Characterization of mouse coagulation (regulatory) genes with use of RNAi

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Chapter 1

General introduction

Blood coagulation and thrombosis

Blood coagulation helps to protect the integrity of the vascular system following tissue injury. The process of blood clotting and then subsequent dissolution of clot, is termed hemostasis. Under physiological circumstances, hemostasis is a tightly regulated process that prevents excessive blood loss upon vascular injury.[1] Upon vascular damage, a hemostatic clot is formed at the site of injury, which is accomplished by a very rapid activation of pre-existing proteins. The coagulation cascade is activated, vessels constrict, platelets aggregate and form a platelet plug. Simultaneously, a series of enzymatic reactions occurs which converts fibrinogen to fibrin and ultimately results in the formation of a cross-linked fibrin-clot which stabilizes platelet thrombi, thus, repairing the vascular injury.[2-4] Anticoagulant factors limit activation of coagulation to the site of injury[1] and fibrinolysis gradually dissolves the fibrin clump at the site of the lesion, and thus results in a normalization of the blood circulation at this level (Figure 1).[5] The equilibrium between procoagulant, anticoagulant and fibrinolytic factors is tightly regulated to provide hemostasis at the site of the injury.[6] However, imbalance between procoagulant, anticoagulant and fibrinolytic factors can lead to conditions such as hypocoagulable or hypercoagulable states, which can subsequently result in bleeding or thrombosis, respectively.[7]

Thrombosis is unwanted thrombus formation in the vessels, obstructing the blood flow through the circulatory system. Thrombosis can occur both in arteries and veins which is associated with arterial (myocardial infarction and stroke) and venous disorders (venous thrombosis and pulmonary embolism), respectively.[8] This dissertation centers on the transcriptional regulation of coagulation factor genes to understand the mechanism(s) by which (risk) factors induce a hypercoagulable state and increase the risk for venous thrombosis.

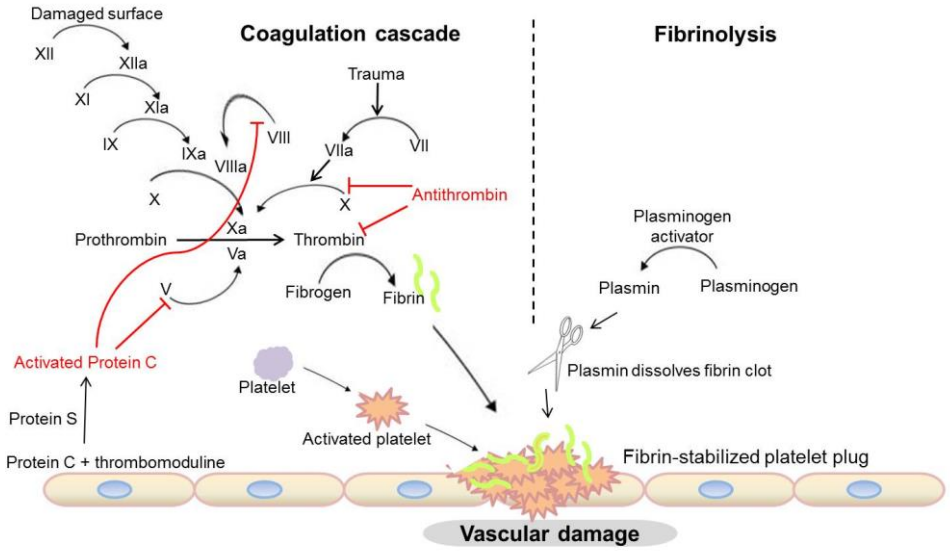

Figure 1: Schematic overview of coagulation cascade. Upon vascular injury, normalized blood circulation is guaranteed by controlled procoagulant, anticoagulant (red lines) and fibrinolysis pathways. (Licenced by GNU Free Documentation Licence, version 1.2) Adapted from: http://commons.wikimedia.org/wiki/File:Coagulation_full.svg

