Chapter 7

General discussion and future perspectives

Venous thrombosis is a common disease, in which a blood clot (thrombus) is formed in a vein, obstructing the blood flow.[1;2] Thrombosis is considered to be a multifactorial disease in which both genetic and acquired risk factors are involved to cause disease.[3] Some acquired risk factors of venous thrombosis are associated with a hypercoagulable state,[4-6] which may be to a certain extent dependent on the dysregulation of gene expression in the liver, as the liver is the major organ that produces coagulation factors. Coagulation gene transcription can be modulated at different levels through hepatic transcription factors, co-regulatory or intermediate proteins, however, the exact contribution of these modulators to coagulation gene transcription is largely unknown. We aimed to study the mechanisms underlying hepatic coagulation gene transcription *in vivo* in mice to advance our understanding of why thrombotic risk coincides with abnormal coagulation profiles.

To investigate the exact contribution of hepatic transcription factors, co-regulatory or intermediated proteins in hepatic coagulation gene transcription, we employed small interfering RNA (siRNA) as a research tool to knockdown gene expression in mouse liver. The siRNA approach allowed us to efficiently knockdown gene expression with minimal methodology- and target-related secondary effects. Thus, we studied i) the direct role of transcription factors HNF4α and CEBPα in mouse coagulation gene transcription ii) the role of co-regulatory or intermediate protein FOXA1 in modulating the impact of nuclear hormone receptor on mouse coagulation gene transcription and iii) the role of two anticoagulation genes (PC and AT) for which loss of function in mice coincides with embryonic lethality.

Relatively rapid knockdown (i.e. two and five days post siRNA injection) of HNF4α and C/EBPα led to altered hepatic (coagulation) gene expression levels and indicated that these transcription factors are directly controlling multiple coagulation genes *in vivo*. In addition, we also showed that hepatic

FOXA1 is required for estrogen bound estrogen receptor α (ERα)-chromatin interactions in mouse liver as it is in (human) estrogen responsive cancer cells. Furthermore, we developed a novel thrombosis mouse model through siRNA-mediated transient depletion of two important anticoagulant factors, i.e. antithrombin and protein C, in normal adult mouse. The novel model achieved by this strategy is technically fast, simple, and easily reproducible.

In summary, we identified a number of genes that are potentially relevant to the etiology of venous thrombotic risk. Overall, the siRNA approach proved to be a valuable tool in studying hepatic genes that are suspected to play a role in the pathophysiology of venous thrombosis.

Hepatic genes contribute to regulate coagulation factor production

Three independent approaches point to an important role for HNF4α in the regulation of mouse coagulation gene expression (**chapter 2** and **3**): i) An *in vitro* approach in which HNF4α was silenced in mouse primary hepatocytes, ii) a conditional knockout mouse approach in which HNF4α was deleted in the liver starting at birth, iii) as well as a novel siRNA approach where HNF4α was silenced in livers of adult animals in a transient and acute fashion. All three approaches yielded essentially the same results i.e. HNF4α is a key gene in the regulation of large panel of mouse coagulation genes. We now speculate that HNF4α is also important to human coagulation, and that variation in HNF4α levels and activity will possibly modulate the risk for coagulation abnormalities such as bleeding and (venous) thrombosis. The potential clinical importance of HNF4α with respect to coagulation has been demonstrated in the past by our department for coagulation factor IX, where disruption of a binding site for HNF4α in the factor IX promoter results in hemophilia B Leyden.[7;8] The clinical importance of HNF4α with respect to other (metabolic) pathways controlled by HNF4α has also been demonstrated. Recent genome-wide

association studies demonstrated that single nucleotide polymorphisms at the HNF4α locus displayed highly significant associations with HDL cholesterol levels,[9] type 2 diabetes (T2DM) susceptibility,[10;11] maturity-onset diabetes of the young (MODY) susceptibility,[12;13] C-reactive protein levels[14] and ulcerative colitis susceptibility.[15-17] Mutations in the HNF4α binding site of the apolipoprotein CII (ApoCII) promoter are associated with hyperchylomicronaemia.[18] Thus, HNF4α can be of clinical importance for diseases related to its target genes. Whether genetic variation in the HNF4α gene is important for coagulation and thereby venous thrombotic disease is unknown. Therefore, we propose to determine whether single nucleotide polymorphisms in the gene encoding HNF4α gene are associated with altered hypercoagulability and increased venous thrombotic risk in large population-based case-control studies such as the MEGA (multiple environmental and genetic assessment of risk factors for venous thrombosis) study.

