Alternative trafficking of Weibel-Palade body proteins in CRISPR/Cas9-engineered von Willebrand factor–deficient blood outgrowth endothelial cells

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Abstract
Background: Synthesis of the hemostatic protein von Willebrand factor (VWF) drives formation of endothelial storage organelles called Weibel-Palade bodies (WPBs). In the absence of VWF, angiogenic and inflammatory mediators that are stored in WPBs are subject to alternative trafficking routes. In patients with von Willebrand disease (VWD), partial or complete absence of VWF/WPBs may lead to additional bleeding complications, such as angiodysplasia. Studies addressing the role of VWF using VWD patient–derived blood outgrowth endothelial cells (BOECs) have reported conflicting results due to the intrinsic heterogeneity of patient-derived BOECs.

Objective: To generate a VWF-deficient endothelial cell model using clustered regularly interspaced short palindromic repeats (CRISPR) genome engineering of blood outgrowth endothelial cells.

Methods: We used CRISPR/CRISPR-associated protein 9 editing in single-donor cord blood–derived BOECs (cbBOECs) to generate clonal VWF−/− cbBOECs. Clones were selected using high-throughput screening, VWF mutations were validated by sequencing, and cells were phenotypically characterized.

Results: Two VWF−/− BOEC clones were obtained and were entirely devoid of WPBs, while their overall cell morphology was unaltered. Several WPB proteins, including CD63, syntaxin-3 and the cargo proteins angiopoietin (Ang)-2, interleukin (IL)-6, and IL-8 showed alternative trafficking and secretion in the absence of VWF. Interestingly, Ang-2 was relocated to the cell periphery and colocalized with Tie-2.

Conclusions: CRISPR editing of VWF provides a robust method to create VWF-deficient BOECs that can be directly compared to their wild-type counterparts. Results

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INTRODUCTION

Von Willebrand factor (VWF) is a large multimeric protein that plays a central role in vascular homeostasis. VWF is synthesized by endothelial cells (ECs) and megakaryocytes and secreted VWF mediates adhesion of platelets to sites of vascular damage and acts as a carrier for coagulation factor VIII.1 The importance of synthesis and secretion of VWF is highlighted by the bleeding disorder von Willebrand disease (VWD), which is caused by mutations in the VWF gene that lead to either qualitative or (full or partial) quantitative deficiencies in VWF.2 Elevated levels of VWF, on the other hand, have been associated with increased risk of thrombosis.3

Following its synthesis in ECs, VWF is stored in rod-shaped storage organelles, called Weibel-Palade bodies (WPBs), where it is stored together with a wide variety of inflammatory and angiogenic mediators.4–6 VWF is the driving force behind the formation of its own storage organelle, illustrated by the lack of WPBs in ECs from a patient with severe VWD type 3 or from VWF null animal models8–10 and the formation of pseudo-WPBs upon heterologous expression of VWF in other cell types.11–12 Upon endothelial triggering, that is, after vascular damage, WPBs undergo exocytosis and deliver their cargo to the cell surface or into the vascular lumen, leading to the formation of long VWF strings to which platelets and also leukocytes and erythrocytes can adhere.14–18

Absence of WPBs not only compromises the hemostatic response of the endothelium but also has consequences for other secretory cargo that relies on WPBs for proper delivery to and across the plasma membrane. Trafficking of several inflammatory mediators, including P-selectin, CD63, and chemokines such as interleukin (IL)-6 and IL-8, is likely to depend on VWF synthesis and WPB formation.19 This is underscored by impaired leukocyte rolling and neutrophil infiltration in wounds in VWF−/− mice, resulting from defective translocation of P-selectin and/or CD63.8,20–22 In addition, storage and secretion of another WPB cargo protein, the proangiogenic mediator angiopoietin (Ang)-223–25 is also disturbed when ECs are depleted of VWF.26 Continuous release of Ang-2 as a consequence of the unavailability of its default storage compartment has been proposed as one of the underlying mechanisms behind angiodysplasia, a clinical complication of VWD that is characterized by recurrent bleeding in the gastrointestinal tract and is associated with vascular malformations of the gut.27,28 However, studies into angiogenic properties of blood outgrowth endothelial cells (BOECs) derived from patients with VWD, which can be regarded as endothelial models of VWD, have failed to unequivocally support this model.27,26,29,30

