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

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Cover illustration: cross section (hematoxyline/eosin staining) of a mouse liver depleted with protein C and antithrombin.

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If you can dream it, you can do it.

(Walt Disney, 1901-1966)

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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.

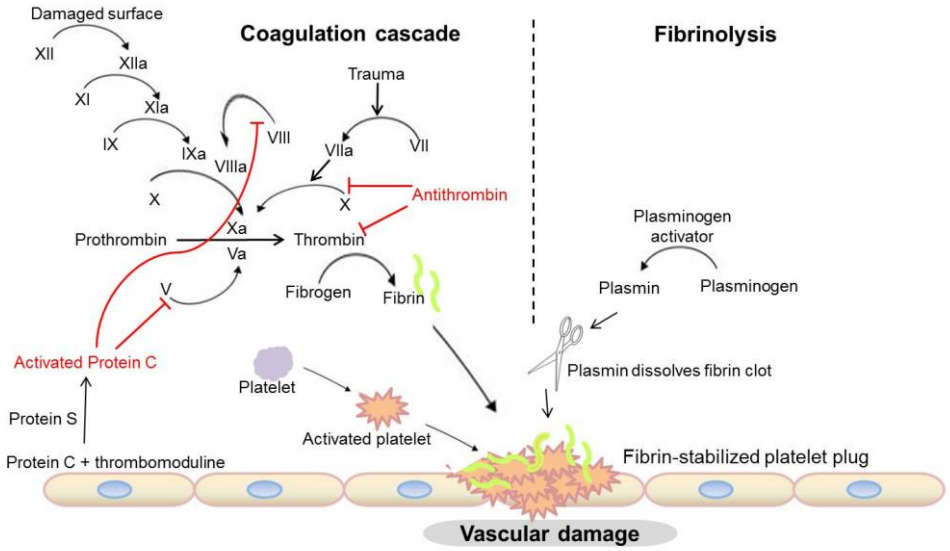

Figure 1: Schematic overview of coagulation cascade. Upon vascular injury, normalized blood circulation is guaranteed by controlled procoagulant, anticoagulant (red lines) and fibrinolysis pathways. (Licenced by GNU Free Documentation Licence, version 1.2) Adapted from: http://commons.wikimedia.org/wiki/File:Coagulation_full.svg

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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Chapter 2

The Role of Hepatocyte Nuclear Factor 4a in Regulating Mouse Hepatic Anticoagulation and Fibrinolysis Gene Transcript Levels

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Dear editors,

Hepatocyte Nuclear Factor 4α (HNF4α) is a transcription factor belonging to the steroid/thyroid hormone nuclear receptor superfamily that is expressed at high levels in the liver, and is suspected to be critical for the synthesis of a large number of hepatic coagulation factors. *In vitro* gene promoter studies identified functional HNF4α binding sites near the genes encoding human procoagulant factors (F) II (F2), VII (F7), VIII (F8), IX (F9), X (F10), XI (F11), XII (F12),[1-7] and anticoagulant factors protein S (PROS1), protein Z (PROZ) and antithrombin III (SERPINC1).[8-10] The *in vivo* importance of HNF4α in regulating hepatic transcription of procoagulant genes was established by examining hepatic mRNA levels from liver-specific HNF4α-null mice.[11] Northern blot analysis demonstrated the impact of *Hnf4*α deletion on expression of *F5, F9, F11, F12* and *F13b*, whereas no effect was observed on expression of *F2*, *F7*, *F8* and *F10*.[11] Although *in vitro* studies suggest that HNF4α may also be critical in regulating transcription of hepatic factors involved in anticoagulation and fibrinolysis,[8-10] here its importance *in vivo* remains presently unknown. Because the HNF4α-null mouse studies demonstrated that *in vitro* promoter analysis studies are not a reliable indicator of a crucial transcriptional role of HNF4α,[11] we decided to study its involvement in the expression of anticoagulation and fibrinolysis genes *in vivo* as well.

The role of HNF4α was studied using liver mRNA samples from 45-days-old male liver specific HNF4α-null mice (HNF4α-floxed/floxed with albumin-Cre; KO) and control mice (HNF4α-floxed/floxed without albumin-Cre; FLOX),[11] by real time RT-PCR on the relevant genes (for methods see)[12]).

We first demonstrated that livers of KO mice were devoid of *Hnf4*α transcript levels and Hnf4α protein levels (Figure 1A and 1C, respectively), we subsequently confirmed strong reductions in procoagulant *F5* and *F12* transcript levels (-64 and -95%, respectively). Regarding anticoagulation

and fibrinolysis genes, male KO mice displayed markedly reduced transcript levels of hepatic protein C inhibitor (*Serpina5,* -100%), protein Z (*Proz,* -97%), and α2-antiplasmin (*Serpinf2,* -77%) (Figure1A). Moderate reductions were observed for Protein Z inhibitor (*Serpina10*, -34%) (Figure 1A). Protein C (*Proc*, -28%), protein S (*Pros1,* -15%), plasminogen (*Plg*, -5%) and antithrombin (*Serpinc1*, +13%) transcript levels were not significantly affected by the hepatic loss of *Hnf4*α, whereas hepatic mRNA levels of Heparin Cofactor II (*Serpind1,* +75%) were significantly increased in KO as compared to FLOX mice (Figure 1A). Hepatic tissue-type plasminogen activator (*Plat*), α2-macroglobulin (*A2m)* and plasminogen activator inhibitor-1 (*Serpine1*) mRNA levels were too low to detect.

Differences in hepatic anticoagulation and fibrinolysis gene transcript levels upon liver specific *Hnf4*α deletion in age-matched littermate female KO and FLOX mice were essentially the same as observed for the males (data not shown).

We also investigated whether the observed HNF4α-mediated changes in anticoagulation and fibrinolysis gene transcript levels *in vivo* would replicate in normal mouse primary hepatocytes following acute *Hnf4*α siRNA-mediated knockdown - thus excluding delayed and/or indirect effects of *Hnf4*α deletion on transcription of coagulation genes. Hepatocytes were isolated from male C57Black/6J mice through retrograde collagenase perfusion[13] and cells were cultured in collagen S-coated 6-well plates in complete DMEM. Twenty four hours after isolation, cells (at ~85% confluency) were transfected with *Hnf4*α-specific (si*Hnf4*α) or control siRNA (siScrambled). Forty-eight hours after siRNA transfection, *Hnf4*α transcript levels in si*Hnf4*α transfected hepatocytes were decreased by 80% as compared to siScrambled transfected cells (Figure 1B), which was paralleled by a comparable reduction in Hnf4α protein levels (Figure 1D).

Reduction in hepatocyte Hnf4α expression coincided with significant reductions in transcript levels of the anticoagulant genes *Serpina5* (-45%),

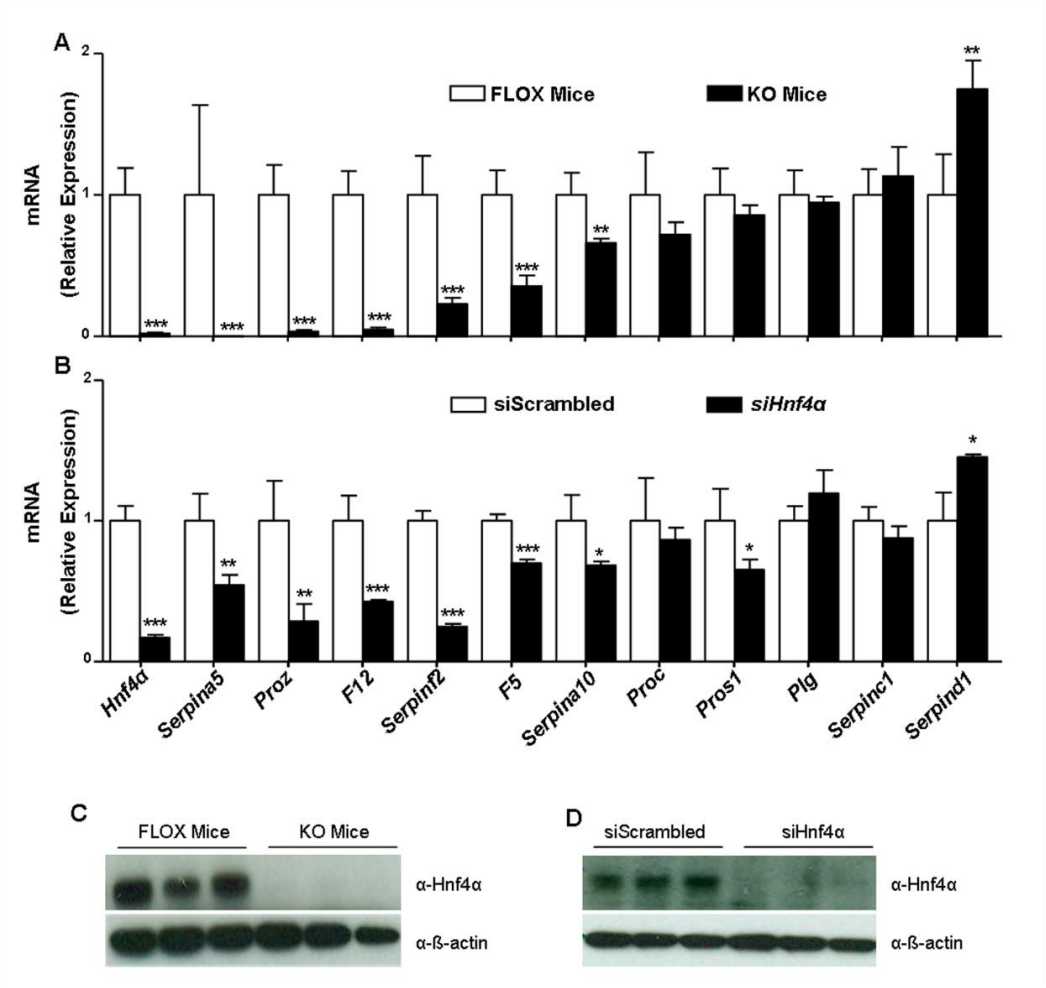

Figure 1: Anticoagulation and fibrinolysis gene transcript levels in livers of HNF4α-null mice and cultured mouse primary hepatocytes transfected with *Hnf4*α-specific siRNA.

Anticoagulation and fibrinolysis gene transcript levels in **(A)** liver tissue from 45-day-old male liver specific *Hnf4*α-null (KO, ■) and control (FLOX, □) mice, and **(B)** in mouse primary hepatocytes 48 hours after transfection with 100nM *Hnf4*α-specific siRNA (■) or control siRNA siScrambled (□) (Dharmacon Lafayette, CO, USA J-065463-07, target sequence GCG AAC UCC UUC UGG AUG A or D001810-01, siScrambled sequence UGG UUU ACA UGU CGA CUA AUU respectively) using the Dharmafect Duo transfection reagent® (Dharmacon, T-2010-03). Quantitative real-time PCR was performed as described (12). Gene-specific primers used were described before (12) with the exception of those for *Serpina5* (forward; TCT GGC ATT ACT GAC CAT ACC AA, reverse; GAC TCT TCA ACC

TCC ATC ATG GA). β-actin was used as internal control for quantification and normalization. The _Ct values of the individual samples were related to the mean _Ct of the reference group (FLOX or siScrambled). On the x-axis the coagulation and fibrinolysis genes are ranked according to the magnitude of effects observed *in vivo*. Data are expressed as mean ± standard error of the mean. For the *in vivo* studies, 8 animals per group were used. For the *in vitro* studies, a representative of 3 individual experiments is shown, each performed in triplicate. Hnf4α protein levels in KO and FLOX mice **(C)** and mouse primary hepatocytes which were transfected with si*Hnf4*α or siScrambled **(D)** siRNA as determined by Western blot analysis on liver or cell homogenates (15µg total protein lysate) using anti-Hnf4α antibody (C-19, sc- 6556, Santa Cruz biotech., Santa Cruz, USA). ß-actin was used as protein loading control.
In vivo and *in vitro* data were statistically analysed using Mann-Witney *U*-test and unpaired t-test, respectively. P-values < 0.05 were regarded as statistically significant. *P<0.05, **P<0.01, ***P<0.001

Proz (-71%), *Serpina10* (-31%), *Pros1* (-35%), the fibrinolysis related gene *Serpinf2* (-75%) and control procoagulant genes *F5* (-30%), *F12* (-57%) (Figure 1B). *Proc, Serpinc1,* and *Plg* transcript levels were not significantly affected by si*Hnf4*α , while mRNA levels of *Serpind1* significantly increased (+46%) in si*Hnf4*α transfected cells as compared to siScrambled cells (Figure 1B). Thus, the HNF4α-mediated changes in anticoagulation and fibrinolysis gene transcript levels in livers of 45-days-old HNF4α-null mice were largely reproduced in wild type mouse primary hepatocytes rapidly following siRNA-mediated *Hnf4*α knockdown.

