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## Personalized treatment for von Willebrand disease by RNA-targeted therapies

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# 6

## **Defective von Willebrand factor multimerization in endothelial colony forming cells with low von Willebrand factor production**

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*In preparation*



## Abstract

Von Willebrand factor (VWF) is a multimeric protein that is dimerized and multimerized in the endoplasmic reticulum and Golgi apparatus, respectively. After synthesis, VWF is either stored as large multimers in rod-shaped organelles, called Weibel-Palade bodies (WPBs), or VWF is constitutively released. Recent studies on cultured endothelial cells showed that constitutively released VWF is secreted faster and consists mainly of low molecular weight VWF. Furthermore, small interfering RNA (siRNA)-mediated downregulation of VWF in cultured endothelial cells resulted in shorter WPBs and increased secretion of VWF from the endothelial cells. The effects of VWF downregulation on the multimerization of VWF has never been studied and we hypothesized that downregulation of VWF not only results in shortening of the WPBs and increased VWF secretion, but also affects the VWF multimerization. This hypothesis was tested in endothelial colony forming cells (ECFCs) isolated from three healthy donors by downregulating the VWF synthesis with siRNAs against *VWF*. WPB quantification of confocal microscopy images confirmed that downregulation of VWF leads to shorter WPBs. The multimeric state of VWF was assessed in conditioned medium and protein lysates by VWF multimerization analysis and the VWF collagen binding assay. Remarkably, we observed in conditioned medium, but not in protein lysates, a decrease in high molecular weight VWF and an increase in the intensity of the VWF dimer. In addition, we confirmed increased VWF secretion from ECFCs treated with siRNA against *VWF* compared to cells treated with a negative control siRNA. Altogether, we show that VWF downregulation results in increased release of mainly low molecular weight VWF.

## Introduction

Von Willebrand factor (VWF) is a large multimeric hemostatic protein mainly produced by endothelial cells. The functionality of VWF in hemostasis is highly dependent on the multimeric size of VWF.<sup>1,2</sup> The process of VWF dimerization and multimerization is regulated in the endoplasmic reticulum and (trans)-Golgi network, respectively.<sup>3,4</sup> Large proportions of the VWF multimers are tightly packaged in the endothelial storage organelles, the Weibel-Palade bodies (WPBs).<sup>5-7</sup> Stimuli released after vascular damage, stress or inflammation enforces the WPBs to release their content.<sup>8</sup> Mediated by vascular shear, VWF multimers will unroll and form ultra-large VWF strings that attract platelets to sites of vascular damage. This process of secretion is called regulated or stimulated secretion and can be induced by amongst others thrombin or histamine that raise intracellular  $\text{Ca}^{2+}$  levels or epinephrine or vasopressin that raise intracellular cAMP levels.<sup>9</sup> Besides regulated secretion, WPBs also continuously secrete VWF without the aid of stimuli and this is known as basal secretion.<sup>10</sup> Furthermore, small proportions of VWF are not stored in WPBs, but directly secreted through the constitutive release pathway.<sup>11</sup> Although it has been known for years that constitutively released VWF mainly consists of low molecular weight (LMW) VWF<sup>12,13</sup>, it was recently described that constitutively released LMW VWF is mainly secreted at the basolateral side of the endothelial cells into the subendothelial matrix.<sup>14</sup> Basal and regulated secretion on the other hand, consists mainly of high molecular weight (HMW) VWF that is secreted at the apical side of the endothelial cells.<sup>14</sup>

Quantitative or qualitative defects in VWF leads to von Willebrand disease (VWD), the most common inherited bleeding disorder.<sup>15</sup> In the past decade, human umbilical vein endothelial cells (HUVECs) and endothelial colony forming cells (ECFCs, previously called blood outgrowth endothelial cells or BOECs) have been used to study VWD and VWF in more detail on a cellular level.<sup>16-20</sup> It was amongst others shown that siRNA-mediated downregulation of *VWF* in HUVECs led to increased secretion of VWF from the endothelial cells and a decrease in the number and length of WPBs.<sup>21,22</sup> Since downregulation of VWF led to decreased WPB size, we hypothesized that also the multimeric state of VWF is affected in VWF downregulated cells. In this study, we test this hypothesis in ECFCs isolated from three different healthy controls by downregulation of the VWF synthesis using siRNAs.