Variation in the genetic background between patients as well as controls may be at the basis of the discrepancy between outcomes in BOECs derived from different individuals. This is further confounded by the broad spectrum of VWD-causing mutations and the residual VWF expression levels that are associated with these mutations.31 To overcome this, there is a need for targeted genetic strategies for long-term complete ablation of VWF in human primary ECs. Others have previously used the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system to target gene expression in ECs in vitro.32,33 In this study, we used CRISPR-Cas9 genetic engineering to knock out VWF expression using guide RNAs that target the first exon of the VWF gene. For this we used cord blood outgrowth endothelial cells (cbBOECs) from a single donor, with which we created multiple VWF-deficient and control BOEC clones with an identical genetic background. cbBOECs possess an increased expansion potential compared to other primary ECs,34 which allowed for prolonged culturing and clonal expansion after single-cell sorting that are necessary to generate genetically homogeneous populations of VWF null cells. True VWF−/− cbBOECs obtained with our model system confirmed alternative trafficking of several WPB proteins in the absence of VWF and support the theory that increased Ang-2/Tie-2 interaction contributes to angiogenic abnormalities in VWD patients.

**KEYWORDS**
endothelial cells, gene knockout techniques, protein transport, secretory vesicles, von Willebrand factor

### Essentials

- Von Willebrand factor (VWF) synthesis is essential for the formation of the Weibel-Palade Bodies (WPBs).
- Patient-derived endothelial cell models of von Willebrand disease and VWF deficiency have shown a high degree of phenotypic heterogeneity.
- We generated VWF knockout endothelial cells (ECs) using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 gene editing on cord blood outgrowth ECs (cbBOECs).
- WPB-associated proteins show alternative localization in the absence of VWF, and angiopoietin (Ang)-2 colocalizes with the receptor Tie-2.
were selected using a high-throughput screen for loss of VWF secretion, and VWF mutations were validated by Sanger and next-generation sequencing (NGS). Morphologic, functional, and proteomic analyses were used to confirm that CRISPR-engineered BOECs retained their endothelial properties. We used this model of VWF deficiency to study alternative trafficking of proteins that are normally trafficked via WPBs. Upon the absence of WPBs, we observed an alternative localization pattern for proteins normally associated with the WPB membrane, such as CD63 and the regulator of WPB exocytosis, syntaxin-3. Alternative targeting was also seen for IL-6, IL-8, and Ang-2, which led to altered storage and secretion of these WPB cargo proteins in VWF−/− BOECs.

2 | METHODS

2.1 | Antibodies and reagents

Antibodies used in this study are listed in Table S1.

2.2 | Isolation of cbBOECs and cell culture

Cord blood was collected from umbilical veins within 48 hours after delivery and was processed for BOEC isolation essentially as described before.35 cbBOECs were cultured in EC growth medium (EGM)-2 (Lonza, Basel, Switzerland, CC-3162) supplemented with 18% fetal calf serum (EGM-18; Bodinco, Alkmaar, The Netherlands).36 Human embryonic kidney 293T (HEK293T) cells were cultured in Gibco Dulbecco’s Modified Eagle Medium, high glucose, pyruvate (ThermoFisher, Landsmeer, The Netherlands) for regular passing and seeding and in EGM-18 for virus production.