Venous thrombosis and venous thrombosis risk factors

The incidence of venous thrombosis is 2-3 per 1000 inhabitants per year, and has a steep age gradient e.g. an annual incidence of 1 per 100 individuals in the elderly may be attained.[9] As early as 1856, Virchow published the famous “Virchow’s triad”, which postulated that thrombosis is caused by at least one of the following factors: alterations in blood flow (stasis), alterations in the composition of the blood and damage to the vascular wall.[10] Virchow’s broad classification remains valid in the 21st century. Traditionally, it has been shown that the risks of venous thrombosis are mainly related to either changes in blood flow (stasis) or the composition of the blood (hypercoagulability), whereas changes in the vessel wall seems to be of lesser importance.[11] One of the common

classifications for risk factors of venous thrombosis is based on genetic and acquired risk factors that result in stasis and/or a hypercoagulable state.[9;11]

Inherited genetic risk factors are often related to abnormal blood composition resulting in hypercoagulability. Deficiencies of anticoagulant factors, such as antithrombin, protein C and protein S, and their association with clinical venous thrombosis were described in the 20th century.[12-15] Homozygous deficiencies of anticoagulant factors are extremely rare and lead to purpura fulminans (disseminated intravascular coagulation) in newborns, which is life-threatening.[16;17] In contrast, the prevalence of heterozygous deficiencies is more common in the general population at about 1 in 500-2000 individuals. Heterozygosity increases the risk for venous thrombosis about 10-fold.[18-20]

Not only the relatively rare deficiencies of natural anticoagulants are of importance, but also more common gain-of-function mutations of procoagulant factors are associated with increased risk of thrombosis. Factor V Leiden and prothrombin-20210A mutations are quite common (2% - 5% in Caucasians populations) and increase the risk of venous thrombosis 2- to 7-fold.[21-23] Another genetic risk factor for venous thrombosis is non-O blood group, which indirectly increases FVIII levels and thrombosis risk.[24-26] Moreover, several common polymorphisms were identified in or near the procoagulant factor XI (FXI) gene that lead to higher levels of FXI which are associated with thrombosis.[27;28] Mutation in fibrinogen gamma gene increased the risk of venous thrombosis 2.4-fold.[29]

Besides genetic risk factors, there are several acquired risk factors that increase the risk of venous thrombosis, such as prolonged immobilization, surgery, obesity, pregnancy, malignancies, increased thyroid hormone levels and use of female hormone (oral contraceptives or hormone replacement therapy).[11;30-37]

A number of these acquired risk factors are associated with a hypercoagulable state. Some (but not all) acquired risk factors, such as

hormones and obesity, coincide with changes in coagulation, probably by modulating gene transcription in the liver.[38-42] Since, the liver is the main organ for the production of plasma coagulation factors, a prothrombotic state may occur when altered transcription of the coagulation genes in the liver leads to an imbalance in the ratio of pro- and anti-coagulant proteins in the plasma. However, the mechanisms by which the aforementioned conditions modulate hepatic transcription of coagulation genes have not been completely elucidated. We hypothesized that hepatic transcription factors can become dysregulated, and that this dysregulation underlies the hypercoagulable state observed for a number of acquired conditions associated with an increased risk for venous thrombosis.

Transcriptional control of blood coagulation genes

Transcription of genes is mediated by transcription factors and their co-regulatory proteins. Transcription factors bind at or near the gene promoter in a sequence-specific manner to regulate gene expression, either as enhancer or as repressor. Under physiological conditions, transcription of genes encoding coagulation factors is regulated to produce sufficient amounts of coagulation factors in order to achieve hemostatic equilibrium and a clot-free blood circulation.

