Personalized treatment for von Willebrand disease by RNA-targeted therapies

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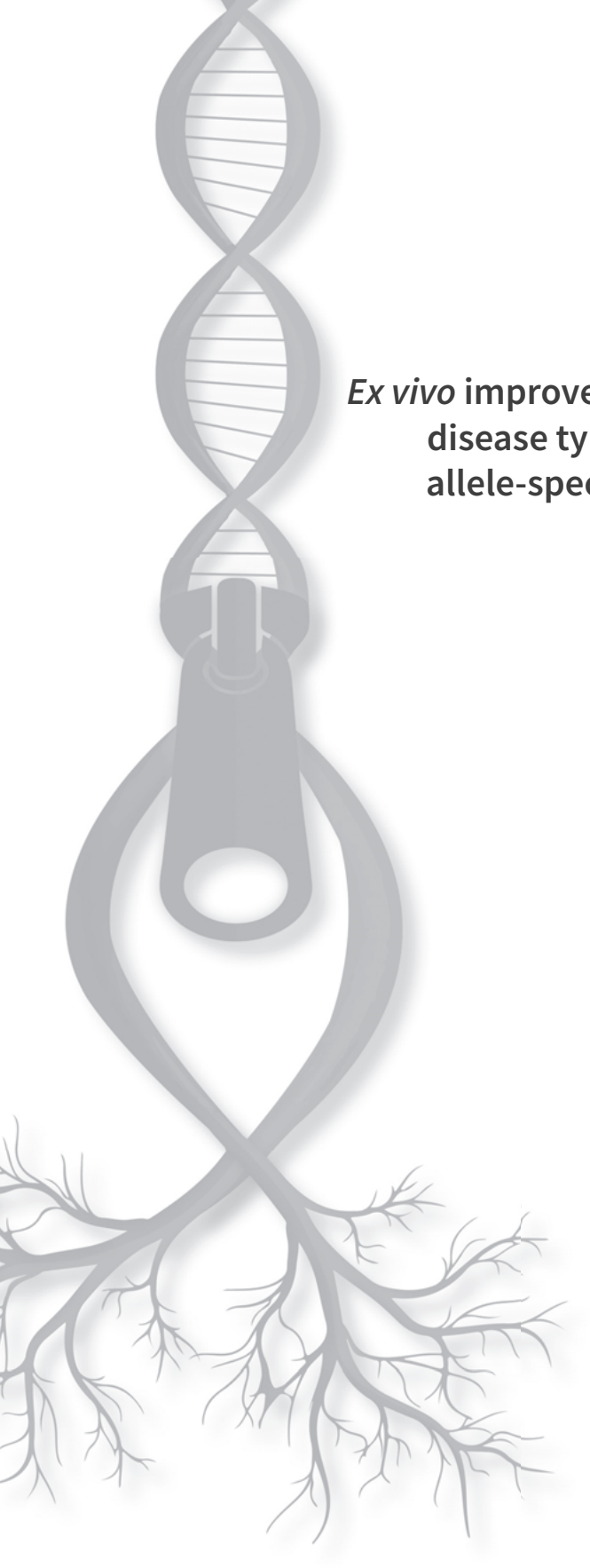

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Ex vivo improvement of a von Willebrand disease type 2A phenotype using an allele-specific small interfering RNA

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Abstract

Von Willebrand disease (VWD) is the most common inherited bleeding disorder and is mainly caused by dominant negative mutations in the multimeric protein von Willebrand factor (VWF). These mutations may either result in quantitative or qualitative defects in VWF. VWF is an endothelial protein that is secreted to the circulation upon endothelial activation. Once secreted, VWF multimers bind platelets and chaperone coagulation factor VIII in the circulation. Treatment of VWD focuses on increasing VWF plasma levels, but production and secretion of mutant VWF remains uninterrupted. Presence of circulating mutant VWF might, however, still affect normal hemostasis or functionalities of VWF beyond hemostasis. We hypothesized that inhibition of the production of mutant VWF improves the function of VWF overall and ameliorates VWD phenotypes. We have previously proposed the use of allele-specific small interfering RNAs (siRNAs) that target frequent *VWF* SNPs to inhibit mutant *VWF*. The aim of this study is to prove the functionality of these allele-specific siRNAs in endothelial colony forming cells (ECFCs). We have been able to isolate ECFCs from a VWD type 2A patient with an intracellular multimerization defect, reduced VWF collagen binding and a defective processing of proVWF to VWF. After transfection of an allele-specific siRNA that specifically inhibited expression of mutant VWF, we showed amelioration of the laboratory phenotype, with normalization of the VWF collagen binding, improvements in VWF multimers, and enhanced VWF processing. Altogether, we prove that allele-specific inhibition of the production of mutant VWF by siRNAs is a promising therapeutic strategy to improve VWD phenotypes.

Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder that clinically affects around 1 in 10,000 people.[1] VWD is mainly characterized by mucocutaneous bleeding, like nose bleeding or menorrhagia.[2] Also, surgical or dental procedures might lead to critical bleeding events. VWD is caused by quantitative or qualitative defects in von Willebrand factor (VWF), a large multimeric glycoprotein produced by endothelial cells and megakaryocytes.[3] VWF primarily functions in hemostasis, where ultra-large VWF strings that are secreted from the endothelial cells attract platelets to sites of vascular damage. Furthermore, in the circulation VWF acts as chaperone for coagulation factor VIII (FVIII), thereby extending the half-life of FVIII.[4] In the past years, also roles of VWF beyond hemostasis have been described. Examples are roles of VWF in inflammation, angiogenesis and wound healing.[5-7]

