Personalized treatment for von Willebrand disease by RNA-targeted therapies

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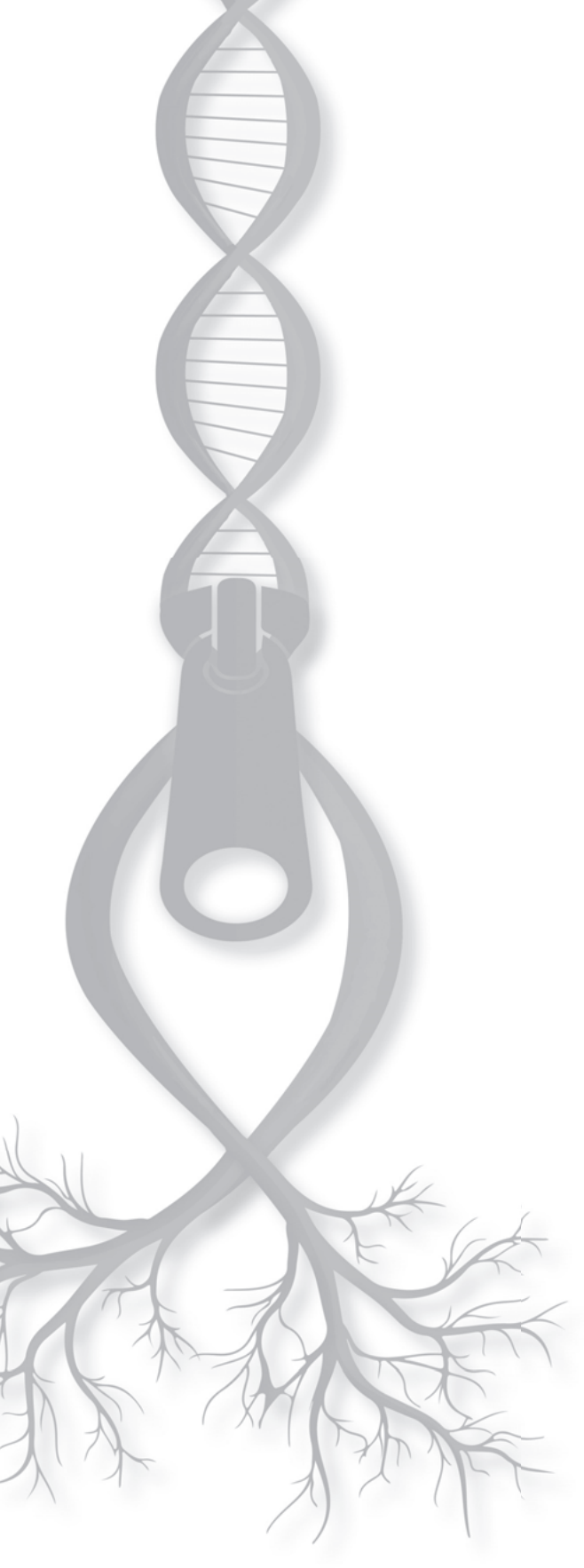

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General introduction and outline of the thesis

In 1998, Andrew Fire and Craig Mello discovered that double-stranded RNA molecules corresponding to the coding region of specific genes, can interfere with the production of proteins.[1] Fire and Mello named this phenomenon RNA interference (RNAi) and the invention of it has had many implications in fundamental as well as translational medical sciences.[2] It is therefore not without merit that both inventors received the Nobel Prize in physiology or medicine for this discovery in 2006. The discovery of RNAi was quickly followed by studies that shed light on the mechanism of RNAi.[3-6] Most importantly, it was established that small double-stranded RNA molecules of about 20-25 nucleotides are responsible for the interference process.[3-5] These short double-stranded RNA molecules are called small interfering RNAs (siRNAs) and can be generated intracellularly as a cleavage product of long double-stranded RNA molecules or can chemically be synthesized and experimentally introduced into cells.[7,8] For fundamental scientific purposes, siRNAs are used as a tool to inhibit the production of proteins and follow the consequences of this inhibition to better understand functional aspects and cellular mechanisms of proteins.[9] In translational science, synthetic siRNAs are used to minimize production of mutant or deleterious proteins and correct for disease phenotypes.[10,11] The use of synthetic siRNAs are also central to this thesis. Here, siRNAs are applied to correct phenotypes of the bleeding disorder von Willebrand disease (VWD).

