Personalized treatment for von Willebrand disease by RNA-targeted therapies

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Correction of a dominant-negative von Willebrand factor multimerization defect by small interfering RNA-mediated allele-specific inhibition of mutant von Willebrand factor

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Abstract

Background Treatment of the bleeding disorder von Willebrand disease (VWD) focuses on increasing von Willebrand factor (VWF) levels by administration of desmopressin or VWF-containing concentrates. Both therapies leave the production of mutant VWF unhindered, which may have additional consequences, such as thrombocytopenia in patients with VWD type 2B, competition between mutant and normal VWF for platelet receptors, and the potential development of intestinal angiodysplasia. Most cases of VWD are caused by dominant negative mutations in VWF, and we hypothesize that diminishing expression of mutant VWF positively affects VWD phenotypes. ***Objectives*** To investigate allele-specific inhibition of *VWF* by applying small interfering RNAs (siRNAs) targeting common single-nucleotide polymorphisms (SNPs) in *VWF*. This approach allows allele-specific knockdown irrespective of the mutations causing VWD. ***Methods*** Four SNPs with a high predicted heterozygosity within *VWF* were selected, and siRNAs were designed against both alleles of the four SNPs. siRNA efficiency, allele specificity and siRNA-mediated phenotypic improvements were determined in VWF-expressing HEK293 cells. ***Results*** Twelve siRNAs were able to efficiently inhibit single *VWF* alleles in HEK293 cells that stably produce VWF. Transient cotransfections of these siRNAs with two *VWF* alleles resulted in a clear preference for the targeted allele over the untargeted allele for 11 siRNAs. We also demonstrated siRNA-mediated phenotypic improvement of the VWF multimerization pattern of the VWD type 2A mutation VWF p.Cys2773Ser. ***Conclusions*** Allele-specific siRNAs are able to distinguish *VWF* alleles on the basis of one nucleotide variation, and are able to improve a severe multimerization defect caused by VWF p.Cys2773Ser. This holds promise for the therapeutic application of allele-specific siRNAs in dominant negative VWD.

Introduction

von Willebrand disease (VWD) is the most common inherited bleeding disorder caused by defects in von Willebrand factor (VWF), a large multimeric glycoprotein produced by endothelial cells and megakaryocytes. VWF is an important hemostatic protein with two main functions: adhesion and aggregation of platelets at sites of vascular damage, and protection of coagulation factor VIII from degradation in the bloodstream.[1] More than 90% of VWD cases are caused by dominant negative VWF mutations.[2] These dominant negative mutations can lead to either quantitative (VWD type 1) or qualitative (VWD types 2A, B, and M) defects in secreted VWF.[2,3]

VWD mainly leads to mucocutaneous or postoperative bleeding, and patients are generally treated on demand upon a bleeding event.[4] The mainstay of treatment is to increase circulating VWF levels by the administration of desmopressin (DDAVP) or VWF-containing concentrates.[4] Administration of DDAVP is the primary choice of treatment, and induces the release of VWF from its endothelial storage organelles, the Weibel-Palade bodies.[5] When DDAVP does not result in the secretion of VWF, or when the secreted VWF is non-functional, DDAVP may be ineffective. The release of mutant VWF may also result in adverse events such as the development of severe thrombocytopenia in patients with VWD type 2B, owing to spontaneous binding of mutant VWF to platelets. When DDAVP is ineffective or contraindicated, the preferred treatment is administration of VWF-containing concentrates.[6,7] Although normal exogenous VWF is administered, endogenous mutant VWF is still being produced and secreted, which might interfere with normal hemostasis. Therefore, administration of VWF-containing concentrates may correct the VWF deficiency, but cannot prevent secondary, negative effects caused by mutant VWF.

We hypothesize that reducing mutant VWF production, while preserving the production of normal VWF, has a positive effect on VWF function and VWD phenotypes. We aim to use allele-specific small interfering RNAs (siRNAs) to inhibit mutant *VWF* alleles. The use of allele-specific siRNAs in VWD has been reported before by Casari *et al*., where siRNAs were designed against the breakpoint of the dominant negative partial VWF deletion p.Pro1127_Cys1948delinsArg.[8] This approach may be effective, however, it is only applicable to patients harboring this specific deletion. Therefore, we choose an approach whereby siRNAs are designed against common single-nucleotide polymorphisms (SNPs) in *VWF*. When a patient is heterozygous for the specific SNP, one can target the SNP that is located on the same allele as the dominant negative *VWF* mutation causing VWD. The full complementarity of the siRNA with the targeted heterozygous *VWF* SNP allele will lead to siRNA-mediated degradation of the dominant negative *VWF* allele. The mismatch of the same siRNA with the other SNP allele is thought to maintain expression of the normal *VWF* allele (Fig. S1A). An approach in which siRNAs are designed against dominant negative mutations may be more straightforward, but

will not be feasible in a therapeutic perspective, because the existence of hundreds of VWD-related mutations.[9] Discrimination of *VWF* alleles by SNPs requires only a small set of SNPs with a high minor allele frequency (MAF) to cover a large group of patients with a wide range of *VWF* mutations.

In this study, we show the proof of principle of siRNA-mediated SNP-based allele-specific inhibition of *VWF*. We demonstrate that it is feasible to discriminate with siRNAs between two *VWF* alleles differing by only one nucleotide, and that this discrimination leads to a clear improvement of a dominant negative multimerization defect caused by VWF p.Cys2773Ser.

Methods

Plasmid expression vectors

Recombinant pcDNA3.1/Zeo (+) containing full-length human VWF (hVWF) cDNA was used and modified in this study. pcDNA3.1/Zeo (+) hVWF contained a *VWF* allele with the following nucleotides at the SNP positions: c.1451G (rs1800378), c.2365A (rs1063856), c.2385T (rs1063857), and c.2880G (rs1800380). Five new hVWF plasmids were generated to create opposite *VWF* alleles (c.1451A, c.2365G, c.2385C, c.2880A, and c.2365G/c.2880A). Both these nucleotide variations and VWF p.Cys2773Ser (c.8318C) were introduced into pcDNA3.1/Zeo (+) hVWF by use of the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA). HA and MYC peptide tags were used to distinguish *VWF* alleles at the protein level and at the mRNA level, and introduced at the C-terminal end of VWF by use of the Q5 Site-Directed Mutagenesis Kit. Tables S1 and S2 show an overview of the generated constructs. Primers for the substitutions and insertions were designed with NEBaseChanger (New England Biolabs). Plasmids were purified with the PureYield Plasmid Maxiprep system (Promega, Madison, WI, USA), and correctness of the sequences was verified by Sanger sequencing (BaseClear, Leiden, the Netherlands).

SNP selection and siRNA design and synthesis

Four SNPs with a high MAF in the Caucasian population were selected from the 1000 Genomes Project.[10] On the basis of provided sequences, two or three siRNA oligonucleotides with the highest predicted efficiency and allele specificity were designed for both alleles of the four SNPs (Ambion, Life Technologies Europe BV, Bleiswijk, the Netherlands). Custom Silencer Select 21-mer siRNA oligonucleotides with a dTdT overhang at the 5′ end of the sense strand were synthesized by Life Technologies (Ambion, Life Technologies Europe BV). Ambion Silencer Select Negative Control siRNA was used as a negative control (siNEG).

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells (ATCC, Rockville, MD, USA) were cultured in Minimum Essential Medium Eagle α (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Sigma-Aldrich), and 50 µg/mL gentamicin (GIBCO, Invitrogen). HEK293 cells stably producing VWF were generated by transfection of hVWF plasmids with FuGENE HD transfection reagent (Promega). Cells stably producing VWF were selected by the use of Zeocin Selection Reagent (Life Technologies Europe BV).

For transient transfections, 100,000 cells were seeded on poly-D-lysine (5 mg/mL; Sigma-Aldrich) coated wells of 24 wells plates to reach 50-60% confluence the next day. Cells were transfected with plasmids and/or siRNA by the use of Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA). Each transfection was performed with 600 ng/mL plasmid and/or 0.5, 1, 5, 10 or 20 nM siRNA in 500 µL of culture medium. Twenty-four hours after transfection, medium was refreshed with 500 µL of culture medium. Conditioned medium and cell lysates were harvested 48 hours after transfection, when siRNAs were transfected into cells stably producing VWF, and 72 hours after transfection, when siRNA and VWF plasmids were cotransfected into transiently transfected cells. Cell lysates were generated by overnight incubation of HEK293 cells at 4 °C in 500 µL of Passive Lysis buffer (Promega) supplemented with cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich).