In conclusion, our *in vivo* data, point to an important role for HNF4α in regulating hepatic transcription of mouse *Serpina5*, *Proz*, *Serpinf2*, *Serpina10* and *Serpind1.* Our *in vitro* data support these findings and suggest that this control is direct and does not involve intermediates. Thus, hepatic HNF4α is critical for regulation of a number of hepatic procoagulant genes[11] as well as anticoagulant and fibrinolysis genes, showing HNF4α importance in blood coagulation homeostasis.

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Chapter 3

Modulation of Mouse Coagulation Gene Transcription Following Acute *In Vivo* Delivery of Synthetic Small Interfering RNAs Targeting HNF4α and C/EBPα

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SUMMARY

Hepatocyte nuclear factor 4α (HNF4α) and CCAAT/enhancer-binding protein α (C/EBPα) are important for the transcriptional control of coagulation factors. To determine *in vivo* the direct role of HNF4α and C/EBPα in control of genes encoding coagulation factors, a synthetic small interfering (si)RNA approach was used that enabled strong reduction of mouse hepatic HNF4α and C/EBPα under conditions that minimized target-related secondary effects. For both HNF4α and C/EBPα, intravenous injection of specific synthetic siRNAs (siHNF4α and siC/EBPα) resulted in more than 75% reduction in their liver transcript and protein levels 2 days post-injection. For siHNF4α, this coincided with marked and significantly reduced transcript levels of the coagulation genes *Hrg*, *Proz*, *Serpina5*, *F11*, *F12*, *F13b*, *Serpinf2*, *F5*, and *F9* (in order of magnitude of effect) as compared to levels in control siRNA injected animals. Significant decreases in HNF4α target gene mRNA levels were also observed at 5 days post-siRNA injection, despite a limited level of HNF4α knockdown at this time point. Compared to HNF4α, C/EBPα knockdown had a modest impact on genes encoding coagulation factors. A strong reduction in C/EBPα transcript and protein levels resulted in significantly affected transcript levels of the control genes *Pck1* and *Fasn* and a modest downregulation for coagulation genes *Fba*, *Fbg* and *F5*. *F5* and *F11* were the sole coagulation genes that were significantly affected upon prolonged (5 day) C/EBPα knockdown. We conclude that in the mouse, HNF4α has a direct and essential regulatory role for multiple hepatic coagulation genes, while a role for C/EBPα is more restricted. In addition, this study demonstrates that synthetic siRNA provides a simple and fast means for determining liver transcription factor involvement *in vivo*.

INTRODUCTION

Hepatocyte nuclear factor 4α (HNF4α) and CCAAT/enhancer-binding protein α (C/EBPα) are two distinct transcription factors that are of key importance in controlling many genes specifically expressed in the liver and associated with a number of critical metabolic pathways.[1-3] In addition, both transcription factors are claimed to be critical for control of the hepatic genes encoding proteins in the coagulation pathway. For HNF4α, first evidence came from *in vitro* gene promoter studies for human coagulation genes. Functional HNF4α binding sites were identified near the genes encoding factor (F) II (F2), VII (F7), VIII (F8), IX (F9), X (F10), XI (F11), XII (F12), protein S (PROS1), protein Z (PROZ) and antithrombin III (SERPINC1).[4-13]

The *in vivo* importance of HNF4α in regulating hepatic transcription of coagulation genes described in studies using hepatocyte-specific HNF4α knockout mice.[14;15] *Hnf4a* disruption affected expression of factor (F) *F5*, *F9*, *F11*, *F12*, *F13b*, protein C inhibitor (*Serpina5*), protein Z (*Proz*), α2-antiplasmin (*Serpinf2*), protein Z inhibitor (*Serpina10*), and heparin Cofactor II (*Serpind1*), whereas no effects were observed for *F2*, *F7*, *F8*, *F10*, protein S (*Pros1*) and antithrombin (*Serpinc1*).[14;15] For C/EBPα, studies on the relevance to coagulation are limited. A recent CHIPseq study determined the genome-wide occupancy of C/EBPα in the livers of human, mouse, dog, opossum, and chicken. Fibrinogen A (FGA) and F2 were identified among 32 genes located near 35 C/EBPα binding events that were conserved among these five vertebrates.[16] In the mouse genome, *Fga* and *F2*, C/EBPα binding sites were 45 and 64 bp from the transcription start site, respectively. However, it should be emphasized that binding does not necessarily correlate with functional activity in controlling gene transcription. Furthermore, carriers of hemophilia B Leiden have a causal mutation in a C/EBPα binding site in F9 promoter,[17] and in line, liver-specific C/EBPα-null mice display reduced hepatic expression of *F9.*[17;18]

Finally, *in vitro* studies demonstrated requirement of C/EBPα for F8 expression, but this involved nonhepatic inflamed cells.[19] Whether other coagulation genes other than FGA, F2, F9 and F8 are regulated by C/EBPα is unknown.

The study of the *in vivo* roles of HNF4α and C/EBPα in control of gene transcription in liver employed a conditional gene knockout approach because conventional gene knockouts for HNF4α and C/EBPα were embryonic lethal.[20;21] In general, a conditional gene knockout approach does not allow a rapid significant deletion of the gene of interest *in vivo* without challenging liver physiology; fast adenovirus-mediated hepatic delivery of the required Cre- recombinase does allow rapid hepatic disruption of 'floxed' alleles but is concomitant with adenovirus-related acute hepatic inflammation.[22;23] Similarly, inducible liver-specific gene disruption based on the MX1-Cre transgene requires a burst of circulating interferon to evoke the necessary activation of MX1-Cre.[24] Meaningful studies of the role of hepatic transcription factors can therefore only start after weaning of the adenovirus or interferon effects. At that time, transcription factor deletion may already have induced (secondary) changes in liver physiology and compensatory changes in expression of other hepatic transcription factors. It may lead to a possible misinterpretation of the direct role of a given transcription factor in gene regulation. Hence, the current observations regarding the role of HNF4α and C/EBPα in regulating coagulation gene transcription obtained in adult mice lacking HNF4α and C/EBPα from birth on (Cre recombinase under control of the albumin promoter),[25] following Cre supplied by means of adenovirus[26] or the MX1-Cre transgene[27] may be in part secondary to changes in liver physiology and changes in expression of other hepatic transcription factors. This may, explain the unexpected transcriptional increase of numerous hepatically expressed genes including the coagulation gene *Serpind1* and absence of effects for the *in vitro* identified

targets *F2*, *F7*, *F8*, *F10*, *Pros1* and *Serpinc1* in livers from mice lacking HNF4α from birth.[14;15]

Recently, lipid-based reagents became available that allow efficient delivery of synthetic small interfering (si) RNAs to livers of adult mice following systemic injection.[28] Thus, transient knockdown of target gene expression can be achieved rapidly (within two days post siRNA delivery) and does not involve changes in liver physiology as a result of harsh methodology. In the present study, we used this *in vivo* siRNA approach to rapidly reduce HNF4α and C/EBPα expression in mouse livers and to determine the impact of these two distinct transcription factors on hepatic coagulation gene transcription.

MATERIAL AND METHODS

siRNA screening and validation

Pre-designed siRNAs for mouse HNF4α and C/EBPα mRNAs were purchased from Ambion Applied Biosystems, Carlsbad, California, USA (Ambion Silencer® Pre-designed for HNF4α; catalogue numbers 67633 (#1), 67634 (#2) and 67635 (#3) with sense sequences GGC AGA UGA UCG AAC AGA UUU, CCA AUG UCA UUG UUG CUA AUU, and AGA GGU CCA UGG UGU UUA AUU, respectively and for mouse C/EBPα; catalogue numbers 63853 (#1), 63854 (#2), 63855 (#3) with sense sequences GCA AAA AUG UGC CUU GAU AUU, AAA GCU GAG UUG UGA GUU AUU, and ACU CAA AAC UCG CUC CUU UUU, respectively). Ambion's siNEG (catalogue number 4404020) was used as control siRNA. This negative control siRNA was selected using a modified blast to account for short sequence length and demonstrated to exclude significant homology to any known gene targets in RefSeq and MirBase (more detailed documentation on this negative control siRNA is available on the manufacturer's website).[29] Hepatocytes from female C57Black6/J mice (Charles River, Maastricht, The Netherlands) were isolated through

retrograde collagenase perfusion and cultured in collagen-coated dishes exactly as previously described.[30] Twenty four hours after isolation 10^6 cells were transfected with the siRNA (final concentration 0.3, 3 or 30 nM) using the Dharmafect Duo transfection reagent® (Dharmacon, T-2010-03) according to the manufacturer's protocol. Twenty-four hours after transfection, levels of HNF4α and C/EBPα mRNAs were determined by quantitative real-time PCR (see below). Sequences from siRNAs yielding maximal reduction of transcript levels at a siRNA concentration of 3 nM were considered for use in *in vivo* studies.

Gene knockdown in mouse liver

Control siNEG, siHNF4α and siC/EBPα (Ambion In-Vivo-Ready for catalogue numbers 4404020, 67633 and 63855, respectively) were complexed with Invivofectamine® 2.0 Reagent (Invitrogen, Life technologies Corporation, USA) exactly according to the manufacturer's protocol. Subsequently, female C57Black/6J mice (weighing 17–19 gram) were intravenously injected via the tail vein with 200µl complexed siRNA at a dose of approximately 7 mg of siRNA per kg body weight (in total 54 animals, 18 animals per siRNA). At two and five days post siRNA injection, animals (9 mice per siRNA for each time point) were anesthetized by a subcutaneous injection with a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg) and atropine (125 µg/kg) after which the abdomen was opened by a midline incision and a blood sample on sodium citrate (final concentration 0.32%) was drawn from the inferior caval vein. Plasma was obtained by centrifugation and stored at -80°C until use. Liver was isolated and weighed, and liver left lobule was snap-frozen for mRNA and protein analyses and stored at -80°C until use. All mice were housed under a 12-h light/dark cycle, with standard chow diet and drinking water provided ad libitum. All experimental procedures were approved by the animal welfare committee of the Leiden University (under registration # 11005).

For HNF4α, as a reference, liver materials from 45-day old female HNF4α-null mice with a liver-specific deletion of exons 4 and 5 of the *Hnf4a* gene (HNF4α-floxed/floxed with albumin-Cre; KO) or control mice (HNF4α-floxed/floxed without albumin-Cre; FLOX)[25] were used.

RNA isolation and real-time RT-PCR

Liver samples (20-30 mg) were homogenized in RNAzol (Tel-Test) and RNA isolation and cDNA synthesis was performed as previously described.[31] Gene-specific quantitative real-time PCR (QPCR) primers for *Pck1*, *Fasn*, *Scd1*, *Lgp* and *Gys2* have been described previously:[32] all other gene specific QPCR primers were designed with the Primer Express software (Applied Biosystems). QPCR primer sequences are presented in Table 1. QPCR was performed on the ABI Prism 7900 HT Fast Real-Time PCR System from Applied Biosystems and data were analysed using the accompanying Sequence Detection System software. The comparative threshold cycle method with β-actin as internal control was used for quantification and normalization. siNEG-injected animals were set as a reference and the ΔCt values of the individual samples were related to the mean ΔCt of the reference group.

