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ORIGINAL ARTICLE

Endothelial characteristics in healthy endothelial colony forming cells; generating a robust and valid ex vivo model for vascular disease

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Abstract

Background: Endothelial colony forming cells (ECFCs) derived from peripheral blood can be used to analyze the pathophysiology of vascular diseases ex vivo. However, heterogeneity is observed between ECFC clones and this variability needs to be understood and standardized for ECFCs to be used as a cell model for applications in vascular studies.

Objective: Determine reference characteristics of healthy control ECFCs to generate a valid ex vivo model for vascular disease.

Methods: Putative ECFCs (n = 47) derived from 21 individual healthy subjects were studied for cell morphology and specific cell characteristics. Clones were analyzed for the production and secretion of von Willebrand factor (VWF), cell proliferation, and the expression of endothelial cell markers.

Results: Based on morphology, clones were categorized into three groups. Group 1 consisted of clones with classic endothelial cell morphology, whereas groups 2 and 3 contained less condensed cells with increasing cell sizes. All clones had comparable endothelial cell surface expression profiles, with low levels of non-endothelial markers. However, a decrease in CD31 and a group-related increase in CD309 and CD45 expression, combined with a decrease in cell proliferation and VWF production and secretion, was observed in clones in group 3 and to a lesser extent in group 2.

Conclusions: We observed group-related variations in endothelial cell characteristics when clones lacked the classic endothelial cell morphology. Despite this variation, clones in all groups expressed endothelial cell surface markers. Provided that clones with similar characteristics are compared, we believe ECFCs are a valid ex vivo model to study vascular disease.

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KEYWORDS
blood coagulation, endothelial cells, hemostasis, von Willebrand diseases, von Willebrand factor

1 | INTRODUCTION

von Willebrand factor (VWF) plays an essential role in primary hemostasis and is exclusively synthesized and stored in endothelial cells and megakaryocytes (platelets). Upon vascular injury, VWF is released into the circulation where this multimeric protein is required for platelet adhesion and serves as a carrier for coagulation factor VIII (FVIII). Defects of VWF lead to the most common inherited bleeding disorder, von Willebrand disease (VWD).[1-3] The pathophysiology of VWD can be investigated by examining the synthesis, storage, and secretion of VWF in cellular models. For many years transfected heterologous cell systems have been used successfully to elucidate many aspects of VWF synthesis and secretion.[4-6] Because these cells do not produce endogenous VWF, models like these do not fully reflect the characteristics of primary VWF producing cells. However, obtaining endothelial cells or megakaryocytes with a VWD phenotype requires invasive procedures, such as vessel collection or a bone marrow biopsy. A more recent and promising development is the isolation of endothelial colony forming cells (ECFCs) as a true-to-nature cell model. These cells, previously also referred to as blood outgrowth endothelial cells (BOECs), were first described by Hebbel's group in 2000.[7] They circulate in the bloodstream and are isolated from mononuclear cells in peripheral blood (PBMCs) via a routine blood draw. Interestingly, their origin remains unknown although recent research excludes bone marrow and suggests a microvascular niche origin.[8]

Cultured ECFCs demonstrate endothelial cell-like characteristics, such as the typical cobblestone morphology, expression of endothelial cell surface antigens, and the production of VWF and storage in Weibel-Palade bodies (WPBs).[9] These cells represent a relatively mature endothelial cell phenotype and have been implicated in postnatal vasculogenesis and endothelial repair at sites of vascular injury.[10] Importantly, ECFCs from patients with VWD and other vascular pathologies demonstrate persistent abnormalities in structure and function after isolation from the blood.[11] Our groups (Eikenboom, Lillicrap, James) and others have applied ECFCs to investigate the pathogenesis of VWD and angiodysplasia.[12-17] Our findings suggest that ECFCs are promising biological tools to understand a variety of hemostatic disorders. In spite of the potential of ECFCs, we and others have also reported broad variations in VWF–related results when ECFCs are in culture.[18] Additionally, we have noticed that the success rates of ECFC isolations is lower than reported, with some donors never yielding any colonies.[18-20]

We recently studied a cohort of ECFC clones derived from six healthy donors and observed large variations in VWF-related results among ECFCs,[18] not only from different donors, but also among clones from individual donors. This variation could be attributed to multiple causes, such as the origin and age of ECFCs, but also donor-specific features such as age and (vascular) disease. Additionally, ECFC isolation and culture methods, which can vary between research groups, might influence and affect the cells' proliferative capacity and possibly phenotype.[19] This variation indicates that there is a need for standardization of protocols in order to compare findings across laboratories.[20] For ECFCs to be a valid cell model for use in disease studies, the extent of variability of cellular phenotype needs to be understood in more detail.

Here we study the isolation, and structural and functional qualities, of these circulating endothelial cells, with standardized protocols between different research groups. We isolated ECFCs from healthy donors, not diagnosed with VWD or other bleeding disorders, and subjected these cells to several assays in order to determine normal reference characteristics of ECFCs. These parameters can be beneficial for a more accurate analysis of ECFCs from patients and therefore lead to the generation of a valid ex vivo disease model for vascular diseases.

Essentials

- Endothelial colony forming cells (ECFCs) show variations in morphology and cell characteristics.
- Putative ECFCs derived from healthy controls can be categorized into three morphological groups.
- There are group-related variations in endothelial cell characteristics.
- ECFCs are a valid ex vivo model to study disease when clones with similar characteristics are compared.