## Methods

### ***ECFC isolation***

ECFCs were isolated from three healthy donors as described before.<sup>23</sup> Peripheral blood was drawn in lithium heparin tubes (ECFCs C1 and C2; BD Biosciences, Erebodgem, Belgium) or in sodium heparin CPT™ Mononuclear Cell Preparation Tubes (C3; BD Biosciences). Blood was diluted 1:1 in phosphate buffered saline (PBS) and layered over Ficoll Paque (LUMC Pharmacy, Leiden, the Netherlands), followed by centrifugation. The mononuclear cell fraction was washed twice in PBS supplemented with 10% fetal bovine serum (FBS) after which cells were taken up in Lonza (ECFC C3) or PromoCell (ECFCs C1 and C2) endothelial culture medium. Lonza endothelial culture medium consisted of 500 ml EBM™-2 medium (Lonza, Breda, the Netherlands) supplemented with the EGM™-2 BulletKit™ (Lonza), 100 ml FBS (GIBCO®, Invitrogen, Carlsbad, CA, USA) and 7 ml Antibiotic Antimycotic solution (Sigma-Aldrich #A5955, st. Louis, MO, USA). PromoCell endothelial culture medium consisted of 500 ml Endothelial Cell Growth Medium 2 (PromoCell C-22111, Heidelberg, Germany), 50 ml FBS (GIBCO®) and 5 ml Antibiotic Antimycotic solution (Sigma-Aldrich). Mononuclear cells were plated in 48 wells plates (Nunc Cell-Culture Treated Multidishes (Nunclon, Roskilde, Denmark) or Sarstedt TS plates (Nümbrecht, Germany)) pre-coated with 50 µg/ml rat tail collagen type I (BD Biosciences). Medium was refreshed every other day until day 21. Passaging and usage of cells is performed as earlier described<sup>23</sup>, but with PromoCell culture medium.

The study was approved by the institutional ethical review board (study registered as NL54591.058.15). Informed consent was obtained from all healthy controls in accordance with the declaration of Helsinki.

### ***siRNA transfection***

For transfections, 100,000-125,000 cells per well were plated on rat tail collagen (50 µg/ml; BD biosciences) coated wells of a 24 wells plate. 24 hours after plating the cells, a negative control siRNA (siNEG, 4404020, Life Technologies Europe BV) or an siRNA against *VWF* (siVWF, s14834, Life Technologies Europe BV, Bleiswijk, the Netherlands) were transfected into ECFCs using DharmaFECT duo transfection reagent (Dharmacon, Lafayette, Colorado, USA). Before transfection, cells were washed twice with Hanks' Balanced Salt solution (Thermo Fisher Scientific, Carlsbad, CA, USA) and once with PromoCell EGM2 culture medium (without extra FBS). siRNAs and DharmaFECT duo were separately diluted in Optimem1 (Thermo Fisher Scientific) and mixed together by pipetting. After 20 minutes of incubation, the siRNA-DharmaFECT mixture was complemented with Optimem1 supplemented with 4% FBS. Cells were incubated with 150 µl transfection mixture containing a high concentration of siRNA for three hours. After the three hour incubation, 250 µl culture medium was added to obtain a final siRNA concentration of 20 nM. Medium was refreshed 24 hours after transfection and

every other day thereafter. Six days after transfection, the medium was harvested and cells were lysed with protein lysis buffer containing OptiMem1, 0.1% triton X-100 (Sigma-Aldrich) and a tablet of cOmplete protease inhibitor cocktail with EDTA (Roche Diagnostics, Mannheim, Germany).