Von Willebrand disease and von Willebrand factor

VWD is the most common inherited bleeding disorder, named after Erik von Willebrand who first described the disease in 1926.[12] VWD is mainly associated with easy bruising, post-surgical or traumatic bleeding, and mucocutaneous bleeding (e.g. nose or menstrual bleeding).[13] The bleeding phenotype of patients varies from mild, with only bruises and sparse nose bleeding, to severe, with bleeding from the gastrointestinal tract or joints.[13,14] In the past, many patients with mild bleeding symptoms have gone unnoticed since bleeding also occurs in the normal population. However, better awareness of the disease among clinicians and developments in the diagnostic procedures for VWD have resulted in better recognition of the disease.[15] This is especially essential when a patient undergoes surgical or dental procedures.

VWD is caused by defects in the multimeric protein von Willebrand factor (VWF), a highly glycosylated protein produced by endothelial cells and megakaryocytes.[16] Upon expression in the endothelial cells, VWF undergoes several modifications before it is constitutively secreted or stored as multimers in the endothelial storage organelles, the Weibel-Palade bodies (WPBs).[17-20] Endothelial activation by vascular damage or inflammation leads to the release of VWF from the WPBs.[21] The released VWF multimers will unroll into ultra-large VWF strings, a process that is mediated by shear stress induced by vascular flow.[22,23] These ultra-large VWF strings expose their A1 domain, allowing platelet binding through the platelet glycoprotein Ibα (GPIbα) receptor followed by platelet activation and aggregation.[24] The elongated form of VWF also opens up the cleavage site for the metalloprotease ADAMTS13

(a disintegrin and metalloprotease with thrombospondin type motifs 13) in the A2 domain. Cleavage of VWF by ADAMTS13 degrades the ultra-large VWF multimers in smaller sized multimers that are present in a globular form in the circulation.[25,26] In the circulation, VWF is the binding partner of coagulation factor VIII (FVIII), thereby increasing the FVIII half-life.[27]

Three types of VWD can be distinguished, VWD types 1, 2 and 3 (an overview of the VWD types is summarized in Table 1).[28] VWD type 1 is associated with decreased VWF plasma levels (<30 IU/dL) and is mainly caused by dominant negative mutations in VWF.[28-30] These mutations are distributed throughout the whole protein and may lead to decreased secretion of VWF from the endothelial cells, or increased clearance of VWF from the circulation.[20] VWD type 2 is associated with qualitative defects in VWF and patients are categorized among VWD types 2A, 2B, 2M and 2N, depending on the specific defect.[28] VWD type 2A patients have abnormal plasma multimers which is the result of either defective intracellular multimerization or increased cleavage of VWF by ADAMTS13.[20] VWD type 2B patients have gain-of-function mutations in the GPIbα binding site in VWF and therefore platelets bind VWF even when VWF is in its inactive and globular form.[20] This VWF-platelet complex is cleared fast from the circulation which results in low platelet counts. In VWD type 2M, loss-of-function mutations in the GPIbα binding site in VWF results in decreased affinity of VWF to platelets. Also, patients with a collagen binding defect are categorized among VWD type 2M.[20,28,31] VWD types 2A, 2B and 2M are all caused by dominant negative mutations in specific domains of VWF. VWD type 2N patients have a decreased binding to coagulation FVIII caused by homozygous or compound heterozygous mutations in the FVIII binding site.[20,32] Lastly, VWD type 3 patients have a complete absence of plasma VWF, which is generally caused by homozygous or compound heterozygous null mutations.[20,28]

Treatment of von Willebrand disease

The choice of VWD treatment depends on the type and severity of VWD. Mild cases of VWD are generally treated on demand using desmopressin (DDAVP).[33] DDAVP mediates the release of VWF from the endothelial cells through the Vasopressin receptor 2.[34] This leads to a short-term increase of endogenously produced circulating VWF, which is usually sufficient to stop bleeding after trauma or prevent bleeding during a planned intervention. DDAVP is, however, not effective in VWD type 3 patients since they do not synthesize VWF at all. Furthermore, DDAVP is contra-indicated in VWD type 2B patients, because increased release of endogenous mutant VWF will capture circulating platelets and therefore induce thrombocytopenia.[35] In cases where DDAVP is not effective or contra-indicated, a second treatment option is the administration of VWF-containing concentrates.[36,37] VWF-containing concentrate is an exogenous source of VWF and is either plasma-derived or recombinant. Recombinant VWF is only recently approved by the FDA and superiority of recombinant VWF over plasma-derived VWF has yet to be proven.[38-41]