2 | MATERIALS AND METHODS

2.1 | Study design

This study is a collaboration between research groups from Leiden, the Netherlands (Leiden University Medical Center, LUMC) and Kingston, Canada (Queen's University). We have standardized the following approach and detailed protocols of this project between the groups (Figure 1). When methods differ, the Leiden approach is described first, followed by Kingston. The study protocol for ECFC isolation was approved by the ethics review boards of both LUMC and Queen's University. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Healthy participants were 18 years or older and had not been diagnosed with or known to have VWD or any other bleeding disorder.

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FIGURE 1 Flowchart of endothelial colony forming cells characterization. Clones were taken from liquid nitrogen and grown for two passages in T75 flasks. When confluent, cells were either stained for fluorescence-activated cells sorting analysis or cells were plated for proliferation, secretion, and immunofluorescence assays

2.2 | ECFC isolation and culture

Blood samples were obtained from healthy donors via venipuncture in sodium heparin Vacutainers or mononuclear cell preparation tubes (CPTs) with Ficoll Paque (BD Biosciences) for PBMC collection. Additionally, for each donor citrated blood was collected in Vacutainers (BD Biosciences) for preparation of plasma.

ECFCs were isolated using a protocol adapted from Martin-Ramirez et al.[21] Peripheral blood samples collected into CPTs were centrifuged directly and samples drawn into sodium heparin tubes were first diluted 1:1 with phosphate buffered saline (PBS, LUMC Pharmacy) before being separated by a gradient centrifugation over Ficoll Paque (LUMC Pharmacy). The PBMC fraction was isolated and washed twice with PBS/10% fetal bovine serum (FBS; Gibco Invitrogen). Cells were resuspended in EGM-10 (EBM-2 Basal Medium with EGM-2 supplements & growth factors (Lonza, Basel, Switzerland or PromoCell); 10% FBS; 1% antibiotic antimycotic solution (Sigma-Aldrich) and cells were seeded at 250 000 to 400 000 cells per well in EGM-10 on 48 wells plates (Sarstedt) precoated with 50 µg/mL collagen type I rat tail (BD Biosciences). Medium was refreshed every day for 7 days, and thereafter every other day for a maximum of 28 days. Cells were checked daily after 10 days of incubation. Typically, ECFC colonies started to appear between days 10 and 21. When a colony covered approximately 80% to 90% of the well, cells were passaged with TrypLE or 0.25% trypsin/0.02% EDTA (Gibco) to the subsequent well size; ie, from a 48 well, to a 24 well, and onward on a collagen I coat (previously described in de Jong et al[18]; all culture vessels from Corning). Isolated individual colonies were grown to full confluence (passage 3) and stored in nitrogen. Some isolations resulted in a high yield of individual ECFC clones. When multiple individual clones appeared, we expanded a maximum of 10 individual clones and pooled the remaining clones before being cryopreserved.

2.3 | Set-up characterization assays

Cells were cultured in EGM-10 on collagen I-coated plates/flasks and grown to 3 to 5 days postconfluency. From previous work in our lab, we have shown that this is the maximum level of VWF antigen (VWF:Ag) secretion (maximum cell density).[18] Therefore, all experiments were initiated on cells at passage 5, 3 to 5 days after reaching maximum confluency. Cells were seeded or processed immediately for flow cytometry (fluorescence-activated cells sorting; FACS) analysis. Cells for VWF:Ag production and confocal microscopy were seeded in 24 well plates and grown to 3 to 5 days confluency, before experiments started. The characterization assays were performed on different clones of individual donors when available, or single clones were repeated. Bright-field images were taken when cells were 3 to 5 days postconfluent and cell density was determined with CellProfiler 3.1.8 software (Broad Institute of Harvard and MIT, Cambridge, MA, USA).

2.4 | Confocal immunofluorescence microscopy

Cells were plated on glass coverslips coated with collagen I in 24 well plates and grown to 3 to 5 days postconfluency. In Leiden, cells were fixed with 4% paraformaldehyde (PFA; Alfa Aesar) without washing, then rinsed with PBS and blocked and permeabilized for 20 minutes in blocking buffer (PBS; 5% Normal Goat Serum [Dako]; 0.02% saponin [Sigma-Aldrich]). In Kingston, cells were washed once with PBS and fixed with Fixation Solution (BD Bioscience). Cells were rinsed with PBS and permeabilized with PBS/0.1% Triton x-100 for 10 minutes on ice, then washed with PBS and blocked for 20 minutes with Protein Block (Dako).

After fixation, permeabilization, and blocking, cells were stained with primary antibodies for VWF and VE-cadherin (Table S1 in supporting information) diluted in either blocking buffer or PBS/1% bovine serum albumin (BSA) (Sigma-Aldrich). Nuclear staining was performed with either Hoechst (Thermo Fisher Scientific) or DAPI (Sigma-Aldrich) diluted in PBS. Coverslips were mounted by Mounting Media (Dako) or ProLong Diamond Antifade Mountant (Thermo Fisher Scientific) and cells were imaged using the Leica TCS SP8 inverted confocal microscope (Leica Microsystems) with the white light laser (WLL), hybrid detectors (HyD), and the HC PL APO CS2 63x/1.40 oil immersion objective. Images were acquired and analyzed using the LAS-X Software (Leica Microsystems).

2.5 | FACS analysis

Cell surface marker expression was analyzed using flow cytometry. Either cells (100 000 cells/antibody mix) or CompBeads

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(Thermo Fisher Scientific) were resuspended in FACS buffer (PBS; 1% BSA; 0.01% sodium azide [Sigma-Aldrich]) and incubated on ice for 30 minutes with labelled primary antibodies or isotype controls (Table S1). Cells and beads were fixed with 2% PFA or with Fixation Solution (BD Bioscience). After washing, samples were resuspended in FACS buffer before being analyzed on the BD™ LSR II (BD Bioscience) or SH800S Cell Sorter (Sony Biotechnology). Data was analyzed with FlowJo software (FlowJo LLC v10.6.1, BD Bioscience).