Quantification of VWF protein levels

VWF antigen (VWF:Ag), VWF-HA and VWF-MYC protein levels in conditioned medium and cell lysates were measured by ELISA. For VWF:Ag measurements, ELISA plates (Greiner, Frickenhausen, Germany) were coated overnight with polyclonal antibody rabbit anti-hVWF (A0082; Dako, Glostrup, Denmark) diluted in coating buffer (100 mM bicarbonate, 500 mM NaCl, pH 9.0). Samples were diluted in wash buffer (50 mM triethanolamine, 100 mM NaCl, 10 mM EDTA, 0.1% Tween-20), and incubated for 2 hours. Polyclonal antibody rabbit anti-hVWF coupled to horseradish peroxidase (HRP) (P0226; Dako) was used as the detecting antibody, and diluted in wash buffer. Wells were incubated with secondary antibody for 2 hours. O-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) was used as substrate, and one tablet of OPD was dissolved in 24 mL of substrate buffer (22 mM citric acid, 51 mM phosphate, pH 5.0) plus 12 µL of 30% H_2O_2. Wells were incubated with substrate solution, and the reaction was terminated by the addition of 2 M H_2SO_4 after 15 minutes. Normal pooled plasma (NPP) was used as the reference.

To quantify VWF-HA protein levels, ELISA plates were coated overnight with a monoclonal antibody against hVWF (CLB-RAg35; Sanquin, Amsterdam, the Netherlands).[11] Plates were

blocked for 30 minutes in blocking buffer (phosphate-buffered saline [PBS], 3% bovine serum albumin, and 0.1% Tween-20). Samples were diluted in blocking buffer, and wells were incubated with diluted sample for 1.5 hour. Wells were incubated with rabbit anti-HA monoclonal antibody (Cell Signaling, Leiden, the Netherlands) diluted in blocking buffer for 1.5 hour. Goat anti-rabbit IgG (H+L)-HRP (Bio-Rad, Veenendaal, the Netherlands) diluted in blocking buffer was used as secondary antibody, and wells were incubated with secondary antibody for 1 hour. OPD conversion was performed as described above. Purified recombinant VWF-HA was used as the reference and normalized to VWF:Ag levels in NPP.

To quantify VWF-MYC protein levels, ELISA plates were coated overnight with rabbit anti-hVWF polyclonal antibody (Dako). Samples were diluted in blocking buffer, and plates were incubated with diluted sample for 2 hours. Wells were incubated with the secondary polyclonal antibody rabbit anti-c-Myc-tag-HRP (GenScript, Piscataway, NJ, USA) for 2 hours. OPD conversion was performed as described above, and the reaction was terminated after 10 minutes by the addition of 2 M H_2SO_4. Purified recombinant VWF-MYC was used as the reference, and normalized to VWF:Ag levels in NPP.

RNA isolation and quantification of VWF mRNA

RNA was generated from cell lysates harvested 72 hours after transfection by use of the RNeasy Micro Kit (Qiagen, Hilden, Germany). Plasmid and genomic DNA was removed with the TURBO DNA-free kit (Invitrogen). Complementary DNA was synthesized by the use of SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) with poly(T) primers (Sigma-Aldrich). Quantitative PCR (qPCR) was performed with Sybr Select Master Mix (Thermo Fisher Scientific) in the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). Allele-specific qPCR primers amplifying the MYC or HA nucleotide sequence were used to distinguish the two *VWF* alleles. The glyceraldehyde-3-phosphate dehydrogenase gene was used as the endogenous reference gene, and analyzed within the same qPCR run. The comparative Ct method was used for analysis, with complementary DNA of HEK293 cells transfected with the two *VWF* alleles and siNEG being used as the control.[12]

Immunofluorescent staining

72 hours after transfection, HEK293 cells were fixed with 4% paraformaldehyde (Alfa Aesar, Karlsruhe, Germany) for 20 minutes. Cells were rinsed in PBS, and blocked and permeabilized for 20 minutes in blocking buffer (PBS, 5% normal goat serum [Dako]; 0.02% saponin [Sigma-Aldrich]). Primary antibodies (rabbit anti-HA and mouse anti-MYC; Cell Signaling) were diluted in blocking buffer, and cells were incubated with primary antibody for 45 minutes. Cells were incubated for 30 minutes with the secondary antibodies goat anti-rabbit IgG (H+L)

AF488 and goat anti-mouse IgG (H+L) AF594 (Thermo Fisher Scientific) diluted in blocking buffer. Coverslips were mounted by the use of ProLong Diamond Antifade Mountant (Thermo Fisher Scientific), and cells were visualized with a Leica TCS SP8 upright confocal microscope equipped with a 63x/1.40 numerical aperture Plan Apo oil immersion objective.

VWF multimer analysis

Conditioned medium was collected 48 hours after refreshment of the medium and 72 hours after transfection. Conditioned medium samples were separated under non-reducing conditions on a 1.5% SeaKem HGT(P) agarose (Lonza, Rockland, ME, USA) separation gel containing 0.4% SDS, and visualized by western blotting with rabbit anti-hVWF polyclonal antibody (A0082; Dako) as described previously.[13] Densitometry images were generated with imageJ 1.51h (National Institutes of Health, Bethesda, MD, USA) to quantify high and intermediate molecular weight multimers. Densitometry images were divided into the five smallest bands and the rest (intermediate and large bands). First, the VWF large multimer ratio was determined by dividing the area of intermediate and large VWF multimers over the total area (Fig. S2). Then, the VWF large multimer index was calculated by dividing the VWF large multimer ratio of cells cotransfected with mutant and normal *VWF* with or without siRNA treatment by the VWF large multimer ratio of control cells (Fig. S2). This is a modified version of the VWF large multimer index described by Tamura *et al*.[14] We divided densitometry images into two instead of three segments because less high molecular weight VWF is produced in *in vitro* cell systems.

Statistics

GraphPad prism version 7.00 (GraphPad Software, La Jolla, CA, USA) was used for graphics and statistical analyses. Error bars in the histograms represent one standard deviation. A two-tailed Mann-Whitney *U*-test was used to check for significance in siRNA allele specificity. For all statistical analyses, significance was set at $P < 0.05$.

Results

SNP selection

A set of four SNPs with a high MAF in the coding region of *VWF* was selected from the 1000 Genomes Project.[10] These SNPs include rs1800378 (c.1451G|A), rs1063856 (c.2365A|G), rs1063857 (c.2385T|C), and rs1800380 (c.2880G|A) (Table 1). rs1800378 results in a non-synonymous substitution (p.His484Arg) and has a MAF of 0.35 in the Caucasian population. rs1063856 also results in a non-synonymous substitution (p.Thr789Ala), and is in linkage

disequilibrium with rs1063857, which results in a synonymous substitution (p.Tyr795=). Both SNPs have a MAF of 0.37 in the Caucasian population. rs1800380 results in a synonymous substitution (p.Arg960=) and has a MAF of 0.26 in the Caucasian population. By counting the Caucasian individuals included in 1000 Genomes (N = 503) who are heterozygous for at least one of the four selected SNPs, we determined that 74% of this population are heterozygous for at least one of these SNPs (Table 1).[10]

Table 1. Single-nucleotide polymorphism (SNP) information in Caucasian population

SNP	cDNA location in *VWF*	Minor allele	MAF*	% heterozygous
rs1800378	c.1451G|A	A	0.35	45.3
rs1063856	c.2365A|G	G	0.37	46.5
rs1063857	c.2385T|C	C	0.37	46.5
rs1800380	c.2880G|A	A	0.26	38.1
All 4 SNPs, at least one heterozygous				74.0

* Based on 1000 Genomes.[10] MAF, minor allele frequency; VWF, von Willebrand factor

siRNA efficiency and allele specificity

Two or three siRNAs per *VWF* allele were screened for their efficiency and allele specificity by siRNA transfections in HEK293 cells stably producing VWF generated for all eight *VWF* alleles (Fig. S1B; Table S1). Specific inhibition and non-specific inhibition were determined by VWF:Ag measurements in conditioned medium and cell lysates after transfection of each siRNA in cells expressing the targeted or untargeted *VWF* allele, respectively. We selected at least one siRNA per SNP allele according to their efficiency in inhibiting *VWF* and their ability to distinguish between the two *VWF* alleles (Fig. 1). These siRNAs included: si1451G-1, si1451G-2, si1451A-2, si1451A-3, si2365A-1, si2365G-2, si2385T-1 si2385T-2, si2385C-2, si2880G-3, si2880A-2 and si2880A-3. Although si2365G-2 did not efficiently inhibit the production of VWF, it was the most effective siRNA for this target and was therefore not excluded from further analyses.