Treatment of VWD is focused on raising VWF plasma levels, most often on demand after a bleeding event, or prior to a planned surgery or dental procedure. Plasma VWF levels can be raised by administration of desmopressin (DDAVP) or VWF-containing concentrates.[8-11] DDAVP provokes the release of endogenous VWF from the endothelial cells and is the primary choice of treatment in most patients with VWD type 1 and some patients with VWD type 2. DDAVP is, however, contra-indicated in VWD type 2B where released mutant VWF might lead to dangerously deep thrombocytopenia caused by the enhanced binding of mutant VWF to platelets.[12] Also, some patients are unresponsive to DDAVP treatment.[9] Those patients that do not respond to DDAVP or for which DDAVP is contra-indicated, can be treated with VWF-containing concentrates.[11] Although DDAVP or VWF-containing concentrates are usually sufficient to stop or prevent bleeding, they have only short-term effects and they do not cope with the continuous release of mutant VWF. Circulating mutant VWF might cause thrombocytopenia in VWD type 2B that cannot be prevented by administration of VWF-containing concentrates. Furthermore, presence of mutant VWF might affect processes beyond hemostasis in which VWF plays a role. An example is the development of intestinal angiodysplasia (as a result of disturbed angiogenesis) resulting in severe intestinal bleeding. This diathesis is more common among VWD patients than the normal population and is suggested to be caused by long-term exposure to mutant VWF that affects angiogenesis.[13,14] VWD patients with recurrent gastrointestinal bleeding are often treated with repeated dosing of VWF-containing concentrates, which is a burden and does not always solve the gastrointestinal bleeding.[15]

Since most VWD is caused by dominant negative mutations in VWF, we and others previously hypothesized that inhibition of the production of only mutant VWF might overcome the abovementioned shortcomings of the current treatment modalities.[16,17] Inhibition of mutant VWF only might be accomplished by small interfering RNAs (siRNAs) that discriminate between two alleles based on one nucleotide mismatch. We recently published the proof of principle

of this approach in human embryonic kidney (HEK) 293 cells overexpressing *VWF* alleles.[16] We showed that allele-specific siRNAs that target a heterozygous single-nucleotide polymorphism (SNP) located on the same allele as a dominant negative VWD type 2A mutation corrects for the VWD type 2A phenotype.[16] For this approach, various siRNAs have been selected to target four frequent SNPs in *VWF*. It was calculated that 74% of the patient population will be heterozygous for at least one of these four SNPs and thus might be a candidate for this approach of allele-specific *VWF* silencing.

HEK293 cells are a good model to prove the principle of allele-specific *VWF* inhibition and select for efficient and specific siRNA candidates, however HEK293 cells do not endogenously produce VWF. We therefore aim in this study to test the approach of allele-specific *VWF* inhibition by SNP-targeted siRNAs in endothelial colony forming cells (ECFCs, previously called blood outgrowth endothelial cells or BOECs). ECFCs are cultured endothelial cells that can be isolated from peripheral blood.[18,19] We show that siRNAs are able to selectively inhibit *VWF* alleles in healthy control ECFCs and that allele-specific siRNAs can improve the VWD phenotype of ECFCs that were isolated from a VWD type 2A patient with the VWF p.Cys1190Tyr mutation.

Methods

Patients and controls

A VWD type 2A patient with the VWF p.Cys1190Tyr (c.3569G>A) mutation and five healthy control subjects were included in the study. Blood was drawn from all subjects for ECFC, plasma and genomic DNA isolation. The patient's plasma was analyzed for VWF antigen (VWF:Ag), VWF activity and FVIII activity. VWF:Ag in plasma was determined using the STA LIA VWF:Ag test (Stago, Leiden, the Netherlands) and was analyzed on the Sta-R Max analyzer (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, Breda, the Netherlands). Samples were analyzed on the BIO-FLASH (Werfen) and a commercial calibrator (supplied with the HemosIL AcuStar VWF:RCo) was used as reference. The 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. Genomic DNA was used to confirm the mutation in the VWD patient and to determine the genotypes in all subjects for four *VWF* SNPs: rs1800378, rs1063856, rs1063857 and rs1800380. Genotypes were determined using the Taqman SNP genotyping assays (Thermo Fisher Scientific, Carlsbad, CA, USA).

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

SNP phasing

PacBio long read single molecule sequencing was used for linkage analysis of the heterozygous SNP and the p.Cys1190Tyr mutation. RNA was isolated from an ECFC cell pellet from the VWD type 2A patient using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands). Complementary DNA (cDNA) was synthesized using SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientific) with primers designed to span a region of 2.8 kb (Forward primer: CACCTTCAGTGGGATCTGCC; Reverse primer: TTTCAAGACCTCGCTGGTGG). *VWF*-specific products were amplified using the KAPA HiFi HotStart ReadyMix PCR kit (Roche Diagnostics, Mannheim, Germany). Amplicons were barcoded and SMRTbell adapters were added using the SMRTbell Barcoded Adapter Complete Prep Kit (Pacific Biosciences, Menlo Park, CA, USA). Barcoded amplicons were sequenced using a P6-C4 SMRT cell on a Pacific Biosciences RSII sequencer. Error-free circular consensus reads were mapped to the reference, followed by variant calling and resolution of variants phase using WhatsHap suit.

ECFC isolation and culture

ECFCs were isolated as described in Chapter 6 of this thesis. Peripheral blood was drawn in lithium heparin tubes (ECFC C1, C2 and C4; BD Biosciences, Erebodegem, Belgium) or in sodium heparin CPT™ Mononuclear Cell Preparation Tubes (ECFC C3, C5 and 2A; BD Biosciences).

siRNA transfections were performed in ECFCs as described in Chapter 6 of this thesis with Custom Silencer Select siRNAs (Ambion, Life Technologies, Bleiswijk, the Netherlands), an siRNA against *VWF* (siVWF, s14834, Life Technologies), or a negative control siRNA (siNEG, 4404020, Life Technologies). siRNAs were transfected into ECFCs in a concentration of 20 nM, unless otherwise stated. RNA lysates were generated 48 hours after transfection. Conditioned medium and protein lysates were harvested six days after transfection, and 24 hours after refreshing the medium. Protein lysates were generated by an overnight incubation of ECFCs in Optimem1 (Thermo Fisher Scientific), 0.1% triton X-100 (Sigma-Aldrich) and a tablet of cOmplete protease inhibitor cocktail with EDTA (Roche Diagnostics)

VWF protein analysis

VWF:Ag levels in conditioned medium and protein lysates were measured by ELISA as described before[16], with the modification that samples were diluted in phosphate-buffered

saline containing 0.1% tween (PBS-tween), or in PBS containing 1% BSA when the VWF:Ag ELISA was performed simultaneous with the VWF collagen binding (VWF:CB) assay. The VWF:CB assay is performed as described in Chapter 6 of this thesis.

