# The endothelial compartment as a disease modifier in bleeding disorders

Laan, S.N.J.

# Endothelial Colony Forming Cells in the spotlight, insights into the pathophysiology of von Willebrand disease and rare bleeding disorders

**Laan SNJ**, Lenderink BG, Eikenboom JC, Bierings R

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## Abstract

Endothelial cells deliver a vital contribution to the maintenance of hemostasis by constituting an anatomical as well as functional barrier between the blood and the rest of the body. Apart from the physical barrier function, endothelial cells maintain the hemostatic equilibrium by their pro- and anticoagulant functions. An important part of their procoagulant contribution is the production of von Willebrand factor (VWF), which is a carrier protein for coagulation factor VIII (FVIII) and facilitates the formation of a platelet plug. Thus, VWF is indispensable for both the primary and secondary hemostasis, which is exemplified by the bleeding disorder von Willebrand disease (VWD) that results from qualitative or quantitative deficiencies in VWF.

A cellular model that was found to accurately reflect the endothelium and its secretory organelles are endothelial colony forming cells (ECFCs), which can be readily isolated from peripheral blood and constitute a robust *ex vivo* model to investigate the donor's endothelial cell function. This review summarizes some of the valuable insights on biology of VWF and pathogenic mechanisms of VWD that have been made possible using studies with ECFCs derived from patients with bleeding disorders.

## Introduction

Hemostasis is the cessation of bleeding through coagulation in the event of vascular injury. The hemostatic balance is an interplay of pro-coagulant and anti-coagulant mechanisms and can be regarded as a balance. Tipping too far to one side without sufficient adjustments in response ultimately leads to defective or overactivated coagulation, which increases the risk of bleeding or thrombosis, respectively. Much of the research in this field has been, and still is, revolving around identifying and investigating (new) hemostatic players. With the aim of unraveling their underlying mechanisms, and how their interplay can give rise to a pathogenic state. In this review we will briefly outline how endothelial cells (ECs) actively participate in preserving the hemostatic balance, with particular focus on their role in regulating acute and steady state levels of the hemostatic protein Von Willebrand factor (VWF). Our molecular understanding of the biosynthesis and secretion of VWF primarily originates from *in vitro* studies in endothelial cells. Here, we will highlight endothelial colony forming cells (ECFCs) as a versatile, *ex vivo* endothelial cell model for studying basic principles of endothelial cell biology and hemostasis in their native environment. ECFCs are uniquely suited to study the links between genetic mutations in patients and their cellular phenotype. This will be illustrated by the insights obtained from patient-derived ECFCs, such as for the bleeding disorder von Willebrand disease (VWD), the process of angiogenesis and for regulated secretion of VWF via Weibel-Palade body (WPB) exocytosis.



### The role of endothelial cells in hemostasis

Endothelial cells passively and actively contribute to anti- and procoagulant as well as fibrinolytic mechanisms (Figure 1). Their anticoagulant function is vital as it prevents blood from unintentional clotting, and in doing so averts the formation of clots that can cause thrombosis, infarcts or stroke. The endothelium acts first and foremost as a physical barrier between blood and tissue. As such, it also prevents contact of platelets and coagulation factors in the circulation with procoagulant components in the subendothelial matrix, such as collagen and tissue factor (TF), thereby restricting initiation of primary and secondary hemostasis pathways, respectively (1).