2.6 | VWF production and secretion

Basal/constitutive VWF secretion of the cells was determined by the release of VWF:Ag over 24 hours in release medium (Opti-MEM™ I Reduced serum media GlutaMAX™ supplement [Gibco]; 10 mmol/L HEPES, pH 7.4, 0.2% BSA). For regulated VWF secretion, cells were incubated for 1 hour in release medium supplemented with 100 μmol/L histamine (Sigma-Aldrich) before medium was collected. To determine intracellular VWF, wells with cells were lysed either overnight at 4°C in Opti-MEM I/0.1% Triton X-100 (Sigma-Aldrich) or for 1 hour at room temperature in Passive lysis buffer (Promega), both supplemented with cOmplete Protease Inhibitor cocktail with EDTA (Roche Diagnostics). Wells were scraped before lysates were collected. VWF production and secretion was measured as VWF:Ag by enzyme-linked immunosorbent assay (ELISA) as previously described.[18]

2.7 | Plasma analysis

Plasma samples from Leiden were measured for VWF:Ag and VWF and FVIII activity by the clinical chemistry laboratory at the LUMC, Leiden. VWF:Ag in plasma was determined using the STA LIA VWF:Ag test (Stago) and was analyzed on the Sta-R Max analyser (Stago) with a commercial STA VWF:Ag calibrator (STA Unicalibrator, Stago) as reference. VWF activity was determined with the VWF ristocetin-triggered GPIb binding assay (VWF:GPIbR) with HemosIL AcuStar VWF:RCo reagent (Werfen IL). Samples were analyzed on the BIO-FLASH (Werfen) and a commercial calibrator (supplied with the HemosIL AcuStar VWF:RCo) was used as reference. FVIII activity was determined using an automated one-stage clotting assay on the STA-R MAX analyzer (Stago) with Sta-immunodef VIII (Stago) and STA-CK Prest 5 (APTT; Stago) reagents. Commercial normal pool plasma (STA Unicalibrator, Stago) was used as reference. At Kingston, only VWF:Ag was measured in the plasma samples, and this was done by ELISA as previously described.[22]

2.8 | Proliferation assay

Proliferation of clones was analyzed with the Cell Counting Kit-8 (Dojindo Molecular Technologies) according to the manufacturer's protocol. Proliferation was measured by evaluating the metabolism of the cells. Cells were seeded in 96 well plates at 5000 cells per well and incubated at 37°C, 5% CO_2 and measurements were taken at 24, 48, and 72 hours. After 24 hours of incubation, media was replaced with medium containing CCK-8 dye and incubated for a further 2 hours. The optical density values were recorded by a microplate reader at 450 nm and this was repeated at 48 and 72 hours.

2.9 | Clonogenic assay

A clonogenic assay was performed following an adjusted protocol.[23] Cells were seeded in duplicate in a serial dilution at 200, 400, and 800 cells in collagen I-coated 6 well plates. Cells were grown in EGM10 for 10 days. Cells were fixed with 4% PFA and stained for 10 minutes with 0.1% crystal violet (Sigma Aldrich). Images were taken on a ChemiDoc (BioRad) and counted with ImageJ.

2.10 | Statistics

GraphPad version 8 (GraphPad Software, La Jolla, CA, USA) was used for graphics and statistical analysis. One-way analysis of variance or, when not normally distributed, Kruskal-Wallis tests, were performed to make comparisons among groups, followed by either Tukey's or Dunn's tests for multiple comparisons. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Cell isolation success rate

In total we obtained blood samples from 50 healthy donors (11 Kingston, 39 Leiden) resulting in the successful isolation and expansion of putative ECFC clones from 23 of these donors, giving a success rate of 46%. During the isolations of 13 donors, multiple clones were obtained and these were cultured separately so that both inter- and intra-donor differences could be studied. Clones were coded by a number representing the donor and a subsequent letter indicating different clones from a single donor. We fully characterized 48 putative ECFC clones from 21 individual donors (Clones 1-5 from Kingston and Clones 6-21 from Leiden), using the standardized procedures and protocols established (Figure 1 and Table 1). We have chosen clones showing a range in the day that they appear, morphology, and growth rates. When available, multiple clones were analyzed with a maximum of five clones per donor. We were unable to revive ECFCs from cryopreservation from two donors and these were therefore excluded from this study. One clone, Clone 1C, was characterized but not included in the final analyses, as the isolated cells proved to be fibroblasts. Additionally, no correlations were observed among blood volumes, PBMC counts, and ECFC colonies ($R^2 = .1621$ and $R^2 = .0033$, Figure S1 in supporting information).

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TABLE 1 Details of isolated putative endothelial colony forming cells clones

Donor details	Kingston	Leiden	Total
Donors	11	39	50
Successful isolations	5 (45%)	18 (46%)	23 (46%)
Expanded clones	16	106	122
M/F (sex)	1/4	6/12	7/16
Age in years, median (range)	27 (24-38)	26 (20-63)	26 (20-63)
Cell characterizations			
Donors	5	16	21
Characterized clones[a]	15[b]	33	48[b]

[a]When available, multiple clones were analyzed with a maximum of five clones per donor. We were unable to revive clones from cryopreservation from two donors and these were therefore excluded from this study.

[b]One clone (Clone 1C) is not included in final analysis as this clone proved to be fibroblast.