Plasma coagulation factors are mainly produced in the liver. A number of ChIP (chromatin immunoprecipitation) and *in vitro* functional studies provided evidence that constitutive expression of plasma coagulation factors is guaranteed by a panel of (liver-specific) transcription factors. For example, mutagenesis studies showed that hepatocyte nuclear factor (HNF)1α, HNF3β, and specificity protein Sp1/Sp3, are important in the regulation of prothrombin expression.[43] Functional promoter activity assays in the human liver-derived cell line HepG2 provided evidence of binding regions of HNF1α and HNF4α to the promoter of protein S.[44] Furthermore, clinical and *in vitro* mutagenesis studies showed that HNF4α and HNF6 are

major factors controlling FIX and protein C expression respectively.[45-47] Other *in vitro* studies reported the transcriptional regulation of coagulation factor X by GATA-4 and Sp1.[48] Amongst others, hepatic transcription factors HNF4α and CCAAT/enhancer-binding protein α (C/EBPα) are of particular interest. These factors are not only abundantly expressed in liver and are important for liver development and function in general, but they also suspected to contribute directly to the transcription of a number of coagulation genes. In addition, liganded estrogen and thyroid hormones receptors may interact with these factors[43;49-55] which suggests that these transcription factors potentially may play a role as an intermediate for increasing the risk of thrombosis associated with these hormones. Thus, several liver transcription factors have the capability to regulate coagulation gene expression. Nevertheless, the exact contribution of these transcription factors in regulating coagulation gene transcription *in vivo* is limited. Further (*in vivo*) studies are required to understand the exact contribution of liver-specific transcription factors in the development of a hypercoagulable state and consequent increased risk for developing VT.

A second mechanism that is used to regulate coagulation genes is mediated via transcription factors that play a relatively minor role in constitutive expression, but inducible through environmental or metabolic pathways. For instance, obesity is associated with hyperinsulinemia, a pro-inflammatory state, and increased transcription of the factor VIII, fibrinogen-gamma and PAI-1 genes that results from increased activity of the transcription factor nuclear factor kappaB (NF-κB).[56;57] Upon inflammation, tissue factor gene expression is induced in monocytes via activator protein 1 (AP-1), NF-κB and Sp1.[58] Thus, obesity and its related inflammation contribute to modulation of above mentioned coagulation gene expression in a multistep process resulting in a hypercoagulable state. Mouse and human studies have shown that the plasma coagulation profile was

affected upon nutritionally induced obesity,[42] but whether the observed effects can be explained by aforementioned mechanisms, is not known yet.

Nuclear hormone receptors are able to bind to hormone response elements in the promoter regions of target genes, and thereby are able to modulate gene transcription. *In vivo* studies have shown that use of exogenous estrogens (component of oral contraceptive pills or hormone replacement therapy which is acquired risk factors of venous thrombosis) modulates hepatic expression of several genes via estrogen receptor α.[38] *In vitro* functional and *in vivo* ChIP-on-chip studies have identified estrogen response elements in promoter regions of murine genes encoding fibrinogen, FII, FVII, FX, FXI, FXIII, α2-antiplasmin, heparin cofactor II, protein S, protein Z and plasminogen.[39;59] Studies of our own group indicated that estrogens are also able to modulate mouse hepatic gene expression of a large number of coagulation genes (encoding fibrinogen-γ, FII, FV, FVII, FX, FXI, FXII, antithrombin, protein C, protein Z, protein Z inhibitor and heparin cofactor II) via estrogen receptor α (ERα).[38] Thus, estrogen hormone modulates transcription of a number of coagulation genes via ERα, and altered transcription coincides with altered plasma activity levels of coagulation proteins.