Immunoblotting

Frozen liver material (10-20 mg) was grounded, liver protein (15 µg) was denatured, separated on 8-10% Novex® Tri-Glycine gels, and immunoblotted using a goat polyclonal IgG against human HNF4α (sc-6556, Santa Cruz Biotechnology) or rabbit polyclonal against rat C/EBPα (sc61, Santa Cruz Biotechnology). ß-actin was detected using rabbit polyclonal against human ß-actin (Ab8227, Abcam) and served as protein loading control. The antibodies are reactive to mouse HNF4α, C/EBPα (both p42 and p30 unit) and ß-actin, respectively. Bound IgG was detected using horseradish peroxidase-labeled anti-goat (sc-2020, Santa Cruz) or

anti-rabbit (172-1019, Bio Rad) IgG followed by enhanced chemiluminescence system (Amersham Pharmacia Biotech) to detect peroxidase activity.

Table 1: QPCR primer sequences

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
Actb	AGGTCATCACTATTGGCAACGA	CCAAGAAGGAAGGCTGGAAAA
Apoc2	AAGATGACTCGGGCAGCCT	CAGAGGTCCAGTAACTTAAGAGGGA
Apoa4	CAGCTGACCCCATACATCCAG	TCATCGAGGTGTGCAGGTTG
Cd36	GTTCTTCCAGCCAATGCCTTT	ATGTCTAGCACACCATAAGATGTACAGTT
Cebpa	ATAGACATCAGCGCCTACATCGA	GTCGGCTGTGCTGGAAGAG
Cebpb	CGGGACTGACGCAACACA	CCGCAGGAACATCTTTAAGTGATTA
Cyp4v3	CTCTCCGAGTTTTCCCATCTGT	TTGTAACCGCCCACTTCACA
F2	GGACGCTGAGAAGGGTATCG	CCCCACACAGCAGCTCTTG
F5	CATGGAAACCTTACCGACAGAAA	CATGTGCCCCTTGGTATTGC
F7	CGTCTGCTTCTGCCTCTTAGA	ATTTGCACAGATCAGCTGCTCAT
F9	GCAAAACCGGGTCAAATCC	ACCTCCACAGAATGCCTCAATT
F10	GTGGCCGGGAATGCAA	AACCCTTCATTGTCTTCGTTAATGA
F11	GAAGGATACGTGCAAGGGAGATT	CAAGTGCCAGACCCCATTGT
F12	GGGCTTCTCCTCCATCACCTA	GCAACTGTTGGTTTTGCTTTCC
F13a1	GATGTCCTGGCCAAACAAAG	GGCAGCACCTCGGACCTT
F13b	GACACTGCCCCCTGAGTGTGTTGAAA	AACAACCACACCGTTTGCTATG
Fasn	CCCTTGATGAAGAGGGATCA	ACTCCACAGGTGGGAACAAG
Fga	TTCTGCTCTGATGATGACTGGAA	GGCTTCGTCAATCAACCCTTT
Fgg	TGCTGCCTGCTTTTACTGTTCTC	TCTAGGATGCAACAGTTATCTCTGGTA
Gys2	GACACTGAGCAGGGCTTTTC	GGGCCTGGGATACTTAAAGC
Hnf4a	AGAGGTTCTGTCCCAGCAGATC	CGTCTGTGATGTTGGCAATC
Hrg	AAAACGGATAATGGTGACTTTGC	TCCCCTCCTCTCGCTCTTATAA
Lgp	CCAGAGTGCTCTACCCCAAT	CCACAAAGTACTCCTGTTTCAGC
Pck1	CTGGCACCTCAGTGAAGACA	TCGATGCCTTCCCAGTAAAC
Plg	TGACATTGCCCTGCTGGAAAC	CAGACAAGCTGGAATGACTTTATCC
Proc	GCGTGGAGGGCACCAA	CCCTGCGTCGCAGATCAT

Pros1	GGTGGCATCCCAGATATTTCC	CACTTCCATGCAGCCACTGT
Proz	GCAGCCAGAGTCAGCCTAGCT	CACGCCGGCACAGAAGTC
Scarb1	GCCAGGAGAAATGCTTTTTGTT	GGCCTGAATGGCCTCCTTA
Scd1	AGCTGGTGATGTTCCAGAGG	GTGGGCAGGATGAAGCAC
Serpina5	TCTGGCATTACTGACCATACCAA	GACTCTTCAACCTCCATCATGGA
Serpina10	TGGCCCTGGAGGACTACTTG	CCATTTTCCTGGTTTTCATATTCTG
Serpinc1	TGGGCCTCATTGATCTCTTCA	CCTGCCTCCAGCAACGAT
Serpind1	GAATGGCAATATGTCAGGCATCT	CACTGTGATGGTACTTTGGTGCTT
Serpinf2	TTCTCCTCAACGCCATCCA	GGTGAGGCTCGGGTCAAAC

Plasma analyses

Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin (total and conjugated) levels were determined using routine clinical chemistry assays. Global coagulability of the plasma was determined by measuring the prothrombin time (PT, Simple Simon PT system, Zafena), and the activated partial thromboplastin time (APTT) by using the STA Neoplastin Plus reagent (Roche) on the STart 4 analyzer (Diagnostica Stago). Plasma F5 activity was analyzed by using chromogenic substrate conversion[33] and activity levels of factor (F) F11 and F12 were measured with APTT-based assays.[31] Plasma fibrinogen antigen levels were assessed with a commercial murine ELISA kit from Affinity Biologicals. In plasma assays of individual coagulation factors, pooled normal mouse plasma was used to generate standard curves and the control siNEG-injected group was set as a reference (100%).

Statistical analyses

Data were analysed with the GraphPad Instat software. Statistical differences between control siNEG and siHNF4α or siC/EBPα groups were evaluated using a Mann-Whitney Rank sum test (animal studies) or

Student's t-test (hepatocyte studies). A *P*-value of <0.05 was considered to be statistically significant.

RESULTS

siRNA screening and validation

To select an effective siRNA, mouse primary hepatocytes were transfected with three different predesigned synthetic siRNAs for HNF4α, and C/EBPα mRNAs. For HNF4α, all three siRNAs were highly and equally effective with over 95% reduction of *Hnf4a* transcript levels at a final concentration of 30 nM (Figure 1A). siRNA duplex #3 had the highest level of knockdown of *Hnf4a* transcript levels at the 3 nM concentration and was selected for use in *in vivo* experiments. For C/EBPα, all three siRNAs tested were highly effective, albeit that the levels of reduction (~80%, Figure 1B) were somewhat lower as compared to the siRNAs for HNF4α. For C/EBPα siRNA duplex #1 was selected, being the most effective at 3 nM. The siHNF4α #3, siC/EBPα #1 and the siNEG were subjected to large-scale preparation in the lipid-based *in vivo* transfection reagent optimized for hepatic delivery, and injected intravenously into C57Black/6J female mice. Two days after injection, siHNF4α and siC/EBPα produced a more than 75% reduction in liver *Hnf4a* and *Cebpa* transcript levels (Figure 2A and 2B, respectively), as well as strongly reduced liver HNF4α and C/EBPα protein levels (Figure 2C). For siC/EBPα this strong level of knockdown of liver C/EBPα mRNA and protein persisted for at least five days (Figure 2B and 2D). For siHNF4α, HNF4α mRNA and protein levels remained reduced at five days, but, as quantified for the transcript, only at a mean level of 36% (Figure 2A and 2D). This relatively quick return to normal HNF4α levels for siHNF4α was also observed in a second independent experiment and could not be overcome by a repeat intravenous siHNF4α injection (7 mg/kg) at day 2 after the first injection (data not shown).

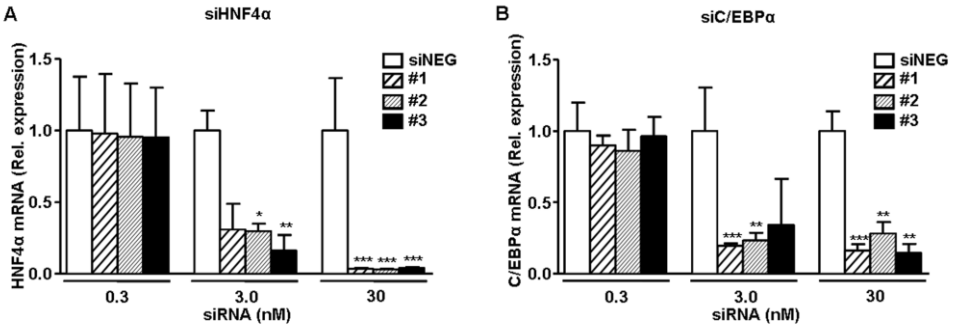

Figure 1. Screening and validation of siRNAs in mouse primary hepatocytes.

Screening of siRNA in mouse primary hepatocytes 24 hours after transfection with 0.3, 3 and 30nM of three *Hnf4a*-specific (#1-3, panel A), three *Cebpa* -specific siRNAs (#1-3, panel B) or a control siRNA (siNEG, panel A and B, open bars). *Hnf4a* and *Cebpa* transcript levels were determined by quantitative real-time PCR. β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group (siNEG). On the x-axis siRNA concentrations are indicated. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (difference 2^ΔΔCt+SEM and 2^ΔΔCt-SEM). Individual experiments were performed in triplicate. Data were statistically analyzed using the Student's t-test. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001

During the 5-day observation period siHNF4α and siC/EBPα did not affect mouse body weight and liver weight as compared to siNEG injected animals or uninjected controls. Gross pathological analysis revealed no abnormalities. Plasma bilirubin, ALT, AST and ALP in siNEG injected animals were comparable to those observed for uninjected controls (Table 2) as expected.[28] As compared to the siNEG injected controls, at two days post siRNA injection, ALP levels were significantly increased by siHNF4α, and at 2 days significantly reduced by siC/EBPα. At 2 and 5 days, plasma bilirubin, AST, ALT levels in siHNF4α and siC/EBPα were below the limits of detection and comparable to siNEG injected animals.

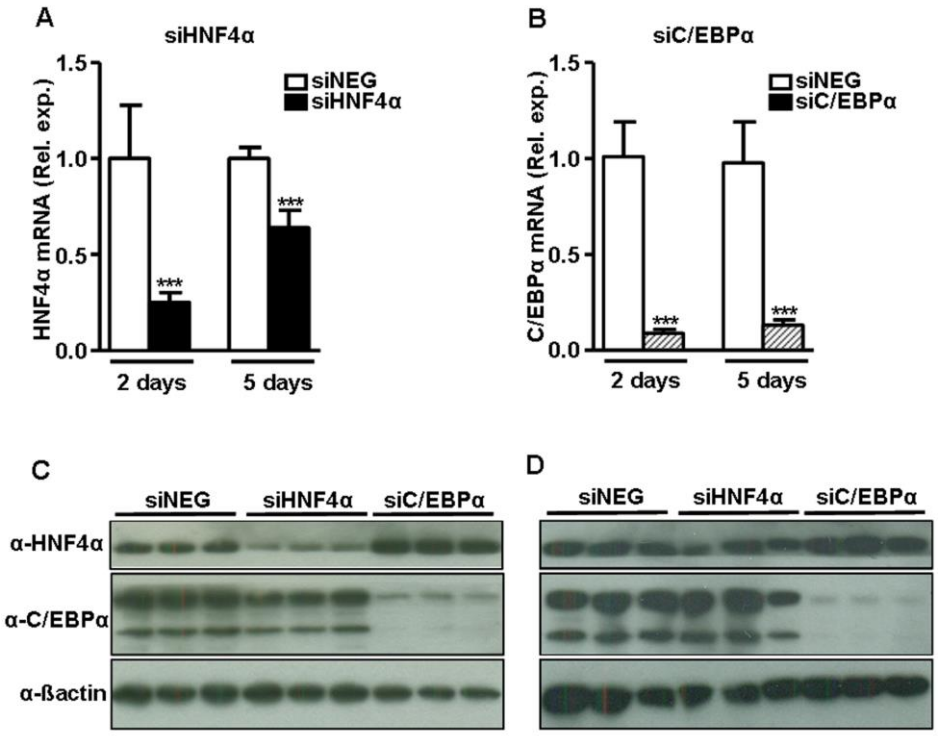

Figure 2. Knockdown of HNF4α and C/EBPα in mouse liver.