3.2 | Morphology and grouping of putative ECFCs

Even though cells were all isolated using the same protocol, and grown in defined endothelial growth medium, the morphology of the clones varied widely, and therefore we chose to categorize the clones into three morphological groups. In total 47 clones were grouped; ECFCs in group 1 (n = 17) displayed the classic cobblestone endothelial cell morphology, with small cells that were tightly packed. Clones in Group 2 (n = 17) were medium sized cells that were less condensed. Colonies in group 3 (n = 13) consisted of cells that were enlarged, spread out, and never reached full confluency (Figures 2A and B, and Table 2). Looking purely at morphology, clones in groups 1 and 2 appear to be ECFCs, whereas clones in group 3 are of unclear lineage.

Multiple clones from 13 individual donors were analyzed. Three of these donors (15, 16, and 19) had all clones classified in the same group, and nine donors had clones in adjacent groups (ie, groups 1-2 or 2-3). Only one donor (Clone 11) had clones in groups 1 and group 3. It should be noted that we isolated cells from donor Clone 11 and also donor Clone 15 on different days. Clone 11A (group 3) was isolated from the first donation, and clones 11B, 11C, and 11D (all group 1) during the second donation and isolation. For donor Clone 15, each donation yielded one clone, which are both in group 3.

3.3 | VWF localization

We have studied the storage of VWF in WPBs and the expression of VE-cadherin in the three morphological groups. Endothelial cells express VE-cadherin and when the cells are in a confluent layer, VE-cadherin expression is upregulated and gets concentrated at cell-cell junctions. We analyzed staining for VWF, VE-cadherin, and cell nuclei by confocal microscopy. Although clones in all groups

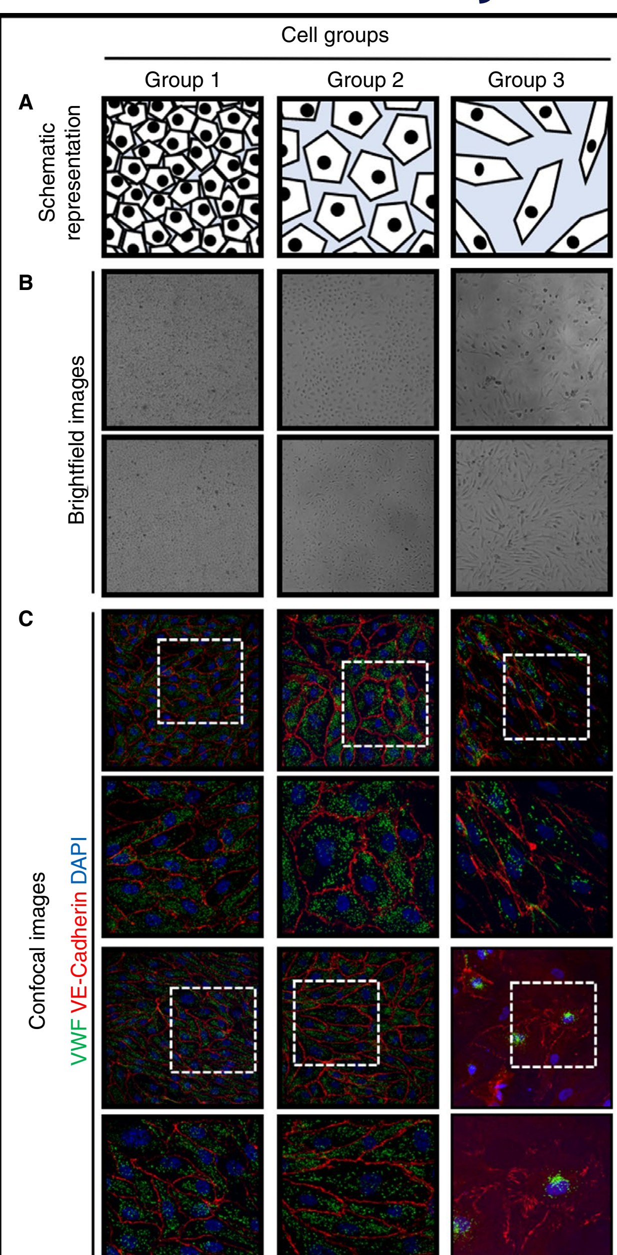

FIGURE 2 Grouping of clones based on morphology. A selection of clones grouped into three groups based on morphology. A, Schematic overview of cell morphology for the three groups. Group 1, classic endothelial (cobblestone) morphology, small cells, tightly packed (n = 17 clones); group 2, medium sized cells, less condensed (n = 17 clones); group 3, enlarged cells, spread out, will not reach confluency (n = 13 clones). B, Bright-field images of selected clones representing the three groups. C, Confocal microscopy images with zoomed in sections of clones representing the three groups: von Willebrand factor (VWF; green), VE-cadherin (red), and nucleus (DAPI)

showed VWF storage in elongated WPBs, differences in numbers and staining patterns were observed. Due to the variation in imaging between labs, we were unable to quantify the immunofluorescence data. However, we did see that most cells in group 1 stained positive for VWF stored in WPBs, which declined to ~50% in cells in group 2,

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and became even lower for cells in group 3, together with a decrease in VE-cadherin expression. As mentioned before, clones in group 3 were generally spread out and never reached full confluency, leading to lower VE-cadherin expression (Figure 2C).