### **VWF analysis**

VWF antigen (VWF:Ag) and VWF collagen binding (VWF:CB) were simultaneously measured by ELISA in conditioned medium and protein lysate samples. The VWF:Ag ELISA was performed as described before<sup>24</sup>, with the only modification that samples were diluted in dilution buffer (PBS containing 1% bovine serum albumin). For VWF:CB analysis, ELISA plates were coated with 0.3% bovine collagen type I (95%) and III (5%) (StemCell technologies, Cologne, Germany) in PBS. Samples were diluted in dilution buffer and incubated for 2 hours. To increase the signal of the ELISA, biotin (Sigma-Aldrich) was conjugated to rabbit anti-VWF-IgG (A0082; DAKO, Glostrup, Denmark) to generate rabbit anti-VWF-IgG-biotin. ELISA plates were incubated with rabbit anti-VWF-IgG-biotin diluted in dilution buffer for one hour. Thereafter, ELISA plates were incubated with Streptavidin-(POLY) horseradish peroxidase (Thermo Fisher Scientific) diluted in dilution buffer for one hour. O-phenylenediamine dihydrochloride (Sigma-Aldrich) was used as substrate and dissolved in 11 ml substrate buffer (22 mM citric acid, 51 mM phosphate, pH 5.0) with addition of 11  $\mu$ l 30% H<sub>2</sub>O<sub>2</sub>. The enzymatic reaction was terminated using 2M H<sub>2</sub>SO<sub>4</sub>. Normal pooled plasma was used as reference.

VWF multimers were analyzed under non-reducing conditions by agarose gel electrophoresis and subsequent western blotting as described before.<sup>24</sup> ECL Western Blotting Substrate (Promega, Madison, WI, USA) was used to detect the VWF multimers. Densitometry images were generated by ImageJ (ImageJ 1.51h, Bethesda, MD, USA).<sup>24</sup> Equal concentrations of VWF:Ag were loaded in each lane of a gel.

### **Immunofluorescent analysis and WPB quantification**

ECFCs were plated on rat tail collagen coated coverslips and transfected as described above. Six days after transfection, cells were fixed using ice-cold methanol for 10 minutes and stained for VWF, VE-cadherin and nuclei as described before.<sup>23</sup> Per cell line, 10 to 13 images were randomly taken (focused on nuclei staining) using the Leica TCS SP8 X WLL converted confocal microscope with an HC PL APO CS2 63x/1.40 OIL immersion objective. The number of cells and number and size of WPBs were quantified using CellProfiler software (v.8.1.3).<sup>25</sup> Using a self-created pipeline, channels (VWF, VE-cadherin and nuclei) were split and converted into black and white images. First, the number of cells were determined by Hoechst positive objects. Then, the interior of a cell was determined by the area that was

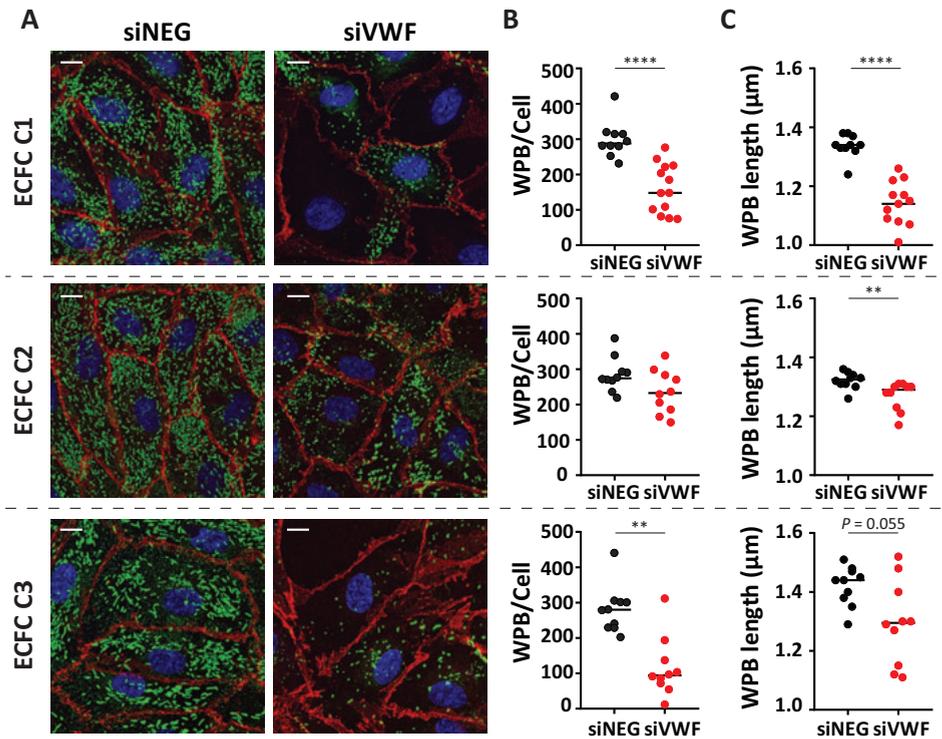
surrounded by VE-cadherin positive staining. Lastly, the number and length of the WPBs were determined per cell by analysis of VWF positive objects. WPBs were only analyzed for cells that were completely visible.