In the normal human situation, two *VWF* alleles are coexpressed in the same cell. Competition between the two alleles might improve the allele specificity of the siRNAs. By using a coexpression system in which two VWF plasmids containing either a MYC or an HA peptide tag were cotransfected into HEK293 cells, we were able to distinguish and quantify the expression of the two respective *VWF* alleles. Although the peptide tags are located at the C-terminal end of VWF, they did not interfere with dimerization and multimerization of VWF (Fig. S3A). Staining of HEK293 cells cotransfected with two VWF plasmids showed coexpression of both conjugated proteins in most cells, confirming real coexpression in the system (Fig. S3B).

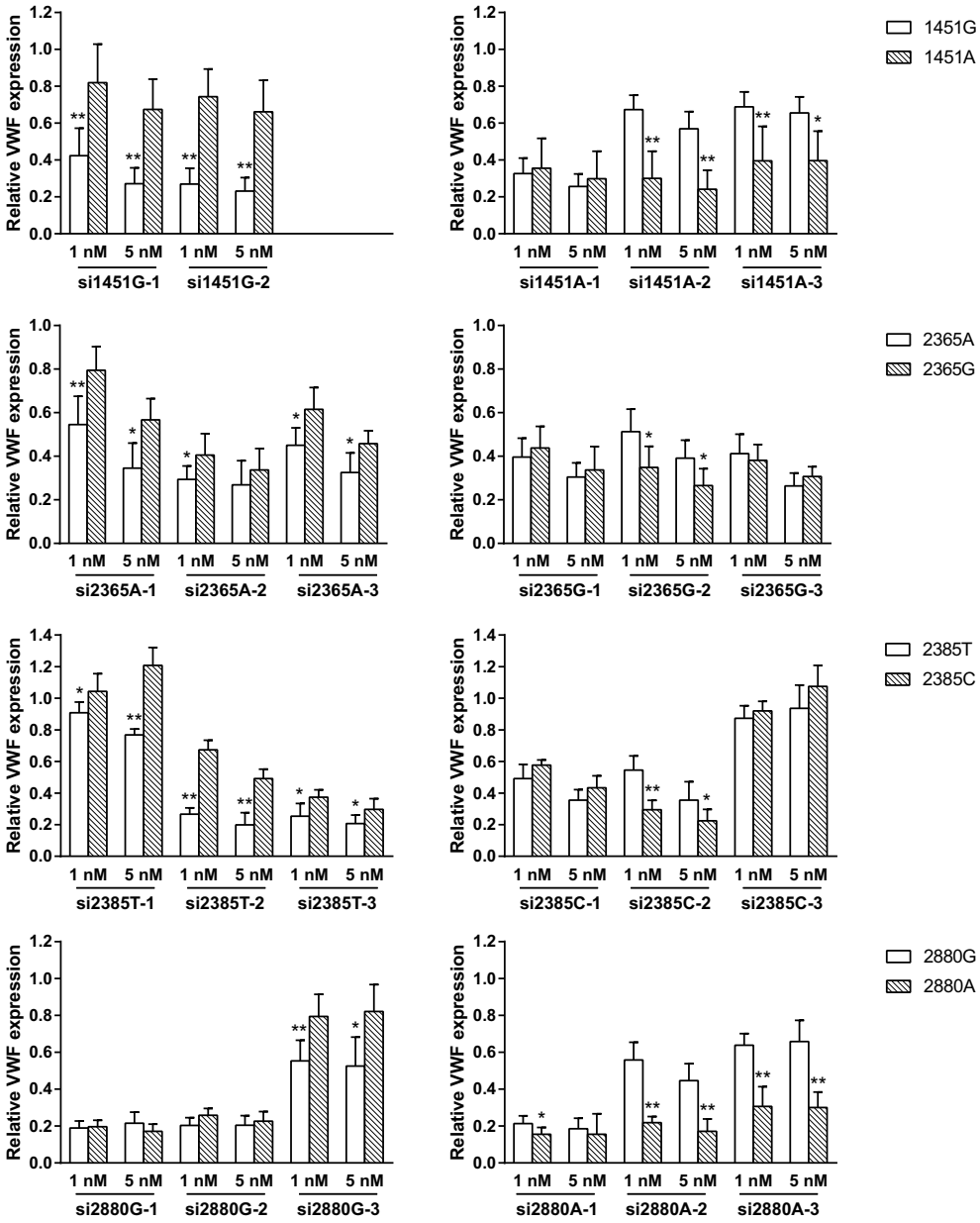

Figure 1. Allele-specific inhibition of von Willebrand factor (VWF) in human embryonic kidney 293 (HEK293) cells stably producing VWF. Normalized total VWF antigen (VWF:Ag) levels measured by ELISA in conditioned medium and cell lysates of HEK293 cells stably producing VWF transfected with allele-specific small interfering RNAs (siRNAs). Allele-specific siRNAs were transfected into HEK293 cells stably expressing either of the *VWF* alleles (Fig. S1B). Transfections were performed at siRNA concentrations of 1 nM and 5 nM, and the VWF:Ag levels measured by ELISA were normalized to the VWF:Ag levels measured in cells transfected with a negative control siRNA. Shown is the mean + one standard deviation of the compiled results of three independent experiments performed in duplicate (N = 6). Mann-Whitney (targeted *versus* untargeted allele), **P* < 0.05, ***P* < 0.01.

Two VWF plasmids containing either of the *VWF* alleles were cotransfected with the 12 most potent siRNAs into HEK293 cells (Fig. S1C). The MYC peptide tag was incorporated in the plasmid containing the *VWF* allele: c.1451G, c.2365A, c.2385T, and c.2880G. The HA peptide tag was incorporated in plasmids containing the opposite *VWF* SNP alleles (Table S1). Coexpression of the siRNA with the two alleles of a SNP clearly led to increased efficiency and allele specificity for all siRNAs, except for si2385T-1, as compared with siRNA transfections in cells stably producing VWF (Figs 2A and S4). This was observed both at the protein level in conditioned medium and in cell lysates (Fig. 2A). Efficient and allele-specific inhibition was observed in a dose-dependent manner at all tested siRNA concentrations, with even upregulation of the untargeted allele being observed at the lowest siRNA concentrations (Fig. S4). These results were obtained reproducibly in two independent experiments. The optimal siRNA concentration was determined according to the lowest percentage of the targeted allele in the total sample, and was found to be 10 nM for si1451A-2, si1451A-3, and si2880A-2, and 5 nM for all other siRNAs (Fig. 2). The effect on mRNA level was determined for the 11 most effective siRNAs in a third independent experiment (Fig. 2B). We observed efficient inhibition of the targeted allele at the mRNA level, although with a lower degree of allele specificity than at the protein level measured in conditioned medium in the same experiment, with the relative mRNA expression of the targeted allele being approximately half that of the untargeted allele for most siRNAs (Fig. 2B).

VWD phenotype improvement by siRNA-mediated allele-specific inhibition

Eleven siRNAs proved to be highly efficient and allele-specific, and a remaining fraction of only 5% of the targeted allele in the whole sample was observed for the most optimal siRNA (data not shown). The presence of these low levels of targeted allele would predict a phenotype improvement in the presence of a dominant negative mutation. We selected a previously characterized VWD type 2A mutation, VWF p.Cys2773Ser, as a model mutation with which to study this hypothesis.[15] VWF plasmids containing all possible SNP alleles and VWF p.Cys2773Ser were generated to test this hypothesis (Table S2).