HNF4α, in general, controls constitutive expression of hepatic genes, but evidence is emerging that HNF4α itself also is subject to regulation. *In vitro* studies demonstrated that HNF4α activity is modulated by selective occupation of its receptor pocket with fatty acids.[19;20] Also changes in cellular lipids and monosaccharaides affect HNF4α activity and transcriptional activation at least as demonstrated for its target steroid hormone binding globulin (SHBG).[21] For SHBG, thyroid hormone was also shown to increase HNF4α-mediated transcription.[22] Dietary saturated fats increase HNF4α binding activity on ApoCIII promoter and enhanced ApoCIII mRNA levels.[23] Moreover, HNF4α can cross-talk with nuclear receptors since HNF4α directly binds to a number of nuclear hormone receptors including estrogen or thyroid hormone receptor by protein-protein interaction.[24;25] HNF4α compete to bind at the response elements with a number of other nuclear receptors including thyroid receptors and estrogen receptors. The possibility that HNF4α may compete with estrogen receptors

for binding to estrogen response elements has for example been described for the estrogen-dependent transcription of the coagulation factor XII gene.[26] Furthermore, the farnesyl X receptor (FXR), a family member of estrogen and thyroid hormone receptors, was able to competitively displace HNF4α from the promoters of ApoCIII[27] and ApoA.[28] Recently, genome-wide binding studies of FXR and HNF4α showed that about 50% of the binding sites of these transcription factors overlap in mouse liver, and FXR cooperates with HNF4α to modulate gene expression.[29] These studies suggest that HNF4α transcriptional activity is subject to modulation by metabolic and hormonal pathways with involvement of nuclear hormone receptors. In our mouse studies on estrogen, progesterone, pregnancy, obesity and thyroid hormone, we repeatedly observed changes in hepatic gene transcription of multiple HNF4α coagulation targets[5;6;30-32] (**chapter 2, 3** and **4**). We speculate that pregnancy, contraceptive pill use, hyperthyroidism, and obesity, all conditions that are associated with increased risk for venous thrombosis, affect HNF4α and thereby control of coagulation targets. Further studies are required to gain insight in the contribution of HNF4α under these conditions known to increase the risk of venous thrombosis.

In **chapter 4**, we have shown that silencing of hepatic FOXA1 had no or minimal impact on (estrogen-induced changes in) hepatic coagulation gene transcription. Surprisingly, this was in a setting where FOXA1 silencing strongly affected estrogen-binding to genomic target DNA, including coagulation factors. Although we were unable to provide an explanation for this discrepancy, this study demonstrated that FOXA1, like in a panel of hormone-responsive cancer cells,[33;34] is able to modulate estrogen receptor DNA-interaction also in normal liver tissue (**chapter 4**). Recently, genetic studies identified mutations within the FOXA1 gene (locus), and mutations in the FOXA1 binding sites were associated with increased risk of (hormone-related) breast and prostate cancers.[35-38] As estrogen hormone

use is associated with a hypercoagulable and prothrombotic state which likely originates from the activation of hepatic estrogen receptors, we propose, as for hormone-related cancers, to study whether genetic variation(s) in FOXA1 contributes to hormone-related venous thrombosis.