2.3 | Lentiviral CRISPR-Cas9 targeting constructs

Guide RNA (gRNA) sequences targeting the first exon of VWF were designed using the MIT CRISPR design tool (http://crispr.mit.edu)37 and the BROAD Institute single guide RNA (sgRNA) designer (http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design)38 by submitting the DNA sequence of VWF exon 1 flanked by 100 bp up- and downstream (chromosome 12, 6123020–6123259 positive strand; Figure 1). gRNA sequences were selected that have a high predicted efficiency (BROAD Institute score) and a high inverted off-target score (MIT CRISPR design tool).37,38 gRNAs used in this study were gRNA-1: 5′-TGCGCTCTATTTTGCCAGGT-3′ and gRNA-2: 5′-AGGCACCAGGCAAATCTGGC-3′. Complementary oligos were hybridized with BsmBI restriction site compatible overhangs on either side (gRNA-1: 5′-CAGCGTGGCCCCCTATTTTGCCAGGT-3′ and 5′-AACACCTGCGAAATGAGGGCCAC-3′, gRNA-2: 5′-CACCGAGCACCAGCAAAATCTGGC-3′ and 5′-AAACGCGCATTTGGCCGGGTGCTC-3′) to facilitate cloning into BsmBI-digested LentCRISPR v2 plasmid39 (a gift from Feng Zhang, Addgene #52961). The resulting constructs LentCRISPR-gRNA-1 and LentCRISPR-gRNA-2 were verified by DNA sequencing analysis.

2.4 | Generation of CRISPR-edited VWF−/− cbBOECs

Lentivirus production in HEK293T cells and transduction of ECs was performed as described.36 cbBOECs (passage number: 4, confluency: 60%-80%) were lentivirally transduced with LentCRISPR v2 as a control (LentCRISPR-CTRL), LentCRISPR-gRNA-1, LentCRISPR-gRNA-2 or a combination of LentCRISPR-gRNA-1 and LentCRISPR-gRNA-2 in 6-well plates (Figure 1). After selection using 1 μg/mL of puromycin for 72 hours, cells were left to recover until confluency. Cells of each condition were single-cell sorted into gelatin-coated 96-well plates by fluorescence-activated cell sorting (FACS) using a BD FACSAria III cell sorter (BD Biosciences, Breda, The Netherlands) and using anti-vascular endothelial (VE) cadherin–fluorescein isothiocyanate as an EC marker. EGM-18 was replaced every 2 to 3 days, and after 7 days colonies started to form in some wells. Conditioned media of wells that were >50% confluent were assayed using VWF ELISA40 to identify candidates no longer secreting VWF. VWF-deficient wells were passaged and expanded for further analysis including immunoblotting of cell lysates and Sanger sequencing and NGS of the genomic DNA isolated with the DNeasy Blood & Tissue kit (Qiagen, Venlo, The Netherlands). Verified clones were cryopreserved as described.35

2.5 | Secretion assay

ECs were seeded in gelatin-coated 6-well plates and cultured at full confluency for 4 to 5 days. Twenty-four hours before the secretion assay, media were replaced for fresh EGM-18 supplemented with 10 ng/mL of IL-1β (I9401, Sigma-Aldrich, St Louis, MO) or vehicle. Twenty-four-hour conditioned media were harvested, cells were pretreated for 15 minutes with serum-free M199 medium (Gibco 22340, ThermoFisher) supplemented with 0.2% (w/v) bovine serum albumin (BSA; Serva, Heidelberg, Germany) and were stimulated with 100 μmol/L of histamine or vehicle for 30 minutes as described previously.41 Releasates were collected, and stimulated and unstimulated ECs were lysed in M199 with 0.2% BSA and 1% Triton X-100 (Sigma-Aldrich). Protein secretion and intracellular content were determined using the DuoSet ELISA kit for Ang-2 (DY623; R&D Systems, Minneapolis, MN), and the Pelikine compact kit for IL-6 and IL-8 (M1916 and M1918, respectively; Sanquin, Amsterdam, The Netherlands). Assays were performed according to manufacturer’s protocol.

2.6 | Statistical analysis

Statistical analyses were performed in Prism 8.1.1 (GraphPad Software, La Jolla, CA). Normal distribution of the data (N = 3) was confirmed using the Shapiro-Wilk test. In case of 1 dependent and 1 independent variable, a 1-way analysis of variance (ANOVA) was used with a Tukey’s multiple comparisons test and Bonferroni correction method for multiple testing. In case of 2 independent variables, a 2-way ANOVA was used with Tukey’s multiple comparisons
**Step 1** - Design guide RNAs (gRNAs) against the first exon of VWF

**Step 2** - Clone gRNAs into LentiCRISPR vector

**Step 3** - Lentivirus production in HEK293T

**Step 4** - Transduction of cbBOEC and selection with puromycin

**Step 5** - Single-cell sorting by flow cytometry

**Step 6** - VWF ELISA of conditioned medium to identify biallelic VWF knockout clones

**Step 7** - Expand biallelic VWF knockout clones

**Step 8** - Confirm biallelic VWF knockout by WB and sequencing
test and Bonferroni-Dunn correction method for multiple testing. Significance values are specified in figure legends.