Transfection of normal VWF only into HEK293 cells leads to a VWF multimer pattern with a full range of VWF multimers (Fig. 3A, first lane). Cotransfection of normal VWF with increasing concentrations of VWF p.Cys2773Ser resulted in a progressively abnormal multimer pattern, with only monomers and dimers being present when only VWF p.Cys2773Ser was transfected (Fig. 3A, last lane).[15] The heterozygous state of the patient is mimicked by the cotransfection of equal amounts of normal and mutant VWF p.Cys2773Ser (Fig. 3A, middle lane). The heterozygous multimer pattern of the cotransfection of equal amounts of mutant and normal VWF (for set-up, see Fig. S1D) was improved towards a normal pattern by most siRNAs (Fig. 3B). The VWF large multimer index was calculated to quantify the increase in the amount of high molecular weight VWF (Table 2).

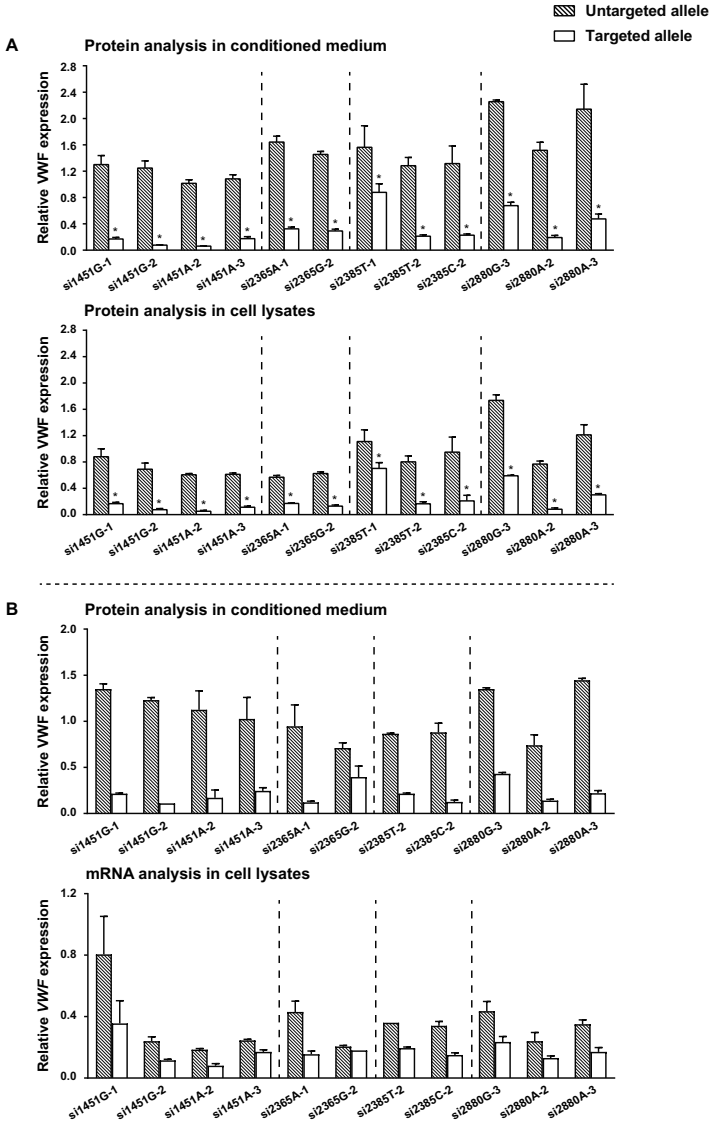

Figure 2. Allele-specific inhibition of von Willebrand factor (VWF) in cotransfected human embryonic kidney 293 (HEK293) cells. HEK293 cells were cotransfected with VWF-HA, VWF-MYC and allele-specific small interfering RNAs (siRNAs) at the optimal siRNA concentration, determined by the percentage of targeted allele in the whole sample. The optimal siRNA concentrations were determined to be 10 nM for si1451A-2, si1451A-3, and si2880A-2, and 5 nM for all other siRNAs. The untargeted and targeted alleles could be either VWF-HA or VWF-MYC, depending on the *VWF* allele (Table S1). VWF-HA and VWF-MYC protein and mRNA levels were normalized to the VWF-HA and VWF-MYC protein and mRNA levels measured in HEK293 cells cotransfected with the two *VWF* alleles and a negative control siRNA (Fig. S1C). (A) Normalized VWF-HA and VWF-MYC protein levels measured by ELISA in conditioned medium and cell lysates. Shown is the mean + one standard deviation (SD) of the compiled results of two independent experiments performed in duplicate (N = 4). (B) Normalized VWF-HA and VWF-MYC protein levels measured by ELISA in conditioned medium and, from the same experiment, the corresponding normalized *VWF-HA* and *VWF-MYC* mRNA levels determined by quantitative PCR on cDNA samples. Shown is the mean + 1 SD of the compiled results of one independent experiment performed in duplicate (N = 2). Mann-Whitney (targeted *versus* untargeted allele), **P* < 0.05.

Cotransfection of normal and mutant VWF into HEK293 cells resulted in a large multimer index of 50.1% ± 4.5% as compared with cells transfected with only normal VWF (100%). Increases in the large multimer index were observed for most siRNAs, but were highest for cells treated with si1451G-2 and si2880G-3, at 82.8% and 85.3%, respectively (Table 2). Combining siRNAs to target different SNPs simultaneously could potentially increase the effect. A combination of two siRNAs targeting either c.2365A and c.2880G or c.2365G and c.2880A did not result in further improvement in multimerization patterns, and nor did the patterns deteriorate (Fig. 3C).

Table 2. VWF large multimer index calculated on densitometry images of multimer analysis

	VWF large multimer index (%)
Normal	100
Normal + mutant	50.1 ± 4.5
Normal + mutant + si1451G-1	70.9
Normal + mutant + si1451G-2	82.8
Normal + mutant + si1451A-2	71.2
Normal + mutant + si1451A-3	66.8
Normal + mutant + si2365A-1	58.8
Normal + mutant + si2365G-2	48.4
Normal + mutant + si2385T-2	75.5
Normal + mutant + si2385C-2	70.9
Normal + mutant + si2880G-3	85.3
Normal + mutant + si2880A-2	59.8
Normal + mutant + si2880A-3	79.5

si1451G-1, indicates 'small interfering RNA 1 against *VWF* c.1451G', all siRNAs are indicated according to this principle; VWF, von Willebrand factor