Apart from HNF4α, C/EBPα and FOXA1, we also investigated a fourth gene which may modulate hepatic gene coagulation transcription i.e. steroid receptor co-activator 1 (SRC1). This factor modulates the activity of a panel of nuclear receptors relevant to coagulation gene transcription i.e. estrogen receptor, thyroid receptor, and HNF4α.[39-43] Following siRNA-mediated silencing in mouse liver (70% reduction in transcript levels) no alterations in coagulation gene transcription were observed, nor did SRC1 silencing affect coagulation gene transcription induced by estrogens (own unpublished observation). Whether this implies that SRC1 is not important for blood coagulation gene transcription, or that compensatory mechanisms by related proteins SRC2 or SRC3 mask a potential role of SRC1, is not known. In general, the number of the genes investigated here possesses related or redundant genes that may provide compensation upon silencing. For example, it has been described that SRC2 partially compensates for SRC1 function.[44] It has been claimed that FOXA1 and FOXA2 have overlapping functions and compensate for each other in the development of multiple organs.[45-48] In **chapter 3** we observed C/EBPβ upregulation upon silencing of C/EBPα. FOXA1 silencing may have been compensated by the action of FOXA2 and/or FOXA3 in our studies, and this has to be evaluated further. For future studies of transcription factors and their modulators, we propose to include silencing of the gene of interest alone or in combination with silencing the genes that may provide a potential backup mechanism. Such studies may yield better insight in the role of the individual factors. Moreover, combined silencing is a feasible strategy as we demonstrated for antithrombin and protein C in **chapter 5**.

Besides transcription factors, their co-regulators and their pioneering factors, coagulation gene transcription may also be affected by genetic factors that modulate the availability of the substrates that activate transcription factors. Genetic variation in glucuronosyltransferase-2B7 (*UGT2B7*),[49] cytochrome P450 3A5 (*CYP3A5*)[50] and nuclear factor erythroid-derived 2-like 2 (*NFE2L2*)[51] showed association with an increased risk of venous thrombosis. These metabolic enzymes are thought to be (directly or indirectly) involved in modulating the inactivation and thereby circulating levels of biologically active estrogen. Silencing *UGT2B7, CYP3A5* and *NFE2L2* in livers of mice, using the siRNA strategy used throughout this dissertation, may help to further delineate the role of these genes in modulating the estrogen activation status and thereby their contribution to prothrombotic states in particular under exposure to estrogen. Furthermore, it may also aid to understand the contribution of these genes to many other diseases in which estrogens are thought to play a positive or negative role, such as arterial disease, osteoporosis, breast and endometrial cancer.

siRNA based thrombosis mouse model

Mice do not spontaneously develop venous thrombosis and, consequently, an ideal thrombosis mouse model does not exist because thrombosis always has to be induced by experimental means. A number of experimental models have been developed that rely on vessel wall damage due to photochemical injury,[52] mechanical trauma,[53;54] by applying an electric current[55;56] or ferric chloride.[57-59] In addition, models are available in which surgically induced stasis triggers thrombus formation.[60] Although these models may be helpful to study therapeutic strategies, by nature, they have limited value in studying the initiation of ‘spontaneous’ venous thrombosis. In addition to these experimental models, several genetic knockout or mutant mouse thrombosis models have been developed which

carry a prothrombotic state, such as Factor V Leiden (hemizygous) mice, thrombomodulin (TM) proline (substitution of Glu 387 with Pro: pro/pro) mutant mice,[61;62] mice deficient for anticoagulant protein C, protein S or antithrombin.[63-66] Protein S serves as cofactor for activated protein C. Activated protein C degrades factor Va and factor VIIIa, while antithrombin inhibits the active site of serine proteases such as thrombin and factor Xa (Figure 1). The level of thrombosis varies from subtle microvascular thrombosis characterized by development of mild fibrin deposition in different tissues (Factor V Leiden and TMpro/pro mice) to a severe thrombotic coagulopathy resulting embryonic or perinatal lethality (anticoagulant deficient mice). We attempted to develop a novel type of spontaneous thrombosis model based on reducing anticoagulation by means of siRNA (**chapter 5**). The spontaneous thrombosis observed following knockdown of antithrombin and protein C provides a technically simple, fast to perform, and easily reproducible model. However, at present the phenotype was always severe and it may be worthwhile to further improve this model especially with respect to tuning the degree of severity. We have observed that severity of the thrombotic phenotype was siRNA and/or dose dependent (**chapter 5**). A lower dose of antithrombin and protein C specific siRNA, and also when excluding protein C siRNA, produced a less severe thrombotic phenotype. Thus, we believe that the thrombotic phenotype can be further adjusted to a less severe and more easily modifiable phenotype. More studies are required regarding the initiation, dynamics and histology of thrombosis in this model, and to what extent these resemble thrombosis in humans. Recently, it has been demonstrated that platelets, neutrophils, and monocytes play an important role in the initiation of venous thrombosis in a damage-free model, based on flow restriction.[67] In addition, neutrophil extracellular traps (NETs) triggering FXII-dependent coagulation contributed to thrombus propagation