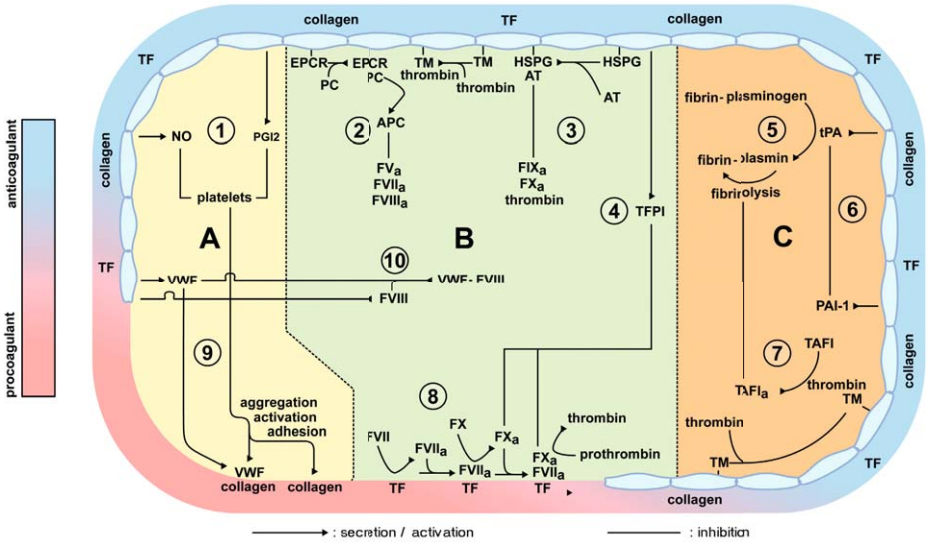


**Figure 1. Anti- and procoagulant functions of endothelial cells.** Endothelial cells (ECs) directly and indirectly contribute to anti- and procoagulant mechanisms in primary and secondary hemostasis (**A**, respectively **B**) and fibrinolysis (**C**). Anticoagulant mechanisms in primary and secondary hemostasis include synthesis of the platelet inhibitors Nitric Oxide (NO) and prostaglandin I2 (PGI2) (**1**), the protein C (PC) pathway supported by the endothelial protein C receptor (EPCR) – thrombomodulin (TM) tandem on ECs (**2**), the antithrombin (AT) pathway on Heparan Sulphate Proteoglycans (HPSGs) on ECs (**3**) and the synthesis of Tissue Factor Pathway Inhibitor (TFPI), which negatively regulates the Tissue factor (TF) pathway (**4**). Endothelial cell control over fibrinolysis includes secretion of tissue plasminogen activator (tPA), which activates plasminogen on fibrin into plasmin, thereby promoting degradation of fibrin (**5**). Activation of fibrinolysis is counteracted by Plasminogen Activator Inhibitor-1 (PAI-1) produced by endothelial cells (**6**) and by the conversion of Thrombin Activatable Fibrinolysis Inhibitor (TAFI) by the thrombin-TM complex on ECs (**7**). Damage to the vessel wall exposes TF within the subendothelial matrix and activates the TF pathway, resulting in the generation of thrombin (**8**). Binding of VWF to exposed collagen promotes adhesion and subsequent, activation and aggregation of platelets (**9**), while binding of VWF to FVIII prevents FVIII from premature clearance (**10**).

Endothelial cells also take more active roles in preserving the hemostatic balance. Nitric oxide (NO) and prostacyclin (PGI2), which are continuously synthesized by endothelial cells, are potent vasodilators and platelet inhibitors that are used for local control of hemodynamic forces in the blood and of adhesion and aggregation of platelets (2,3). ECs also produce Tissue Factor Pathway Inhibitor (TFPI), which blocks the TF-activated factor VII (TF-FVIIa) complex and prothrombinase of the extrinsic pathway (4). Additionally, ECs can act on the intrinsic pathway by producing and presenting anticoagulants like heparan sulphate proteoglycans (HSPG) on their membranes, which bind liver-produced antithrombin (AT) to the vessel wall. This induces conformational changes of AT and enhances its interaction with the blood clotting cascade proteinases

factor Xa and IXa to inhibit their activity (5). Additionally, ECs modulate the common pathway through their production of thrombomodulin, which is a thrombin-specific receptor on the endothelial cell membrane that decreases circulating thrombin levels. Moreover, ECs express endothelial cell protein C receptor (EPCR) on their membranes, which can bind to protein C (PC). This facilitates activation of PC into activated protein C (APC) by the thrombin-thrombomodulin complex, which leads to the subsequent inactivation of coagulation factors such as FV, FVII and FVIII (6).



Stimulated and constitutive secretion of tissue plasminogen activator (tPA) from endothelial cells (7,8) leads to the conversion of fibrin-bound plasminogen to plasmin, which is a serine protease that degrades fibrin during fibrinolysis and clot resolution (9). Endothelial cells also express inhibitors of fibrinolysis, such as the serpin plasminogen activator inhibitor-1 (PAI-1) (10), which inhibits tPA by binding its active site thereby preventing the interaction of tPA with plasminogen and the latter's subsequent conversion to fibrin (11). Furthermore, the thrombin-thrombomodulin complex, which is found on the surface of endothelial cells, activates the thrombin-activatable fibrinolysis inhibitor (TAFI). TAFI cleaves C-terminal lysine residues from fibrin, which decreases its affinity for plasminogen and tPA, reduces plasmin generation and thus attenuates fibrinolysis (12).