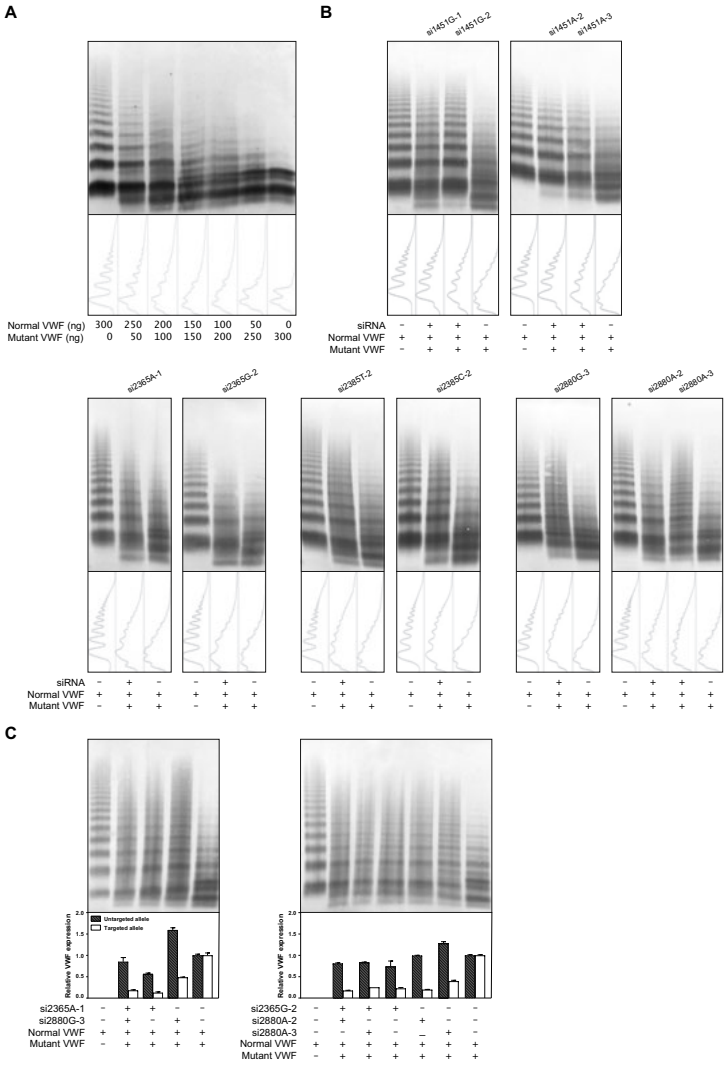

Figure 3. Improvement of a multimerization defect by allele-specific small interfering RNAs (siRNAs). von Willebrand factor (VWF) multimer analysis was performed on conditioned medium of human embryonic kidney 293 (HEK293) cells cotransfected with normal VWF and mutant (p.Cys2773Ser) VWF plasmids. Shown are conditioned medium samples 72 hours after transfection and 48 hours after refreshment of the medium, loaded on a 1.5% SDS-agarose gel. (A) HEK293 cells were transfected with different concentrations of normal and/or mutant VWF plasmids. Increasing concentrations of mutant VWF resulted in a severe dimerization and multimerization defect.[15] (B) HEK293 cells were transfected with only normal VWF or with normal and mutant VWF in a 1:1 ratio. Allele-specific siRNAs were transfected into HEK293 cells cotransfected with normal and mutant VWF (Fig. S1D). Addition of allele-specific siRNAs resulted in clear improvement of the VWF multimer patterns for several siRNAs. (C) HEK293 cells were transfected with only normal VWF or with normal and mutant VWF in a 1:1 ratio. A single siRNA or a combination of two siRNAs with different targets was transfected into HEK293 cells cotransfected with normal and mutant VWF. A combination of two siRNAs targeting c.2365A and c.2880G or c.2365G and c.2880A did not clearly improve the multimerization pattern as compared with single siRNA transfections. A slight improvement in allele-specific inhibition at the protein level was observed with combined targeting of c.2365A and c.2880G as compared with targeting of only c.2365A. This was not observed when siRNAs with the targets c.2365G and c.2880A were combined.

Discussion

In this study, we selected a set of allele-specific siRNAs with the *in vitro* ability to mediate allele-specific inhibition of *VWF*. Inhibition of mutant VWF production, while preserving the production of normal VWF, improved the multimerization pattern of the dominant negative VWF mutation p.Cys2773Ser. This approach could improve several phenotypes caused by dominant negative VWF mutations, which are present in > 90% of the VWD population.[2]

Various studies have proven that siRNAs, microRNAs, antisense oligonucleotides and CRISPR-Cas9 can discriminate two alleles on the basis of one nucleotide variation.[16-22] This discrimination could be based on the dominant negative mutation itself, or on a SNP located on the same allele as the dominant negative mutation. For this study, we chose to use allele-specific siRNAs that target frequent SNPs in *VWF*. siRNAs were chosen to prove the feasibility of allele-specific inhibition in VWD, and because of their safe temporary effects. By the selection of four SNPs with a high MAF in *VWF*, it is possible to design a treatment applicable to a major part of the Caucasian population, as 74% will be heterozygous for at least one of these four SNPs. By increasing the number of SNPs, the population coverage can even be increased up to 93% (data not shown). A much larger number of siRNAs would be required to target each individual *VWF* mutation.

The proof of principle of allele-specific inhibition of *VWF* was tested by overexpression of *VWF* alleles in HEK293 cells. Two methods were used to screen for effective siRNAs. First, siRNAs were transfected into HEK293 cells stably producing VWF. This setting resembles a normal situation, in which there is continuous production of VWF. Using this experimental set-up, we selected at least one effective siRNA per SNP allele. However, the ratio of the targeted and untargeted allele was not yet optimal. We reasoned that discrimination between the two *VWF* alleles present in the heterozygous situation, as in patients, might improve allelic discrimination by siRNAs. To mimic the coexistence of two *VWF* alleles in one cell, we performed cotransfections of two *VWF* alleles and an siRNA targeting one of the two alleles in HEK293 cells. This did indeed lead to an increase in specificity of the siRNAs for the targeted allele. The increase in allele specificity suggests that competition between two alleles leads to preference of the siRNA for its specific target. Remarkably, we observed at the protein level, but not at the mRNA level, upregulation of the untargeted *VWF* allele for most siRNAs at the lowest siRNA concentrations (Figs S4 and 2B). This might have been caused by transfection of an excess of plasmids, which may lead to a maximum production capacity of the translational machinery. siRNA-mediated inhibition of the targeted allele may then provide greater access of the translational machinery to the untargeted allele, and enhance translation of the untargeted allele. An increase in the siRNA concentration would inhibit this process by increasing non-specific binding of the siRNA to the untargeted allele. Whether this upregulation also occurs in cells endogenously producing VWF could be studied in blood outgrowth endothelial cells (BOECs).[23] As BOECs are primary cells, they differ from heterologous cell systems in the

processing and maturation of pre-mRNA. Although siRNAs bind and process mature mRNA in the cytoplasm, differences in mRNA modifications between primary and heterologous cell systems should be kept in mind. We and others have shown the feasibility of isolating BOECs from VWD patients.[24-27] Genotyping patients and family members for the patients' mutation and the four selected SNPs will identify which SNPs the patient is heterozygous for, and which of the two SNP alleles is linked to the dominant negative mutation.

In this study, we investigated the effect of the most potent siRNAs on VWF p.Cys2773Ser, a fully characterized dominant negative mutation causing defective intracellular multimerization.[15] Cotransfection of normal VWF and VWF p.Cys2773Ser into HEK293 cells did indeed lead to a severe multimerization defect and a decrease in the amount of high molecular weight VWF. The addition of the most potent siRNAs resulted, for almost all siRNAs, in clear improvements in VWF multimerization. The multimer pattern could not be further improved by the simultaneous transfection of two siRNAs with different targets (Fig. 3C). Inhibition of mutant *VWF* as therapy for VWD is especially beneficial for VWD patients in whom the unhindered production of mutant VWF has detrimental effects that are not prevented by current therapies. The most clear example is the development of thrombocytopenia in VWD type 2B patients, which is often provoked by stress responses during surgery or pregnancy.[28,29] However, apart from those patients with a clear unmet clinical need, inhibition of mutant *VWF* might also benefit patients with other VWD phenotypes, e.g. patients with a very fast clearance rate or a severe secretion defect caused by intracellular retention. In those cases, inhibition of the mutant allele will increase circulating levels of normal VWF. Furthermore, recent findings suggest a role for VWF in angiogenesis, i.e. the formation of new blood vessels from existing vessels.[30,31] The negative regulation of VWF on the process of angiogenesis potentially results in increased blood vessel formation, and may lead to intestinal angiodysplasia and intractable intestinal bleeding in VWD patients.[32,33] As VWF is thought to have an intracellular and extracellular signaling function in the process of angiogenesis, mutant VWF itself may be responsible for maintaining the aberrant angiogenesis.[31,32] Inhibiting mutant VWF expression may attenuate all of these effects, and at the same time increase circulating levels of normal VWF.

Allele-specific siRNAs could be used in a prophylactic setting, either to prevent spontaneous bleeding or prior to scheduled interventions, or they could convert a DDAVP-unresponsive patient into a DDAVP-responsive patient. The exact application of allele-specific inhibition of *VWF* will depend, among other things, on the patient's phenotype and the duration of siRNA inhibition *in vivo*. *In vivo* use of siRNAs requires a delivery vehicle. Until now, delivery of siRNAs has been mainly focused on targeting the liver.[34,35] Recently, delivery vehicles targeting the endothelium have also shown positive results regarding inhibition of several endothelial genes.[36,37] These results are promising regarding the possibility of endothelium-targeted siRNA therapies for VWD.

Altogether, the recent developments in the field of siRNA delivery, the ability of the designed siRNAs to inhibit *VWF* in an allele-specific manner and the ability of these siRNAs to improve a severe VWD phenotype hold promise for the use of allele-specific siRNAs as therapy for dominant negative VWD.