Quantification of unprocessed proVWF in protein lysates was performed by a sandwich ELISA. ELISA plates were coated with rabbit anti-VWF (Dako) in 100 mM bicarbonate, 500 mM NaCl at pH 9.0. ELISA plates were incubated with protein lysates diluted in PBS-tween. Mouse anti-VWFpp conjugated to horseradish peroxidase (CLB-Pro 14.3, Sanquin, Amsterdam, the Netherlands) was used as detection antibody and ELISA plates were incubated with detection antibody diluted in PBS-tween. OPD was used as substrate and dissolved in substrate buffer (22 mM citric acid, 51 mM phosphate, pH 5.0) with addition of 11 µl 30% H_2O_2. The enzymatic reaction was followed kinetically for 5 minutes. The ratio of unprocessed VWF over the total VWF:Ag levels was used as a measure for the processing of VWF. The average of control samples was set to one.

VWF multimers were visualized using agarose gel electrophoresis under non-reducing conditions as described before.[16] VWF multimers were visualized using ECL Western Blotting Substrate (Promega, Madison, WI, USA).

VWF monomers were visualized by western blot under reducing conditions. Protein lysates were reduced using 50 mM NuPAGE™ Sample Reducing Agent (Thermo Fisher Scientific) and proteins were separated on a 4-12% Bis-Tris gel (Thermo Fisher Scientific). Proteins were transferred to a PVDF membrane (BioRad, Veenendaal, the Netherlands) using a Trans-Blot Turbo Transfer System (BioRad, 1.5A, 15 minutes). Rabbit anti-VWF conjugated to horseradish peroxidase (DAKO, P0226) was used as detection antibody. The membrane was incubated with detection antibody diluted in PBS-tween containing 5% non-fat milk (Nutricia, Zoetermeer, the Netherlands). ECL Western Blotting Substrate was used to visualize VWF. Densitometry images were generated and quantified by ImageJ (ImageJ 1.51h, Bethesda, MD, USA).

RNA analysis

RNA isolation, cDNA synthesis and qPCR analysis with *GAPDH* as endogenous reference gene were performed as described before.[19] Quantitative PCR (qPCR) was performed to quantify *VWF*, but also separate *VWF* alleles. Allele-specific qPCR primers were designed, containing the SNPs on the second last position of the forward primer. Primer sequences and annealing temperatures are shown in Table S1.

Immunofluorescent staining

ECFCs were fixated using ice-cold methanol and stained as described before.[19] Rabbit anti-VWF (DAKO) and mouse anti-protein disulphide isomerase (PDI, Stressgen biotechnologies, San diego, CA, USA) were used as primary antibodies.

Statistical analysis

Graphical images and statistical analysis were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). Mann-Whitney *U* tests were performed to determine significance between specific and aspecific inhibition. One-way ANOVA with Dunnett's multiple comparisons test was used to determine significance between three or four groups. $P < 0.05$ was considered significant.

Results

Time course of VWF inhibition by siRNAs in ECFCs

siRNAs are used in this study to inhibit *VWF* (alleles) in ECFCs. To assess the moment of strongest VWF inhibition by siRNAs on protein level, but also the duration of VWF inhibition, we transfected ECFCs with siVWF or siNEG and followed the VWF secreted in 24 hours for 28 days. Strongest VWF inhibition was observed in conditioned medium, six days after transfection of siVWF (Fig. 1). Furthermore, only after 28 days the VWF levels in siVWF transfected ECFCs were at the same level as ECFCs transfected with siNEG (Fig. 1). Since the strongest effects were observed six days after transfection, we decided to consistently measure the effects of the siRNAs on protein level, six days after transfection.

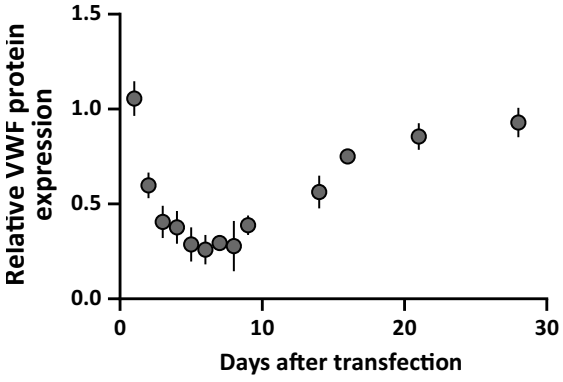

Figure 1. Time course of VWF inhibition by siRNAs in ECFCs. ECFC C3 was transfected with siVWF or siNEG at a concentration of 10 nM. 24 hour medium was taken at the time points indicated in the figure. Shown are the VWF:Ag levels measured in cells transfected with siVWF, normalized to the VWF:Ag levels measured in cells transfected with siNEG. Lowest relative VWF:Ag protein expression was observed six days after transfection. At this day the relative VWF:Ag expression was 26 percent. 28 days after siRNA transfection, the relative VWF:Ag expression was 93 percent and almost back to baseline. The experiment under these conditions was performed three times in duplicate. For some time points, a sample was only taken once. Similar results were observed with different siRNA concentrations (data not shown). ECFC, endothelial colony forming cell; nM, nanomolar; siNEG, negative control siRNA; siVWF, siRNA against *VWF*; VWF, von Willebrand factor; VWF:Ag, VWF antigen

Allele-specific siRNA inhibition in healthy control ECFCs

We previously tested the efficiency and specificity of allele-specific siRNAs that target four common *VWF* SNPs in VWF overexpressing HEK293 cells.[16] For the current study, we selected the most effective allele-specific siRNA per SNP-target from the previous study. To evaluate the efficiency and specificity of the selected allele-specific siRNAs on a protein level in ECFCs, siRNAs were transfected in ECFCs that were homozygous for specific SNPs (ECFCs used per SNP-target are shown in Table 1). For example, an siRNA that is designed to target *VWF* c.1451G (si1451G) was tested for its efficiency in ECFC C1, an ECFC line that is homozygous for *VWF* c.1451G (Fig. 2A). si1451G was tested for its specificity in ECFC C3, an ECFC line that is homozygous for *VWF* c.1451A (Fig. 2A). The same ECFCs were used to assess the efficiency and specificity of si1451A. For all siRNAs tested, no or only minor reduction of the production of the untargeted VWF allele was observed as is shown by the relative VWF expression in ECFCs that did not harbor the SNP variant corresponding to the transfected siRNA (Fig. 2B, untargeted allele). On the other hand, a strong VWF knockdown of the targeted allele was observed in ECFCs that contained the SNP variant corresponding to the transfected siRNA (Fig. 2B, targeted allele). Knockdown was especially strong and efficient for si1451A and si1451G.