### **Statistical analysis**

Graphical images and statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). The Mann-Whitney *U* test was used to assess significance between siNEG and siVWF treated cells. For all analyses, a  $P < 0.05$  was considered significant.

## **Results and discussion**

Previous findings showed that downregulation of the production of VWF in HUVECs by siRNA nucleofection leads to decreased WPB length.<sup>21,22</sup> To investigate whether downregulation of VWF by liposomal siRNA transfection also leads to decreased WPB length in ECFCs, we transfected three proliferative healthy control ECFC lines with either siNEG or siVWF. Transfected cell lines were stained for VWF and VE-cadherin, after which the number and length of WPBs per image were quantified (Fig. 1A). Quantification of the length and number of WPBs confirmed that downregulation of VWF results in a decreased size of WPBs as well as a decreased number of WPBs per cell (Fig. 1B and 1C). For all three cell lines, the median number of WPBs per cell in siNEG treated cells was comparable and around 280 (Fig. 1B). The median length of a WPB in siNEG treated cells slightly varied per cell line from 1.33  $\mu\text{m}$  for C2, to 1.44  $\mu\text{m}$  for C3 (Fig. 1C). WPB length ranged in all cell lines from 0.6 - 5  $\mu\text{m}$  (data not shown), which is in line with previous findings.<sup>6,7,21,22</sup> Here, the images for quantification are generated by a maximum projection of the z-stacks of a frame. However, since most WPBs do not perfectly align with the plain, it is likely that we underestimate the actual length of the WPBs, especially for the larger WPBs. The actual difference in WPB length between siNEG and siVWF could therefore be bigger than is indicated in the graphs (Fig. 1C). The effects of siVWF on the length and number of WPBs depended on the efficiency of siVWF to inhibit VWF, and this varied between the three cell lines (Table 1). E.g. downregulation of VWF in C2 was less efficient, which translated in the less reduction in WPB length. Furthermore, not all areas on the coverslips showed the same degree of VWF downregulation. In some images of siVWF treated cells, cells with normal WPBs were observed, which suggests that the siRNA has not been internalized in these cells. When these cells divide, larger areas with untransfected cells are visible. Fig. S1 shows all images that were taken from the cells. Indeed, some images taken from siVWF treated cells show normal WPBs. This is also clearly indicated in the bar graphs where the median length of WPBs per image was unchanged for some areas (Fig. 1C, especially ECFC C3).