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References

1. Leebeek FWG, Eikenboom JCJ. Von Willebrand's Disease. *N Engl J Med*. 2016;**375**(21):2067-2080.
2. Goodeve AC. The genetic basis of von Willebrand disease. *Blood Rev*. 2010;**24**(3):123-134.
3. Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *J Thromb Haemost*. 2006;**4**(10):2103-2114.
4. Curnow J, Pasalic L, Favaloro EJ. Treatment of von Willebrand Disease. *Semin Thromb Hemost*. 2016;**42**(2):133-146.
5. Mannucci PM, Ruggeri ZM, Pareti FI, Capitanio A. 1-Deamino-8-d-arginine vasopressin: a new pharmacological approach to the management of haemophilia and von Willebrands' diseases. *Lancet*. 1977;**1**(8017):869-872.
6. Franchini M, Mannucci PM. Von Willebrand factor (Vonvendi(R)): the first recombinant product licensed for the treatment of von Willebrand disease. *Expert Rev Hematol*. 2016;**9**(9):825-830.
7. Lillicrap D. von Willebrand disease: advances in pathogenetic understanding, diagnosis, and therapy. *Blood*. 2013;**122**(23):3735-3740.
8. Casari C, Pinotti M, Lancellotti S, et al. The dominant-negative von Willebrand factor gene deletion p.P1127_C1948delinsR: molecular mechanism and modulation. *Blood*. 2010;**116**(24):5371-5376.
9. de Jong A, Eikenboom J. Von Willebrand disease mutation spectrum and associated mutation mechanisms. *Thromb Res*. 2017;**159**:65-75.
10. Auton A, Brooks LD, Durbin RM, et al. A global reference for human genetic variation. *Nature*. 2015;**526**(7571):68-74.
11. Romani de Wit T, Rondaij MG, Hordijk PL, Voorberg J, van Mourik JA. Real-time imaging of the dynamics and secretory behavior of Weibel-Palade bodies. *Arterioscler Thromb Vasc Biol*. 2003;**23**(5):755-761.
12. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *Biotechniques*. 2005;**39**(1):75-85.
13. Tjernberg P, Vos HL, Castaman G, Bertina RM, Eikenboom JC. Dimerization and multimerization defects of von Willebrand factor due to mutated cysteine residues. *J Thromb Haemost*. 2004;**2**(2):257-265.
14. Tamura T, Horiuchi H, Imai M, et al. Unexpectedly High Prevalence of Acquired von Willebrand Syndrome in Patients with Severe Aortic Stenosis as Evaluated with a Novel Large Multimer Index. *J Atheroscler Thromb*. 2015;**22**(11):1115-1123.
15. Tjernberg P, Vos HL, Spaargaren-van Riel CC, et al. Differential effects of the loss of intrachain- versus interchain-disulfide bonds in the cystine-knot domain of von Willebrand factor on the clinical phenotype of von Willebrand disease. *Thromb Haemost*. 2006;**96**(6):717-724.
16. Miniarikova J, Zanella I, Huseinovic A, et al. Design, Characterization, and Lead Selection of Therapeutic miRNAs Targeting Huntingtin for Development of Gene Therapy for Huntington's Disease. *Mol Ther Nucleic Acids*. 2016;**5**:e297.
17. Novelli F, Lena AM, Panatta E, et al. Allele-specific silencing of EEC p63 mutant R304W restores p63 transcriptional activity. *Cell Death Dis*. 2016;**7**:e2227.

18. Miller VM, Xia H, Marrs GL, et al. Allele-specific silencing of dominant disease genes. *Proc Natl Acad Sci U S A*. 2003;**100**(12):7195-7200.
19. Pfister EL, Kennington L, Straubhaar J, et al. Five siRNAs targeting three SNPs may provide therapy for three-quarters of Huntington's disease patients. *Curr Biol*. 2009;**19**(9):774-778.
20. Skotte NH, Southwell AL, Ostergaard ME, et al. Allele-specific suppression of mutant huntingtin using antisense oligonucleotides: providing a therapeutic option for all Huntington disease patients. *PLoS One*. 2014;**9**(9):e107434.
21. Southwell AL, Skotte NH, Kordasiewicz HB, et al. In vivo evaluation of candidate allele-specific mutant huntingtin gene silencing antisense oligonucleotides. *Mol Ther*. 2014;**22**(12):2093-2106.
22. Yoshimi K, Kaneko T, Voigt B, Mashimo T. Allele-specific genome editing and correction of disease-associated phenotypes in rats using the CRISPR-Cas platform. *Nat Commun*. 2014;**5**:4240.
23. Martin-Ramirez J, Hofman M, van den Biggelaar M, Hebbel RP, Voorberg J. Establishment of outgrowth endothelial cells from peripheral blood. *Nat Protoc*. 2012;**7**(9):1709-1715.
24. Hawke L, Bowman ML, Poon MC, Scully MF, Rivard GE, James PD. Characterization of aberrant splicing of von Willebrand factor in von Willebrand disease: an underrecognized mechanism. *Blood*. 2016;**128**(4):584-593.
25. Wang JW, Bouwens EA, Pintao MC, et al. Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease. *Blood*. 2013;**121**(14):2762-2772.
26. Starke RD, Paschalaki KE, Dyer CE, et al. Cellular and molecular basis of von Willebrand disease: studies on blood outgrowth endothelial cells. *Blood*. 2013;**121**(14):2773-2784.
27. Selvam SN, Casey LJ, Bowman ML, et al. Abnormal angiogenesis in blood outgrowth endothelial cells derived from von Willebrand disease patients. *Blood Coagul Fibrinolysis*. 2017;**28**(7):521-533.
28. Hultin MB, Sussman II. Postoperative thrombocytopenia in type IIB von Willebrand disease. *Am J Hematol*. 1990;**33**(1):64-68.
29. Rick ME, Williams SB, Sacher RA, McKeown LP. Thrombocytopenia associated with pregnancy in a patient with type IIB von Willebrand's disease. *Blood*. 1987;**69**(3):786-789.
30. Groeneveld DJ, van Bekkum T, Dirven RJ, et al. Angiogenic characteristics of blood outgrowth endothelial cells from patients with von Willebrand disease. *J Thromb Haemost*. 2015;**13**(10):1854-1866.
31. Starke RD, Ferraro F, Paschalaki KE, et al. Endothelial von Willebrand factor regulates angiogenesis. *Blood*. 2011;**117**(3):1071-1080.
32. Castaman G, Federici AB, Tosetto A, et al. Different bleeding risk in type 2A and 2M von Willebrand disease: a 2-year prospective study in 107 patients. *J Thromb Haemost*. 2012;**10**(4):632-638.
33. Makris M. Gastrointestinal bleeding in von Willebrand disease. *Thromb Res*. 2006;**118 Suppl 1**:S13-17.
34. Pasi KJ, Rangarajan S, Georgiev P, et al. Targeting of Antithrombin in Hemophilia A or B with RNAi Therapy. *N Engl J Med*. 2017;**377**(9):819-828.

35. Heestermans M, van Vlijmen BJ. Oligonucleotides targeting coagulation factor mRNAs: use in thrombosis and hemophilia research and therapy. *Thromb J*. 2017;**15**:7.
36. Dahlman JE, Barnes C, Khan O, et al. In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. *Nat Nanotechnol*. 2014;**9**(8):648-655.
37. Fehring V, Schaeper U, Ahrens K, et al. Delivery of therapeutic siRNA to the lung endothelium via novel Lipoplex formulation DACC. *Mol Ther*. 2014;**22**(4):811-820.