The procoagulant function of the endothelium is mainly mediated by Von Willebrand factor, which is secreted by endothelial cells and takes a central role in primary and secondary hemostasis. At steady state, VWF is critical for the maintenance of sufficient levels of circulating FVIII by physically preventing the latter's premature clearance from plasma (13). Following vascular injury and exposure of the subendothelial matrix, VWF circulating in plasma as well as VWF that is locally secreted by activated endothelial cells binds to collagen via its A3 domain. As a result of the hemodynamic forces of the blood flow, these tethered VWF strings unfold and expose a binding site for the platelet GP1bα receptor within the VWF A1 domain that supports the adhesion and subsequent activation and aggregation of platelets to sites of vascular injury, a critical step in the formation of a platelet plug (14). The importance of VWF is highlighted by the mild to severe bleeding abnormalities that occur in patients with (partial) quantitative reduction or a qualitative defect of VWF circulating in plasma, as seen in VWD and in what was formerly known as "Low VWF" (15,16). Partial deficiencies of functionally normal VWF are often the result of *VWF* missense mutations (17) that reduce its synthesis and/or secretion or lead to enhanced clearance from the circulation as evidenced by altered VWF propeptide (VWFpp)/VWF Antigen (VWF:Ag) and FVIII activity (FVIII:C)/VWF:Ag ratios in plasma (18). However, approximately 30-50% of patients with quantitative VWF reductions lack pathogenic *VWF* variants (18–21). In these cases other genetic modifiers,

including those acting on clearance, synthesis and secretion, may be responsible for the reduced VWF levels (22).

**Cell biology of Von Willebrand factor**

Endothelial cells are responsible for the production of nearly all VWF that is found in plasma (23). VWF is first synthesized as a pre-proVWF monomer, consisting of an N-terminal signal peptide, the VWFpp and the mature VWF subunit, which contains its ligand-binding domains and domains responsible for dimerization and multimerization (24). Following signal peptide cleavage and dimerization of proVWF monomers in the ER, the acidic conditions in the trans-Golgi network (TGN) promote concatemerization of VWF dimers along helical self-templates, leading to the formation of long tubules that consist of ultra-large VWF multimers (25–27). Expression of VWF drives the formation of WPBs, EC-specific secretory organelles that are formed at the TGN and store VWF for basal or stimulated release (28–30). WPBs are generally 1-5 µm long, 0.1 to 0.3 µm wide and owe their characteristic rod shape to the dimensions and parallel arrangement of the VWF tubules (26,30,31).

Newly synthesized WPBs are considered immature and lack the ability to undergo stimulus-induced secretion (32). WPBs go through a post-Golgi maturation process that involves the recruitment of proteins to the WPB membrane and a further acidification to ~pH 5.4 (30,33). A key step in this process is the acquisition of Rab GTPases Rab27A and isoforms of Rab3 (32,34,35), which depends on their activation by the guanine nucleotide exchange factor MAP kinase activating death domain (MADD) (36). In turn, active Rabs on the membrane of mature WPBs recruit effectors that interact with Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins and other exocytotic components that perform essential functions in cytoskeletal interactions, organelle acidification and exocytosis of WPBs (32,37–46). As a result of recruitment of their secretory machinery WPBs become secretion competent and can undergo exocytosis upon activation of the endothelial cell, such as during vascular injury (30). Apart from VWF, WPBs are known to store a variety of other vasoactive agents, including chemokines, angiogenic mediators such as Insulin-like growth factor binding protein 7 (IGFBP7) and Angiopoietin-2 (Ang-2), as well as hemostatic proteins FVIII and tPA (reviewed in (30)). Co-release of these proteins together with VWF may help the endothelium to further fine-tune its hemostatic responses while simultaneously initiating inflammatory and blood vessel repair pathways.