Table 1. Overview of the VWD types, detailing the phenotype, pathophysiological mechanism, main VWF defect, affected domains and main inheritance

VWD type:	Phenotype	Pathophysiological mechanism:	Main VWF defect:	Affected domains:	Main inheritance pattern:
1	Decreased VWF plasma levels	• Decreased synthesis of VWF	Null alleles	Any domain	Autosomal dominant
		• Decreased secretion of VWF from endothelial cells	Missense mutations	Any domain	
		• Increased clearance of VWF from circulation	Missense mutations	D3, A1, A3	
2A	Decreased high molecular weight VWF multimers	• Affected intracellular VWF dimerization/ multimerization	Missense mutations	Propeptide, D3, A1, A2, CK	Autosomal dominant
		• Increased cleavage of VWF by ADAMTS13	Missense mutations	A2	
2B	Increased VWF-platelet binding	• Mutant GPIbα binding site	Missense mutations	A1	Autosomal dominant
2M	Decreased VWF-platelet binding	• Mutant GPIbα binding site	Missense mutations	A1	Autosomal dominant
	Decreased VWF collagen binding	• Mutant collagen binding site	Missense mutations	A1, A3	
2N	Decreased VWF-FVIII binding	• Mutant factor VIII binding site	Missense mutations	D', D3	Autosomal recessive
3	Absence of VWF plasma levels	• No VWF synthesis	Null alleles	Any domain	Autosomal recessive

Although both treatment modalities are generally sufficient to stop bleeding, multiple limitations can be described. Firstly, repeated dosing of DDAVP has been associated with tachyphylaxis, and side-effects like flushing of the face, nausea and headaches.[42] Incidentally, also deep-vein thrombosis has been reported as adverse event to administration of VWF-containing concentrates.[43] Secondly, the effects of both treatments are short-term and, unless being on long-term prophylaxis of VWF-containing concentrates, patients are not protected for sudden bleeding which decreases the quality of life.[44,45] Then, mutant VWF remains to be produced and secreted into the circulation. This may lead to dangerously deep thrombocytopenia in VWD type 2B patients during stress-induced VWF release of mutant VWF.[46-48] Lastly, both DDAVP and VWF-containing concentrates cannot correct for phenotypes developed by long-term exposure to mutant VWF. An important example is the development of intestinal angiodysplasia, resulting in severe intestinal bleeding. A trait that is more common among VWD patients compared to the normal population.[49,50] Recent evidence showed that angiodysplasia is the result of defective VWF and cannot be corrected by DDAVP or VWF-containing concentrates.[9,51-54]

Since presence of mutant VWF is detrimental in several situations, we hypothesized that inhibition of mutant VWF in dominant negative VWD improves the function of VWF and ameliorate VWD phenotypes. In this thesis, we propose the use of allele-specific siRNAs to target mutant *VWF* alleles.

Small interfering RNAs

siRNAs are small double-stranded RNA molecules, containing a passenger (sense) strand and a guide (antisense) strand. siRNAs can intracellularly be generated by cleavage of long double-stranded RNA molecules by the endo-ribonuclease dicer, or can chemically be synthesized and experimentally introduced into cells.[8] In this thesis, we make use of synthetic siRNAs. Synthetic siRNAs generally have a length of 21 nucleotides and are asymmetric because of a two nucleotide overhang at the 3' end of both strands. When an siRNA enters a human cell, it associates with proteins from the RNA-induced silencing complex (RISC; Fig. 1).[55] Although siRNAs were discovered already 30 years ago, the precise mechanism of siRNA loading into RISC is not yet fully elucidated. In humans, it is known that RISC consists of at least Dicer, a double-stranded RNA binding protein, the endonuclease Argonaute 2 (Ago2) and the chaperone heat shock protein 90 (HSP90).[56] Dicer and the trans-activation responsive RNA binding protein (TRBP) bind the asymmetric siRNA and attract Ago2 and HSP90.[57] HSP90 facilitates direct binding of Ago2 to the siRNA, which is followed by unwinding of the duplex and passenger strand removal.[58] Ago2 then directs the guide strand to the targeted mRNA. The guide strand of the siRNA binds the target mRNA through Watson-Crick base pairing.[59] Ago2 then cleaves the mRNA, preventing translation of the mRNA into protein.[60]