in this mouse model.[67]To what extent inflammatory cells, NETs and FXII also contribute to initiation and propagation in our siRNA-based thrombosis mouse model needs to be evaluated. Studies in prothrombotic mouse models (Factor V Leiden and TMpro/pro) demonstrated that exposure to human VT risk conditions (estrogen, obesity, thyroid hormone, and pregnancy) did not translate into a more manifest thrombotic phenotype (increased fibrin deposition, large vessel thrombosis).[31;68;69] Whether estrogen, obesity or pregnancy exacerbates the thrombotic phenotype induced by siRNA-induced inhibition of antithrombin and protein C is one of the issues to be addressed. More knowledge on the mechanisms involved and on the responsiveness to challenges will make clear whether the novel model forms a good alternative to existing venous thrombosis models, which will be of value to further investigating the pathophysiology of venous thrombosis, and contribute to the development of novel (bleeding-free) strategies for treatment and prevention of (recurrent) thrombosis.

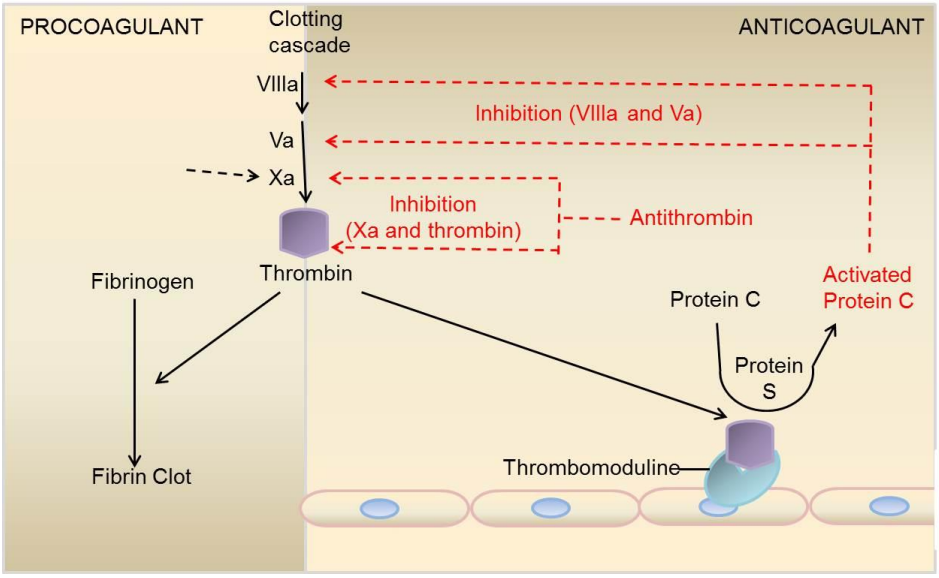

Figure 1: Anticoagulation by Protein C/Protein S and antithrombin pathway. Adapted from http://what-when-how.com/acp-medicine/hemostasis-and-its-regulation-part-1/

Synthetic siRNA as a tool to study gene function

Gene inactivation in mice via homologous recombination in mouse ES cells is the classical approach for exploring gene function[70;71] and has proven to be highly valuable in biomedical research.[72] Despite its value, this approach has also a number of limitations. Many 'knockout' mutations are embryonic lethal because of the vital role of the gene in development[63-66;73;74], thus precluding the analysis of the function of the gene in adult mice. In addition, full gene deletion may induce adaptative responses, which complicates functional analyses and potential masking of the primary role of the gene. Mice conditionally lacking the gene of interest i.e. in an inducible and/or tissue specific fashion may result in circumvention of embryonic lethality. Though, absence of an acute induction of deficiency may still induce adaptational response which masks primary gene function. Finally, production of these gene targeted mice (both conventional knockout and conditional mice) remains an extremely labor intensive and highly expensive procedure.

