Personalized treatment for von Willebrand disease by RNA-targeted therapies

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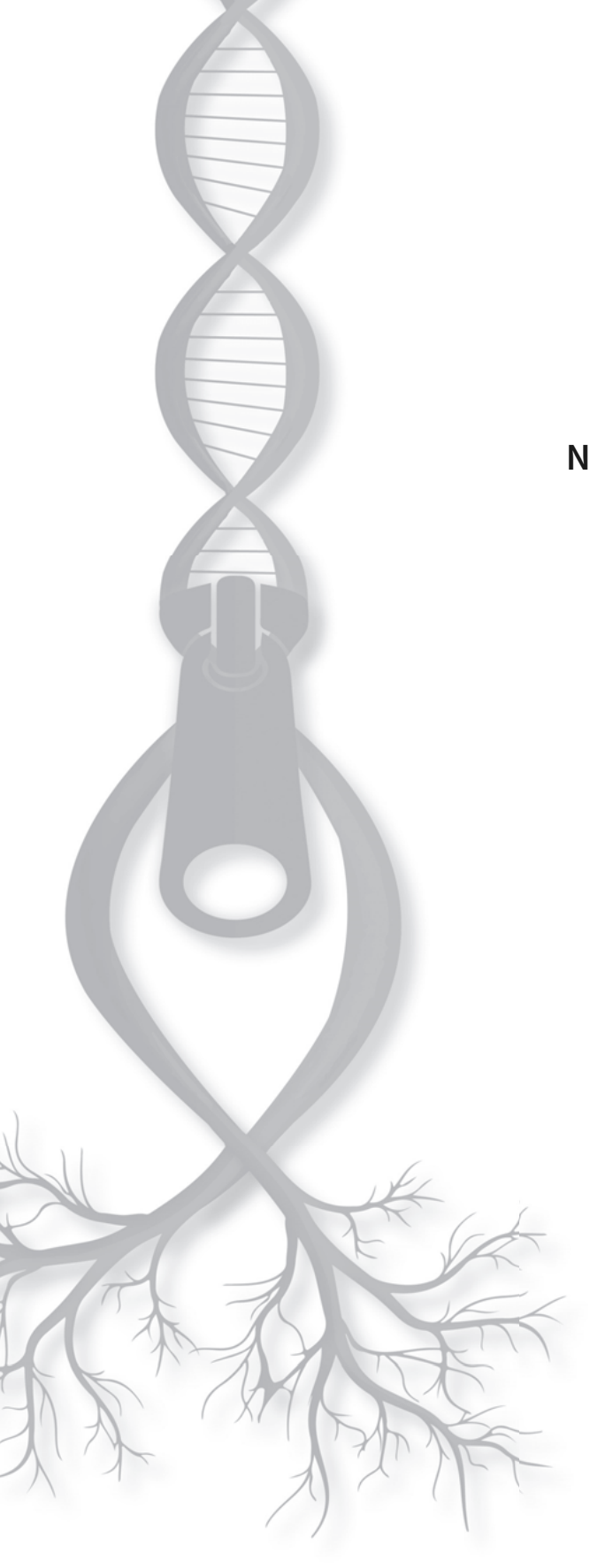

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English summary

Nederlandse samenvatting

English summary

Von Willebrand disease (VWD) is the most common inherited bleeding disorder that clinically affects around 1 in 10,000 people. Patients mainly develop mucocutaneous bleeding, i.e. bruises, epistaxis, gum bleeding and menorrhagia. The more severely affected patients may also develop joint bleeding, or bleeding from the gastrointestinal tract. Also, trauma, surgery or dental procedures may lead to critical bleeding events. VWD-related bleeding are caused by defects in von Willebrand factor (VWF), and these defects can be quantitative or qualitative of nature. VWF is a large multimeric protein that is produced by endothelial cells and megakaryocytes. In the endothelial cells, VWF multimers are generated by C-terminal dimerization of two VWF monomers in the endoplasmic reticulum, followed by N-terminal multimerization in the (trans)-Golgi network. The produced VWF multimers are subsequently stored in storage organelles, the Weibel-Palade bodies (WPBs), or constitutively secreted from the endothelial cells. Vascular damage triggers the release of VWF from the WPBs into the circulation. At sites of vascular injury VWF multimers attach to the exposed collagen, and the presence of shear in the circulation results in the elongation of VWF into ultra-large VWF strings. The primary function of VWF in hemostasis is the attraction of platelets to sites of vascular damage. These platelets can adhere via their glycoprotein Ib alpha receptors to the VWF A1 domain when it is in its open conformation. This subsequently results in platelet activation and platelet plug formation. The ultra-large VWF multimers are cleaved in the A2 domain of VWF by the metalloprotease ADAMTS13, leaving VWF multimers with variable sizes in the circulation. In the circulation, VWF is bound to coagulation factor VIII (FVIII), thereby enhancing the FVIII half-life. VWD can be the result of defects in all mechanisms described above. Patients with a partial VWF deficiency are categorized among VWD type 1. This partial deficiency may result from defective production, reduced secretion or enhanced clearance of VWF. VWD type 2A patients are associated with affected VWF multimers in the circulation, which may result from an intracellular dimerization or multimerization defect, or enhanced cleavage of VWF by ADAMTS13. The binding of VWF to platelets is affected in VWD type 2B and 2M, with enhanced VWF-platelet binding in VWD type 2B, and reduced VWF-platelet binding in VWD type 2M. VWD type 2N results from a decreased binding of VWF to coagulation FVIII. Lastly, VWD type 3 is associated with a complete absence of VWF.