Supplemental data
Figure S1

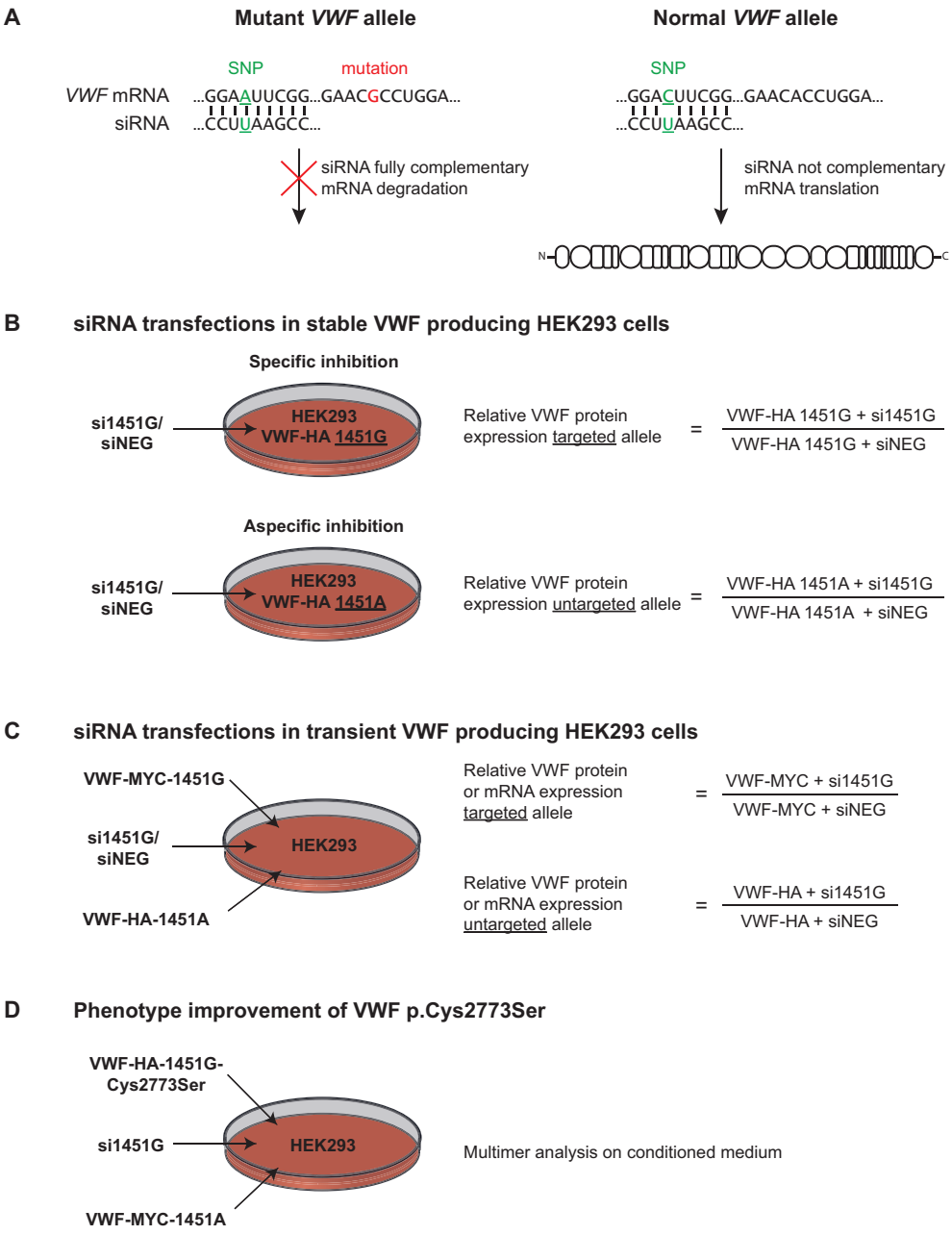

Figure S1. Schematic representation of the hypothesis and experimental setup. (A) siRNAs designed to target a heterozygous SNP (in this example an adenine, green) located on the same allele as the dominant-negative mutation (in this example a guanine, red) are expected to inhibit expression of the mutant *VWF*

allele. The mismatch of the same siRNA with the other allele of the SNP (in this example a cytosine, green) is hypothesized to render the siRNA ineffective and therefore lead to normal mRNA translation. (B) Experimental setup of siRNA transfections to stable VWF producing HEK293 cells. siRNAs and siNEG were transfected to cells stably expressing the targeted *VWF* allele and to cells stably expressing the untargeted allele. Relative VWF expression was determined by dividing the VWF:Ag levels measured in conditioned medium and lysates of cells transfected with a specific siRNA (in this example si1451G) to cells transfected with siNEG. (C) Experimental setup of HEK293 cells cotransfected with siRNAs and two *VWF* alleles. siRNAs were transiently cotransfected to HEK293 cells with two *VWF* alleles containing either an HA or MYC peptide tag. Relative VWF expression of both alleles is determined by dividing the VWF-HA or VWF-MYC protein or mRNA levels measured in conditioned medium and lysates of cells transfected with a specific siRNA (in this example si1451G) to cells transfected with siNEG. (D) Experimental setup of HEK293 cells cotransfected with siRNA and two *VWF* alleles, of which the *VWF-HA* allele also contained the VWF p.Cys2773Ser mutation. Improvements in multimerization were determined by multimer analysis. VWF, von Willebrand factor; SNP, single-nucleotide polymorphism; siRNA, small interfering RNA; mRNA, messenger RNA; si1451G, indicates 'small interfering RNA against *VWF* c.1451G'

Figure S2

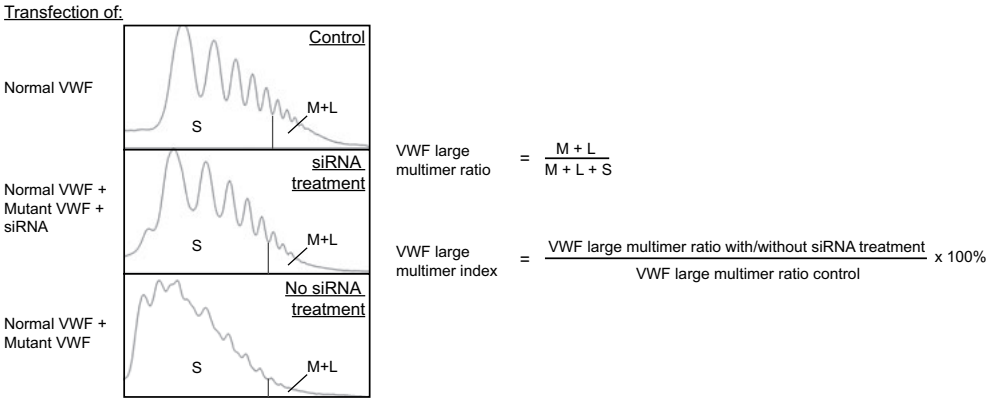

Figure S2. Calculation of the VWF large multimer index. Densitometry images are divided in the five smallest (S) bands and the rest (intermediate and large bands (M+L)). The VWF large multimer ratio is calculated for each densitometry image by dividing the area of intermediate and large VWF multimers (M+L) over the total area (M+L+S). The VWF large multimer index is calculated by dividing the VWF large multimer ratio of cells cotransfected with normal and mutant VWF (with or without siRNA treatment) over the VWF large multimer ratio of cells transfected with normal VWF only. VWF, von Willebrand factor; S, small multimers; M, intermediate multimers; L, large multimers

Figure S3

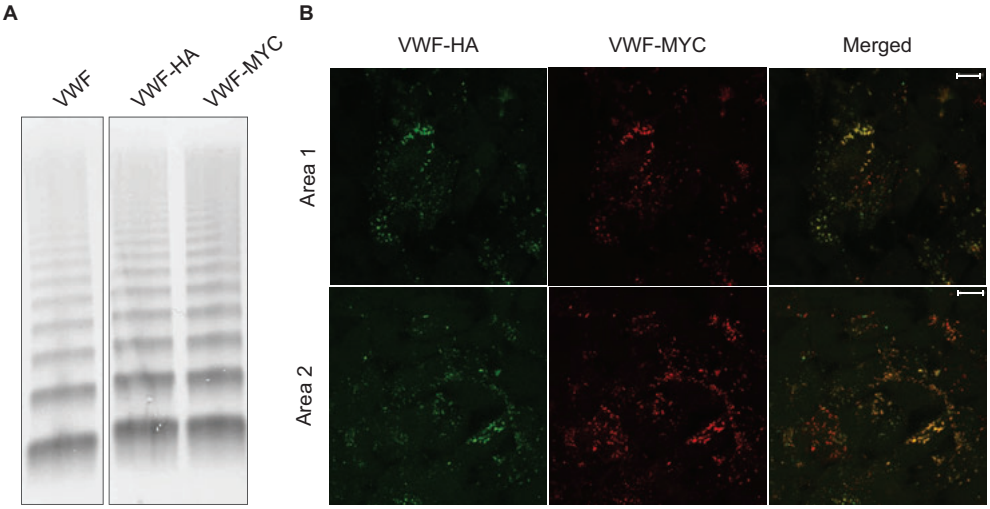

Figure S3. Effect of HA and MYC peptide tags on VWF processing and transfection. (A) VWF multimer analysis on conditioned medium of HEK293 cells transfected with VWF-HA, VWF-MYC or normal VWF plasmids. Shown are conditioned medium samples 72 hours after transfection and 48 hours after refreshing the medium, loaded on a 1.5% SDS-agarose gel. All three samples in the picture were run on the same gel. (B) Representative images of immunofluorescent staining of HEK293 cells cotransfected with VWF-HA (green) and VWF-MYC (red) plasmids. Most cells stain both VWF-HA and VWF-MYC, indicating a true coexpressing system. Bar represents 10 µm. Images were taken with the Leica TCS SP8 upright confocal microscope with a 63x/1.40 NA Plan Apo oil immersion objective. VWF, von Willebrand factor