In the present dissertation, we demonstrated that RNA interference mediated by synthetic small interfering RNAs (siRNA) may provide an alternative to the gene targeting approach when studying gene function at the level of the liver, more specifically the hepatocyte. This siRNA-mediated gene knockdown demonstrated to be an effective strategy allowing transient but selective and robust knockdown of genes of interest in livers of adult mice, without any genomic manipulations.[75-79] Depending on the target gene, an acute and almost complete hepatic knockdown can be provided for a short period of time. The acute knockdown (within 2 days) strongly increases the possibility to detect primary gene function, while knockdown lasts sufficiently long to allow also monitoring of the induction of mechanisms compensating gene knockdown. Although one should be aware that in some cases such adaptations are very rapid followed by the siRNA-mediated drop in target gene expression. As an example, we

observed for C/EBPα that acute knockdown was rapidly followed by C/EBPβ upregulation (**chapter 3**). Thus, caution should be taken and siRNA may not be the 'holy grail' for studying hepatic gene function in absence of adaptation. In addition, the siRNA approach does not allow studies on the impact of long-term absence of the gene of interest (more than one week). Multiple siRNA injections may overcome this limitation.[80] Advantages of siRNA-mediated knockdown over the targeted knockout may further include the possibilities of studying gene dosage effects (by lowering the siRNA dose)[81] and the relative ease to knockdown multiple hepatic genes in one single siRNA administration (**chapter 5**). On the other hand, the siRNA approach also has disadvantages that are inherent to siRNA use. Although highly specific, off-target effects i.e. the unintended suppression of non-target genes, cannot be excluded and may hamper analysis of gene function.[82;83] Modification of nucleotides in siRNA, lower doses of siRNA, and advances in bioinformatics for designing of siRNAs may lead to reduction in off-target effects.[84-86] Furthermore, exogenous siRNA may compete for endogenous and critical RNAi components such as RNA induced silencing complex (RISC), thereby, altering the regulatory functions of some endogenous cellular microRNAs.[87] This may also hamper the analysis the function of the target gene. Competition of siRNA with cellular microRNAs for RISC may be overcome by using lower doses of siRNA.

We demonstrated in **chapter 3, 4** and **5** that lipid-based complexation of siRNAs allows efficient delivery of the siRNA to the hepatocyte. This approach is preferred when studying genes that are mainly active in this cell type such as coagulation genes, apoplipoprotein genes, drug detoxification genes, and genes involved in complement etc. However, studying genes that are expressed in other liver cell types i.e. Kupffer and endothelial cells cannot be investigated with the siRNA delivery vehicle used in this dissertation.

In general, many tissues and cell types other than liver/hepatocytes are difficult to target by siRNA. It has been demonstrated that use of dedicated chemicals may provide delivery of siRNA to organs other than the liver. For example, heart and kidney can be targeted by complexing siRNA with polyethylenimine (In vivo-jet PEI®), and when injected in the mouse jugular or tail vein this allows siRNA delivery to heart and kidney, respectively.[88;89] Furthermore, siRNA delivery to macrophages/microglial cells has been achieved through siRNA coupled to a cell specific targeting peptide. siRNA was coupled to rabies virus glycoprotein (RVG)-9dR peptide to target α7 subunit of the acetylcholine receptor (AchR) expressed by macrophages/microglial cells.[90] siRNA delivery to monocytes was achieved with nanoparticles encapsulated with fluorescent labelled siRNA. The nanoparticles were formed in a spontaneous vesicle procedure using C12-200 lipid, disteroylphosphatidyl choline, cholesterol, PEG-DMG and siRNA.[91] Systemic treatment with nanoparticles-encapsulated with siRNA resulted in reduction of target gene (monocytic chemokine receptor, CCR2) expression in monocytes.[91]

As is evident from the above, so far, a siRNA approach is suitable when addressing hepatic gene function *in vivo*. Future technological improvements enabling delivery of siRNA to other organs and cell types, in absence of off-target effects, and without interference of regulatory microRNA pathways, will increase the application of RNA interference in the analysis of gene function. Ultimately, these improvements may also open the possibility of therapeutic gene silencing.