VWD is a heterogeneous disease, and diagnosis of VWD can therefore be challenging. The workflow of VWD diagnosis is reviewed in **Chapter 2** of this thesis. Diagnosis starts with a questionnaire into the bleeding and family history. When a bleeding disorder is suspected, several general tests are performed to exclude different bleeding disorders. When these tests hint towards VWD, several VWD specific diagnostic tests are performed, which can confirm or exclude VWD. These include VWF:Ag, VWF activity, and FVIII activity measurements. When VWD is confirmed, specific tests are performed to correctly classify patients to the right VWD type. VWF multimerization, VWF-FVIII binding, VWF collagen binding, ristocetin induced

platelet aggregation (RIPA), and VWF propeptide measurements are examples of these subtyping tests.

VWD is mainly caused by mutations in VWF, and VWD-associated mutations have been described in literature since the beginning of the 1990s. An overview of all VWF mutations that have been described in literature until the beginning of 2017 have been reviewed in **Chapter 3**. In total, around 750 different variations have been described to be associated with VWD. From these variations, the disease-causing mechanism was proven for approximately 220 variants. The disease-causing mechanisms have been identified using cellular or animal disease models. These include transfection of mutant VWF constructs in heterologous cell systems, like Human Embryonic Kidney 293 (HEK293), COS-7 and AtT-20, or the use of hydrodynamic gene transfer of VWF plasmids in VWF deficient mice. Most mutations that cause VWD types 1, 2A, 2B and 2M are dominant negative mutations. This means that only one *VWF* allele has to be affected to cause VWD. VWD types 2N and 3 on the other hand are caused by recessive mutations.

Since most VWD is caused by dominant negative mutations in VWF, we hypothesized that inhibition of the mutant *VWF* allele only, without affecting the wild type *VWF* allele, would improve the function of VWF and ameliorate VWD phenotypes. And that this concept may be a new therapeutic approach for a subgroup of VWD patients for which the current treatment modalities are insufficient. As a tool to inhibit specific *VWF* alleles, we made use of small interfering RNAs (siRNAs). siRNAs are small double stranded RNA molecules that can be designed to complement mRNA sequences. Full complementarity between the siRNA and the mRNA will lead to mRNA degradation. A mismatch between the siRNA and the mRNA may preclude the possibility of mRNA degradation by the siRNA, and result in normal translation of this mRNA into protein. siRNAs can be designed to target the nucleotide variation causing the dominant negative mutation itself, or a single-nucleotide polymorphisms (SNP) that is linked to the dominant negative mutation. We have chosen for a SNP-based approach. SNPs as target were chosen since the ultimate goal is to develop a new treatment strategy for VWD, and it is not feasible to design and test siRNAs for the hundreds of mutations that cause VWD. Therefore, four SNPs with a high minor allele frequency in *VWF* have been selected and siRNAs have been designed to target both alleles of the four SNPs. With these four SNPs it is possible to target 74 percent of the population. In **Chapter 4**, we used VWF overexpressing HEK293 cells, as *in vitro* cell model to prove the concept of allele-specific inhibition of *VWF*. Using this cell model, we selected allele-specific siRNAs that efficiently inhibited the targeted allele, but were not or less efficient in inhibiting the untargeted allele. When these siRNAs targeted a *VWF* allele that also contained the dominant negative VWD type 2A mutation, p.Cys2773Ser, clear improvements were made in the VWF multimerization pattern.