siRNAs are able to downregulate genes when the siRNA sequence is in full complementarity with the mRNA sequence. Mismatches between the siRNA and the mRNA might prevent the cleavage ability.[59] This feature enables the use of siRNAs for allele-specific inhibition of mutant alleles in diseases caused by dominant negative mutations. This approach already proved successful *in vitro* as well as *in vivo* for several dominant negative diseases.[61-65] Two approaches of allele-specific inhibition based on single nucleotide mismatches might be applied. You could target the dominant negative mutation itself, because a patient is always heterozygous for that mutation.[61,63,64] Or you could target a single-nucleotide polymorphism (SNP) for which a patient is heterozygous.[63,65] In the latter, linkage analysis should be performed to identify which of the SNP-alleles is located on the diseased allele. Both approaches have been described in this thesis to target dominant negative VWD.

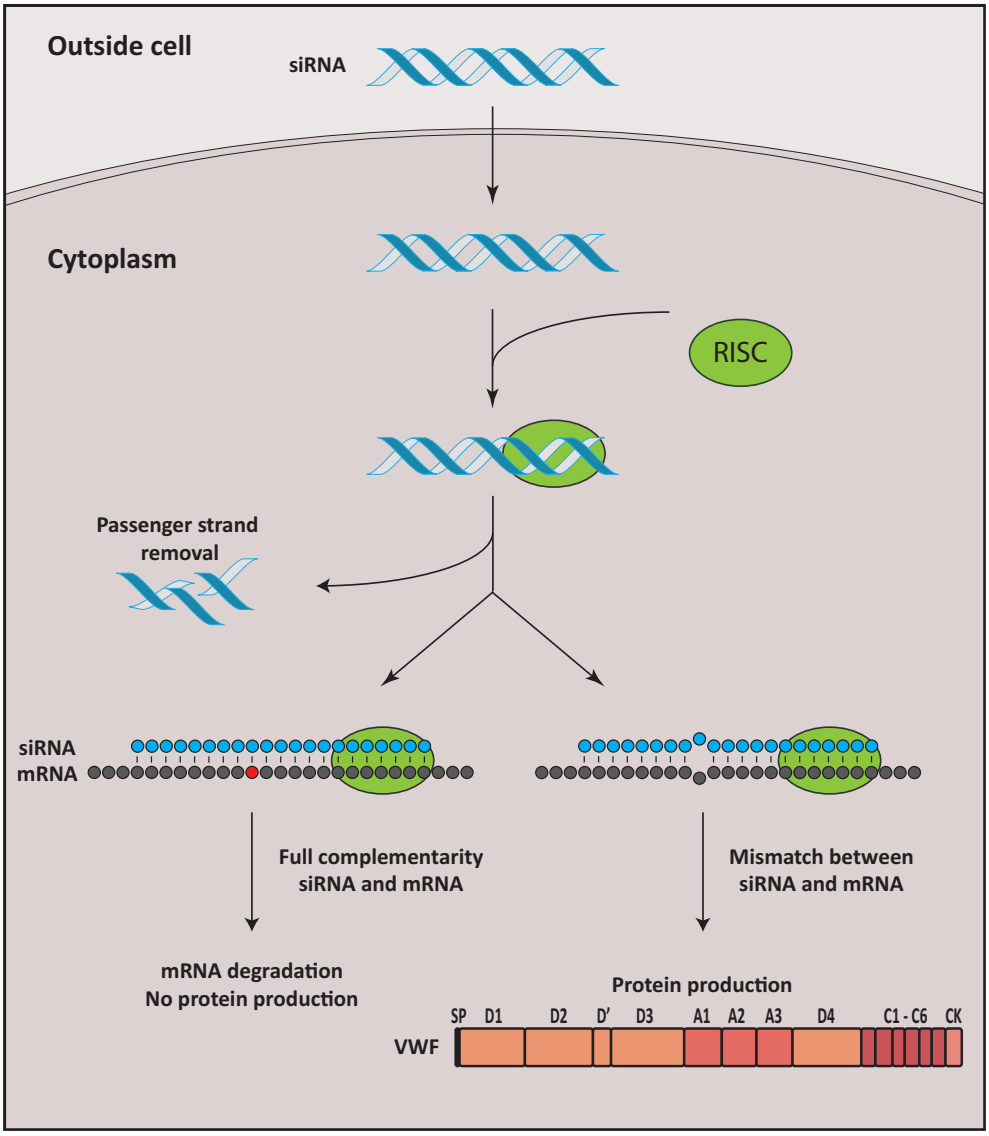

Figure 1. Allele-specific siRNA mechanism. After experimental introduction of a synthetic siRNA into the cell, it associates with the RNA-induced silencing complex (RISC). In humans, RISC consists of at least Dicer, Argonaute 2, the trans-activation responsive RNA binding protein (TRBP) and heat shock protein 90 (HSP90). RISC binding induces unwinding of the siRNA and passenger strand removal. The guide strand is then transported by RISC to the targeted mRNA. Full complementarity of the guide strand to the mRNA leads to mRNA degradation. A nucleotide mismatch between the siRNA and the mRNA might prevent cleavage of the mRNA, which results in normal translation of the mRNA into protein. This feature allows the use of siRNAs to inhibit single (mutant) alleles when a heterozygous variation within a gene is targeted. mRNA, messenger RNA; siRNA, small interfering RNA; VWF, von Willebrand factor

Outline of the thesis

This thesis brings together the fields of the hemorrhagic disorder VWD and RNA therapeutics. First, in **Chapter 2**, the developments in the diagnostic procedures for VWD are reviewed. This chapter explains the diagnostic tests that are required for correct diagnosis of VWD patients. Diagnosis of VWD can especially be challenging because of the heterogeneity of the disease.

The heterogeneity of VWD and the disease-causing protein VWF clearly emerges from **Chapter 3.** In this chapter, all VWF mutations that have been described in literature until early 2017 are reviewed and assessed with respect to pathogenic mechanisms. The disease-causing mechanisms of the mutations are explained throughout this chapter per VWD type. Also, an elaborative description of the VWF synthesis is included.