siRNA based clinical therapy in near future

In this dissertation we have demonstrated that hepatic gene expression can be reduced in mouse liver using RNAi. The liver is an important organ responsible for many vital functions e.g. metabolism of lipids and carbohydrate, synthesis of a number of coagulation factors, hormone

production and detoxification.[92-95] Many diseases originate in the liver; including fatty liver, cirrhosis, cancer, hepatitis (viral infection), hypercholesterolemia, amyloidosis and hemophilia.[96-101] Moreover, several siRNA lipid-based vehicles are documented to target hepatic genes in rodents and non-human primates.[102;103] Therefore, siRNA-mediated targeting of hepatic genes may provide the first proof of concept for siRNA as a therapeutic strategy in general. siRNA-specificity, safety, increased circulation time, biodistribution, effectiveness, ease of production and manufacturing are important consideration for siRNA therapeutics. Moreover, siRNA as therapeutic drug should be patient friendly administrated. Although advancements in bioengineering and nanotechnology have already led to improved control of delivery and release of the siRNA but further developments are required to optimize siRNA technology as therapeutic. In this respect, a number of interesting clinical trials are going on.

Tekmira Pharmaceuticals corporation developed a stable nucleic acid lipid particle (SNALP) formulation as siRNA delivery vehicle. SNALP-siRNA formulations effectively silence gene(s) in several rodents and non-human primates.[102-104] SNALP formulated ApoB siRNA (TKM-ApoB) is in clinical trial for the treatment of hypercholesterolemia by targeting ApoB gene. This trial was terminated, as one of the patient (out of 23) with high doses experienced potential immune stimulation.[105] In September 2010, Tekmira Pharmaceuticals started another Phase I trial for the siRNA drug TKM-PKL_1 based on SNALP lipid nanoparticles for patients with solid tumors by targeting polo-like kinase 1 (PKL1).[106] This phase trial I showed that four out of nine dosed patients have achieved clinical benefit. A phase II clinical trial will be held in 2014.[107]

Alnylam Pharmaceuticals started a phase I trial with another siRNA therapy, (ALN-TTR01), for the treatment of transthyretin-mediated amyloidosis (ATTR). The results showed the safety and tolerability in ATTR

patients.[108] Development of improved and potent ALN-TTR02 is in progress. Another dual siRNA therapeutic (ALN-VSP02) is in Phase I clinical trial for the treatment of liver cancer and metastatic disease of liver. ALN-VSP02 is composed of two separate siRNAs specifically targeting mRNA of vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) mRNA. There are about 20 siRNA therapeutics targeting different genes such as RRM2, PKN3, BCR-ABL and PKN3 in ongoing Phase I clinical trials.

Hemophilia is hereditary genetic disorder with deficiencies of procoagulant factor VIII or factor IX, impairing hemostatic balance.[109;110] There is no cure for hemophilia, but a replacement therapy with regular infusion of deficient clotting factor is effective.[111;112] More recently, Alnylam Pharmaceuticals is developing siRNA therapeutics for the treatment of hemophilia and bleeding disorders by a hemostatic rebalancing approach i.e. knockdown of an anticoagulant factor (antithrombin). Alnylam Pharmaceuticals showed that potent and durable knockdown of antithrombin via ALN-AT3 resulted in normalized thrombin generation and improved hemostasis in hemophilia models.[113] However, a clinical trial has still to be started. Our data support this hemostatic rebalancing approach as we have shown that siRNA-mediated transient and strong knockdown of hepatic antithrombin is able to shift the coagulation balance and induces thrombosis in mouse (**chapter 5**). However, overdosage of antithrombin siRNA may have great impact on hemophilia patients; strong knockdown of antithrombin in wild type mouse tilted the hemostatic balance towards thrombosis (**chapter 5**). Therefore, siRNA doses and extend of antithrombin knockdown should be carefully monitored.

These clinical trials hold promise for the development of therapeutic gene silencing. Successful siRNA mediated knockdown has been demonstrated for liver of rodents and non-human primates. A number of clinical trials are ongoing to silence the siRNA in the liver. Therefore, success of clinical

trials targeting liver genes is an important test case for implication of siRNA based therapeutics. In this dissertation we have shown that RNAi is an efficient method to study gene function in liver, however, the question remains open, “Whether utility of siRNA-based drugs will become a reality as conventional therapeutic medicine in clinic or not?”

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