Studies in HEK293 cells resulted in the selection of allele-specific siRNAs that were efficient and specific in inhibiting single *VWF* alleles. However, HEK293 cell do not produce VWF endogenously, and therefore we aimed to prove the concept of allele-specific *VWF* inhibition in a more physiologic disease model. Endothelial colony forming cells (ECFCs) are cultured endothelial cells that can be isolated from the mononuclear cell fraction of peripheral blood. These cells harbour the typical endothelial cobble stone morphology, and are positive for CD31 and CD146, and negative for CD14 and CD45. Since these cells can be isolated from patients, they are an interesting disease model to test the effectiveness of the allele-specific siRNAs to improve VWD phenotypes in a patient-specific environment. In **Chapter 5**, we optimized this model by characterization of 16 ECFC lines derived from six different ECFC isolations for several VWF-related parameters. We observed clear variations between the cell lines with respect to cell morphology and VWF production. Most importantly, we showed that all cell lines had a different cell density at maximum confluency and that this cell density at maximum confluency positively correlated with VWF production. Furthermore, the tube formation capacity in Matrigel, as a measure for the angiogenic potential, was higher in cells with a low maximum cell density and low VWF production. The possible underlying mechanism behind the variations in ECFCs was investigated by gene expression analysis of several aging and endothelial to mesenchymal transition (EndoMT) genes. ECFCs with a low maximum cell density showed higher expression of both aging and EndoMT markers. It is important to acknowledge the variations between ECFC lines and it is suggested to compare ECFC lines only when they have the same cellular characteristics. When investigating, for example, the effects of siRNA treatment in patient-derived ECFCs, the treated patient-derived ECFCs are compared to the same ECFCs treated with a negative control siRNA. In these situations, ECFCs are used as their own internal control, and can therefore be confidently used.

Before investigating (allele-specific) siRNA treatment in patient-derived ECFCs, it is important to first investigate the overall effects of siRNA-mediated downregulation of VWF on the function and processing of VWF in control ECFCs. Especially since it has been reported that siRNA-mediated inhibition of VWF in human umbilical vein endothelial cells result in shorter WPBs and enhanced secretion of VWF from the endothelial cells. We reproduced these previous findings in **Chapter 6**, but also showed that siRNA-mediated downregulation of VWF resulted in enhanced secretion of mainly low molecular weight VWF. These are important changes in VWF processing and multimerization to keep in mind when performing siRNA treatments in patient-derived ECFCs.

The ability of the allele-specific siRNAs that were selected from the screen in HEK293 cells (in Chapter 4) to also induce allele-specific *VWF* inhibition in ECFCs was investigated in **Chapter 7**. The siRNAs that were selected in Chapter 4 were also effective in ECFCs, which was proven both on protein and RNA level. Furthermore, we successfully isolated ECFCs from

a VWD type 2A patient with the VWF p.Cys1190Tyr mutation. These ECFCs showed decreased VWF collagen binding, defective VWF multimerization and defective processing of proVWF into VWF. Genotyping of this patient revealed that the patient is heterozygous for one of the four selected SNPs and downregulation of the mutant allele by targeting the associated SNP resulted in improvements in VWF collagen binding, VWF multimerization and the processing of VWF.

The effects of the allele-specific siRNAs were promising *in vitro* (HEK293) and *ex vivo* (ECFCs), but several aspects of VWD physiology cannot be investigated in these static cell systems. The last step of this research, discussed in **Chapter 8** of this thesis, was to investigate the effects of allele-specific inhibition of mutant *VWF* in a heterozygous VWD mouse model. Heterozygous VWD type 2B mice were generated by hydrodynamic injection of wild type and mutant mouse *Vwf* cDNA in VWF deficient mice. As a model mutation, the VWD type 2B mutation VWF p.Val1316Met, was chosen. This mutation is associated in men and mice with decreased platelet count, increased platelet size and an increase in VWF that circulates in its active conformation. Inhibition of the mutant allele only by an siRNA that targets the dominant negative mutation p.Val1316Met itself, resulted in clear phenotypic improvements, with correction of platelet count, normalization of platelet size and a decrease in VWF that circulated in its activated conformation.

Altogether, work described in this thesis proved that allele-specific inhibition of the production of mutant VWF by siRNAs results in improvements of several VWD phenotypes in HEK293 cells, in ECFCs and in a VWD mouse model. These results are promising for further development of allele-specific siRNAs as a new treatment strategy for VWD.