Besides transcription factors, coregulatory proteins may have an impact on constitutive coagulation gene transcription. Transcription factors typically regulate gene expression by binding to DNA and recruiting multiple regulatory proteins (co-activators, co-repressors and pioneer factors) and RNA polymerase II to the site of transcription initiation. Co-regulators and pioneer factors alter the transactivation properties of (nuclear hormone) transcription factors by promoting several events, such as chromatin remodeling, recruitment of the pre-initiation complex and movement of RNA polymerase and thereby make the associated DNA more or less accessible for transcription. A coregulator group of steroid receptor co-activators (SRCs); SRC1 (NCOA1; nuclear receptor co-activator 1), SRC-2 (NCOA2)

and SRC-3 (NCOA3) are reported to interact with ERα and to alter ERα transcriptional properties.[60;61] These coregulators possess histone acetyltransferase activity and lead to relaxing of the chromatin, which then allows the basal transcription machinery and other factors to access the promoter of the target genes.[62] Amongst others, FOXA1 (Hepatocyte Nuclear Factor 3α), a pioneer factor that has recently been extensively studied in prostrate, breast and non-breast cancer cells (osteosarcoma cell line), is able to maintain euchromatic conditions (loosely coiled and transcriptionally active DNA) at specific *cis*-regulatory elements.[63;64] Several reports have described that FOXA1 participates in several gene regulatory events with nuclear hormone receptors including ERα.[65-67] Co-regulators may not affect the basal transcriptional activity on its own, as they modulate gene expression together with transcription factors, although co-regulators may contain an autonomous activation function. Thus, nuclear hormone receptors together with their coregulatory proteins may form intermediates leading to changes in coagulation gene transcription, subsequent changes in the plasma coagulation profile, thereby contributing to a prothrombotic condition.

From the above it follows that there are several levels at which coagulation gene expression can be modulated. The exact contribution of transcription factors, co-regulatory and intermediate proteins in regulating the transcription of the coagulation genes is largely unknown. Studying the mechanisms underlying hepatic coagulation gene transcription in more detail may increase our understanding why a thrombotic risk condition coincides with abnormal coagulation profiles. Experimental model systems in which transcriptional regulation of coagulation is conserved are required to obtain such insights.

Model systems to study transcriptional control of coagulation genes

In vitro and ex vivo models

Different model systems to study the control of coagulation genes are available, ranging from *in vitro* and *ex vivo* to *in vivo* (mouse) models. Most of the coagulation factors are produced by the liver (hepatocytes), and several hepatocytic cell lines are available such as HepG2 (human liver-derived cell line), Hepa 1-6 and Hepa-1c1c7 (both mouse liver-derived cell lines). Many *in vitro* studies aimed at understanding the transcriptional control of coagulation genes have been performed in the HepG2 cell line via functional promoter activity assays and mutagenesis studies.[43;44;48] A disadvantage of hepatocytic cell lines is that they poorly express coagulation factors. We have also observed that both mouse hepatocyte cell lines (Hepa 1-6 and Hepa1c1c7) poorly express coagulation genes as compared to mouse liver, which limits the use of hepatocyte cell lines for the study of coagulation gene transcription. Primary (mouse) hepatocyte cultures form an *ex vivo* model system that expresses coagulation factors may be useful to study coagulation control. The disadvantage of primary hepatocyte cultures is that expression of coagulation factors only remains stable for a limited period after isolation; thereafter expression of a number of genes including coagulation genes start decreasing. Reduction in gene expression may be due to the experimental procedures like hepatocyte isolation and artificial culturing conditions. Therefore, primary hepatocyte cultures also are of limited usefulness. Shortcomings of *in vitro* and *ex vivo* cultures can be circumvented by using animal models. These may provide a model system with physiological expression and regulation of hepatic coagulation genes closest to the human situation, including all necessary known and unknown, but essential factors, relevant to coagulation gene transcriptional regulation.

In vivo

As compared to *in vitro* and *ex vivo* cultures, *in vivo* models allow studying in the intact physiological system. Among the animal models available, the

mouse is the most used mammalian model organism in biomedical research. About 85% of genes are conserved between humans and mice and the animal's small size facilitates large scale, high throughput studies making this animal model a cost-efficient.[68] Furthermore, liver-specific gene regulation is to a large extent conserved between humans and mice.[53] Regarding coagulation, murine coagulation factors and their functions in the hemostatic system are comparable to their human counterparts.[69;70] Hence, mice may form a suitable *in vivo* model organism to study the regulation of hepatic coagulation genes and how thrombotic risk conditions coincide with hypercoagulable state.