Table 1. Genotypes of subjects of ECFCs included in this study. The grey cells indicate the SNPs that were targeted per ECFC line

	rs1800378		**rs1063856**		**rs1063857**		**rs1800380**	
	c.1451A|G		**c.2365A|G**		**c.2385T|C**		**c.2880G|A**	
ECFC C1	G	G	A	A	T	T	G	G
ECFC C2	G	A	G	G	C	C	G	A
ECFC C3	A	A	A	A	T	T	G	G
ECFC C4	G	G	G	G	C	C	A	A
ECFC C5	G	A	G	A	T	C	G	A
ECFC 2A	G	A	A	A	T	T	G	G

ECFC, endothelial colony forming cell

The allele-specific siRNAs have been designed with the aim to target heterozygous SNPs located on the same allele as a dominant negative mutation and thereby ultimately correct for a disease phenotype. The ability of the selected siRNAs to inhibit *VWF* alleles in heterozygous ECFCs could, however, only be assessed on RNA level. We therefore tested the allele-specific siRNAs in an ECFC line that is heterozygous for all four SNPs (ECFC C5) and performed allele-specific qPCR to determine the relative RNA expression of either of the *VWF* alleles (Fig. 2C). On RNA level, a stronger overall knockdown of *VWF* was observed compared to what was observed on protein level, both for the targeted and untargeted allele (Fig. 2D). However, for most siRNAs clear specificity for its targeted allele remained. Only si2365A and si2365G showed minor specificity for its targeted allele. This is in line with results observed in HEK293 cells.[16]

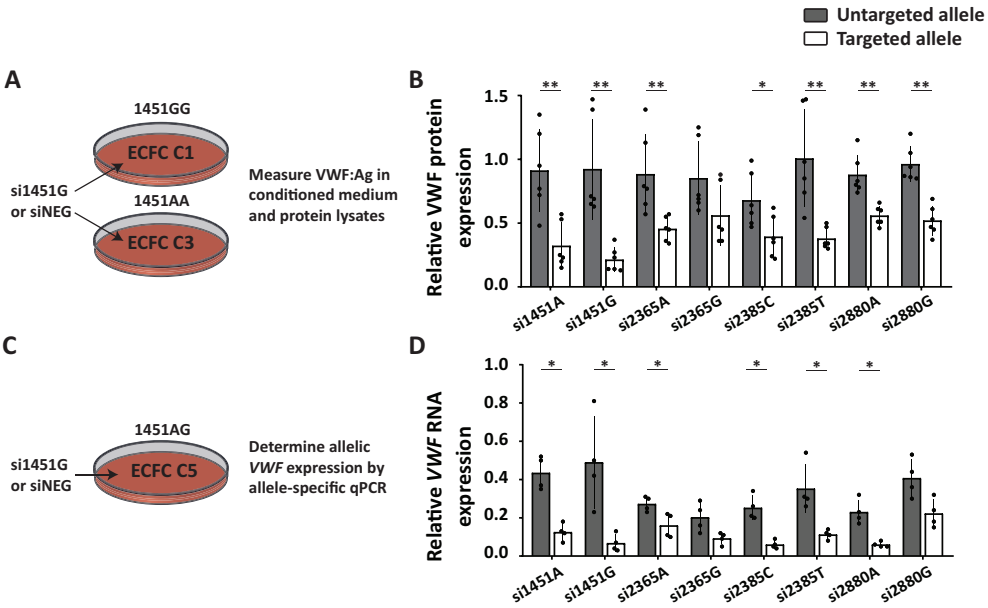

Figure 2. Allele-specific inhibition of VWF in healthy control ECFCs. (A) Experimental set-up of siRNA transfections in ECFCs that were homozygous for specific SNPs to test the efficiency and specificity of allele-specific siRNAs on protein level. As an example, si1451G or siNEG were transfected in ECFC C1 (homozygous for *VWF* c.1451G) and in ECFC C3 (homozygous for *VWF* c.1451A). The efficiency of si1451G is determined by the degree of VWF inhibition in ECFC C1. The specificity of si1451G is determined by the degree of VWF inhibition in ECFC C3. VWF:Ag levels were measured in 24 hour conditioned medium and protein lysates harvested six days after transfection. (B) Relative VWF protein expression of the untargeted (grey bars) and targeted allele (white bars) of ECFCs transfected with allele-specific siRNAs. si1451A and si1451G were tested for their efficiency and specificity in ECFC C1 and ECFC C3. si2365A, si2365G, si2385C and si2385T were tested for their efficiency and specificity in ECFC C2 and ECFC C3. si2880A and si2880G were tested for their efficiency and specificity in ECFC C1 and ECFC C4. Shown are the total VWF:Ag levels (conditioned medium + protein lysates) measured in ECFCs transfected with specific siRNAs, normalized to the total VWF:Ag levels measured in the same ECFCs transfected with siNEG. Shown are the mean ± 1 SD of three independent experiments performed in duplicate. Mann-Whitney, * $P < 0.05$, ** $P < 0.01$ (C) Experimental set-up of siRNA transfections in ECFC C5 (heterozygous for all four *VWF* SNPs), to test the efficiency and specificity of allele-specific siRNAs on RNA level. As an example, si1451G or siNEG were transfected in ECFC C5. RNA lysates were generated 48 hours after transfection and the allelic VWF expression was determined by allele-specific qPCR. (D) Relative *VWF* RNA expression of the untargeted (grey bars) and targeted allele (white bars) of ECFC C5 transfected with allele-specific siRNAs. Shown are the RNA expression levels of *VWF* alleles of ECFC C5 transfected with specific siRNAs, normalized to the expression level of the same allele measured in ECFC C5 transfected with siNEG. Shown are the mean ± 1 SD of two independent experiments performed in duplicate. Mann-Whitney, * $P < 0.05$. ECFC, endothelial colony forming cell; nM, nanomolar; qPCR, quantitative PCR; siNEG, negative control siRNA; siVWF, siRNA against *VWF*; VWF, von Willebrand factor; VWF:Ag, VWF antigen