3.4 | Endothelial cell surface markers

The expression of endothelial cell surface markers was studied in the three groups. Using flow cytometry, a panel of selected endothelial cell surface markers (CD31, CD34, CD51/61, CD144, CD146, CD309) and several non-endothelial markers (CD14, monocytes; CD45, leukocytes) was assessed. We have also screened for CD133, which is a hematopoietic stem cell marker. This marker, however, is a controversial endothelial progenitor marker as previous studies have shown inconsistent results.[11,24,25]

FACS analysis showed that all clones expressed the endothelial cell surface markers, with low expression of the selected negative markers (Figure 3 and Table S2 in supporting information). The great spread seen in the expression data of all markers tested might suggest that the individual colonies are heterologous cell populations. However, there was a significant decrease in CD31 expression and an increase of the non-endothelial marker CD45 combined with an increasing trend in CD14 (P = .1201) when groups 1 and 3 are compared. This can suggest that the cells are transitioning into a more mesenchymal phenotype. Interestingly, a trend was shown in increased expression of CD51/61 ($\alpha_v\beta_3$ integrin) (P = .0657), together with a significant increase in the expression of marker CD309 (VEGFR2/KDR) among groups 1, 2, and 3, which is in agreement with previous mRNA data for this marker.[18]

TABLE 2 Grouping of clones

Group 1	Group 2	Group 3
(Small cells, condensed)	(Medium cells, less condensed)	(Enlarged cells, non-confluent)
Clone 2A	Clone 1A	Clone 1B
Clone 3A	Clone 2B	Clone 1D
Clone 4A	Clone 2C	Clone 7
Clone 4B	Clone 3B	Clone 8A
Clone 4E	Clone 4C	Clone 8B
Clone 5	Clone 4D	Clone 10
Clone 6C	Clone 6A	Clone 11A
Clone 6D	Clone 6B	Clone 15A
Clone 11B	Clone 8C	Clone 15B
Clone 11C	Clone 9	Clone 17
Clone 11D	Clone 12	Clone 18A
Clone 14C	Clone 13	Clone 20
Clone 16A	Clone 14A	Clone 21B
Clone 16B	Clone 14B	
Clone 19A	Clone 18B	
Clone 19B	Clone 18C	
Clone 19C	Clone 21A	

3.5 | VWF production, storage, and secretion

VWF is either secreted constitutively or stored in WPBs and secreted as basal release or after stimulation. Both medium (secreted VWF) and cell lysates (intracellular VWF) were collected, with basal release in medium measured over a period of 24 hours. A significant difference in basal secretion was observed between clones in groups 1 and 3, with an intermediate level of VWF:Ag in group 2. The lysates of ECFCs in group 1 contained more VWF compared to both other groups with a significant difference with group 3 (Figures 4A and B), which indicates that ECFCs in group 1 produce and store more VWF in their WPBs.

We also investigated whether the active release of VWF stored in WPBs differed among the groups. Therefore, medium was collected from cells that were stimulated with 100 μmol/L histamine

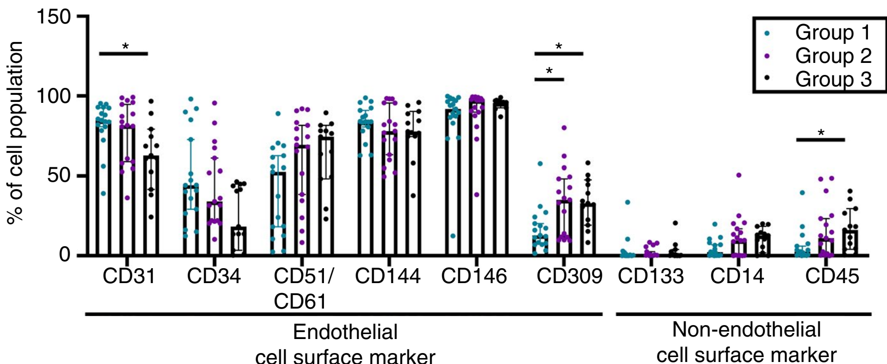

FIGURE 3 Endothelial cell surface marker analysis. Fluorescence-activated cells sorting analysis for cell surface markers CD31, CD34, CD51/61, CD144, CD146, CD309, CD133, CD14, and CD45 per group. Each dot represents an individual clone and data is shown as percentage positive cells in the total cell population. All clones show expression of endothelial cell surface markers and low expression for the non-endothelial markers. CD31, PECAM; CD34, hematopoietic marker; CD51/CD61, $\alpha v\beta 3$ integrin; CD144, VE-cadherin; CD146, MCAM; CD309, KDR/VEGFR2; CD133, hematopoietic stem cell/EPC marker; CD14, monocyte marker; CD45, leukocyte marker. Data shown as median, Kruskal-Wallis test, followed by a Dunn's multiple comparisons test, $^*P < .05$

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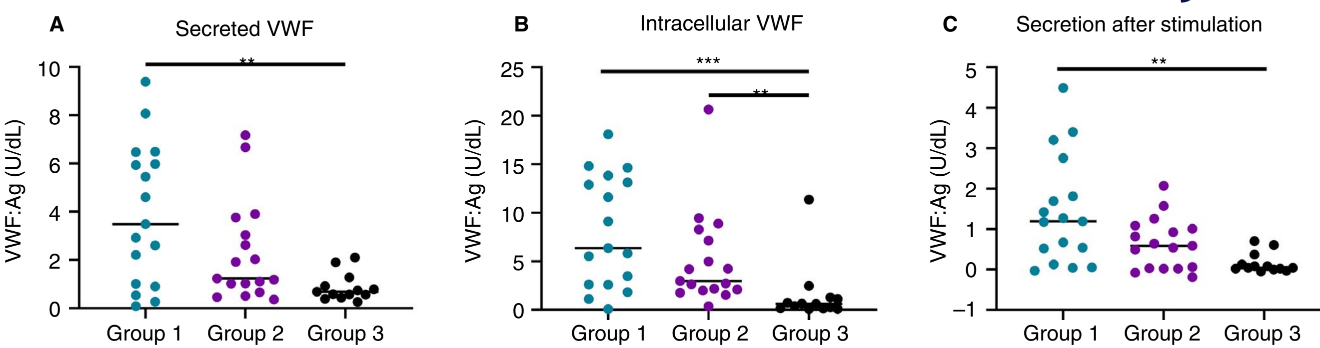