**Figure 1. Quantification of number and length of WPBs in three healthy control ECFCs.** (A) Confocal images of ECFC C1, C2, and C3 treated with siNEG or siVWF. Cells were stained for VWF (green), VE-cadherin (red) and nuclei (blue). Scale bar represents 10  $\mu\text{m}$  (B) Quantification of the number of WPBs per cell. Downregulation of VWF by siVWF resulted in a decreased number of WPBs per cell for ECFC C1 and C3. Mann-Whitney, \*\*  $P < 0.01$ , \*\*\*\*  $P < 0.0001$  (C) Quantification of the WPB length. Downregulation of VWF resulted in a significant decrease in the length of WPBs for ECFCs C1 and C2. A trend towards a significant decrease in WPB length was observed for ECFC C3. Mann-Whitney, \*\*  $P < 0.01$ , \*\*\*\*  $P < 0.0001$ . For all bar graphs: each dot represents the median number of WPBs or the median WPB length per image. ECFC, endothelial colony forming cells; siNEG, negative control siRNA; siVWF, siRNA against *VWF*; VWF, von Willebrand factor; WPB, Weibel-Palade body

**Table 1.** VWF:Ag concentration measured in conditioned medium or protein lysates of ECFCs treated with either siNEG or siVWF

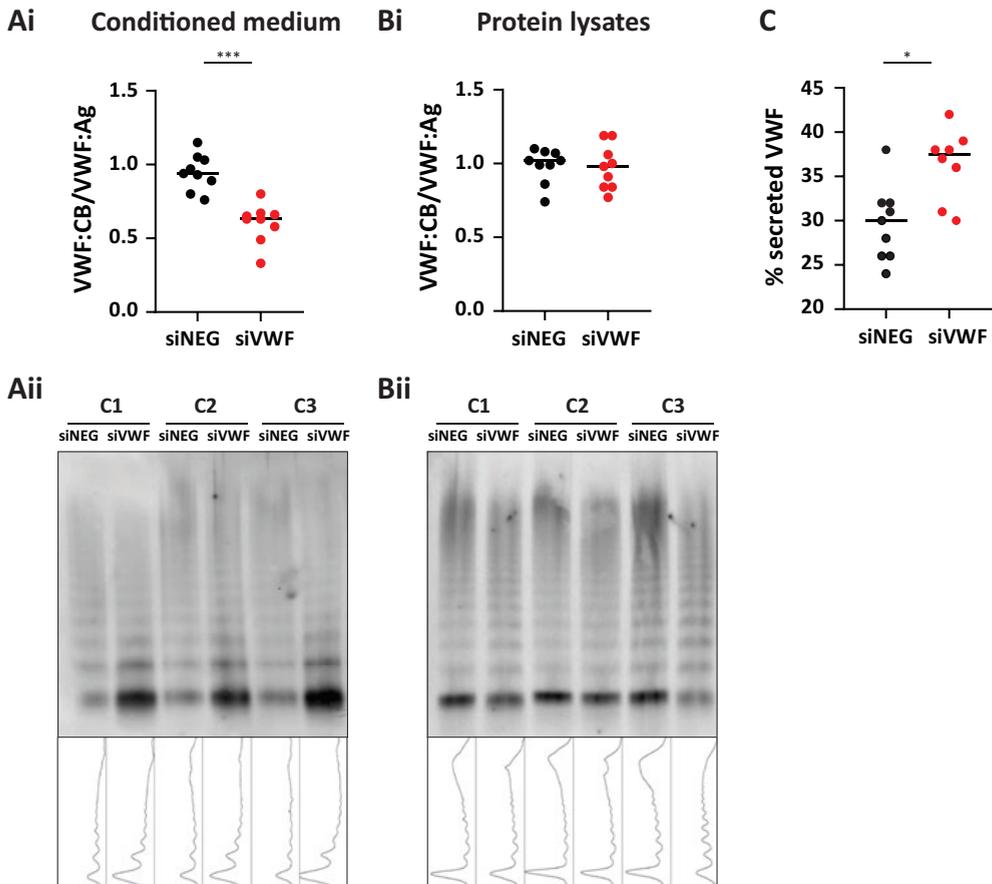
VWF:Ag (mU)	Conditioned medium			Protein lysates		
	ECFC C1	ECFC C2	ECFC C3	ECFC C1	ECFC C2	ECFC C3
siNEG	15.4 $\pm$ 1.9	12.3 $\pm$ 3.2	6.2 $\pm$ 1.5	44.6 $\pm$ 11.0	38.9 $\pm$ 10.5	27.5 $\pm$ 9.2
siVWF	1.9 $\pm$ 0.3	2.8 $\pm$ 0.6	1.4 $\pm$ 0.2	3.8 $\pm$ 0.7	7.6 $\pm$ 1.1	3.1 $\pm$ 0.6
% VWF downregulation	89%	77%	78%	92%	81%	89%