2.7 | Mass spectrometry

Cells were lysed, processed into tryptic peptides and analyzed by mass spectrometry using an Orbitrap Fusion Tribid mass spectrometer (ThermoFisher). Data were processed using the Maxquant computational platform essentially as described. The raw MS files and search/identification files obtained with MaxQuant were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD013857. A more detailed description of the mass spectrometry sample acquisition and data analysis is provided in Data S1.

3 | RESULTS

3.1 | Generation of CRISPR/Cas9 mediated VWF knockout cord blood outgrowth endothelial cells

Two gRNAs (gRNA-1 and gRNA-2) were designed to target the first exon of VWF and were cloned into the lentiviral LentiCRISPR expression vector (Figure 1), which simultaneously expresses gRNAs and the double strand break inducing Cas9 endonuclease. cbBOECs were transduced with LentiCRISPR without targeting gRNA (CTRL), gRNA-1, gRNA-2, or a combination and were then selected for effective virus transduction by puromycin. Introduction of double strand breaks using CRISPR/Cas9 gene editing generally results in a heterogeneous pattern of insertions and deletions, which may not all lead to knockouts. To enable separation and isolation of biallelic knockouts from the pool of cells that most likely also contains nonedited and monoallelic knockouts, we single-cell sorted the transduced cells and grew them up in 96-well plate format. We performed a first screen for VWF-deficient clones by measuring secreted VWF in conditioned media (Figures 1 and 2A). From this, we selected 10 clones that lost the capacity to secrete VWF, as well as 4 control clones, and expanded those to 6-well plates. It must be noted that it is important to initially select multiple clones, as viability of both control and knockout cells appeared to be decreased in some clones after virus transduction, single-cell sorting, and repeated passaging. Immunoblotting was used to confirm absence of VWF in cell lysates (Figure 2B). We selected 2 knockout (VWF−/− A and VWF−/− B) and 2 control clones (CTRL A and CTRL B) with comparable growth characteristics and expansion potential, which were then further expanded for cryopreservation.

Sanger sequencing and NGS of genomic DNA of the 2 selected clones revealed a number of CRISPR-induced mutations, which were found directly adjacent to the gRNA hybridization sites (Figure 2C, Table S2). One allele of clone VWF−/− A contained a single nucleotide insertion (c.13insA), causing a frameshift that leads to a premature stop codon in the second exon (p.R5KfsX41). The other allele contained a 184-bp deletion starting at position −169 until position 15 in exon 1, which led to the removal of its start codon. In line with this large deletion, we observed a smaller PCR fragment next to the normal-sized fragment when amplifying VWF exon 1 from the genomic DNA of this clone (Figure S1). Clone VWF−/− B contained the same c.13insA mutation as VWF−/− A on both alleles and possibly a c.55insA mutation on a single allele, which combined with the c.13insA mutation would cause a frameshift causing a premature stop codon in exon 3 (Figure 2C, Table S2). Other low-frequency variants that were picked up by NGS were most probably the result of sequence read errors (Table S2). These results show that the loss of VWF expression is the direct result of targeted mutations brought about by CRISPR/Cas9-induced double strand breaks in the first exon.

We next sought to confirm that our CRISPR-edited BOEC clones had retained their EC characteristics. CTRL and VWF−/− BOEC clones all formed confluent monolayers with a typical cobblestone morphology (not shown). All clones expressed endothelial markers VE-cadherin (CD144; Figure 3) and platelet EC adhesion molecule-1 (CD31; Figure S2) at cell-cell junctions with no obvious differences between VWF−/− and CTRL clones. Immunostainings using a selection of monoclonal and polyclonal antibodies directed against VWF showed normal distribution and morphology of WPBs in CTRL clones A and B, whereas VWF−/− clones were completely devoid of WPBs or remaining VWF immunoreactivity (Figure 3). Finally, all clones were able to form networks in a Matrigel-based morphogenesis assay in the presence of vascular endothelial growth factor (VEGF; Figure S3A) and maintained LDL uptake capacity (Figure S3B). These results show that we have generated VWF null BOEC clones that have otherwise preserved their normal endothelial properties.