Although the use of mice in hemostasis research may be advantageous, there are also limitations; they do not develop spontaneous thrombosis thereby hampering the impact of altered regulation on clinical end points, i.e. thrombosis. However, several genetic and experimental thrombosis models are available which showed a thrombotic phenotype and used in thrombosis studies.[71;72] Hence, results from mice models offer the possibility to study mechanistic insight into biological processes and allow for example to study gene function in the *in vivo* situation. Therefore, the mouse was used as a model system for studies described in this dissertation.

Gene function in *in vivo* models

Gene knockout

A powerful method to analyze gene function and gene regulation is by making ‘a knockout’ mouse of your (regulatory) gene of interest. Gene inactivation is obtained via homologous recombination in mouse ES cells[73-76]. With this method an essential part of the gene of interest is completely removed or destroyed. Although this approach provides the possibility to study gene function *in vivo*, there are some limitations. For example, (unexpected) compensatory and/or counter regulatory mechanisms might

become activated when a gene is missing, and this complicates the interpretation of the contribution of the gene under study. Moreover, products of many genes may be essential for normal function and embryonic development, and inactivating such genes might induce gross morphological or physiological abnormalities or may be fetal. As an example, conventional knockout mice of two important hepatic transcription factors, HNF4α and C/EBPα, were embryonic or neonatal lethal, respectively.[77;78] This can be explained by the fact that these transcription factors regulate genes that are also required for embryonic development.[54;55;79]

Not only deletion of transcription factors but also deletion of coagulation genes like antithrombin, protein C and protein S appeared embryonic lethal due to severe coagulopathy.[80-83]

Conditional gene knockout using the Cre-loxP system[84-87] allows to knockout genes of interest in specific tissues, and at a particular time point, circumventing early lethality. Cre recombinase, is a site specific integrase that catalyzes recombination between recognition (loxP) sites that encompass the target gene. Promoter choice is important in controlling the site and timing of Cre recombinase expression. To generate conditional knockout mice, a mouse line containing modified Cre recombinase under the control of an inducible tissue specific and/or time-dependent promoter (which stayed non-functional until inducing agent is produced or administrated) is crossed with a mouse line in which the target gene is flanked by loxP sites (flox).[86] The resulting offspring containing both Cre transgene and (loxP) flanked target gene, Cre recombinase will excise the targeted gene segment, through promoter dependent Cre-mediated recombination in specific tissue.[86] For example, HNF4α and C/EBPα liver-specific conditional knockout mice were generated by using albumin-Cre transgenic mice to direct recombination in hepatocytes.[84;85] The efficiency of liver specific albumin-Cre is dependent on mouse age. At the time of

birth recombination is about 40% and completes by the age of six weeks. Therefore, HNF4α and C/EBPα liver-specific conditional knockout mice are viable and have proven to be a very useful tool to study gene function in development, several metabolic pathways, and to some extent their role in coagulation gene transcription.[52;55;84;88]