Correction of a VWD type 2A multimerization phenotype by an allele-specific siRNA

After proving that allele-specific siRNAs can discriminate between *VWF* alleles in healthy control ECFCs, we investigated whether allele-specific VWF inhibition could also correct a VWD phenotype in ECFCs. ECFCs from a VWD type 2A patient with the VWF p.Cys1190Tyr mutation were successfully isolated (ECFC 2A). This patient is clinically characterized by

normal VWF:Ag levels (1.07 IU/mL) and FVIII activity (0.78 IU/mL), but with a reduced VWF activity (0.27 IU/mL). Also, reduced high molecular weight (HMW) VWF is observed in the patient's plasma. Heterozygous SNPs are essential for allele-specific inhibition of the mutant allele, and genotyping of genomic DNA revealed that the patient is heterozygous for one of the four selected SNPs, *VWF* c.1451A|G. PacBio long read sequencing was performed to identify the phasing of this SNP, and showed that *VWF* c.1451A was located on the same allele as the dominant negative mutation p.Cys1190Tyr (c.3569A) (Fig. 3A). Therefore, ECFCs should be transfected with si1451A to inhibit the mutant allele and correct for the VWD type 2A phenotype. On the other hand, treatment of ECFC 2A with si1451G should reduce expression of the wild type allele and is expected to deteriorate the phenotype. Treatment of ECFC 2A with either si1451A or si1451G resulted in allele-specific *VWF* inhibition, as is shown by the allelic *VWF* expression in Fig. 3B. The VWF p.Cys1190Tyr mutation is associated with defective plasma multimers, and a defective intracellular multimerization defect was demonstrated for another mutation at the same amino acid (VWF p.Cys1190Arg).[20,21] We first subjected protein lysates and conditioned medium to the VWF:CB assay, an assay that is able to detect VWF multimerization defects.[22] As expected, we observed both in protein lysates and in conditioned medium of ECFC 2A a lower VWF:CB/VWF:Ag ratio compared to samples derived from healthy control ECFCs (VWF:CB/VWF:Ag in protein lysates of ECFC 2A 0.70 (± 0.14) *versus* 0.98 (± 0.15) in healthy control ECFCs, and in conditioned medium of ECFC 2A 0.64 (± 0.04) *versus* 0.89 (± 0.16) in healthy control ECFCs; Fig. 3Ci and 3Di) This VWF:CB defect was almost corrected in protein lysates of ECFC 2A transfected with si1451A. Contrarily, inhibition of expression of the wild type allele by si1451G clearly worsened the VWF:CB phenotype (Fig. 3Ci). Remarkably, in conditioned medium of ECFC 2A transfected with si1451A, no correction of the VWF:CB defect was observed. Nevertheless, inhibition of the wild type allele by si1451G did result in significantly lower VWF:CB/VWF:Ag as compared to siNEG transfected ECFC 2A (Fig. 3Di). We wondered why inhibition of mutant VWF did not result in correction of VWF:CB/VWF:Ag in conditioned medium as it did in the protein lysates. We hypothesized that downregulation of VWF in general leads to reduced VWF:CB/VWF:Ag in conditioned medium. To assess this hypothesis, VWF:CB/VWF:Ag was determined in conditioned medium and protein lysates of ECFC C1, C2 and C3 transfected with allele-specific siRNAs, siNEG and siVWF. We observed in conditioned medium, but not in protein lysates, that inhibition of the production of VWF by (allele-specific) siRNAs resulted in a gradual decrease of VWF:CB/VWF:Ag (N = 6 for each siRNA in control ECFCs; Fig. 3Cii and 3Dii). When the VWF:CB/VWF:Ag of ECFC 2A transfected with siNEG, si1451A or si1451G were plotted against the VWF:CB/VWF:Ag ratios of healthy control ECFCs, we observed that the VWF:CB/VWF:Ag of ECFC 2A transfected with si1451A (inhibition of mutant allele) shifted towards the reference line of the healthy control ECFCs (N = 9 for ECFC 2A). Whereas inhibition of the normal allele by si1451G resulted in further deterioration of the VWF:CB/VWF:Ag ratio (in conditioned medium as well as in protein lysates, Fig. 3Cii and 3Dii).