3.2 | Whole-proteome analysis of VWF-deficient BOEC

To determine whether, apart from morphologic or functional differences, loss of VWF induces changes in the overall protein expression
profile of ECs. We compared expression profiles of 2 independent CTRL and 2 independent VWF−/− BOEC clones using label-free mass spectrometry-based protein quantification. From this, we quantified the abundance of 4371 proteins, of which only a limited number (17) was significantly up- or downregulated in VWF−/− BOECs (Figure 4A; a separate, side-by-side comparison of the individual clones is shown in Figure S4). As expected, the largest difference was seen for VWF, further confirming that we knocked out its expression in our CRISPR-engineered BOECs. Proteins with statistically significant changes in expression are shown in Figure 4B, which includes among others

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**FIGURE 2** Screening and mutation analysis of VWF knockout clones. (A) Conditioned medium of single-cell clones that had reached >50% confluence was collected, and an ELISA for (secreted) von Willebrand factor (VWF) was performed as a first screen for VWF-deficient clones. Arrows indicate control (CTRL) and gRNA targeted clones that were selected for further screening. (B) After selected clones had been expanded to 6-well plates and reached confluence, cells were lysed and VWF deficiency was assayed using immunoblotting with polyclonal anti-VWF and anti-β-actin as a loading control. Two control clones (CTRL A and CTRL B) and two VWF−/− clones (VWF−/− A and VWF−/− B) were selected, as indicated by the arrows. (C) Sanger sequencing and next-generation sequencing on exon 1 of VWF were used to identify CRISPR/Cas9-induced mutations. Sanger sequence traces are shown for all clones, and major mutations in both VWF−/− clones are indicated in the figure.
retinal dehydrogenase 1 (ALDH1A1), nucleoredoxin (NXN), and collagen XII alpha chain (COL12A1) (Figure 4B). In contrast, expression of the WPB-associated proteins CD63 and syntaxin-3 (STX3), which were represented in our data set, remained unchanged (Figure 4C). This suggests that these proteins are still present, although they may display alternative localizations in the absence of WPBs. The expression profiles of a select array of WPB and endothelial markers presented no differences between CTRL and VWF−/− clones (Figure 4C), establishing that apart from lacking VWF, these cells have retained their endothelial characteristics.

3.3 | Alternative routing of Weibel-Palade body cargo proteins

As genetic ablation of VWF also leads to the absence of WPBs, we hypothesized that proteins that are normally stored in or associated with these granules would now be subject to alternative trafficking pathways, resulting in a change in cellular expression. To test this, we investigated the localization of a number of WPB (cargo) proteins that are involved in inflammation, angiogenesis, and WPB trafficking using immunocytochemistry. In VWF-deficient ECs, 2 chemokines that are normally stored in WPBs upon upregulation with IL-1β, IL-6, and IL-8, were now primarily found in small punctate structures, which may represent small constitutive secretory vesicles and a ribbon-like pattern consistent with their accumulation in the Golgi (Figure 5A,C).44‒47 Because only a small portion of IL-6 and IL-8 is stored inside WPBs,18,46 we addressed whether loss of WPBs has an effect on functional release of these cytokines. Therefore, we stimulated CTRL and VWF−/− for 24 hours with IL-1β and assayed their unstimulated and histamine-evoked release by ELISA (Figure 5B and D). Overall, no significant differences were found in terms of intracellular content or absolute release between control and knockout ECs. In VWF−/−, B cytokine release relative to intracellular content appears to be increased, but not in VWF−/− A. However, the production
of IL-6 and IL-8 is dramatically increased in all clones upon IL-1β, and release over 24 hours is clearly considerably higher than the stored fraction. Another trend was visible, which showed that an induction of cytokine release upon histamine stimulation that was seen in CTRL clones was less pronounced in VWF−/− clones, although the data showed limited statistically significant differences due to variation between experiments.