Despite the many advantages of conditional knockout animals, even these remain imperfect. For example, HNF4α liver-specific conditional knockout mouse model, (based on albumin-Cre promoter) requires four to six weeks for significant and complete deletion of HNF4α in mouse liver, thereby possibly missing the primary role of this gene because unwanted compensatory mechanisms are activated in the meantime. Moreover, HNF4α liver-specific conditional knockout mouse liver had not only visibly gray, molted appearance but also pathological lesions were observed in liver.[84] Thus, prolonged deletion of HNF4α may change the liver physiology and alter expression of other transcription factors, thereby leading to misinterpretation of a direct role of gene in mouse. Alternative to albumin-Cre, fast and rapid hepatic deletion of (part of) the target gene can be achieved by means of adenovirus-mediated hepatic delivery of the required Cre-recombinase or by inducible liver-specific gene disruption based on the Mx1-Cre system.[89-91] The Mx1-Cre system utilizes the inducible Mx1 promoter to control the expression of the Cre recombinase transgene and can be transiently activated to high levels of transcription by introducing interferon and thus delete the target gene rapidly. However, experimental factors are concomitant with acute inflammation or a burst of interferon application[89-91] which may affect the organ physiology and may lead to possible misinterpretation of the direct role of specific targeted gene in gene regulation. Furthermore, even with the emergence of high-throughput gene knockout methods (conventional and conditional), the production of gene-targeted mice remains time-consuming and labor-intensive. New

strategies may be useful not only to improve time- and labor-efficiency but also to achieve a fast and acute *in vivo* inactivation of the gene of interest.

Gene knockdown – RNA interference (RNAi)

For a number of research questions, RNA interference (RNAi) may serve as an alternative to the (conditional) knockout approach. RNAi is a natural process that mammalian cells use to reduce the expression of specific gene(s) at the post-transcriptional level and this mechanism was discovered by Fire, Mello and colleagues in 1998.[92] RNAi is a process by which gene expression is reduced/regulated by endogenous double stranded RNA (dsRNA) in which one strand is partially complementary to a section of a gene's mRNA.[93-95] The dsRNA precursor is cleaved in the cytoplasm by an enzyme of the Dicer family into effector microRNAs (miRNAs), which are single-stranded 21-23 nucleotide long RNA molecules.[96-99] Next, miRNAs are assembled into an RNA-induced silencing complex (RISC).[99] Finally, the antisense miRNA strand guides the RISCs to the complementary mRNA, where they cleave and destroy the cognate RNA and inhibit protein synthesis.[100] It has been reported that miRNAs incorporated into RISC are recycled, thus down regulating gene expression with only a small amount of miRNA.[101]

Methods of mediating the RNAi effect for experimental purposes, involve synthetic small interfering RNA (siRNA) and short hairpin RNA (shRNA). shRNA can be produced inside the cell from a DNA construct, it is first processed by Dicer to small interfering RNA and continues along the RNAi pathway via RISC to silence the gene. siRNA and shRNA are exogenous, synthetic and double-stranded RNA, whereas miRNA on the other hand is an endogenous, natural and single-stranded RNA, often derived from the intronic region of a gene. However, processing and behavior of both miRNA and siRNA is same. siRNAs (manufactured by chemical synthesis) and shRNAs (clones produced in laboratory) can be targeted to any desired

mRNA. siRNA can be used to knock down gene expression selectively without any genomic manipulations *in vitro* and *in vivo.*[102] The gene silencing via siRNA in a sequence specific manner has caused it to attract much attention for application in biosciences and medicine.[102-105] Although living up to their apparent promise there are some challenges like siRNA delivery, knockdown efficiency and off-target effects, which are addressed later in this chapter.

RNAi as an alternative approach to study gene function

Expression of a gene(s) can be modulated with RNAi *in vivo* to study gene function. RNAi can be achieved by means of shRNA or siRNA. shRNA contains a hairpin like stem-loop that resembles intermediates of the endogenous miRNA pathway.[106;107] shRNA can be incorporated into an adenoviral vector, and upon infection with these vectors *in vivo* efficient knockdown of gene expression can be achieved in mouse liver.[108] Knockdown of a target gene may persist for weeks, as the viral shRNA can be continuously synthesized by the host cell. However, viral vectors containing shRNA raised concerns about hepatotoxicity in mice.[109;110] Additionally, shRNA may interfere with gene regulation mediated by endogenous miRNAs.