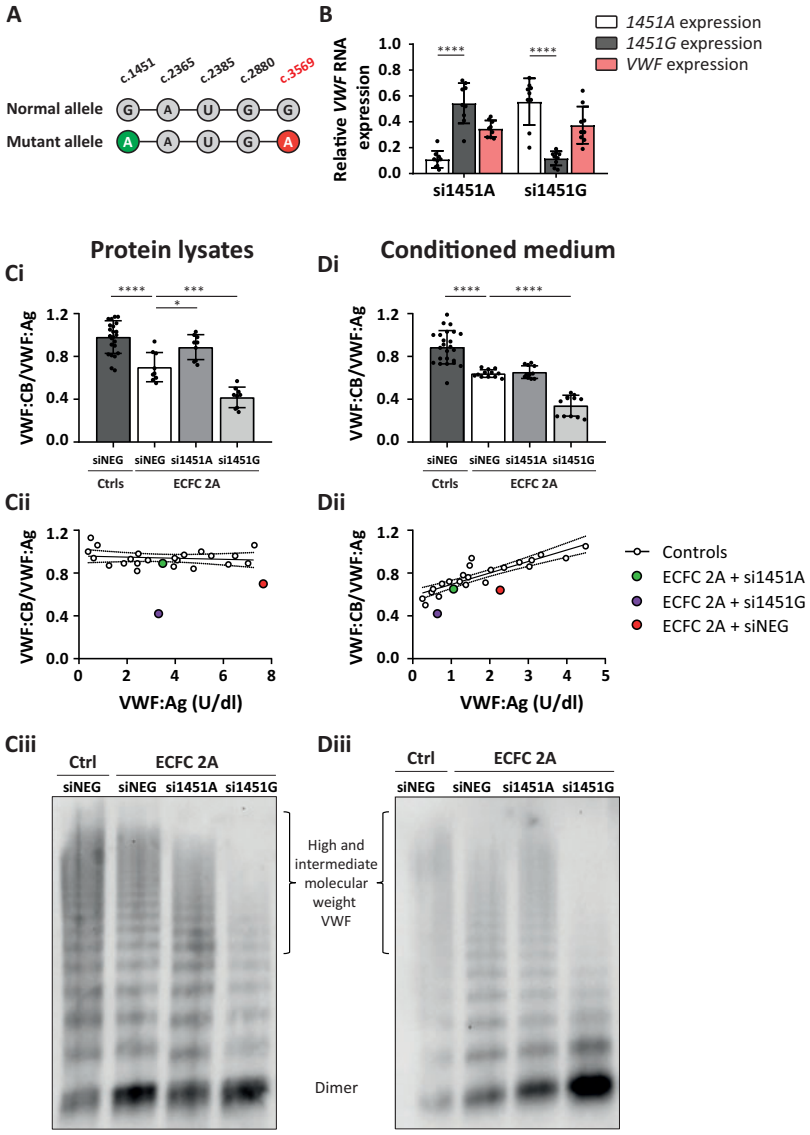

Figure 3. Correction of the VWF multimerization defect in ECFC 2A. (A) Phasing of the heterozygous SNP, *VWF* c.1451A|G, with the dominant negative mutation VWF p.Cys1190Tyr (c.3569A). PacBio sequencing revealed that *VWF* c.1451A is located on the same allele as *VWF* c.3569A. (B) Relative *VWF* RNA expression of ECFC 2A transfected with 20 nM si1451A or si1451G. Shown are the RNA expression levels of *VWF* alleles c.1451A and c.1451G as well as the total *VWF* expression. Expression levels were determined in RNA lysates of ECFC 2A transfected with si1451A or si1451G, normalized to the expression level of the same allele measured in ECFCs transfected with siNEG. Shown are the mean ± 1 SD of three independent experiments performed in triplicate. Mann-Whitney (c.1451A *versus* c.1451G expression), **** $P < 0.0001$. VWF:CB/VWF:Ag determined in (Ci) protein lysates and (Di) conditioned medium of ECFC C1, C2 and C3, transfected with siNEG and ECFC 2A transfected with siNEG, si1451A and si1451G. Shown are the mean ± 1 SD of three independent experiments performed in triplicate for ECFC 2A and the mean ± 1 SD of three independent experiments performed in duplicate for ECFC C1, C2 and C3. One-way ANOVA, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. Normal reference line of VWF:CB/VWF:Ag

plotted against the VWF:Ag levels measured in (Cii) protein lysates and (Dii) conditioned medium of ECFC C1, C2 and C3 transfected with siNEG, siVWF and various allele-specific siRNAs. Every white dot represents the average of three independent experiments performed in duplicate of an ECFC line transfected with a specific siRNA. Included in the graphs are the average of VWF:CB/VWF:Ag of three experiments performed in triplicate in which ECFC 2A was transfected with si1451A (green), si1451G (purple) or siNEG (red). VWF multimerization analysis of (Ciii) protein lysates and (Diii) conditioned medium harvested from a healthy control ECFC line transfected with siNEG and ECFC 2A transfected with si1451A, si1451G and siNEG. ECFC, endothelial colony forming cell; qPCR, quantitative PCR; siNEG, negative control siRNA; siVWF, siRNA against *VWF*; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:CB, VWF collagen binding

Although multimerization defects can be detected with the VWF:CB ELISA, the assay gives no information on the exact multimerization pattern. To assess the multimerization pattern, conditioned medium and protein lysate samples were subjected to the VWF multimer analysis. In conditioned medium, a slight decrease of HMW VWF was observed in the patient-derived ECFCs compared to control ECFCs (Fig. 3Diii). Inhibition of production of mutant VWF resulted in a slight increase in HMW VWF. Inhibition of production of wild type VWF clearly worsened the multimerization pattern in conditioned medium (Fig. 3Diii). No clear decrease of HMW VWF was observed in protein lysates of ECFC 2A compared to protein lysates of healthy control ECFCs. However, a clear increase in the intensity of the dimer band was apparent (Fig. 3Ciii). Also, a slight change in running pattern is visible, i.e. VWF from ECFC 2A seem to migrate a bit slower than VWF from the control ECFC. When the mutant allele was inhibited by si1451A, the intensity of the dimer band clearly decreased, however this coincided with a small decrease in HMW VWF. Furthermore, VWF migration shifted towards the pattern of the control ECFC. When the wild type allele was inhibited by si1451G, the multimerization pattern deteriorated with a decrease in HMW VWF and an increase in the intensity of the dimer band.

Improved VWF processing after allele-specific inhibition of mutant VWF in ECFC 2A

The multimerization pattern of protein lysates of ECFC 2A showed an increase in the intensity of the dimer band. This suggests that the processing of VWF into VWF multimers is affected. Dimerization of VWF takes place in the endoplasmic reticulum (ER).[23] To test whether VWF might be retained in the ER, we performed a costaining of VWF and the ER marker PDI. Indeed, a clear overlap of VWF and PDI staining is observed in ECFC 2A, indicating ER retention (Fig. 4A, ECFC 2A + siNEG, second column). This was not observed in control ECFCs (Fig. 4A, control, first column). When the mutant allele was inhibited by si1451A, retention of VWF in the ER was clearly decreased (best observed in greyscale image) and many cells showed no ER retention at all (Fig. 4A, ECFC 2A + si1451A, third column). Inhibition of the wild type allele by si1451G resulted in a rather severe cellular phenotype, with increased retention of VWF in the ER suggesting increase in severity of the cellular phenotype (Fig. 4A, ECFC 2A + si1451G, fourth column).