The selected siHNF4α-specific siRNA #3 (67635), siC/EBPα-specific siRNA #1 (63853) and a control siNEG were subjected to large-scale preparation in the lipid-based in vivo transfection reagent optimized for hepatic delivery and intravenously injected in C57Black/6J mice (7 mg siRNA per kg mouse, injection volume 200 µl, 18 animals per siRNA). At two and five days post siRNA injection, mice (n=9 per siRNA for both time points) were sacrificed and livers were subjected to HNF4α (panel A) or C/EBPα (panel B) transcript analysis by QPCR. β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group (siNEG). Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data were statistically analysed using Mann Whitney Rank Sum test. *P*-values < 0.05 were regarded as statistically significant. ****P*<0.001. In addition, immunoblotting for HNF4α and C/EBPα was performed for liver homogenates that were prepared for three randomly selected mice per siRNA for both two days (panel C) and five days post injection (panel D). The C/EBPα antibody used, reacts with both C/EBPα p42 and p30.

Changes in transcription of control genes following liver HNF4α and C/EBPα knockdown

In livers of 6-wk-old liver-specific HNF4α deficient animals[25] apolipoprotein C2 (*Apoc2*), apolipoprotein A4 (*Apoa4*), and cytochrome P450 family member 4v3 (*Cyp4v3*) are among the genes that are highly expressed in the liver and are strongly down-regulated by prolonged HNF4α ablation, whereas in these mice scavenger receptor B1 (*Scarb1*) and CD36 (*Cd36*) are among the genes that are strongly upregulated[25;34] (Figure 3B).

The upregulation is suspected to be secondary and may result of prolonged hepatic HNF4α ablation. In livers of mice two days post siHNF4α injection, like in livers of liver-specific HNF4α deficient mice, strongly reduced *Apoc2, Apoa4* and *Cyp4v3* transcript levels were observed (Figure 4A). Also at five days, *Apoc2, Apoa4,* and *Cyp4v3* remained significantly reduced in siHNF4α compared to the siNEG injected animals, despite the limited level of HNF4α knockdown at this time point (Figure 4C and 2A). *Scarb1* and *Cd36* transcript levels were not affected by siHNF4α injection at two or five days (Figure 4A and 4C). Mice in which hepatic C/EBPα has been targeted by recombinant adenovirus encoding siRNA against C/EBPα mRNA demonstrated a role in (fasted) liver glucose and fat metabolism by affecting amongst others transcription of phosphoenolpyruvate carboxykinase (*Pck1*), glycogen synthase (*Gys2*), fatty acid synthase (*Fasn*), stearoyl-CoA-desaturase 1 (*Scd1*) and liver glycogen phophatase (*Lgp*).[32] As demonstrated in Figure 4B, two days post siC/EBPα injection, the livers of (non-fasted) mice displayed significant changes in transcript levels of *Pck1* and *Fasn, Scd1* displayed a 40% non-significant decrease, whereas *Gys2* and *Lgp* were unaffected as compared to siNEG injected animals. As in C/EBPα knockouts[35], siC/EBPα injection resulted in a significant increase in transcript levels of *Cebpb* (+73%, Figure 5) which is considered a compensatory response to C/EBPα knockdown.[35] *Cebpb, Pck1, Fasn* and *Scd1* transcript levels in siC/EBPα-injected mice were

Table 2: Plasma analysis of siHNF4α and siC/EPBα injected mice

	2 days post siRNA injection			5 days post siRNA injection		
	siNEG	siHNF4α	siC/EPBα	siNEG	siHNF4α	siC/EPBα
PT (sec)	11.4 ± 0.1	11.4 ± 0.2	11.6 ± 0.1	12.2 ± 0.1	11.8 ± 0.1*	12.5 ± 0.1*
aPTT (sec)	28.2 ± 0.6	28.6 ± 0.8	27.8 ± 0.4	31.0 ± 0.4	31.9 ± 0.7	30.6 ± 0.6
F5 (%)	100 ± 7	80 ± 11	90 ± 5	100 ± 7	126 ± 6	83 ± 5
F11 (%)	100 ± 6	80 ± 4*	N.D.	100 ± 5	90 ± 8	N.D.
F12 (%)	100 ± 2	100 ± 2	N.D.	100 ± 4	85 ± 8	N.D.
Fbg (%)	100 ± 5	98 ± 3	72 ± 2**	100 ± 10	121 ± 8	70 ± 3**
Tbil (µmol/L)	8.7 ± 0.7	8.5 ± 0.2	8.4 ± 0.4	8.6 ± 0.3	9.0 ± 0.2	8.6 ± 0.4
ALT (U/L)	<20	<20	<20	<20	<20	<20
AST (U/L)	43 ± 8	31 ± 4	32 ± 2	34 ± 3	40 ± 4	39 ± 5
ALP (U/L)	120 ± 8	181 ± 5**	98 ± 4*	90 ± 11	107 ± 7	69 ± 4

Data are represented as mean ± SEM with the group injected with negative siRNA set as a reference. *P<0.05 and **P<0.01 versus mice injected with control siNEG. Prothrombin time (PT), activated partial thromboplastin time (aPTT), Factor activity (F11), Factor 12 activity (F12), Fibrinogen antigen (Fbg) and circulating liver enzymes alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosfatase (ALP) and plasma total bilirubin (Tbil). N.D. = not determined. Values for uninjected control mice: bilirubin 8.3 ± 0.2 µmol/L, ALT < 20 U/L, AST 28 ± 3 U/L, ALP 96 ± 6 U/L

comparable to levels in siNEG animals at five days after injection, despite the persistent strong level of knockdown (Figure 2B and 4D).

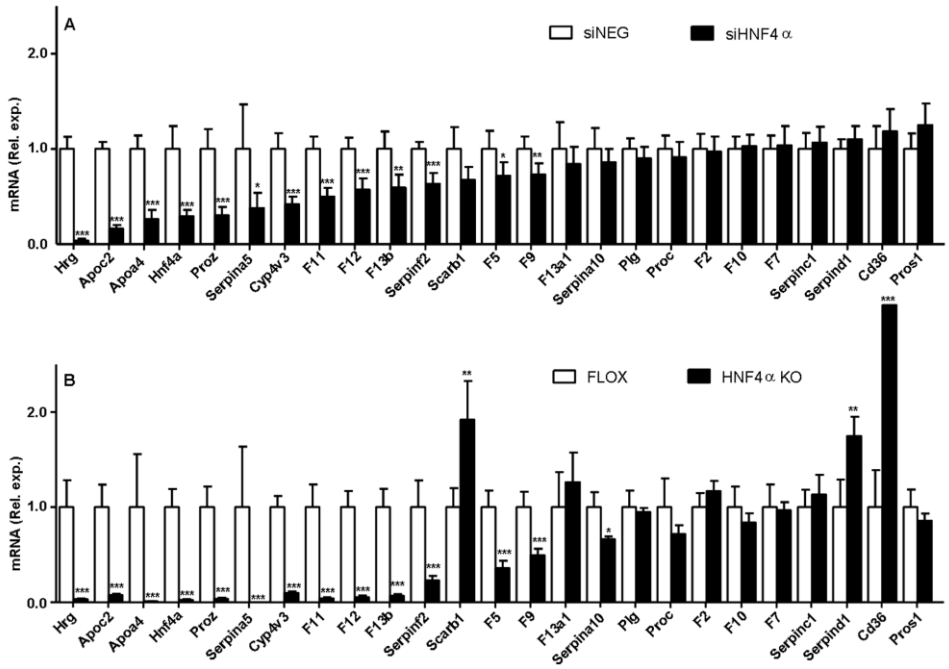

Figure 3. Comparison of gene transcript levels in mouse livers following HNF4α knockdown and liver-specific targeted deletion of *Hnf4a*

Livers from siHNF4α injected animals (panel A, black bars) and siNEG injected animals as controls (panel A, open bars) were subjected to control and coagulation gene transcript levels by QPCR. In panel B, coagulation transcript analysis for liver materials from HNF4α-null mice (KO) or control mice (FLOX) is included for comparison [25]. Data are presented for mice two days post siRNA injection or app. 6 weeks gene ablation (i.e. 6 weeks old HNF4α-null mice). β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the siNEG or FLOX control group. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data were statistically analysed using Mann Whitney Rank Sum test. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001. On the x-axis the coagulation genes are ranked according to the magnitude of effects observed in the siRNA injected animals.

Changes in liver coagulation gene transcription following HNF4α and C/EBPα knockdown

Livers of siHNF4α injected animals with strong reduction in HNF4α transcript and protein levels (two days) displayed markedly and significantly reduced transcript levels of *Hrg* (-97%), *Proz* (-70%), *Serpina5* (-62%), *F11* (-50%), *F12* (-46%), *F13b* (-41%), *Serpinf2* (-36%), *F5* (-38%), *F9* (-27%) (Figure 6A). For the other coagulation genes that were analysed (Figure 3A) we did not observe significant changes as compared to siNEG injected animals. At five days, transcript levels of *Hrg, Proz, Serpina5, F11* and *F12* (-73%, -24%, -79%, -26%, and -27%, respectively) remained reduced, despite the limited level of HNF4α knockdown at this time point (Figure 2A, 6C). Interestingly, at five days, significant elevations in transcript levels of *Serpinf2, Serpind1 a*nd *Pros1* (+20%, + 22%, +32%, respectively) were observed (Figure 6C). Overall, the HNF4α-mediated downregulation of coagulation gene transcript levels in livers of siHNF4α injected animals seemed to largely reproduce those observed in 6 week old HNF4α-null mice with prolonged HNF4α ablation in the liver from birth on (compare Figure 3A, 3B), albeit at a lower extent and, importantly, with two-day siHNF4α injected animals not showing any upregulating effects.

Compared to HNF4α, C/EBPα knockdown had a more modest impact on coagulation gene transcription. Strong reduction in C/EBPα transcript and protein levels resulted in small though significantly reduced transcript levels of fibrinogen α and γ (*Fga* and *Fgg* -25% and -24%, respectively) and *F5* (-27%) at two days post siC/EBPα injection. Only, the reduction in *F5* transcript levels persisted upon prolonged C/EBPα knockdown (-31%). At this time point also *F11* transcript levels became significantly reduced (-21%). For the many other coagulation genes that were analyzed, neither at two (Figure 5) nor at five days (data not shown) post siC/EBPα injection, we observed changes as compared to siNEG injected animals that reached statistical significance.