Most mutations that cause VWD are dominant negative mutations, meaning that only one of the two alleles is affected. This allows the hypothesis that allele-specific inhibition of the mutant *VWF* allele would improve VWF function and ameliorate VWD phenotypes. This hypothesis is tested in several cellular and animal models.

Chapter 4 describes the first *in vitro* proof of principle studies of allele-specific *VWF* inhibition in Human Embryonic Kidney 293 (HEK293) cells. In this study, we show that allele-specific siRNAs that target frequent SNPs in *VWF* can discriminate between two *VWF* alleles in VWF overexpressing HEK293 cells. When we targeted a SNP located on the same allele as a known *VWF* mutation causing a VWD type 2A phenotype, we were able to correct for the VWD type 2A multimerization defect.

HEK293 cells are a good model to prove the principle of allele-specific inhibition and to select a set of effective siRNAs. They, however, have no endogenous VWF expression. An endothelial VWD cell model is therefore necessary to extend the proof of principle to a more physiological relevant environment. Endothelial colony forming cells (ECFCs) are cultured endothelial cells that can be isolated from peripheral blood. These cells can be isolated from VWD patients and are therefore an ideal model to test the approach of allele-specific inhibition to correct for a patient's phenotype.

Chapter 5 describes the validation of the ECFCs as *ex vivo* model for VWD. In this study we standardized the experimental procedures of ECFCs. With this standardized experimental set-up, we show clear variations in cell morphologies and VWF expression. These variations are important to keep in mind when performing experiments with (patient-derived) ECFCs.

The effects of siRNA-mediated inhibition of *VWF* on healthy control ECFCs are described in **Chapter 6**. Interestingly, we observed that downregulation of the production of VWF in

healthy control ECFCs does affect the processing of VWF. In VWF downregulated ECFCs, a larger proportion of the produced VWF is secreted from the cells and this VWF has decreased high molecular weight VWF multimers.

Chapter 7 describes allele-specific inhibition of *VWF* in healthy control and patient-derived ECFCs. We show that the allele-specific siRNAs that were selected in Chapter 4, are also effective in ECFCs. Furthermore, we show the characterization and correction of ECFCs that have been isolated from a VWD type 2A patient.

In **Chapter 8**, we extend the proof of principle of allele-specific *VWF* inhibition to a heterozygous VWD type 2B mouse model. We use a mouse model based on hydrodynamic injection of *Vwf* cDNA in a VWF deficient mouse resulting in hepatic VWF expression, and show that an siRNA against mouse *Vwf* p.Val1316Met is able to improve the VWD type 2B platelet phenotype.

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