The angiogenesis mediator Ang-2 is another protein that, like IL-8, is thought to be coackaged in the WPB during its formation at the trans-Golgi network (TGN), possibly through noncovalent interaction with VWF.48 Also for Ang-2, we observed a punctate pattern in the absence of WPBs, as well as an overall decrease in Ang-2 signal (Figure 6). However, whereas the punctae of IL-6 and IL-8 were diffusely distributed throughout the cell, Ang-2 shows enrichment at cell-cell junctions (Figure 6 see also Figure S5). It has been suggested previously that altered angiogenic properties of ECs lacking VWF may be caused by increased constitutive release of Ang-2, which would then lead to autocrine/paracrine regulation of Tie-2 signaling.28 In line with this, we observed that Ang-2, which in CTRL cells primarily localizes to WPBs and shows minimal overlap with Tie-2, is primarily found on Tie-2 positive structures that are enriched at cell-cell junctions of VWF−/− cells (Figure S5). This suggests that in the absence of its storage compartment, constitutively released Ang-2 associates with Tie-2 on the plasma membrane. Next, we performed secretion assays with the CTRL and VWF−/− clones and measured the Ang-2 concentration in the releasates vs. the lysates by ELISA. Strikingly, lysates of VWF−/− clones contained less Ang-2 than CTRL clones (Figure 6Bii),
**Figure 5** Interleukin (IL)-6 and IL-8 show alternative localization in VWF<sup>−/−</sup> cord blood outgrowth endothelial cells (cbBOECs). (A and C) Two control clones (CTRL A and B) and 2 VWF<sup>−/−</sup> clones (VWF<sup>−/−</sup> A and B) were cultured for 5 to 7 days after reaching confluence, the last 24 hours including 10 ng/mL IL-1β. Immunostainings were performed with mouse monoclonal anti-human VWF (magenta) and (A) anti-IL-6 (cyan) or (C) anti-IL-8 (cyan). Boxed areas are magnified on the right. Scale bars represent 15 μm.

(B and D) Cytokine storage and secretion in IL-1β-treated CTRL and VWF<sup>−/−</sup> BOECs. IL-6 (Bi) and IL-8 (Di) intracellular content were measured by ELISA of lysates. (Bii-iii) Steady-state release of IL-6 (Bii-Biii) and IL-8 (Dii-Diii) was measured by ELISA in 24-hour unstimulated release samples and was expressed as absolute levels (Bii and Dii) or normalized to intracellular content (Biii and Diii). (Biv and Div) Stimulus-induced Ang-2 release was assayed using 30 minutes of unstimulated vs. histamine-treated CTRL and VWF<sup>−/−</sup> BOECs. Data are shown as mean ± standard error of the mean of 3 independent biological replicates, performed in triplo. Statistical analysis was 1-way analysis of variance with a significance level of *P < 0.05, **P < 0.01, ***P < 0.001.
FIGURE 6 Angiopoietin-2 (Ang-2) localizes to the cell-cell junctions in VWF−/− cord blood outgrowth endothelial cells (cbBOECs). (A) Two control clones (CTRL A and B) and 2 VWF−/− clones (VWF−/− A and B) were fixed after 5 to 7 days of confluence and immunostained with mouse monoclonal anti-human VWF (cyan), anti-Ang-2 (green) and anti-β-catenin (magenta). Boxed areas are magnified on the right. Scale bars represent 10 μm. (B) Dysregulation of Ang-2 storage and secretion in the absence of von Willebrand factor (VWF). (Bi) Ang-2 intracellular content was measured by ELISA of lysates. (Bi-iii) Steady-state release of Ang-2 was measured by ELISA in 24-hour unstimulated release samples and was expressed as absolute levels (Bi) or normalized to intracellular content (Bi-iii). (Biv) Stimulus-induced Ang-2 release was assayed using 30 minutes of unstimulated vs. histamine-treated CTRL and VWF−/− BOECs. Data are shown as mean ± SEM of 3 independent biological replicates, performed in triplo. Statistical analysis was one-way analysis of variance with a significance level of *P < 0.05, **P < 0.01, ***P < 0.001.
FIGURE 7  CD63 and syntaxin-3 show loss of localization to elongated vesicles but unaltered pattern on rounded vesicles in VWF<sup>−/−</sup> cord blood outgrowth endothelial cells (cbBOECs). Two control clones (CTRL A and B) and 2 VWF<sup>−/−</sup> clones (VWF<sup>−/−</sup> A and B) were fixed after 5 to 7 days of confluence and immunostained with mouse monoclonal anti-human VWF (magenta) and (A) anti-CD63 (cyan) or (B) anti-syntaxin-3 (STX3, cyan). Boxed areas are magnified on the right. Scale bars represent 10 μm.
while the basal release in 24 hours increased in VWF<sup>+/−</sup> B (Figure 6Bii), substantiating that in the absence of WPBs Ang-2 is not stored intracellularly but is continuously released. Both VWF<sup>+/−</sup> clones exhibited higher Ang-2 release relative to intracellular content compared to CTRL clones (Figure 6Biii). In response to histamine, CTRL clones showed a strong increase of Ang-2 release, whereas in the VWF<sup>+/−</sup> clones, Ang-2 release remained unchanged to unstimulated cells, suggesting that no Ang-2 is stored in histamine-responsive vesicles. Absolute Ang-2 release in response to histamine was indeed decreased in VWF-deficient clones.