The SNARE proteins syntaxin-2 (STX2) and syntaxin-binding protein 5 (STXBP5), which are part of this machinery, have previously been identified in quantitative trait locus mapping studies for genetic associations with VWF (47–50), suggesting that alterations

in the exocytotic process of WPBs contribute to the wide range of plasma VWF levels in the population. Together, this highlights the potential role of the endothelial secretory pathway as a major determinant of circulating VWF levels and bleeding phenotype.

**Cellular model systems**

With the growing insight from large-scale genomic screenings into the genetic mechanisms that underpin VWF abnormalities, investigators now face the challenge to find experimental evidence of the functional impact of variants in *VWF* or other genetic determinants that explains alterations in VWF found in patients. Over the years, a number of approaches have been developed to test these genotype-phenotype relationships in model systems that, to a varying extent, reflect the cellular context in which these pathogenic mechanisms play out. Expression of VWF in non-endothelial cell types such as Chinese hamster ovary (CHO) and human embryonic kidney 293 (HEK293) cell lines has long been the method of choice to study how pathogenic VWF variants affect its biosynthesis, post-translational modification and molecular composition. Because ectopic expression of VWF induces the formation of so-called pseudo-WPBs (51,52), elongated storage organelles that have a striking resemblance to WPBs in terms of morphology and composition, these studies can also reveal how mutations translate to altered storage and secretion of VWF and other WPB constituents (52–54). Such studies do require *a priori* knowledge of the mutations involved and can be difficult to interpret considering that the vast majority (up to 90%) of pathogenic *VWF* variants in VWD are heterozygous dominant-negative mutations (17) that express in conjunction with a normal functional allele in patients. Moreover, other caveats of studying VWF biology outside of its proper cellular context include lack of endothelial cell specific gene expression, non-physiological expression levels from expression constructs with constitutive promoters, as well as the absence of a regulated secretory pathway and many components of the associated exocytotic machinery. This makes ectopic systems such as HEK293 cells of very limited value for functional studies of stimulated VWF secretion or angiogenesis.

To study these processes in their native cellular context it is inevitable to turn to endothelial cells, ideally from the patients themselves. Primary endothelial cells from a multitude of vascular beds, such as from the dermis, brain, lungs, heart, aorta, retina, umbilical cords and foreskin, are nowadays commercially available. Nonetheless, the invasive procedures involved to isolate these endothelial subtypes from patients would make most of them impractical for study purposes. Differentiation of endothelial cells from patient-derived induced pluripotent stem cells (iPSC-ECs) could potentially overcome such hurdles, but varying levels of VWF synthesis between clonal lines and

impaired maturation of WPBs (55) are currently still precluding iPSC-ECs from becoming a viable model system for this purpose.

Endothelial cells can also be derived from a population of cells that circulates in blood, so-called endothelial progenitor cells (EPCs), which can be divided into two populations, early and late, based on their appearance in culture. Late appearing EPCs, first described by Lin *et al.* (56), are of *bona fide* endothelial lineage as judged by the expression of a set of canonical endothelial markers, including VWF, VEGFR2, PECAM and VE-cadherin, and several endothelial cell specific properties such as angiogenic capacity. These cells have been referred to by various other names including endothelial outgrowth cells (EOCs) and blood outgrowth endothelial cells (BOECs). To bring order to the nomenclature conundrum, a consensus was reached to rename them endothelial colony forming cells (ECFCs) (57), which is also the terminology we will use in this review.

ECFCs can be regarded as liquid biopsies of the endothelium (58) and are excellent model systems to investigate pathogenic cellular mechanisms that affect the vasculature, since they carry the genetic background of the patient (59). Despite the fact that their exact origin is still a matter of debate (60,61), ECFCs can be acquired quite reliably from various sources, including venous blood (56,62,63), cord blood (64,65) and even from cryopreserved peripheral blood mononuclear cells (PBMCs) (66). However, some concerns exist regarding their intra- and interindividual phenotypic heterogeneity (67,68), which can potentially influence outcomes of studies and may have its basis in a progression of some ECFC lines into an endothelial-to-mesenchymal transition (EndoMT) state (69).

Despite these challenges, ECFCs are a robust model to directly assess how disease genotype translates to endothelial phenotype *ex vivo,* which is invaluable for research on pathologies that are characterized by an affected endothelium, such as VWD.