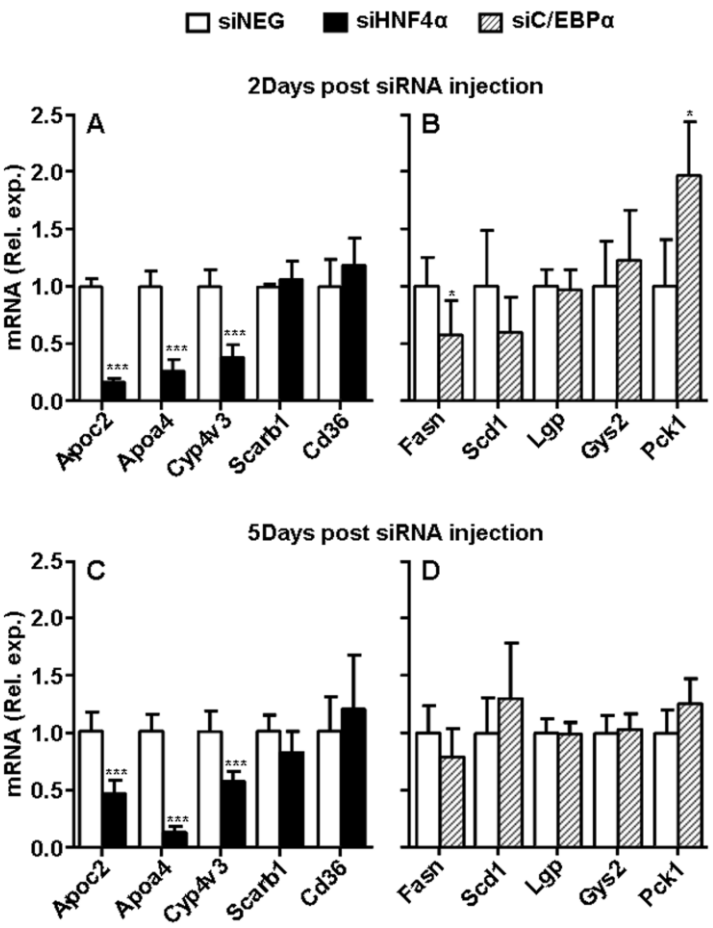

Figure 4. Hepatic transcription of control genes following HNF4α and C/EBPα knockdown

At two (upper panels) and five days (lower panels) post siRNA injection, mouse livers were subjected to transcript levels by QPCR for a panel of control genes. Data are presented for siHNF4α (panel A and C, black bars) and siCEBPα (panel B and D, hatched bars) with siNEG injected animals as controls (panel A, B, C and D, open bars). β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the siNEG group. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data and were statistically analysed using Mann Whitney Rank Sum test. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ****P*<0.001.

At two days, plasma from siHNF4α and siC/EBPα injected animals had an APTT and PT comparable to siNEG injected animals (Table 2). Although analyzed for only a limited number of individual coagulation factors, at this time point, HNF4α knockdown coincided with a significant reduction in plasma F11 activity levels, but not F12 activity levels. C/EBPα knockdown coincided with significant reduction of plasma fibrinogen antigen but not F5 activity levels. At five days, siHNF4α injected animals displayed a minimal but significant shortening of the PT, while that of siC/EBPα injected animals was minimally but significantly prolonged. Plasma fibrinogen antigen levels remained reduced upon prolonged C/EBPα knockdown.

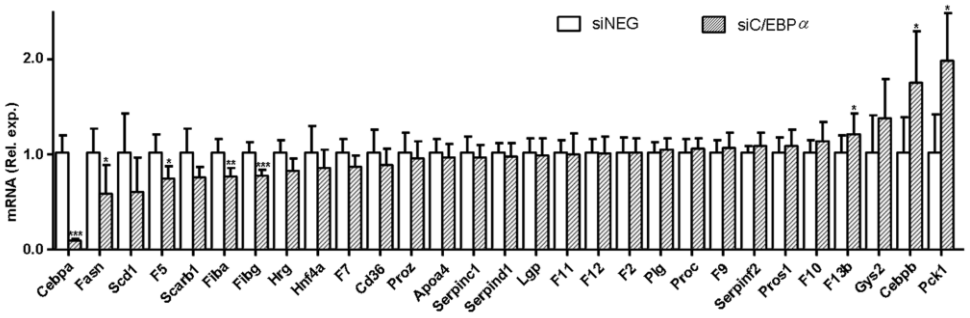

Figure 5. Overview of gene transcript levels in mouse livers following C/EBPα knockdown

Livers from siCEBPα injected animals (hatched bars) and siNEG injected animals as controls (open bars) were subjected to control and coagulation gene transcript levels by QPCR. Data are presented for mice two days post siRNA injection. β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the siNEG group. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data were statistically analysed using Mann Whitney Rank Sum test. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001. On the x-axis the coagulation genes are ranked according to the magnitude of effects observed in the siRNA injected animals.

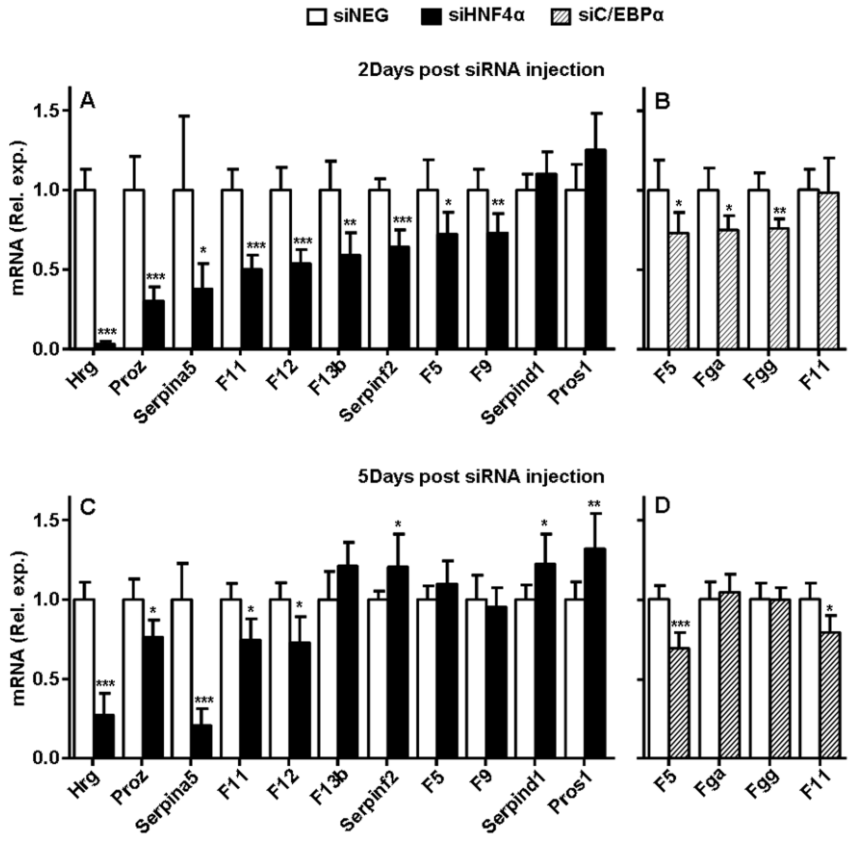

Figure 6. Hepatic coagulation gene transcription following HNF4α and C/EBPα knockdown

At two (upper panels) and five days (lower panels) post siRNA injection, mouse livers were subjected to coagulation gene transcript levels by QPCR. Data are presented for siHNF4α (panel A and C, black bars) and siCEBPα (panel B and D, hatched bars) with siNEG injected animals as controls (panel A, B, C and D, open bars). β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the siNEG group. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data were statistically analysed using Mann Whitney Rank Sum test. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001. Only those coagulation genes are presented for which transcript levels were significantly affected by siHNF4α or siC/EBPα (in two or five days study group). On the x-axis the coagulation genes are ranked according to the magnitude of effects observed in the two day groups.

DISCUSSION

In the present study we used an *in vivo* synthetic small interfering RNA approach to determine in mice the direct role of the liver transcription factors hepatocyte nuclear factor 4α (HNF4α) and CCAAT/enhancer-binding protein α (C/EBPα) in coagulation gene transcription under conditions minimizing methodology or target-related secondary effects. Shortly (two days) post intravenous siHNF4α injection, we observed (strong) reductions in transcript levels of the *Hrg, Proz, Serpina5, F11, F12, F13b, Serpinf2, F5, and F9* genes indicating that these coagulation genes are under direct regulatory control of HNF4α. The relatively modest but fast reduction in transcript levels of *Fga, Fgg* and *F5* observed two days post-siC/EBPα injection indicating that these genes likely targets for direct regulatory control by C/EBPα. However, siC/EBPα injection also rapidly induced increased C/EBPβ transcription, indicating a rapid onset of C/EBPα-related secondary (compensatory) effects and therefore we cannot exclude an underestimation of C/EBPα's direct role in coagulation gene transcription. Overall, we conclude that in the mouse, HNF4α has a direct and essential regulatory role for multiple hepatic coagulation genes. For C/EBPα, such a role is more restricted, but may be underestimated as result of an unexpectedly fast compensatory upregulation of *Cebpb* transcription.

Analyzing the impact of liver HNF4α deficiency on a genome-wide scale using microarrays identified an estimated 20% of the hepatically expressed genes affected by HNF4α deficiency to be upregulated.[32;34] *Scarb1* and *Cd36* were among the genes that were strongly upregulated in liver-specific HNF4α deficient mice while unaffected upon acute siRNA-mediated HNF4α knockdown in mice. It was speculated that *Scarb1* increase may be a secondary consequence of altered lipid homeostasis (e.g., due to changes in intracellular lipid levels in liver of HNF4α-null mice).[25] A similar

mechanism may account for *Cd36* albeit that this gene is predominantly in the liver Kupffer cells. The absence of any statistically significant upregulating effects regarding transcription in livers of mice two days post siHNF4α injection, including *Scarb1* and *Cd36*, suggests that at least a large portion of the upregulating effects observed in the liver-specific knockouts are secondary to prolonged HNF4α disruption. This supports the use of our siRNA approach for *in vivo* studies on the regulating role of liver transcription factors. In this light, it is worthwhile to denote that prolonged knockdown i.e. 5 days post siHNF4α, despite a limited efficacy of hepatic HNF4α knockdown (Figure 2A and 2D), resulted in likely secondary upregulating effects for a number of the coagulation genes (*Serpinf2, Serpind1, Pros1,* Figure 6C).

In the present study, C/EBPα was selected as the second liver transcription factor in this first synthetic siRNA study on transcriptional control of liver coagulation genes given the observations from a recent ChIPseq study on the genome-wide occupancy of C/EBPα in livers of multiple species.[16] In this study, fibrinogen A (FGA) and prothrombin (F2) were among the few genes (from a total of 32 in mouse genome) that were located near ultraconserved C/EBPα binding regions. Indeed, in our mouse study we identified *Fga* as one of the few genes likely to be under direct transcriptional control of C/EBPα.

However, *F2* was clearly not affected following siC/EBPα, suggesting that the C/EBPα binding site located 64 bp from the transcription start site in the mouse *F2* promoter is not critical for physiological control of *F2* transcription. In addition, we were surprised not to find an effect of siC/EBPα on mouse hepatic *F9* transcription. In humans, carriers of hemophilia B Leiden have a causal mutation in a C/EBPα binding site in F9 promoter,[17] and in line, mice with prolonged C/EBPα ablation the liver display reduced hepatic expression of F9.[18] Possibly, C/EBPα interaction with the *F9* promoter, and also that of *F2*, is of high affinity, requiring only

limited levels of C/EBPα binding to drive transcription. Despite the strong level C/EBPα reduction of transcript (-92% and -87% at 2 and 5 days respectively) and protein levels (Figure 2B and 2D) by our synthetic siRNA approach this may not be sufficient to unmask a role for C/EBPα for these type of targets. Alternatively, the observed unexpectedly rapid upregulation of C/EBPβ transcript following siC/EBPα injection, may functionally replace C/EBPα in liver.[36] Indeed, C/EBPβ has been shown to compensate for loss of C/EBPα in the regulation of *Pck1* gene expression.[37] Thus, provided the C/EBPβ transcript rapidly translates to protein (which we did not determine) a compensation for loss of C/EBPα by C/EBPβ cannot be excluded and may explain the absence of effects of siC/EBPα on F2 and F9 and possibly other genes.

The *in vivo* siRNA delivery procedure used had low toxicity without effects on circulating liver enzymes tested ((Table 2) as expected[28]). However, as compared to the control siRNA, both siHNF4α and siC/EBPα had a mild transient effect on serum alkaline phosphatase levels, the circulating marker for biliary obstruction. This suggests the presence of target-related (mild) hepatotoxic effects. Alternatively, changing in ALP levels may reflect a specific transcriptional regulatory role of HNF4a and C/EBPa for genes involved in regulation of bile acid biosynthesis, as has been reported for HNF4α [25]. In the present study, whether siHNF4α or C/EBPα had an immediate effect on bile acid biosynthesis genes like *Cyp7a1*, *Cyp27a1* and *Cyp8b1* was not investigated.