ECFC, endothelial colony forming cells; mU, milli-units; siNEG, negative control siRNA; siVWF, siRNA against *VWF*; VWF, von Willebrand factor; VWF:Ag, VWF antigen

Since mainly HMW VWF is stored in the WPBs<sup>14</sup>, we questioned whether downregulation of VWF not only affects the length of the WPBs, but also the multimeric state of VWF. The multimeric state of VWF was first assessed by the VWF:CB assay. The VWF:CB assay is able to detect multimerization defects, since HMW VWF binds collagen more efficiently than LMW VWF.<sup>26</sup> Conditioned medium and protein lysate samples were simultaneously subjected to VWF:Ag and VWF:CB ELISAs. Interestingly, we observed a significant decrease in VWF:CB/VWF:Ag in conditioned medium samples of cell lines transfected with siVWF compared to siNEG treated cells ( $P < 0.001$ , Fig. 2Ai). On the other hand, the VWF:CB/VWF:Ag in protein lysates was unaltered in siVWF treated ECFCs (Fig. 2Bi). To confirm that the multimeric state of VWF is indeed altered in siVWF treated cells and to assess the exact VWF multimerization pattern, we performed a VWF multimerization assay on all three cell lines transfected with either siNEG or siVWF. In conditioned medium samples of ECFCs treated with siVWF, we clearly observed an increase in intensity of the dimer band and decrease of HMW VWF (Fig. 2Aii). In the protein lysates, the difference between siNEG and siVWF treated cells is less apparent (Fig. 2Bii). However, also in protein lysates a slight decrease in HMW VWF, but also a slight decrease in the intensity of the dimer band, seems notable in siVWF treated cells (Fig. 2Bii). Furthermore, as was observed previously<sup>22</sup>, we noted an increase in the percentage of secreted VWF in siVWF treated cells compared to siNEG treated cells in 24 hours ( $P < 0.05$ , Fig. 2C). This suggests that VWF is secreted faster in cells with low VWF production.

Altogether, we show that VWF downregulation results in a decrease in the number and length of the WPBs and increased secretion of mainly LMW VWF. Since mainly LMW VWF is secreted through the constitutive release pathway, and secretion of VWF through this pathway is faster than secretion through basal release, it is possible that relatively more VWF is secreted through the constitutive release pathway in siVWF treated cells. In our experimental set-up, we could not discriminate between constitutively and basal released VWF. Studies in which the distribution of VWF secretion is determined, by for example Transwell membranes, are needed to confirm this hypothesis.

Whether the effects of VWF downregulation on the multimerization of VWF can be translated to the human body are unknown. To our knowledge, only a small percentage of patients are known to develop VWD because of reduced VWF production. These patients have either one null allele or mutations in the promoter region of *VWF*.<sup>27</sup> These patients, however, do not show affected plasma multimers. Furthermore, our group recently published on the use of allele-specific siRNAs as a therapeutic approach to correct dominant negative VWD. With these studies we aim to inhibit the production of the mutant *VWF* allele only, and thereby correct for VWD phenotypes. This approach would ultimately lead to a VWF production of 50% compared to normal, similar to VWD patients with one null allele. Although these patients do not show affected multimerization, awareness of this *ex vivo* data is critical when studying the effects of allele-specific siRNAs in preclinical VWD models.