A different localization pattern was observed for syntaxin-3 and CD63. 2 proteins that are normally found both on WPBs and on endosomes.<sup>40,49</sup> It has previously been shown that CD63 is trafficked to the WPBs via the endosomal system in an annexin A8- and AP-3-dependent manner.<sup>21,50‒52</sup> As expected, whereas CD63 localization to the elongated WPBs is lost in VWF<sup>+/−</sup> clones, its localization to spherical vesicles that are most likely endosomes remains similar (Figure 7). Interestingly, syntaxin-3 shows a similar pattern, suggesting that this protein may normally follow a similar route as CD63 to arrive at a WPB.

4 | DISCUSSION

In this study, we generated stable VWF knockout ECs using CRISPR/Cas9 gene editing, resulting in ECs entirely devoid of WPBs. To our knowledge, this is the first study to report the ablation of an entire organelle using CRISPR/Cas9. A number of studies have previously employed CRISPR/Cas9 to knock out expression of targets in ECs. In most of those studies, no single-cell selection and clonal expansion was performed, but a bulk population was used in which a proportion of the cells may still express the targeted protein.<sup>33,53</sup> Single-cell cloning is needed to arrive at genetically homogeneous populations of ECs that have lost expression of the target gene, but the extensive passaging involved places high demands on the proliferative capacity of modified endothelial cells. Our approach was similar to a methodological study in which clonal endothelial lines were generated after single-cell sorting of CRISPR-edited cbBOECs,<sup>32</sup> primary ECs that have increased expansion capacity compared other primary ECs such as human umbilical vein ECs or peripheral BOECs.<sup>34,54</sup> Similar to what we find in our study, clonal CRISPR-engineered BOEC lines had retained sufficient expansion capacity for downstream experimental applications. As our target of interest is a secreted rather than a cell surface protein, we used an assay for secreted protein as our initial clone selection followed by cell expansion for further clone selection through analysis of intracellular protein. For knockout of intracellular, nonsecreted proteins of interest, an extra expansion step would be required for immediate clone selection through western blotting, as we have previously described.<sup>52</sup>