Negative control siRNAs - siRNAs with sequences that do not target any gene products - are essential to control for the effects of siRNA delivery, and to determine whether a siRNA is considered to have a positive, negative, or neutral effect in a particular assay. In our animal studies, we included a commercially available negative control siRNA that was designed to have no significant sequence similarity to mouse, rat, or human

transcript sequences (for description see methods and [29]). This negative control siRNA incorporates the same chemical modifications and is purified to the same rigorous specifications as the target-specific siRNAs (siHNF4α and siCEBPα). In addition, this negative control siRNA virtually lacked effects on gene transcription as determined for multiple cell lines following exposure of relatively high doses of negative control siRNA and analyzed by whole genome expression arrays. Despite the careful design of the negative control, we cannot exclude that in our experiments the control siRNA itself had influence on our genes of interest, and thereby leading to misinterpretation of the findings. To fully exclude such misinterpretation, in vivo experiments should be expanded with multiple carefully designed negative and multiple target specific siRNA. The HNF4α-mediated (downregulatory) changes in control and coagulation genes in livers of siHNF4α injected animals largely reproduced that of 45-day-old HNF4α-null mice and wild-type mouse primary hepatocytes rapidly after siRNA-mediated HNF4α knockdown, indicating that the single negative control siRNA approach used in the present study allowed reliable estimation of the (direct) effects of siHNF4α and also that of siC/EBPα.

In vitro siRNA screening and validation in (primary) mouse hepatocytes showed that the siRNAs targeting HNF4α had higher efficacy than those targeting C/EBPα (Figure 1). Remarkably, *in vivo* we observed the opposite i.e. the siC/EBPα were more effective than siHNF4α (Figure 2). Both HNF4α and C/EBPα displayed normal and stable expression in primary mouse liver cells i.e. Ct comparable to fresh livers. This suggests that the discrepancy between the *in vitro* and *in vivo* findings could not be attributed to a rapid decline in HNF4α *in vitro* and thereby the efficacy of the siHNF4α is over estimated. Whether the difference in efficacy is due to differences in *in vivo* siRNA delivery, processing and or stability of the siRNA is unknown. However, it emphasizes that the *in vitro* experiments (as performed) are useful for identifying siRNAs with *in vivo* potential, but are not fully

predictive for identifying most effective siRNA for *in vivo* use. Although, these aspects of *in vivo* siRNA approach should be improved, this study demonstrates that synthetic siRNA provides a simple and fast means for determining direct transcription factor involvement *in vivo* under conditions minimizing secondary effects. Here, *in vivo* siRNA-mediated knockdown enabled us to establish the direct contribution of HNF4α and C/EBPα to the regulation of coagulation gene transcription.

ACKNOWLEDGEMENTS

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Chapter 4

The Role of FOXA1 in Mouse Hepatic Estrogen Receptor α Signaling

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(Manuscript in preparation)

SUMMARY

The Forkhead Box A1 (FOXA1) protein either activates transcription directly, or modulates the activity of other transcription factors, in particular nuclear hormone receptors. In breast cancer cell lines, FOXA1 facilitates binding of estrogen-bound estrogen receptor α (ERα) to chromatin and represents an important determinant of ERα signaling by estrogen. FOXA1 is also expressed in normal human and mouse liver, an estrogen responsive tissue expressing ERα. To investigate the role of FOXA1 on estrogen signaling in liver, FOXA1 was silenced in normal mouse livers, and ERα- chromatin interaction and ERα-mediated transcription were studied both in the absence and presence of an estrogenic stimulus.

In the absence of endogenous estrogen production (i.e. conditions of ovariectomy), silencing of mouse hepatic FOXA1 transcript levels (>60% reduction) resulted in altered transcription of 12 out of 19 hepatic genes analyzed. ChIP analysis confirmed that stimulation of hepatic ERα by 17β-ethinylestradiol increased binding (up to 5-fold) of ERα to genomic regions of representative estrogen responsive genes such as *Shp*, *Gpx3*, *Serpinc1*, *Pck1* and *F5.* This increased binding was close to background level when FOXA1 was silenced. This reduction in ERα-chromatin interaction upon FOXA1 silencing did not translate into altered hepatic transcription of estrogen-responsive genes. We concluded that in the absence of estrogen, FOXA1 contributes to regulation of mouse hepatic (coagulation) gene transcription. FOXA1 can modulate the estrogen-induced ERα interactions with hepatic chromatin. However, despite the modulatory potential at the chromatin level, under the present study conditions, lowered hepatic FOXA1 levels did not modify the estrogen-induced transcriptional changes of a panel of representative hepatic genes.

INTRODUCTION

The FOXA subfamily of winged-helix transcription factors includes Forkhead Box protein A1 (FOXA1), A2 (FOXA2) and A3 (FOXA3), which are encoded by separate genes.[1;2] FOXA factors contain a 100 amino acids long DNA-binding domain that is highly conserved (at least 92%) within the human and rodents FOXA family.[3] The FOXA protein family is critical in a variety of processes, both during development and in postnatal life.[4-12] Previous gene ablation studies of FOXA factors in mice have shown that FOXA2 and FOXA3 altered transcription of genes encoding proteins involved in bile acid and glucose metabolism, respectively.[7;11;13] FOXA1 knockout mice showed postnatal growth retardation with a hypoglycemic state although expression of FOXA1 target genes involved in hepatic gluconeogenesis remained unchanged.[14] Mouse studies claimed that FOXA proteins can bind nucleosomes,[15;16] and open compact chromatin structures, thereby facilitating the binding of other transcription factors.[17] *In vitro* studies reported the presence of FOXA1 binding sites near estrogen receptor α (ERα) binding sites and requirement of FOXA1 for efficient ERα binding to estrogen response elements.[18-20] Recent studies have shown that in breast cancer cells, but also in other types of cancer cells, FOXA1 act as a 'pioneer factor', and FOXA1 is an important determinant of almost all ERα-chromatin interactions and of estrogen-ERα mediated transcriptional activity.[21] Thus, FOXA1 can modulate gene transcription including that regulated by ERα, at least in cancer cells.

Both, ERα and FOXA1 are expressed in normal human and mouse liver.[9;22-24] Several studies, including our own, demonstrated that for the liver, ERα is involved in regulating gene transcription.[23;25] Whether FOXA1 has the potential to directly modulate hepatic gene transcription, and whether FOXA1 can modulate ERα-dependent gene transcription in liver tissue, is unknown.

The aim of this study was to investigate the role and interaction of FOXA1 with ERα in normal mouse liver. RNA interference was used to silence FOXA1 expression in mouse liver, and ERα-chromatin interaction and ERα-dependent transcription were studied both in the presence and absence of an estrogenic stimulus. The genes that were selected for analysis were hepatic (expressed in liver) estrogen-responsive genes including genes of the blood coagulation pathway and hepatic genes that previously were demonstrated to be affected by FOXA proteins.[7;23;25-28] The results showed that FOXA1 had the ability to alter hepatic gene expression independent of estrogen hormone in mouse liver. We also showed that silencing of hepatic FOXA1 inhibited 17β-ethinylestradiol (EE)-bound ERα chromatin binding for a panel of representative hepatic genes. However, despite the modulatory potential of FOXA1 on chromatin binding, FOXA1 silencing did not alter estrogen-induced transcriptional changes of a panel of representative hepatic genes under the present study conditions.

MATERIALS AND METHODS

siRNA screening in in vitro

Pre-designed ON-TARGET Plus siRNAs (synthetic small interfering RNAs) for mouse *Foxa1* mRNAs (designated as siFoxa1) or control siRNA (designated as siScrambled) were purchased from Dharmacon Lafayette, CO, USA (#1: J-046238-05, #2: J-046238-06, #3: J-046238-07, and #4: J-046238-08 or siScrambled, D001810-01). Target sequence of siFoxa1 #1-4 and siScrambled were 5'-CCA GAC CCG UGC UAA AUA C-3', 5'-UCU AUG AAC UCC AUG AAC A-3', 5'-CCA CGA AUC UCA GCU GCA U-3', 5'-GGA GCC AGC CUA CUA CCA A-3' and 5'-UGG UUU ACA UGU CGA CUA AUU-3', respectively. The most Effective siRNAs targeting *Foxa1* were selected using mouse (C57Black/6J, Charles River) primary hepatocytes 24 hours after transfection with 100 nM as described previously.[29] Levels of *Foxa1* mRNA were determined by quantitative real-

time PCR (QPCR, see below). siRNAs were selected for *in vivo* studies based on maximal reduction of *Foxa1* transcript levels as compared to control (siScrambled) siRNA.

mRNA knockdown and hormone treatment in vivo

ON- TARGET Plus Control siScrambled and siFoxa1 #2 (Dharmacon Lafayette, CO, USA, catalogue numbers D001810-01 and J-046238-06 respectively) were complexed with Invivofectamine® 2.0 Reagent (Invitrogen, Life technologies Corporation, USA) according to the manufacturer's protocol. Eight weeks old female C57Black/6J mice (weighing 17–19 gram), were bilaterally ovariectomized under isoflurane anesthesia. After two weeks of recovery, the ovariectomized mice were intravenously injected via the tail vein with 200 µl complexed siRNA at a dose of 7 mg of siRNA per kg body weight (in total 48 animals, 24 animals per siRNA).

Two days post siRNA injection all mice were subjected to liver biopsy (see below), to study the effect of FOXA1 silencing in the absence of an estrogenic stimulus. Mice (12 mice per treatment/siRNA) were treated with vehicle or 1 µg ethinylestradiol (EE, Sigma Aldrich, Steinheim, Germany) per mouse d^{-1}. Vehicle/EE treatment was continued for 3 more days, and then mice were sacrificed for blood and liver collection. Blood samples on sodium citrate (final concentration 0.32%) were drawn from the inferior caval vein. Plasma was obtained by centrifugation and stored at -80°C until use. Liver was isolated and was snap-frozen for chromatin immunoprecipitation (ChIP), mRNA and protein analyses, and stored at -80°C until use.

All mice were housed under a 12-h light/dark cycle, with standard chow diet and drinking water provided ad libitum. All experimental procedures were approved by the animal welfare committee of the Leiden University.

Liver biopsy

For liver biopsy, mice were anaesthetized by isoflurane inhalation, and the upper ventral abdomen up to the sternum was shaved. An abdominal skin incision (for ~1 cm) caudal from the sternum slightly left of the midline was followed by incision of the abdominal muscle (0.5 cm). Using a forceps, the anterior edge of the left lobule was located, held, and a specimen (40-50 mg) was taken using a cauterizer with a stainless steel 0.35 mm diameter tip (Fine Science Tools). The site of cauterisation was visually inspected for absence of bleeding and the left lobule was repositioned in the abdominal cavity. From the liver biopsy, 30-50 mg of tissue that appeared free of damage caused by cauterisation was immediately snap-frozen for mRNA and protein analyses and stored at -80°C until use. The opening in the abdominal cavity was closed by a normal running suture (Silkam 2xDS12 6/0 0,7 45cm 18" Black Silk, braided coated, non-absorbable) for the abdominal musculature and the skin. Post-operative analgesia was achieved by a single subcutaneous injection with buprenorphine (0.05 mg/kg).