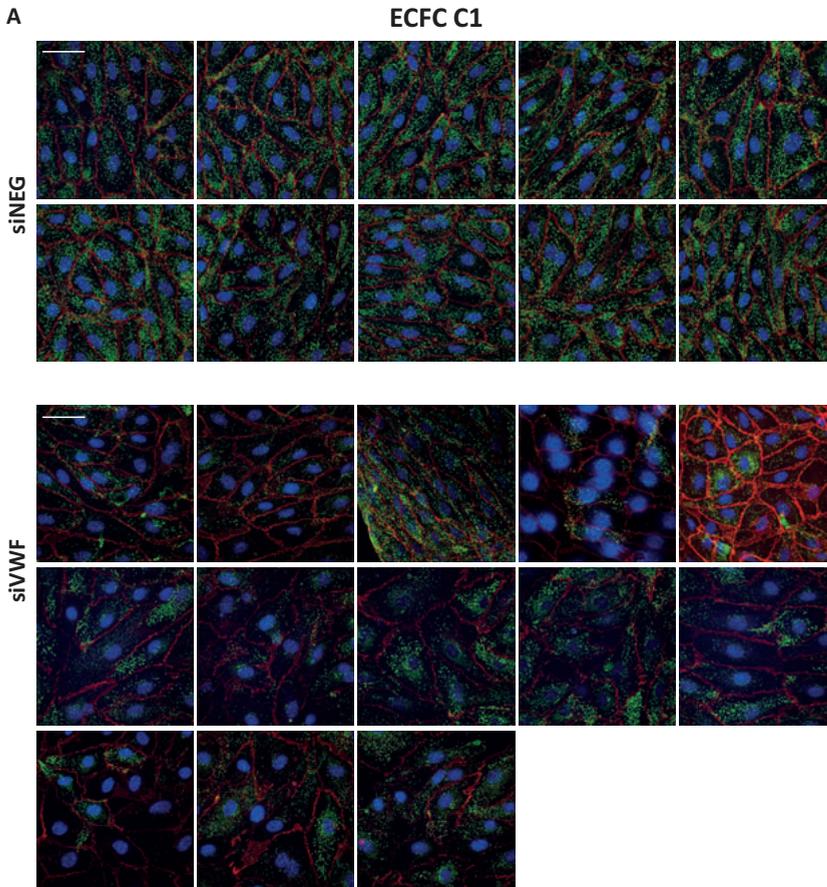


**Figure 2. Effects of VWF downregulation on the multimerization and secretion of VWF.** (Ai) Downregulation of VWF production results in a significant decrease in VWF:CB/VWF:Ag in conditioned medium compared to siNEG treated cells. (Aii) VWF multimerization analysis of conditioned medium samples obtained from ECFC C1, C2, and C3 treated with siNEG or siVWF. Downregulation of VWF production by siVWF resulted in a decrease of high molecular weight VWF and an increase in the intensity of the dimer band. (Bi) Downregulation of VWF production did not alter the VWF:CB/VWF:Ag in protein lysates. (Bii) VWF multimerization analysis of protein lysate samples obtained from ECFC C1, C2, and C3 treated with siNEG or siVWF. A small decrease of high molecular weight VWF seem apparent in siVWF treated cell lines. Also a slight decrease in the intensity of the dimer band is observed. (C) Downregulation of VWF production by siVWF leads to an increase in VWF secretion in 24 hours. (A-C) Graphs show the combined data of VWF:CB and VWF:Ag measurements of ECFC C1, C2, and C3. Transfection experiments were performed three times in duplicate. Every dot represents the average of the duplicate experiment. Mann-Whitney, \*  $P < 0.05$ , \*\*\*  $P < 0.001$ . ECFC, endothelial colony forming cell; siNEG, negative control siRNA; siVWF, siRNA against *VWF*; VWF, von Willebrand factor; VWF:CB, VWF collagen binding; VWF:Ag, VWF antigen

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**Supplemental Figure**

**Figure S1. Effects of VWF downregulation on WPB formation. Confocal images of ECFC C1 (A), C2 (B), and C3 (C) treated with siNEG or siVWF.** ECFCs were stained for VWF (green) and VE-cadherin (red). Per condition, 10-13 images were taken. Scale bar represents 50  $\mu$ m. ECFC, endothelial colony forming cell; siNEG, negative control siRNA; siVWF, siRNA against *VWF*; VWF, von Willebrand factor