To ensure that phenotypic differences that we observed are not the result of (1) variation in genetic background between donors, (2) clonal effects that are not directly linked to the inactivation of the target, or (3) clonal expansion of single cells, we used BOECs from a single donor, and we selected multiple clones from CRISPR-edited cells as well as control cells that were subjected to the same single-cell sorting and expansion procedure in parallel. Even with these precautions, we observed that a substantial number of clonal candidates were generated that ceased to expand shortly after the clonal selection procedure or that suffered from severely reduced proliferative capacity. Whether this is the result of lentiviral transduction and/or expression of Cas9 protein or whether this is an inherent problem of a primary cell system being pushed to the limit of its proliferative capacity by clonal expansion is at this point unclear. Careful surveying of a large number of candidates for their proliferative capacity may therefore be necessary to generate enough clones for the intended experimental analysis. This also complicates the use of (CRISPR-edited) BOECs in assays that model angiogenesis, which are highly dependent on proliferation of ECs, and their outcomes would be influenced by a rapid drift in proliferative capacity between individual clones. For that particular purpose, it may be more suited to use ECs derived from induced pluripotent stem cells (iPSCs). CRISPR/Cas9 gene editing in iPSCs as well as their differentiation into ECs has been well established.<sup>55,56</sup>

Genetic removal of WPBs revealed alternative trafficking pathways for WPB cargo and associated proteins. Contrary to most WPB cargo proteins, which are copackaged with VWF in newly forming WPBs, CD63 first traffics via the endosomal system, and that is why it is not observed in immature WPBs.<sup>50,51</sup> We have recently shown that CD63, but also the WPB v-SNARE protein vesicle-associated membrane protein 8 (VAMP8), depend on the AP-3 complex for their targeting to the WPBs.<sup>52</sup> The WPB regulating t-SNARE protein syntaxin-3, one of the cognate SNARE partners of VAMP8, has also been observed both on WPBs and on round vesicles that are most likely endosomes, but how it is trafficked to WPBs remains unknown. Interestingly, we observe a similar localization pattern for CD63 and syntaxin-3, with localization to WPBs and endosomes in control cells and only to endosomes in VWF<sup>+/−</sup> cells. This shows that syntaxin-3 targeting to endosomes is not dependent on WPBs and is therefore most likely not initiated by a journey through the secretory pathway. Instead, it may be directly incorporated into the endosomes from the Golgi, from which it will then commence its cycling between WPBs, plasma membrane, and endosomes in a similar manner as CD63.

Traffic of the chemokines IL-6 and IL-8, which are incorporated directly into WPBs during their formation at the TGN, is also substantially altered in the absence of WPBs. This was mainly reflected in a loss of the stimulus-sensitive pool of these chemokines in response to agonist triggering (Figure 5B and D). The lack of stimulus-induced release of IL-6 and particularly IL-8, which is a potent neutrophil chemoattractant, that we observe in VWF<sup>+/−</sup> BOECs suggests that apart from the direct<sup>57</sup> and indirect (via P-selectin surface presentation)<sup>58</sup> role that VWF plays in adhesion of leukocytes, impaired regulated secretion of chemokines could further contribute to defects in neutrophil recruitment in the absence of
VWF. Many interleukins, including IL-6 and IL-8, have been implicated in cardiovascular disease such as atherosclerosis (reviewed in Apostolakis et al28). This raises the question of whether dysregulated endothelial chemokine release as a result of VWF deficiency may also affect atherogenesis. Several studies in murine models have indeed shown that VWF-deficient mice are protected from experimental atherosclerosis,59-61 However, firm evidence for a similar protective effect in patients with VWD has so far not been found (reviewed in62). In vitro IL-6 and IL-8 constitutive secretion did not show large differences between CTRL and VWF−/− BOECs (Figure 5), which is consistent with previous observations that the bulk of newly synthesized IL-6 and IL-8 does not enter WPBs but is released via the constitutive secretion pathway.19,46 As far as we are aware, no quantitative analyses have been performed on IL-6 and/or IL-8 levels in VWD patients, but based on our in vitro observations, the likelihood of these deviating significantly from healthy subjects is limited, given that most EC-derived IL-6 or IL-8 is not released via the WPBs.

WPBs also facilitate the long-term storage of the angiogenesis mediator Ang-2 and ensure its on-demand availability during proangiogenic conditions, for instance, in response to triggering of WPB mediator Ang-2 and ensure its on-demand availability during proangiogenesis, the likelihood of these deviating significantly from healthy subjects is limited, given that most EC-derived IL-6 or IL-8 is not released via the WPBs.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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