moreover, retroviral FVIII-transduced human umbilical cord-derived ECFCs are superior to transduced LSECs (liver sinusoidal endothelial cells) in terms of mRNA production, protein levels, and procoagulant activity.[96]

Overall, these results demonstrate that ECFCs are feasible target cells for developing competent gene therapy strategies and ex vivo cell therapy application as a promising instrument for the treatment of bleeding disorders in addition to thrombotic disorders.

Prevention and Treatment of Thrombosis Using ECFCs

Stent thrombosis occurring short or long term after coronary artery stenting has a significant clinical impact owing to the high risk of myocardial infarction, with a reported mortality of 45%.[97,98] Mediating an antithrombotic effect, one approach for stent thrombosis prevention is rapid endothelialization of the metal, for example, by seeding autologous ECFCs on the graft before implantation[99] or the design of ECFC capture stents coated with ECFC-binding antibodies.[100–103] In an atherosclerotic rabbit model combining both ECFC capture stent placement and local EPC transplantation, successful ECFC transplantation could be shown and compared with a drug-eluting stent receiving group revealed lower in-stent restenosis rates in the ECFC capture stent receiving group.[103] The GENOUS stent, a bioengineered stainless steel coronary stent with a biocompatible circumferential coating of anti-CD34 antibody, was approved after showing the safety and efficacy of the ECFC capturing approach for the prevention of stent thrombosis in human clinical trials.[100,104]

The promotion of neovascularization and direct incorporation of ECFCs into ischemic tissues has been demonstrated in animal models of hind limb ischemia,[105,106] ischemic retinopathy, and myocardial infarction[107,108] after transplantation of human ECFCs. In a mouse model of induced thrombosis, Modarai et al showed penetration of circulating endothelial cells into the thrombus and a twofold increase of circulating EPCs (VEGFR-2^+/$CD34^+$) compared with control mice.[109] These results indicate increased ECFC proliferation in thrombotic conditions and a potential ECFC contribution to thrombus resolution. Therefore, autologous ECFCs are promising candidates for thrombotic prevention and potential treatment of ischemic disorders in the future.

Conclusion and Perspectives

ECFCs represent the endothelial precursor, which can be derived from both peripheral blood and the umbilical cord. They can give rise to mature endothelial cells with a high proliferative potential to generate large amounts of autologous ECs in vitro. In culture, ECFCs closely imitate vascular ECs' features phenotypically, genetically, and functionally, making them ideal ECs surrogates. Findings from the multiple studies reviewed here demonstrate the value of ECFCs as a patient-derived endothelial cell model, which provides a more in-depth insight into the pathogenesis mechanisms of hemostatic disorders and thrombotic disease.

The outcomes obtained from former studies presented here provide evidence that ECFCs are promising candidates for regenerative therapy of ischemic and thromboembolic disorders. Additionally, multiple studies reviewed here demonstrate that ECFCs could be used as an instrument for gene therapy to treat bleeding (including VWD and hemophilia A) and thrombotic disorders. Although the use of autologous ECFCs for vascular regenerative therapy and gene therapy remains mostly preclinical, future research strategies, with a greater emphasis on standardization of isolation and culture strategies besides validation of ECFCs identity, can accelerate the prospect for clinical trials. Furthermore, future research utilizing a more significant number of patient-derived ECFCs and cutting-edge molecular technologies such as wide-transcriptome and proteomic profiling will help understand molecular mechanisms of vascular diseases and to develop the potential application of ECFCs for diagnostic purposes.

Conflict of Interest

The authors declare that they have no conflict of interest.

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