FIGURE 4 Storage and secretion of von Willebrand factor (VWF). A, Secreted VWF from cells (medium). VWF release in medium was measured over a period of 24 hours by enzyme-linked immunosorbent assay (ELISA). B, Intracellular VWF storage measured in cell lysates. VWF in cell lysates was measured over a period of 24 hours by ELISA. C, Secretion of VWF into media after histamine stimulation. Absolute VWF release was measured by stimulation with histamine (100 μmol/L) for 1 hour with ELISA (basal-stimulated). Values are given as median. Kruskal-Wallis test was performed, followed by a Dunn's multiple comparisons test; $^{**}P < .01$, $^{***}P < .001$

for 1 hour. VWF:Ag was measured and again, significantly lower levels of VWF:Ag secretion was observed for group 3 compared to group 1 (Figure 4C). These results have not been corrected for cell count, as wells from group 1 clones contain more cells than in groups 2 and 3. However, when we look at total VWF:Ag per cell (conditioned medium and cell lysates), we observed a similar trend showing a decrease in VWF levels among the three groups (Figure S2 in supporting information, $P = .0961$). This is in accordance with previous results showing that ECFCs with the classical endothelial morphology secrete higher levels of VWF per cell.[18]

3.6 | Plasma data

Plasma was collected from the donors (not always on the same day as the PBMC isolation) and measured for VWF:Ag, and VWF and FVIII activity. The samples from Kingston were only measured for VWF:Ag, and plasma samples from two donors (1 and 5) were not available. VWF plasma levels and activity (Figure 5A and B) of the samples measured are in the normal range, resulting in normal VWF activity/Ag ratios (Figure 5C). Finally, FVIII activity was measured, and these were also in the normal range (Figure 5D). No significant differences were observed in any plasma measurements among the three groups. This suggests that there is no clear correlation between the differences seen in ECFC characteristics and the VWF and FVIII plasma levels from the donors in this study.

3.7 | Demographic of donors and cell characteristics

Previously, groups have looked at several factors, such as age and gender of the donors, and disease populations. We have assessed several aspects and we did not see any significant differences with respect to age and gender based on the morphology groups (Figure S3 in supporting information, Kruskal-Wallis, followed by a Dunn's multiple comparisons test, $P = .1158$ and a contingency chi-square test, $P = .6147$, respectively).

3.7.1 | Proliferation capacity and cell density

To assess cell density of the clones, bright-field images were obtained 3 to 5 days postconfluence and cells were counted with CellProfiler. As expected, we detected significantly lower cell numbers in groups 2 and 3 compared to group 1 (Figure 6A). A similar observation was also reflected in the proliferation rates of the clones, which was measured at 24, 48, and 72 hours after seeding. Proliferation of clones in the three groups was comparable for the period between 24 and 48 hours ($P = .1204$), but decreased significantly in group 3 clones in the second time period (48-72 hours) compared to group 1 (Figure 6B). The total proliferation over 72 hours was also significantly lower in group 3 than in group 1 ($P = .0076$). We have also evaluated clonogenicity performing a clonogenic assay[23] on a selection of ECFCs from all three groups. We observed similar numbers in colonies being formed, indicating that ECFC clones from all three groups show proliferation potential (data not shown).

3.7.2 | Day of appearance

It has been reported that ECFCs normally appear 10 to 21 days after PBMC isolation and plating.[26,27] Cells were checked daily after day 10 by bright-field microscope and when the first endothelial cell-like cells were detected, this indicated the day of appearance of a colony. When this day was compared among groups, there was a significant delay in appearance of colonies in group 3 compared to group 1 ($P = .0236$; Figure 6C).

4 | DISCUSSION

In recent years, we and others have been using ECFCs as an endothelial cell model, to study different diseases.[11,13,17,28] However, heterogeneity is often observed between ECFC clones both between and within individual donors. This inter- and intra-donor variability requires further understanding and standardization

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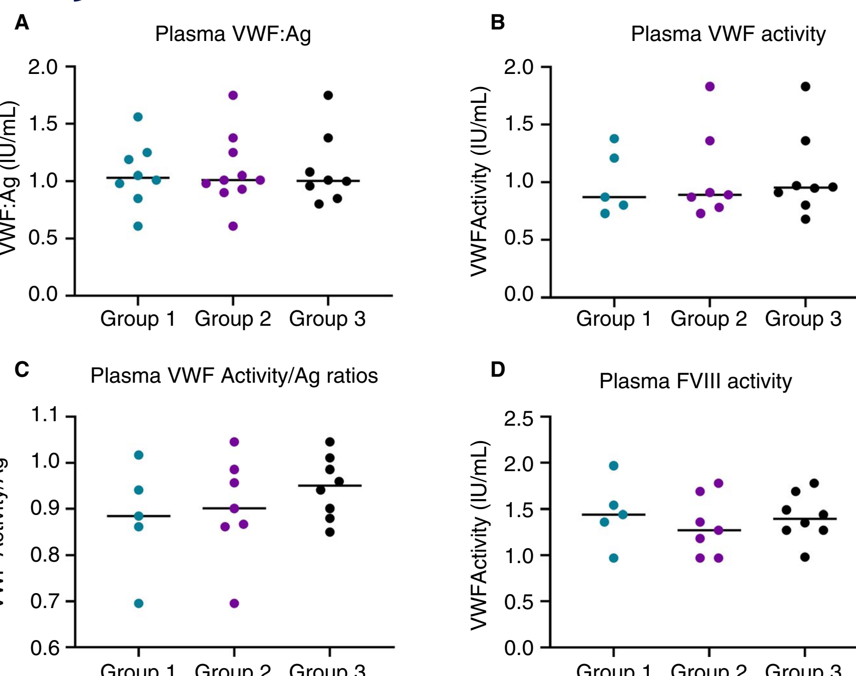