RNA isolation and real-time RT-PCR

Liver (biopsy) samples (20-30 mg) were homogenized in RNAzol (Tel-Test, Friendswood, Texas, USA) and RNA isolation and cDNA synthesis was performed as previously described.[25] Gene-specific quantitative real-time PCR (QPCR) primers have been described previously;[25;29] with exception of those for *Foxa1* (forward; 5'-ATG AGA GCA ACG ACT GGA ACA G-3', reverse; 5'-TGC TGA CAG GGA CAG AGG AGT A-3'), *Foxa2* (forward; 5'-CGG GGA CCC CAA GAC ATA CC-3', reverse; 5'-CCA TGG TGA TGA GCG AGA TG-3'), *Shp* (forward; 5'-CAT GGA AAT GGG CAT CAA TA-3', reverse; 5'-CGT GGC CTT GCT ATC ACT TT-3') and prekallikrein *Klkb1* (forward; 5'-TGG TCG CCA ATG GGT ACT G 3', reverse; 5'-ATA TAC GCC ACA CAT CTG GAT AGG-3'). QPCR was performed on the ABI Prism 7900 HT Fast Real-Time PCR System from Applied Biosystems and

data were analysed using the accompanying Sequence Detection System software (Applied Biosystems, Foster city, CA, USA). The comparative threshold cycle method with β-actin as internal control was used for quantification and normalization. siScrambled and/or vehicle treated animals were set as a reference and the ΔCt values of the individual samples were related to the mean ΔCt of the reference group.

Immunoblotting

Frozen liver material (10-20 mg) was grounded, and lysed in RIPA-lysis buffer. Cell lysate (25µg) was denatured, separated on 8-10% Novex® Tri-Glycine gels (Invitrogen®, Carlsbad, CA, USA) (city country), and immunoblotted using a goat polyclonal IgG against human FOXA1 (Ab-5089, Abcam, Cambridge, USA). ß-actin was detected using rabbit polyclonal against human ß-actin (Ab8227, Abcam) and served as protein loading control. These antibodies are cross-reactive to mouse FOXA1 and ß-actin, respectively. Bound IgG was detected using horseradish peroxidase-labeled anti-goat IgG (sc-2020, Santa Cruz Biotechnologies, Santa Cruz, USA) or anti-rabbit IgG(172-1019, BioRad, Hercules, CA, USA) IgG followed by enhanced chemiluminescence system (Amersham Pharmacia Biotech, Amersham, UK) to detect peroxidase activity.

Chromatin immunoprecipitation

Chromatin was prepared from liver samples as described previously.[30] Briefly, about 0.5cm^3 liver tissue obtained five days post siRNA injection was fixed in 1% formaldehyde and quenched for 5 minutes with glycine (0.125M) at room temperature. After washing twice with PBS, the material was dounced and filtered through a 100 µm cell strainer. Cells were lysed in Lysis-buffer 'LB1' (50mM Hepes [pH 7.5], 14mM NaCl, 1mM EDTA, 10% glycerol, 0.5% Nonidet-P40, 0.25% Triton-X-100, protease inhibitor [cOmplete, EDTA-free, #11873580001; Roche Diagnostics, Mannheim,

Germany]) and the nuclei were pelleted by spinning at 2000 × rcf (relative centrifugal force) for 5 min at 4 °C. Nuclear pellets were lysed in 2 ml Lysis-Buffer 'LB2' (150mM NaCl, 50mM Tris-HCl, pH 7.5, 5mM EDTA, 0.5% Nonidet P-40, 1% Triton X-100, protease inhibitor [cOmplete, EDTA-free, #11873580001; Roche Diagnostics, Mannheim, Germany]. Chromatin was sheared in an ultrasonic bath (Branson), three times 10 minutes, 15 sec on/off cycle with high power at 4^{o}C. Samples were immunoprecipitated with 2 µg ERα antibody MC-20 (Santa Cruz Biotechnology, Santa Cruz, CA) according to a published protocol.[31] Unspecific binding of protein to beads was measured using 2 µg normal rabbit IgG (Millipore, Billerica, Massachusetts, USA) as control. Immunoprecipitated and purified DNA was amplified by QPCR genomic primer pairs (designed with Primer3Plus software) of *Shp*, *Serpinc1* and *Pck1* (Supplementary table 1)*, Gpx3* and *F5* (EpiTect ChIP qPCR Assay 200, GPM1029444(+)09A, GPM1028370(-)17A, respectively, Qiagen, Benelux B.V., Venlo, Netherlands). QPCR was performed as mentioned above. The comparative threshold cycle method with IgG as internal control was used for quantification and normalization. Relative enrichment values were calculated by dividing the ChIP values obtained with ERα antibody by the ChIP values obtained with Input. ChIP was performed independently on liver samples from three mice per group and QPCR was performed in duplicate for each sample.

Plasma analyses

Thrombin generation was assessed as described.[32] Briefly, thrombin generation reaction was initiated in 1:6 diluted plasma, triggered with 1 pM tissue factor (TF) and measured with calibrated automated thrombogram (Thrombinoscope B.V., Maastricht, the Netherlands). Accompanying software program enabled the calculation of thrombin activity against the calibrator and displayed thrombin activity with the time.

Statistical analyses

Data were analysed with the GraphPad Instat software (La Jolla, CA, USA). Statistical differences were evaluated using a Mann-Whitney Rank sum test (animal studies; transcript data) or Student's t-test (hepatocyte and ChIP studies). A *P*-value of <0.05 was considered as statistical significant.

RESULTS

FOXA1 silencing in absence of an estrogenic stimulus

To investigate the role of FOXA1 in mouse liver gene transcription (both estrogen dependent and independent), we followed an RNA interference (RNAi) strategy. Previously, we demonstrated that this allows fast and acute silencing of hepatic (control) genes and analysing their function *in vivo*.[29] First, an effective FOXA1-specific siRNA was selected by transfecting mouse primary hepatocytes with four different FOXA1 specific predesigned synthetic siRNAs. As shown in Figure 1A, both siRNA duplex #2 and #3 resulted in ~90% reduction of FOXA1 transcript levels compared to siScrambled control transfected hepatocytes. siRNA duplex #2 was selected for large-scale preparation and complexation with a lipid-based *in vivo* transfection reagent optimized for hepatic delivery. First, the role of FOXA1 was investigated in absence of estrogen hormone. To this end, female ovariectomized C57Black/6J mice were intravenously injected in the tail vein with siFoxa1 or siScrambled (7 mg/kg). The extent of *in vivo* FOXA1 silencing was determined both at 2 at 5 days after siRNA injection, using liver biopsy (50 mg) and whole liver material, respectively.

Two days post-siRNA injection, reduction in *Foxa1* transcript levels ranged from 49% to 72% of that of control siRNA injected animals (Figure 1B). At this time point, FOXA1 protein levels were modestly affected, as analysed for mice with relatively strong levels of silencing at the transcript level (> 57%; Figure 1C). Five days after injection, reduction in *Foxa1* transcript levels ranged from 44% to 73% of control siRNA injected animals (Figure

1B). At this time point, relatively low levels of *Foxa1* transcript levels were associated with low levels of FOXA1 protein in liver (Figure 1C). Although quantitative protein data are lacking, this suggests that at 2 days, *Foxa1* transcript reduction did not yet translate into reduced protein levels, probably because of a relatively long half-life of the FOXA1 protein.

We opted to analyze the expression levels of a panel of hepatic genes that either have FOXA binding sites in their enhancer region (e.g. *F2*) or genes are regulated by FOXA targets (e.g. *Hnf4a*, *Klkb1*, *Pck1* and *Proc)*.[7;26-28;33] As shown in Figure 2A and 2B, FOXA1 silencing (both at two and 5 days post siFoxa1) did affect hepatic expression levels of *F2,* but not of *Hnf4a*, *Klkb1*, *Pck1* and *Proc,* (Figure 2B). *F2* transcript levels showed modest, but significant, correlation with level of residual hepatic *Foxa1* transcript levels (r=0.4436, *P*=0.0460, n=11).

Two and five days post- siRNA injection, livers samples were also analyzed for a panel of known estrogen-responsive genes.[23;25] Hepatic *Fiba, Src1* and *Hrg* transcript levels remained unaffected by siFoxa1 injection (both at two or five days post siRNA injection), while hepatic expression of *F2, F5, F7, F10, F11, F13b, Serpinc1, Gpx3*, *Apoa4*, *Apoc2* and *Esr1* were significantly altered as compared to control siRNA injected animals (either two or five days post siFoxa1 injection); at two days, a reduction in hepatic transcript levels of *Gpx3* (-37%) and *Apoa4* (-22%) was observed, while an elevation in hepatic transcript levels of *F13b* (20%), *Esr1* (32%), *F11* (35%), and *Apoc2* (43%) was observed (Figure 2C). At five days, hepatic transcript levels of *F2* (-20%), *F5* (-21%), *F7* (-22%), *F10* (-39%), *F11* (-28%), *Serpinc1* (-19%) and *Apoa4* (-10%) were reduced, while *Gpx3* (43%) levels were increased (Figure 2D).

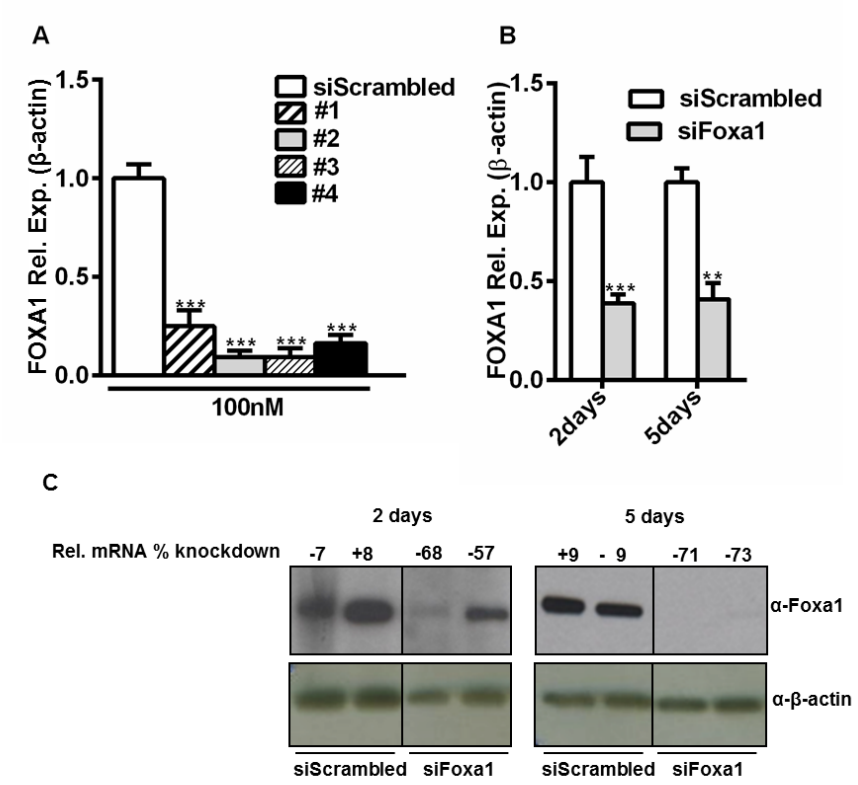

Figure 1. Screening and validation of siRNA in *in vitro* and *in vivo*. Screening of siRNA in mouse primary hepatocytes 24 hours after transfection with 100nM of four *FOXA1*-specific (#1-4), or a control siRNA (siScrambled, panel **A**). *FOXA1* transcript levels were determined by quantitative real-time PCR (QPCR). On the x-axis the siRNA concentrations are indicated. The selected siFoxa1-specific siRNA #2 and a control siScrambled were subjected to large-scale preparation in the lipid-based in vivo transfection reagent optimized for hepatic delivery and intravenously injected in ovariectomized C57Black/6J mice (7 mg siRNA per kg mouse, injection volume 200 µl, 12 animals per siRNA). Two days post siRNA-injection, liver biopsies were performed for transcript analysis (B). Five days post siRNA-injection mice (n=12 per siRNA) were sacrificed and livers were subjected to *FOXA1* transcript analysis by QPCR (panel **B**). β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group (siScrambled). On the x-axis days post siRNA-injections are indicated. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (difference 2^ΔΔCt+SEM and 2^ΔΔCt-SEM).

Individual experiments were performed in triplicate. *In vitro* and *in vivo* data were statistically analyzed using the Student's t-test and Mann Whitney Rank Sum test, respectively. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001. In addition, immunoblotting for FOXA1 was performed for liver homogenates that were prepared for two selected mice (with most efficient knockdown at the mRNA level) per siRNA for both two days and five days post injection (panel **C**). β-actin was used as loading control. The uncropped protein blot is in **Supplementary Figure 1**.