**ECFCs: von Willebrand disease**

The first use of VWD patient-derived ECFCs was by Berber and colleagues who investigated the common R924Q polymorphism in ECFCs of a compound heterozygous VWD type 2N patient (R816W/R924Q), but failed to find conclusive evidence for the pathogenic nature of this variant (70). Back-to-back publications by Starke *et al.* and Wang *et al.* followed shortly after, which used ECFCs from type 1 and type 2 VWD patients to investigate the cellular phenotypes that are associated with a variety of exonic mutations within *VWF* (71,72). This marked a key advance in the field as the consequences of the pathogenic mechanisms involved in VWD could be revealed to their full extent for the first time. Depending on the mutations involved, these

mechanisms include retention of VWF inside the endoplasmic reticulum (ER), incorrect proteolytic processing, reduced high molecular weight VWF multimers, impaired stimulus-induced secretion and the loss of the characteristic elongated morphology of WPBs, and reduction or loss of these organelles entirely. Moreover, Wang *et al.* showed that in selected cases these defects can lead to reduced ability to generate long VWF strings upon release from the endothelium, which is expected to exacerbate bleeding complications in patients that already have reduced circulating VWF levels. Later studies focusing on ECFCs from VWD type 3 patients, as well as patients with mutations in the VWF propeptide region, the C-terminal cysteine knot domain or with large in-frame deletions, also reported retention of VWF in the ER in conjunction with reduced numbers of small, spherical WPBs (73–76), underlining that this is a common pathogenic mechanism in quantitative and qualitative VWD (Figure 2). Since unrestrained progression of VWF through the secretory pathway is important for the generation of sufficient numbers of elongated WPBs that are capable of secreting long VWF strings (77), any variant of VWF which causes (partial) retention in the ER can be expected to be accompanied by impaired secretory responses.



The VWD mutational spectrum also includes a large number of non-coding and splice site mutations for which the pathogenic mechanisms long remained elusive. An early study using VWD ECFCs detailed the mechanism by which a heterozygous 13 bp deletion in the *VWF* promoter in a VWD type 1 patient brought on reduced VWF transcript production from that allele, thereby showing for the first time how VWF promoter mutations can lead to quantitative VWF deficiencies (78). Additional studies using VWD patient ECFCs unraveled the pathogenic mechanisms of splice site mutations, a silent exonic mutation that led to intron retention and a deep intronic mutation (79–81). It is worth noting that some of these studies involving *VWF* mutations in non-coding regions or splice site mutations would have been impossible in ectopic expression systems.

VWD ECFCs have also been used to identify new genetic modifiers of *VWF* that could contribute to quantitative deficiencies of VWF. Two studies used transcriptomic analysis of ECFCs from patients with "Low VWF" and VWD type 1 (82,83), identifying the transcription factor friend leukaemia integration 1 (FLI1) and the microRNA miR-23b as potential modifiers of *VWF* transcription and stimulated VWF secretion, respectively. Such screenings are of a hypothesis-generating nature and will require further experimental validation of candidates to assess their biological and clinical relevance.

Finally, patient-derived ECFCs have significant potential as an ex vivo model for the development of new therapeutics for VWD, for instance as a platform to test strategies

for permanent or temporal correction of VWF defects. Proof of principle for phenotypic correction was shown in VWD type 3 dog ECFCs that were transduced with lentivirus carrying VWF cDNA (84). More recently, siRNAs targeting exonic single nucleotide polymorphisms (SNPs) in *VWF* were used to allele-selectively silence a heterozygous p.C1190Y mutation in ECFCs of a VWD type 2A patient, resulting in loss of mutant allele expression and phenotypic correction *ex vivo* (85). This could pave the way for more individualized approaches by using ECFCs as an *ex vivo* validation model for possible VWD treatments.