RNAi can also be achieved by means of 20-22 nucleotide long double stranded synthetic siRNA that specifically mediates degradation of mRNAs transcribed from a certain gene.[111] Use of synthetic siRNA has advantages over shRNA; first, they are easy to synthesize and different chemical modifications can be introduced with ease to increase the stability in biological systems. Second, siRNAs are less likely to interfere with gene regulation mediated by endogenous miRNAs, because siRNA enters the RNAi pathway later.[112] However, siRNAs exhibit low membrane permeability, because of a poor hydrophobicity and they are sensitive to nuclease degradation. This poses challenges for their delivery and uptake

in cells, particularly when systemic administration is necessary. Therefore, suitable delivery systems are required for *in vivo* applications. Only hepatic siRNA delivery systems with or without vehicles will be briefly described below, as the aim of this dissertation is to study the transcriptional control of (blood) coagulation factors solely at the level of the (mouse) liver.

Targeting siRNA to the liver

The biggest challenge of using siRNA as a tool to study gene function *in vivo* is the difficulty of siRNA delivery (e.g. delivery to the liver).[113;114] In order to overcome delivery barriers, several strategies with non-viral systems have been developed, from naked siRNA (without any vehicle) to formulation of siRNA with lipids or synthetic polymers to deliver siRNA to the liver.[115;116] Each delivery system comes with its own *in vivo* application (system) with varying margins of efficiency.

Naked siRNA delivery without vehicle

Several research groups used hydrodynamic delivery of naked siRNA to achieve gene silencing in the mouse liver. Two parameters are critical for hydrodynamic delivery, injection volume (9% body weight by volume) and speed of intravenous tail vein injection.[117-119] This method of siRNA delivery to mouse hepatocytes results in enlargement of the liver fenestra and the generation of transient pores in the plasma membrane of hepatocytes. Hydrodynamic delivery enables siRNA to get into the cells in highly vascularized organs, such as the liver, and to efficiently knockdown endogenous gene expression in mice.[120] However, in contrast to what has been reported, in our hands the typical gene knockdown achieved by this method is only 20-40%[121] [and own unpublished observations]. Furthermore, hydrodynamic injection requires a high level of technical skill and causes transient liver damage, characterized by cell swelling, some necrosis and modestly elevated serum liver transaminase levels.[122]

siRNA delivery with vehicles

A variety of vehicles have been documented to deliver siRNA, including peptide-mediated delivery and complexed glycan encapsulated siRNA particles, liposomes (lipid bilayer vesicle) formulations e.g. lipoplexes (complexes of cationic lipids and nucleic acids), or lipid nanoparticles (LNPs) typically containing ionizable amino acids.[116;123;124] However, liposome-based vehicles can cause inflammatory toxicity through activation of the innate immune response.[125] LNPs are advanced siRNA delivery systems. These are colloidal carriers that consist of a lipid matrix which is composed of physiological components, thereby overcoming the innate immune responses. Alternatively, siRNAs can be linked to ligands of cell surface receptors for endocytosis to target the delivery of the siRNAs to cells that bear a specific receptor. For example, complexing siRNA with polyethyleneimine-hyaluronic acid (PEI-HA) induces efficient gene silencing in the tissues expressing hyaluronic acid receptors (e.g. liver).[126] These strategies deliver siRNAs via receptor-mediated endocytosis, although the trafficking of siRNAs into and within cells has not been well studied.

Another delivery strategy is to complex siRNA with lipidoids. Lipidoids belong to the most mature class of lipid-based systemic delivery vehicles and are synthesized by conjugate addition of an acrylate or acrylamide to primary or secondary amines of lipids.[127] These lipidoids resulted in effective hepatic siRNA delivery and gene knockdown *in vivo.*[127;128] Three years ago, a user friendly lipidoid-like, liposome-based siRNA delivery tool became available through Life Technologies under the name of Invivofectamine® 2.0 which delivers siRNA to mouse liver. Invivofectamine may contain physiological components as Life Technologies claimed minimal toxicity using this delivery vehicle without releasing the full specifications. Single intravenous tail vein injection of siRNA complexed using Invivofectamine efficiently delivers siRNA to the liver and can result in efficient knockdown of hepatic gene expression. A single dose of 3-7mg

siRNA per kilogram body weight was claimed to provide an efficient knockdown of the hepatic target gene. Moreover, Invivofectamine as a delivery vehicle offers the opportunity to knockdown multiple hepatic target genes (up to four) simultaneously.[129]