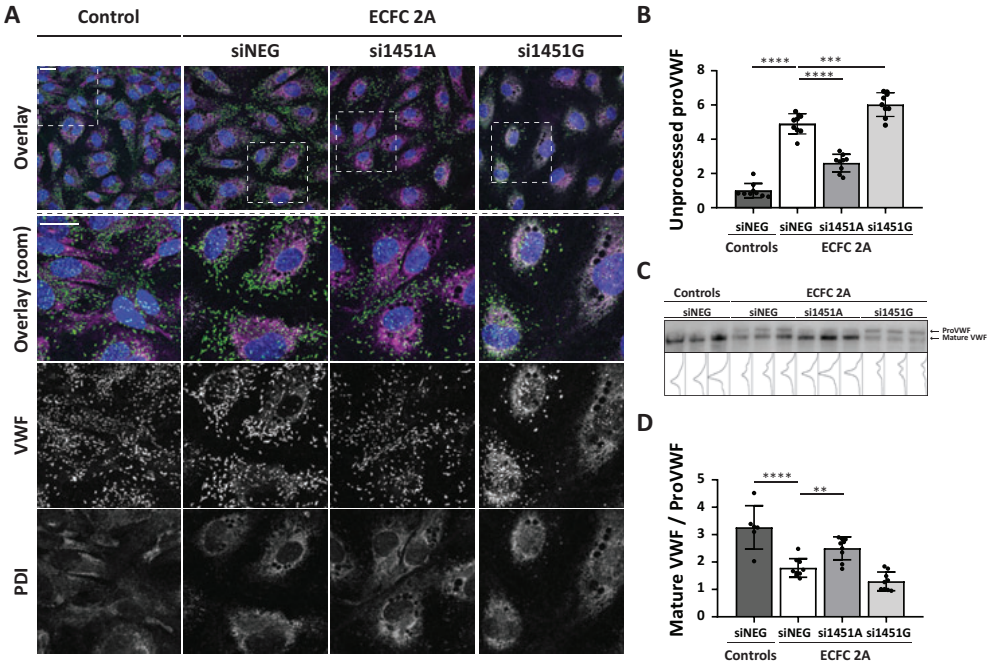

Figure 4. Correction of the VWF processing defect in ECFC 2A. (A) Confocal images of an untreated healthy control ECFC line and ECFC 2A transfected with siNEG, si1451A and si1451G. ECFCs were stained for VWF, PDI and nuclei. The upper row shows an overview image with VWF (green), PDI (magenta) and nuclei (blue). The second row shows a zoom-in of the upper image. Colocalization between VWF and PDI is shown in grey. The third and fourth rows show greyscale images of VWF and PDI staining, respectively. Scale bar represents 20 µm. (B) Quantification of unprocessed proVWF measured by ELISA in protein lysates of control ECFCs transfected with siNEG or ECFC 2A transfected with siNEG, si1451A or si1451G. Shown are the mean ± 1 SD of three independent experiments performed in triplicate for ECFC 2A and the mean ± 1 SD of nine randomly selected protein samples of ECFC C1, C2 and C3. The average of the control ECFCs is set to one. One-way ANOVA, *** $P < 0.001$, **** $P < 0.0001$. (C) Western blot of protein lysates of control ECFCs transfected with siNEG or ECFC 2A transfected with siNEG, si1451A or si1451G. Protein lysates were run under reduced conditions on a 4-12% Bis-Tris gel. Shown are protein lysates of ECFC C1, C2 and C3 transfected with siNEG and the three samples of a triplicate experiment for ECFC 2A transfected with siNEG, si1451A and si1451G. (D) Quantification of the western blot shown in panel C. Shown is the mean ± 1 SD of quantified western blots performed on protein lysates of three independent experiments performed in triplicate for ECFC 2A and the mean ± 1 SD of six protein lysate samples of ECFC C1, C2 and C3. One-way ANOVA, ** $P < 0.01$, **** $P < 0.0001$. ECFC, endothelial colony forming cell; PDI, protein disulphide isomerase; siNEG, negative control siRNA; siVWF, siRNA against *VWF*; VWF, von Willebrand factor; VWF:Ag, VWF antigen; VWF:CB, VWF collagen binding

VWF is produced as proVWF that is dimerized in the ER. After dimerization of proVWF in the ER, VWF is translocated to the Golgi where the propeptide is cleaved from VWF by furin.[24] Increased ER retention of VWF results in an increased amount of unprocessed proVWF in the cells. This can be quantified by an ELISA that measures the amount of propeptide that is still bound to VWF. Protein lysates of ECFC 2A showed a 4.9 fold higher level of unprocessed VWF compared to protein lysates of control ECFCs. This was decreased 1.9 fold after inhibition of production of the mutant allele by si1451A (Fig. 4B). Compared to control ECFCs, 6 fold

more unprocessed VWF was present in ECFC 2A in which the wild type allele was inhibited by si1451G (Fig. 4B).

A western blot in which the samples are run under reducing conditions can be used as an alternative method to identify defects in the processing of VWF. In this western blot, an increase in the intensity of the unprocessed proVWF band was detected in protein lysates of ECFC 2A compared to control ECFCs (Fig. 4C). The intensity of the unprocessed proVWF band decreased after inhibition of the mutant allele by si1451A. An increase in the intensity of the unprocessed proVWF band was observed when the wild type allele was inhibited by si1451G (Fig. 4C). Quantification of the western blot confirms the defective processing of VWF and correction of the phenotype after transfection of si1451A that inhibited expression of the mutant allele (Fig. 4D).