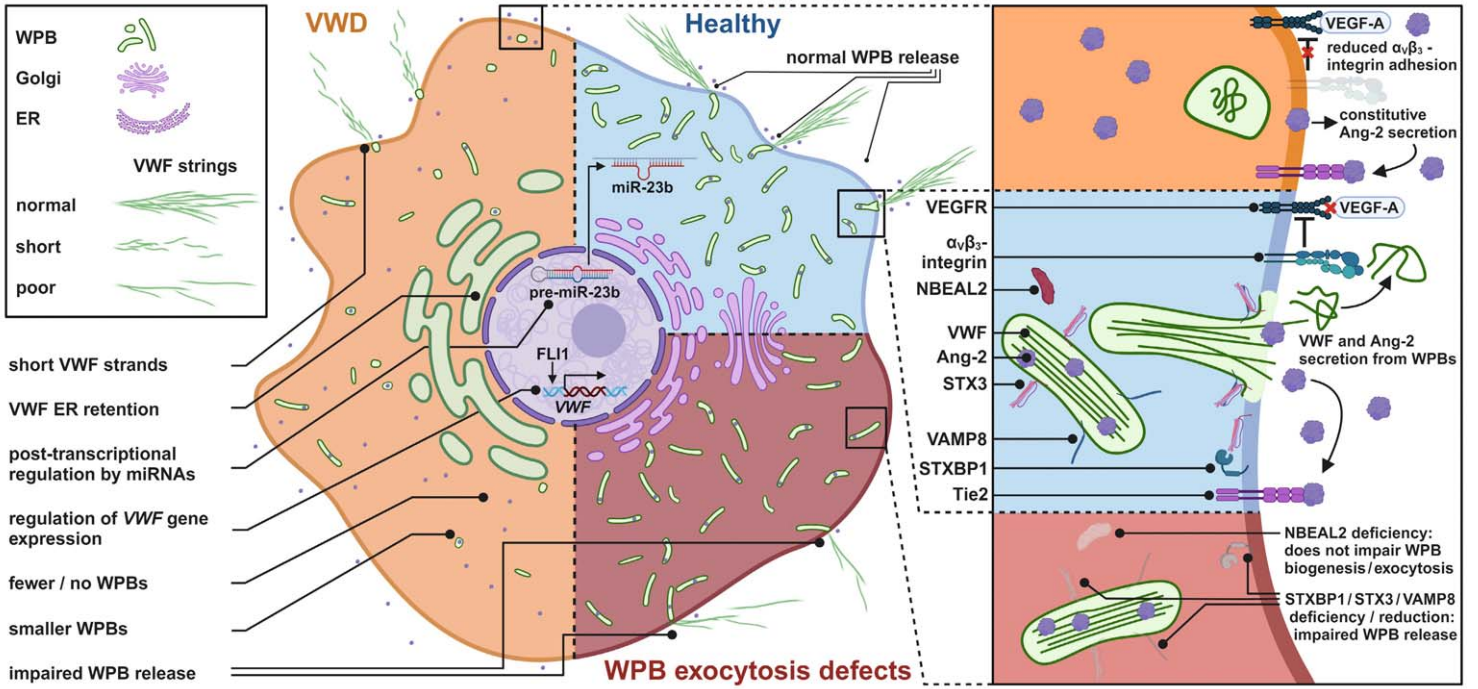


**Figure 2. Insights into the pathophysiology of bleeding disorders from studying endothelial colony forming cells.** The model depicts three states of an endothelial cell; in healthy condition (blue), when derived from a patient with VWD (orange) or in other disease where WPB secretion defects occur (red). On the right, a zoomed in panel displays components of the secretory pathway and how it may be affected in patients with bleeding disorders. αVβ3, alphaVbeta3-integrin; Ang-2, angiopoietin-2; ER, endoplasmic reticulum; FLI1, Friend leukemia integration 1; NBEAL2, Neurobeachin-Like 2; miR-23b, microRNA 23b; STX3, Syntaxin 3; STXBP1, Syntaxin-Binding Protein 1; VAMP8, Vesicle-Associated Membrane Protein 8; VEGF-A, Vascular Endothelial Growth Factor A; VEGFR, Vascular Endothelial Growth Factor Receptor; VWF, von Willebrand factor; VWD, von Willebrand disease WPB; Weibel-Palade body. Created with BioRender.com