Figure S4

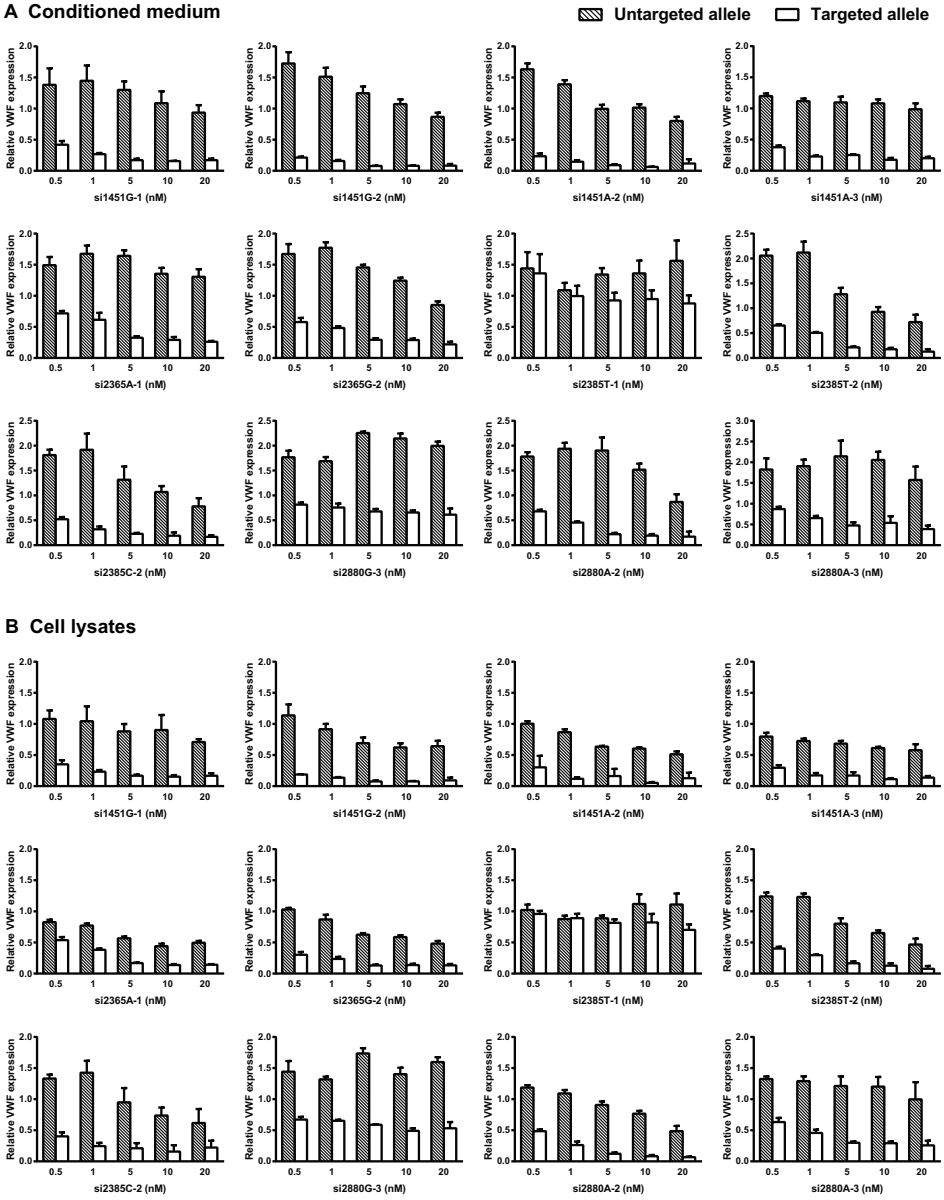

Figure S4. Dose response of allele-specific siRNAs cotransfected with two *VWF* alleles into HEK293 cells. Normalized VWF-HA and VWF-MYC protein levels measured by ELISA in (A) conditioned medium and (B) cell lysates of HEK293 cells cotransfected with VWF-HA, VWF-MYC and allele-specific siRNAs at five different concentrations to determine the dose-response. The untargeted and targeted allele could either be VWF-HA or VWF-MYC, depending on the *VWF* allele (Table S1). VWF-HA and VWF-MYC protein levels were normalized to the VWF-HA and VWF-MYC protein levels measured in HEK293 cells cotransfected with the two *VWF* alleles and siNEG. Shown are the mean + 1 SD of the compiled results of two independent experiments performed in duplicate (N = 4). si1451G-1, indicates 'small interfering RNA-1 against *VWF* c.1451G', all siRNAs are indicated according to this principle; siRNA, small interfering RNA; VWF, von Willebrand factor; nM, nanomolar

Table S1. VWF plasmids used to determine efficiency and degree of allele-specificity in stable VWF producing HEK293 cells and transiently cotransfected HEK293 cells

pcDNA™3.1/Zeo (+)	rs1800378 c.1451G|A	rs1063856 c.2365A|G	rs1063857 c.2385T|C	rs1800380 c.2880G|A	Used for:
hVWF-HA	G	A	T	G	Stable cell line
hVWF-MYC	G	A	T	G	Cotransfection
hVWF-HA-1451A	A	A	T	G	Stable cell line/cotransfection
hVWF-HA-2365G	G	G	T	G	Stable cell line/cotransfection
hVWF-HA-2385C	G	A	C	G	Stable cell line/cotransfection
hVWF-HA-2880A	G	A	T	A	Stable cell line/cotransfection

VWF, von Willebrand factor

Table S2. VWF plasmids used for allele-specific inhibition of hVWF p.Cys2773Ser

	pcDNA™3.1/Zeo (+)	rs1800378 c.1451G|A	rs1063856 c.2365A|G	rs1063857 c.2385T|C	rs1800380 c.2880G|A	p.Cys2773Ser c.8318G|C
To target c.1451G	hVWF-HA-Cys2773Ser	G	A	T	G	C
	hVWF-MYC-1451A	A	A	T	G	G
To target c.1451A	hVWF-HA-1451A-Cys2773Ser	A	A	T	G	C
	hVWF-MYC	G	A	T	G	G
To target c.2365A	hVWF-HA-Cys2773Ser	G	A	T	G	C
	hVWF-MYC-2365G	G	G	T	G	G
To target c.2365G	hVWF-HA-2365G-Cys2773Ser	G	G	T	G	C
	hVWF-MYC	G	A	T	G	G
To target c.2385T	hVWF-HA-Cys2773Ser	G	A	T	G	C
	hVWF-MYC-2385C	G	A	C	G	G
To target c.2385C	hVWF-HA-2385C-Cys2773Ser	G	A	C	G	C
	hVWF-MYC	G	A	T	G	G
To target c.2880G	hVWF-HA-Cys2773Ser	G	A	T	G	C
	hVWF-MYC-2880A	G	A	T	A	G
To target c.2880A	hVWF-HA-2880A-Cys2773Ser	G	A	T	A	C
	hVWF-MYC	G	A	T	G	G
To target c.2365A and c.2880G	hVWF-HA-Cys2773Ser	G	A	T	G	C
	hVWF-MYC-2365G/2880A	G	G	T	A	G
To target c.2365G and c.2880A	hVWF-HA-2365G/2880A-Cys2773Ser	G	G	T	A	C
	hVWF-MYC	G	A	T	G	G

VWF, von Willebrand factor