Discussion

Allele-specific siRNAs may be used to selectively inhibit mutant alleles to improve disease phenotypes. Previously, we have shown that allele-specific siRNAs that distinguish two *VWF* alleles based on one nucleotide mismatch of SNPs can specifically inhibit *VWF* alleles in HEK293 cells, and that these siRNAs can improve a VWD phenotype. Here, we aimed to extend the proof of concept of allele-specific VWF inhibition to an *ex vivo* setting by the use of patient-derived and healthy control ECFCs. In this study, we prove that allele-specific siRNAs can inhibit single *VWF* alleles based on one nucleotide mismatch *ex vivo* in healthy control ECFCs. This was shown both on protein and RNA level. Also, inhibition of the mutant allele in ECFCs isolated from a VWD type 2A patient resulted in clear improvements in the patient's cellular phenotype.

ECFCs are used in this study as *ex vivo* cell model for VWD. ECFCs are the only source of proliferative endothelial cells that can be isolated from patients by a peripheral blood venepuncture.[25] The use of ECFCs has the advantage that the cells are proliferative, have generally a high VWF production and are easy to transfect.[26] However, also clear variations between various ECFC cell lines emerges.[19] Most important, clear variations in VWF expression exists between ECFC lines and it was therefore suggested to be cautious when comparing different ECFC lines. When allele-specific siRNAs are tested in (patient-derived) ECFCs, the effects are determined in ECFCs transfected with either an allele-specific siRNA or a negative control siRNA. Cells are therefore used as their own internal control, and do not have to be compared to control ECFCs. To assess the efficiency and specificity of the allele-specific siRNAs on a protein level, we had no choice but to compare different healthy control ECFC lines (Fig. 2A). And although the ECFC lines in this study were carefully selected based on their proliferative state and VWF production, differences between ECFC lines could not be avoided.

This was also reflected in variations in the efficiency of siVWF to inhibit VWF production in ECFCs. For example, we reproducibly observed a stronger relative VWF inhibition by siVWF in ECFC C1 compared to ECFC C2 (data not shown). No firm conclusions can therefore be drawn on the efficiency and specificity of the allele-specific siRNAs on protein level that have been tested in the different healthy control ECFC lines (Fig. 2B).

SNPs in *VWF* with a high minor allele frequency have been used in this study as target to inhibit *VWF* alleles. This approach was chosen since with only a small number of SNPs, a large percentage of the patient population can be reached. It was for example calculated that 74% of the Caucasian population is heterozygous for at least one of the four SNPs used in this study.[16] From this study it became evident that especially si1451A and si1451G have high potency as allele-specific siRNAs, and both siRNAs proved, depending on the allele targeted, successful to, respectively correct or deteriorate a VWD type 2A phenotype *ex vivo*. Whether the other siRNAs have the same ability to correct for VWD phenotypes, remains to be unanswered. It is however highly unlikely that si2365A or si2365G will have similar effects as si1451A or si1451G. Fortunately, exclusion of si2365A and si2365G does not reduce the fraction of the patient population that can be reached, since *VWF* c.2365A|G is in almost complete linkage disequilibrium with *VWF* c.2385C|T.[27] Furthermore, the efficacy of the allele-specific siRNAs in this study may still be improved by for example alternating the chemical modification of the siRNAs.[28] Also, more SNP-targets could be tested to increase the percentage of the targeted patient population.

The ability of the allele-specific siRNAs to correct a VWD phenotype was tested in ECFCs isolated from a VWD type 2A patient with the VWF p.Cys1190Tyr mutation. This mutation is characterized by a clearly defined laboratory phenotype with reduced HMW multimers and reduced VWF collagen binding.[20] Inhibition of the mutant allele by si1451A resulted in clear improvements in the processing of VWF and the VWF:CB/VWF:Ag in protein lysates. Remarkably, no increase of VWF:CB/VWF:Ag was observed in conditioned medium of ECFC 2A in which expression of the mutant allele was inhibited by si1451A (Fig. 3Di). It appeared, however, that also a reduced VWF:CB/VWF:Ag was observed in healthy control ECFCs transfected with siRNAs that reduced the overall expression of VWF (Fig. 3Dii). When the VWF:CB/VWF:Ag of ECFC 2A transfected with si1451A was plotted against the normal reference line, the VWF:CB/VWF:Ag was almost normalized. Also, the multimerization pattern of VWF in conditioned medium showed an increase in HMW VWF in ECFCs transfected with si1451A compared to siNEG transfected cells. The reason for discrepancy between de VWF:CB assay and the VWF multimer analysis remains uncertain. It might be possible that the VWF:CB is sensitive for altered ratios between HMW and low molecular weight VWF. Further *in vivo* studies should reveal whether reduced VWF:CB after downregulation of VWF is also apparent *in vivo* and what the potential consequences of this effect are.

The use of siRNAs as a therapeutic application requires persistence of the siRNA activity over a longer period of time. Clinical trials with siRNAs that target liver-expressed genes showed persistent siRNA-mediated downregulation of several genes for more than a month.[29-31] In ECFCs, downregulation of VWF was observed for up to 28 days, even though the cells were still proliferating in the first week after transfection. Furthermore, siRNA-mediated inhibition of *Icam2* and *Tie2* in mice by siRNAs that were complexed in polymeric nanoparticles or a cationic lipoplex delivery system, respectively, resulted in persistent, and around 80 percent, *Icam2* and *Tei2* inhibition for more than 21 days after a single dose.[32,33] These results of long-term gene inhibition are promising for further developments of siRNA therapeutics for endothelial genes, like *VWF*. For VWD patients, long-term correction of VWF may especially be beneficial for selected patients with a severe bleeding phenotype that currently require repeated dosing with VWF-containing concentrates.

To conclude, we show that allele-specific siRNAs are effective in the inhibition of single *VWF* alleles in ECFCs. Inhibition of expression of the mutant allele in ECFCs isolated from a VWD type 2A patient with the VWF p.Cys1190Tyr mutation resulted in clear improvements of the cellular phenotype. Further studies in VWD mouse models are needed to translate the positive *ex vivo* results to an *in vivo* model, and to show whether the allele-specific siRNAs are able to correct a bleeding phenotype.

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