Scope of the dissertation

The scope of this dissertation was to study the mechanisms by which hepatic coagulation gene transcription is regulated, in order to increase our understanding of how thrombotic risks conditions coincide with hypercoagulable state. We investigated the contribution of transcription factors, co-regulatory and intermediate proteins (HNF4α, C/EBPα and FOXA1) in hepatic transcriptional regulation of coagulation factors. In the present dissertation, we employed synthetic siRNAs in mice to further detail our knowledge on hepatic genes that are part of (anti)coagulation genes and/or are suspected to contribute in regulation of coagulation factor production.

Outline of the dissertation

We proposed that synthetic siRNA could serve as a tool to study the exact contribution of hepatic transcription factors, co-regulatory and intermediate proteins in regulating the transcription of coagulation genes. In **chapter 2**, we successfully used synthetic siRNAs to target HNF4α in mouse primary hepatocytes, determined the impact on mouse coagulation gene transcription, and compared the findings to those observed in livers of mice conditionally lacking HNF4α. Synthetic siRNA appeared a relatively simple and fast approach as compared to a (conditional) knockout approach to study gene function, which encouraged us to use synthetic siRNAs in an *in vivo* setting.

Next, we set out to develop a siRNA-based approach to efficiently knockdown hepatic transcription factors *in vivo* in mice. Successful and fast

knockdown of hepatic gene expression of our first target genes, i.e. HNF4α and C/EBPα, was achieved with synthetic siRNAs complexed with Invivofectamine 2.0® (**chapter 3**). We demonstrated the direct role of these two important hepatic transcription factors *in vivo* in controlling gene regulation of blood coagulation factors. We also compared the hepatic gene expression of HNF4α knockdown and liver-specific conditional HNF4α knockout mice to explore the direct targets of HNF4α and validation of usefulness of siRNA technology. These studies showed that HNF4α and C/EBPα are important transcription factor in controlling hepatic gene transcription of a number of coagulation genes. Moreover, these studies revealed that our approach is suitable as an alternative to knockout approach when studying hepatic mouse genes.

siRNA-mediated knockdown of genes may not only be useful to study hepatic transcription factors but it can also be a useful method to study intermediate co-regulatory proteins such as FOXA1 in mouse liver. FOXA1 is a major determinant of the estrogen response in breast and non-breast cancer cells. The study described in **chapter 4** was aimed to investigate the role of FOXA1 as an intermediate factor in estrogen hormone response in regulating (more specifically coagulation) gene transcription in mouse liver. siRNA-mediated knockdown of FOXA1 modulated estrogen-induced ERα chromatin interaction in mouse liver.

Next, the siRNA approach was used to explore the function of anticoagulant genes for which the knockout appeared embryonic lethal. In **chapter 5** we described the successful hepatic knockdown of antithrombin and protein C (alone or in combination). This shed new light on the function of these anticoagulants and provided a novel mouse model featuring a spontaneous (venous) thrombotic phenotype.

In **chapter 6**, we investigated the regulation of coagulation factor 11 (*F11*) gene cluster which includes *F11*, *Klkb1* and *Cyp4v3*. Regulation of mouse

F11 gene cluster was analyzed under several metabolic conditions, and included studies on the role of HNF4α by using our *in vivo* siRNA approach. In **chapter 7**, all the generated experimental data are discussed in a broader context and future perspectives are described. In the last and final chapter, **chapter 8**, the main findings of studies described in this dissertation are summarized.

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