FIGURE 5 Plasma parameters. A, von Willebrand factor antigen (VWF:Ag) levels measured in plasma. B, VWF activity levels measured in plasma. C, Plasma VWF ratios were calculated by the VWF activity measurements divided by VWF antigen levels. D, Factor VIII (FVIII) activity levels measured in plasma. Each dot represents an individual donor. VWF:Ag levels were measured for donors included at both Leiden and Kingston (values for donors 1 and 5 not available). VWF activity and FVIII levels were only measured in samples from Leiden. Values are given as median and a one-way analysis of variance test was performed, followed by a Tukey's multiple comparisons test or a Kruskal-Wallis test was performed, followed by a Dunn's multiple comparisons test

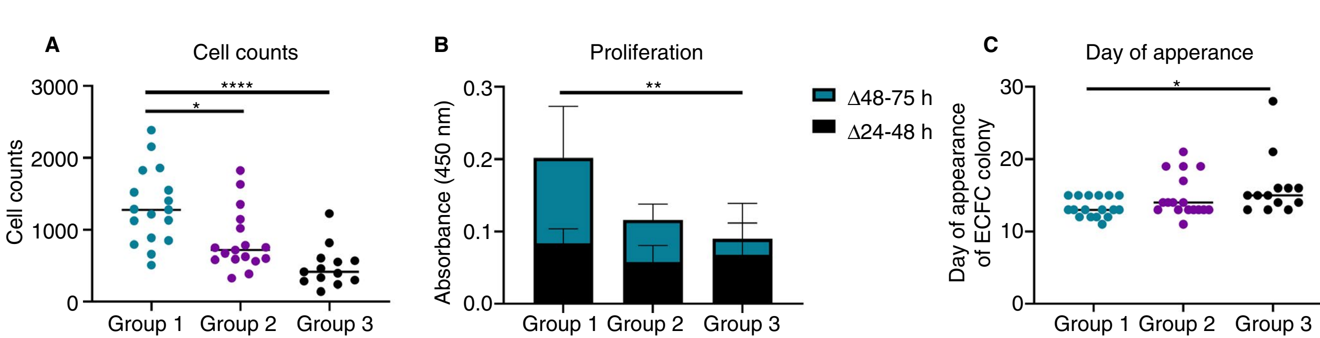

FIGURE 6 Demographic of donors and cell characteristics. A, Cell counts for clones. Pictures were taken 3 to 5 days postconfluency by bright-field microscopy and cells were counted using CellProfiler. B, Proliferation rates of cells per group. Proliferation rates of clones in the three groups were measured over a period of 72 hours after plating. Proliferation is indicated by absorbance, measured on a plate reader at 450 nm. Proliferation in the first period was at comparable levels; however, a significant difference was shown between groups 1 and 3 for the second time period. C, Day of appearance of clones. Isolated mononuclear cells in peripheral blood were checked daily by bright-field microscope after 10 days of culture after isolation. Day of appearance was determined when the first endothelial like cells were noted, indicating the start of a colony. Values are given as median and a one-way analysis of variance test was performed, followed by a Tukey's multiple comparisons test or a Kruskal-Wallis test was performed, followed by a Dunn's multiple comparisons test; $^{*}P < .05$, $^{**}P < .01$ $^{****}P < .0001$

in order for ECFCs to be used as a robust cellular model for applications in vascular studies. In this study we sought to understand and standardize normal reference characteristics for ECFCs in order for these cells to become a valid ex vivo model for the study of vascular diseases, such as VWD. We have analyzed and characterized 47 putative ECFC clones from 21 individual healthy donors. Cell morphology, proliferation, VWF production, storage and secretion, cell surface markers, and other cell-related parameters were assessed.

As expected, there was variation observed in cell morphology. Therefore we opted to use these differences in cell morphology as the primary means for defining individual putative ECFC clones prior to conducting further structural and functional assays. Clones in group 1 exhibited the classical cobblestone endothelial cell morphology, with increasing cell size and decreasing confluency in groups 2 and 3. Even though clones in group 3 did not show the classical endothelial cell morphology, FACS data showed that all clones in this study expressed, to some degree, endothelial cell markers and had low expression of non-endothelial cell markers. However, based on the significant range seen in the expression profiles, the clones are likely heterogeneous endothelial cell populations as some positive endothelial surface markers, such as CD31, showed a trend in decreasing expression among groups.

The increase of $\alpha_v\beta_3$ integrin (CD51/CD61) expression on clones in group 3 is interesting as $\alpha_v\beta_3$ integrin plays a role in endothelial cell adhesion to basement membrane matrix and endothelial cell

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migration. This particular integrin is a receptor for several components of the ECM, including vitronectin, and is found on normal smooth muscle cells, osteoclasts, monocytes, and platelets.[29] Although it is minimally expressed on normal resting endothelial cells, it is significantly upregulated in newly formed blood vessels within human tumors, in healing wounds, and in response to certain growth factors.[30-33] This could indicate that the clones in group 3 are in a more angiogenic state and further characterization is needed to assess for markers such as VEGF, VEGFR1, Ang-1, and Ang-2. Furthermore, a group-related decrease in CD34 combined with an increase in CD14 (monocytes) and CD45 (leukocytes) expression was observed in group 3, which could indicate an endothelial to mesenchymal transition (EndoMT) in clones in group 3 as we have suggested before.[18] Furthermore, an increase in CD309 (VEGFR2/KDR) expression on ECFCs in group 3 was observed. Previous studies addressing gene expression of CD309 have shown a similar trend and a significant negative correlation with maximum cell density, with higher levels of CD309 in lines with lower maximum cell densities.[18]