**ECFCs: von Willebrand disease and angiogenesis**

Angiodysplastic lesions in the gastrointestinal (GI) tract are fragile vascular networks that are associated with recurrent GI bleeding, which can result in anemia and a decrease in quality of life (86). In up to 38% of VWD patients with GI bleeding, angiodysplastic lesions are present in the GI tract (87), which pose significant challenges in combination with the bleeding disorder (88). Angiodysplasia is the result of abnormal angiogenesis, the process that generates new blood vessels from existing vasculature. Directed migration and reorganization of endothelial cells during angiogenesis is highly dependent on growth factors such as vascular endothelial growth factor (VEGF) and Angiopoietin-1 and -2 (Ang-1 and Ang-2). VEGF is a potent stimulus of VWF secretion from WPBs (89), which also store the Tie2 receptor ligand Ang-2 and other pro-angiogenic components such as IGFBP7 (90,91). Targeted release of Ang-2 by exocytosis of WPBs activates autocrine and paracrine Tie2 signalling, which contributes to vessel lumen formation and shaping of new blood vessels (44,92,93). VWF is also a ligand for adhesion receptors such as $\alpha_V\beta$-3integrin, which is an important regulator of endothelial cell migration that can also quench VEGFR-2 signalling when in complex with ligands such as VWF (94). VWF binds a large array of pro-angiogenic growth factors via its heparin-binding domain (95), and also binds Ang-2 and IGFBP7 post-release (91,96,97), which may serve as a mechanism to focus these factors in areas of wound healing and active blood vessel formation.

The first evidence for a role in angiogenesis for VWF was presented by Starke and co-workers, who found increased migration, proliferation and angiogenesis in endothelial cells that were depleted of VWF using RNAi and in ECFCs from a cohort of VWD type 1, 2M and 2A patients (98). This was accompanied by reduced β3-integrin expression, reduced αVβ-3integrin-dependent adhesion and increased constitutive Ang2- secretion, possibly as a result of reduced intracellular retention of Ang2- due to loss of WPBs. A second study by Groeneveld et al. reported that compared to control ECFCs, most type 1 and type 2 VWD ECFCs had a slightly lower directionality of migration in a wound-healing assay, while VWD type 3 ECFCs, which are entirely devoid of VWF synthesis, had a higher migration velocity (99). However, no clear effects of *VWF* mutations on overall angiogenic potential were seen in this VWD cohort. Selvam *et al.* found wide variation in parameters such as migration, proliferation and tube formation in VWD patient-derived ECFCs, but also reported that type 1, 2 and some type 3 VWD ECFCs had significantly increased constitutive Ang-2 secretion compared to controls (100). A similar observation was made in cord blood ECFCs in which CRISPR/Cas9-mediated VWF gene knockout led to increased constitutive secretion of Ang-2 and association of Ang-2 with the Tie2 receptor (101). Since these cells are also devoid of WPBs and no longer capable of storing Ang-2, stimulus-induced Ang-2 release was severely reduced.

All together, these (patient-)ECFC based studies have provided some evidence for a role for VWF in angiogenesis (Figure 2). However, the exact function remains unclear perhaps due to the limitations of this model which are associated with the phenotypic heterogeneity and gradual loss of proliferative capacity of patient and control ECFCs.

**ECFCs: Weibel-Palade body exocytosis defects and Storage Pool Disorders**

The cellular machinery involved in biogenesis and secretion of WPBs is complex (30). Because regulated secretion is fundamental to the function of numerous different cell types, many components of the WPB machinery are also involved in similar secretory processes in non-endothelial cells. Defects in shared secretory components can therefore affect more than one cell type and may lead to complex, multi-system manifestations in patients. In several cases, endothelial cells isolated from rare patients with abnormalities in non-endothelial cell functions have contributed to identification and functional characterization of new regulators of WPB secretion (Figure 2). The SNARE proteins syntaxin-2 (STX2), STX3 and syntaxin-binding protein 1 (STXBP1) were identified as hits in an interactomic screen of downstream effectors of the Rab27A-Slp4-a complex in endothelial cells. *De novo* mutations in *STXBP1* are associated with early infantile epileptic encephalopathy type 4 (EIEE4), an epileptic disorder that is thought to result from impaired neurotransmitter release due to STXBP1 haploinsufficiency. In keeping with the defective secretory responses in neurons in these patients, STXBP1 haploinsufficient EIEE4 patient-derived ECFCs also showed severely impaired $Ca^{2+}$- and cAMP-mediated VWF secretion, confirming the role of STXBP1 in stimulus-induced WPB exocytosis (39). Homozygous nonsense mutations in *STX3* are causative for microvillus inclusion disease (MVID), a rare congenital disorder of the gut characterized by severe diarrhoea, which is the result of incorrect targeting of microvilli that normally migrate/move to the apical side of intestinal epithelial cells (102). MVID patient-derived ECFCs, which were entirely devoid of STX3, had reduced VWF and VWFpp secretion at submaximal stimulation and strikingly showed signs of loss of polarity of VWF and VWFpp release during basal secretion (42).

Storage Pool Disorders (SPDs) are a heterogeneous group of disorders that affect the formation of lysosome related organelles (LROs), which include platelet alpha- and dense granules, but also WPBs (103). Due to the universal mechanisms involved in the formation of LROs, SPDs often present as multi-system disorders and affect secretory function of both platelets and a variety of other cell types. Hermansky-Pudlak syndrome 2 (HPS2) is a rare genetic SPD characterized by interstitial lung disease, neutropenia and bleeding, which may find its origin in the lack of dense granules in platelets. HPS2 is caused by mutations in *AP3B1* which encodes for the beta subunit of the AP-3 complex, a cargo sorting complex that traffics secretory cargo and membrane

proteins from endosomes to LROs. Whole proteome analysis of ECFCs derived from HPS2 patients revealed that loss of the AP-3 complex due to compound heterozygous *AP3B1* mutations was accompanied by loss of VAMP8 (104). VAMP8 is an R-SNARE that cycles via an endosomal compartment to WPBs, where it supports exocytotic fusion of WPBs via interaction with plasma membrane based Q-SNAREs. Lacking VAMP8 on their WPBs, HPS2 ECFCs showed severely reduced stimulus-induced WPB exocytosis, suggesting that WPBs acquire secretion competence partly by recruitment of membrane proteins such as SNAREs from the endosomal compartment in an AP-3 dependent manner. It also indicates that the bleeding abnormalities that are seen in some SPDs and that were generally attributed to impaired platelet function, may in some cases be further compounded by decreased endothelial secretory responses. This can however not be extrapolated to all SPDs: Kat *et al.* found that NBEAL2–deficient ECFCs from patients with Gray Platelet Syndrome, a bleeding disorder characterized by loss of platelet alpha-granules as well as abnormalities in formation of secretory organelles in neutrophils, had normal biogenesis, maturation and exocytosis of WPBs (105). This also underscores the divergent mechanisms that control VWF storage in endothelial WPBs and in alpha-granules in megakaryocytes. In aforementioned studies, the ECFCs originate from individuals suffering from rare genetic disorders. While these results come from very small sample sizes, they can still be very useful to unravel complex cellular mechanisms of disease.

**The road ahead for ECFCs**

ECFCs are unique personalized endothelial model systems to study endothelial cell function in health and disease and have already led to major advances in our understanding of VWD and WPB biology (Figure 2). While they have generated great interest in the hemostasis community and various other focus areas of vascular biology, their use is still complicated by poor standardization of isolation and culturing methods and substantial intra- and inter-individual phenotypic variability. This can negatively impact the interpretability of experimental results, which for now may preclude their wider adaptation within the scientific community. Future efforts should be aimed at understanding the source of this variation and designing strategies that can prevent or minimize its impact on experimental outcome. Disease modeling using ECFCs, such as described here for VWD, has so far been limited to studying these cells in isolation using 2D cell culture platforms. To fully appreciate endothelial cell function within its authentic (patho-)physiological context, for instance within an injured blood vessel or during organotypic function, it is pertinent that the physical and chemical milieu that is induced by blood flow or neighbouring cells is present. Integration of ECFCs with vessel- or organ-on-a-chip technologies offers the exciting opportunity to develop miniature *ex vivo* personalized disease models that include the patient's own diseased endothelium.

## Acknowledgements

The SYMPHONY consortium, which aims to orchestrate personalized treatment in patients with bleeding disorders, is a unique collaboration between patients, health care professionals, and translational and fundamental researchers specializing in inherited bleeding disorders, as well as experts from multiple disciplines (106). It aims to identify best treatment choice for each individual based on bleeding phenotype. To achieve this goal, work packages (WP) have been organized according to 3 themes (e.g. Diagnostics [WPs 3 and 4], Treatment [WPs 5–9], and Fundamental Research [WPs 10–12]). Principal investigator: M.H. Cnossen; project manager: S.H. Reitsma.

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