Confocal imaging revealed that while all clones showed the presence of both VWF and VE-cadherin, cells in groups 2 and 3 showed a lower frequency of cells that stained positive for these two endothelial cell proteins. There were also differences in WPB numbers, but no differences were observed in the morphology of the WPBs among groups. This was in line with the production/storage and basal secretion data of VWF:Ag, also after stimulation. The average cell proliferation rates, VWF production and secretion, both constitutive and after histamine stimulation, were all highest in clones designated to group 1. Again, these parameters were lower in group 2 and lowest in group 3. We also had access to plasma samples from most of the donors included in this study. The differences observed in the endothelial cells from the three groups did not correlate with the plasma levels measured, which were all in the normal range and are therefore not cell group-specific. Previously we have reported the correlation of secreted VWF:Ag levels, mRNA expression, and maximum cell density and showed that ECFC lines with a high maximum cell density secreted more VWF per cell than lines with a low maximum cell density.[18] Here, the same trend is shown with decreasing VWF secretion in the three groups, when taking the secretion and cell count data into account.

In conclusion, this study indicates that although clones in all three groups show endothelial markers and produce and secrete VWF, clones in group 3 might prove to be fully differentiated endothelial cells at the start of isolation, or transition to a more mesenchymal phenotype (EndoMT) during cell culture in vitro, which is important when using ECFCs as a cell model in disease studies. We do not believe there are mixed cell populations within the individual clones in group 2 and group 3. Cells in these groups show a uniform morphology of enlarged cells, without any cells showing the cobblestone morphology like ECFCs in group 1. Besides this, the enlarged cells in group 3 express VWF with storage in WPBs, indicating these cells are endothelial cells with a changing phenotype.

For this project we used detailed standardized protocols between groups in Leiden and Kingston, which gave us an opportunity to investigate the inter-laboratory differences on the isolation of ECFCs. We have adapted our ECFC isolation protocol according to Martin-Ramirez et al,[21] a commonly used approach to isolate ECFCs. In both study locations there is an ECFC isolation success rate of approximately 46%, which is considerably lower than previous reported (~70%).[20] We are uncertain why the success rate of isolation is lower than previously reported and lower than our own experience. In this study we have used 48 well plates instead of larger plates or vessels for the isolations. We chose this setup because we wanted to look at the intra-donor variability of clones from individual donors and with this approach, we could easily separate the clones. However, we were not expecting lower success rates, as we have used the same coating (collagen I) and similar cell seeding densities as other protocols.

Even though we used standardized protocols, with identical or near identical reagents and equipment, some practical differences persisted between the Leiden and Kingston laboratory. This applies mainly for FACS analysis, which is difficult to standardize due to the equipment availability at the different facilities. Even though we use the same antibody panel for characterization, the manufacturers and fluorophores differed for some antibodies.

Here we included healthy donors to analyze ECFCs for normal reference characteristics. The term "healthy" for this study meant donors who were not diagnosed for VWD or any other bleeding disorder. However, this does not exclude any other factors and (vascular) diseases that might have an influence on the isolation process, and morphology, numbers, and circulation of ECFCs in peripheral blood in general.[34,35] Furthermore, we have looked at other parameters that could have an effect on the ECFC isolations such as age and gender, but did not find any correlation. As previously reported, we have noted that some donors consistently fail to yield any ECFCs in repeated isolations and generally donors who yield ECFCs appear to do so consistently.[20] This means that the circulation and isolation of ECFCs is donor specific and further investigation is needed to identify other factors that might have an influence, such as diseases, medication, or smoking.[35,36]

This brings us to the discussion of the origin of ECFCs, which remains unclear but could contribute to the phenotypical differences seen in these endothelial cells. Several sites of origin have been proposed such as bone marrow[7] or tissue vascular niches.[37] Either way, different origins can lead to different types of endothelium that result in the differences in cell morphology and phenotypes of ECFCs. However, it is clear that there needs to be additional research focused on the origin of ECFCs.

Because the isolation and collection of ECFCs is variable and with success rates around 50%, we are constantly working to adjust and improve the isolation protocol, but are also exploring new approaches for VWD ex vivo models. In this study all experiments were performed under static conditions on postconfluent cells. However, it would be interesting to see if the results change when cells are grown under flow ,which represents a more natural environment.

To conclude, this project gives an indication of ECFC characteristics based on their morphology and other cell parameters. These results highlight the importance of understanding VWF parameters in

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ECFCs from healthy controls. Even though all clones seem from the same endothelial lineage, based on their endothelial surface marker profiles, there is a variation seen in morphology, proliferation rates, and in other endothelial characteristics such as VWF production and secretion. For clones in group 3, the exact origin remains uncertain. Therefore, for ECFCs to be used as a valid vascular disease model, patient ECFCs should be analyzed in parallel with control ECFCs showing comparable morphology based on the groups discussed in this project.

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CONFLICTS OF INTEREST

JE, PJ, and DL received research funding from CSL Behring. The other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

S. de Boer: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. M. Bowman: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. C. Notley: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. A. Mo: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. P. Lima: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. A. de Jong: collection and/or assembly of data, final approval of manuscript. R. Dirven: collection and/or assembly of data, final approval of manuscript. E. Weijers: conception and design, collection of data, final approval of manuscript. D. Lillicrap: conception and design, data analysis and interpretation, final approval of manuscript. P. James: conception and design, data analysis and interpretation, final approval of manuscript. J. Eikenboom: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

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SUPPORTING INFORMATION

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