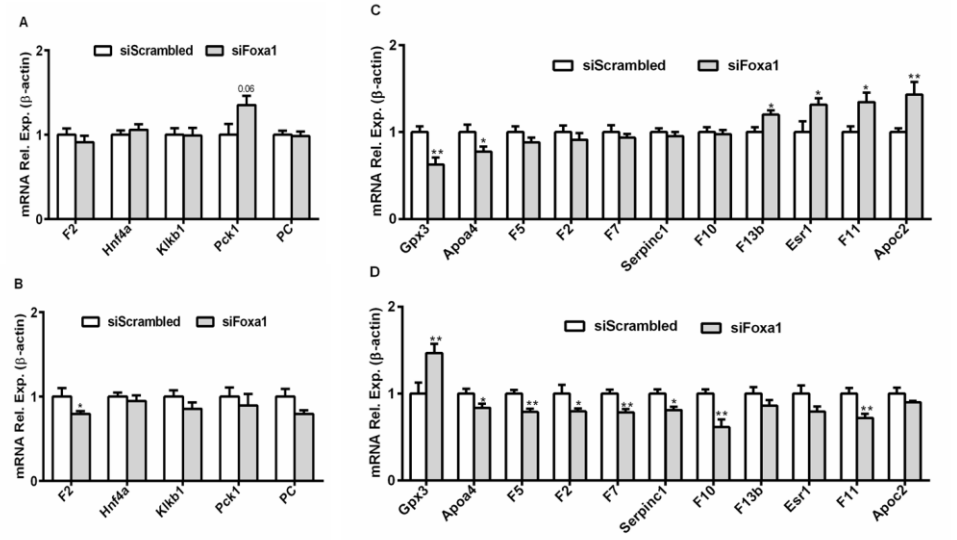

Figure 2. Hepatic gene transcription following FOXA1 knockdown in mouse liver. At two (upper panels, **A** and **C**) and five days (lower panels, **B** and **D**) after siRNA injection, mouse livers were subjected to gene transcript analyses by QPCR. Data are presented for siFoxa1 (gray bars) with siScrambled injected animals as controls (open bars). β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the siScrambled group. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data were statistically analyzed using the Mann Whitney Rank Sum test. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001. On the x-axis the coagulation genes are ranked according to the magnitude of effects observed in the 2-days groups.

Effect of FOXA1 silencing on estrogen-mediated ERα-chromatin binding and transcriptional activity

Two days post-siRNA injection mice were treated with vehicle or 1 µg EE per mouse d^{-1}. A dose of 1 µg EE per mouse d^{-1} was chosen, as this is known to result in significant changes in hepatic gene transcription.[25] Liver samples were collected five days post siRNA injection i.e. after three days of daily EE treatment.

Hepatic transcript levels of *Foxa1* were significantly decreased upon EE treatment in control siRNA (-36%) as well as in siFoxa1 (-74%) injected animals as compared to control siRNA injected animals (Figure 3A). Thus, estrogen treatment downregulates hepatic FOXA1 expression in mice, adding to the siFoxa1 effects.

To investigate whether FOXA1 modulates estrogen-induced binding of ERα to mouse liver genomic DNA, we performed chromatin immunoprecipitation (ChIP) for ERα, followed by QPCR analysis of promoter regions for *Shp*, *Gpx3*, *Serpinc1*, *Pck1* and *F5.* These genes are established estrogen responsive genes and contain ERα binding motifs.[23;25] ERα-ChIP followed by real-time PCR was used with primers flanking the promoter regions of the selected genes. In animals injected with control siRNA, estrogen treatment induced a 2 to 4-fold enrichment at selected promoter regions of studied genes (*Shp, Gpx3, Serpinc1, Pck1* and *F5*: Figure 3B). ERα binding at promoter regions of studied genes remained unaffected in animals injected with FOXA siRNA and vehicle treatment (data not shown). As shown in Figure 3B, silencing of hepatic FOXA1 strongly and significantly reduced estrogen-induced ERα binding to *Shp*, *Gpx3*, *Serpinc1*, *Pck1* and *F5*. Thus, silencing of FOXA1 inhibits estrogen-induced binding of ERα to chromatin for all genes selected for this analysis.

We next assessed the effect of FOXA1 silencing on ERα-mediated hepatic gene transcription. In control siRNA injected animals treated with EE, we observed significant altered hepatic transcript levels of *F7*, *F2*, *Serpinc1*,

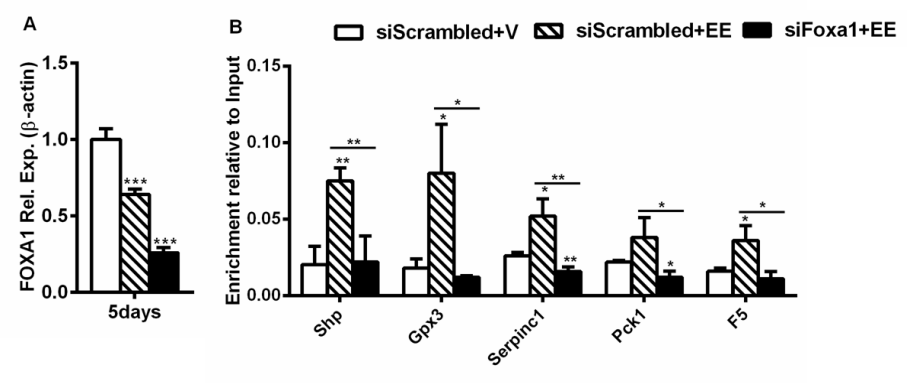

Figure 3. Binding of ERα to chromatin is influenced by expression levels of FOXA1 in mouse liver. Ovariectomized C57Black/6J mice were injected with siScrambled or siFoxa1 (7 mg siRNA per kg mouse) and two days post siRNA injection mice were treated for three days with 1µg EE d^{-1} or vehicle per mouse. (A) FOXA1 hepatic transcript levels were analyzed using QPCR (n=12 mice per group). β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group (siScrambled-V). Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). On the x-axis days post siRNA-injections are indicated. (B) ERα chromatin immunoprecipitaion (ChIP) was performed on liver samples followed by QPCR of known ERα binding regions. Normal rabbit IgG was used as internal control for quantification and normalization. The promoter occupancy was analyzed for three individual animals in each group and each sample was assayed in duplicate. Data are presented as ERα enrichment relative to Input as mean ± standard error (SD). Transcript and ChIP data were statistically analyzed using the Mann Whitney Rank Sum test and Student's t-test, respectively. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001.

F5, *PC*, *Gpx3* and *Apoa4,* thereby confirming earlier observations (Figure 4).[25] *Pck1* and *Hnf4α* were not affected by hormone, while *Shp* hepatic transcript levels were too low to allow quantification (Figure 4). Despite, the relatively strong effect of FOXA1 silencing on ERα-binding to the genomic DNA encoding promoter region of *Shp*, *Gpx3*, *Serpinc1*, *Pck1* and *F5*

genes, an effect of FOXA1 silencing on EE-induced transcription was absent. Silencing of FOXA1 did not modulate the estrogen induced transcription of these genes, nor did it affect the transcription of other estrogen responsive genes (*F7, F5, Hnf4a, Pck1, Gpx3* and *Apoa4,* Figure 4).

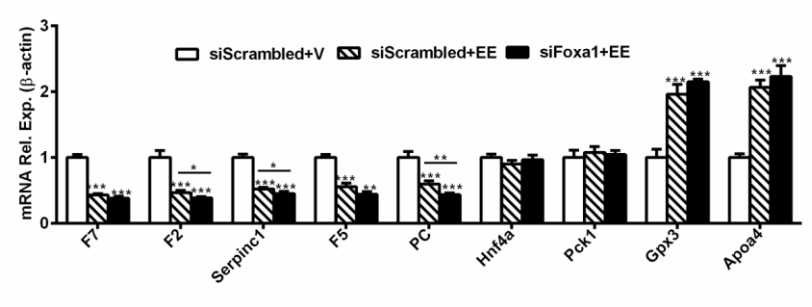

Figure 4. EE-bound ERα hepatic gene transcription is dependent on FOXA1 expression. Livers from ovariectomized mice, five days post siRNA and after three days of EE/vehicle treatment (as mentioned in the legends of Figure 3) were subjected to transcript analysis by performing QPCR (see figure 1 legends). Data are presented for siScrambled+Vehicle (Open bars), siScrambled+EE (hatched bars) and siFoxa1+EE (Black bars). On the x-axis the coagulation genes are ranked according to the magnitude of effects observed in siScrambled+EE group. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data were statistically analyzed using the Mann Whitney Rank Sum test. *P*-values < 0.05 were regarded as statistically significant. **P*<0.05, ***P*<0.01, ****P*<0.001

In addition the impact of FOXA1 silencing on EE-induced transcription (of *Pck1*, *Serpinc1*, *F5* and *Gpx3*) was analysed for a subset of animals that were used for ChIP analysis and displayed a relatively strong level of FOXA1 'knockdown' (<80%, n=3). Also in these samples, FOXA1 silencing did not modulate the estrogen-induced alterations in transcription (*Pck1*, *Serpinc1* and *F5:* Supplementary figure 2). For *Gpx3*, hormone–induced

gene expression was reduced upon FOXA1 silencing, but this did not reach statistical significance (Supplementary figure 2).

Thrombin generation assay

Hormone-induced ERα-dependent changes in hepatic coagulation gene transcription lead to estrogen-induced changes in plasma thrombin generation.[34] We used this global plasma coagulation assay to assess whether subtle effects of FOXA1 silencing on (hormone-induced) transcript impacted the hemostatic balance. In the absence of hormone, FOXA1 silencing (5 days post siRNA injection) results in a significant increase in plasma thrombin generation (increased peak height (nM FIIa) 43±6 vs 37±6, p=0.032 and increased endogenous thrombin potential (ETP) 290±44nM vs 242±17nM, p=0.0049, respectively). Estrogen treatment also increased ETP compared to vehicle treated animals (306±14nM vs 242±17nM P=<0.0001; Supplementary figure 3B) and with decreased propagation of thrombin generation, which is in line with the overall decrease in liver coagulation gene transcript levels and confirms the earlier observations.[34] Estrogen-induced effects on thrombin generation were not affected by FOXA1 silencing (310±13 vs 306±14; Supplementary figure 3C). Thus, the absence of a modulatory effect of FOXA1 silencing on hormone-induced hepatic coagulation gene transcription, was paralleled by absence of an effect of plasma thrombin generation (Figure 4).

DISCUSSION

In the present study, we examined the role of FOXA1 in mouse hepatic gene transcription in the presence and absence of estrogen. We observed that in the absence of endogenous estrogen, FOXA1 directly regulated mouse hepatic (coagulation) gene transcription. In addition, we found that changes in FOXA1 expression impacted the interaction between ERα and hepatic chromatin. Unexpectedly, we observed that FOXA1 silencing only

minimally impacted ERα-dependent hepatic gene transcription. Despite the negative findings regarding transcription, we conclude that FOXA1 has the ability to modulate estrogen-induced ERα chromatin interaction in normal mouse liver, similar to what has been observed for estrogen-responsive (human) cancer cell lines.

Previous studies provided evidence that *F2*, *Hnf4a, Klkb1, Pck1* and *Proc* genes are regulated by FOXA proteins (as determined by *in vitro* reporter and mutagenesis studies or *in vivo* knockout mouse models).[7;26;27;35] Hence these genes were used as controls in analyzing the impact of silencing hepatic FOXA1. Remarkably, only *F2* was confirmed as being under direct transcriptional control of FOXA1, while confirmation was absent for *Hnf4a, Klkb1, Pck1* and *Proc* gene. In contrast to the previous studies, our study was under conditions of ovariectomy (absence of endogenous estrogen); and FOXA1 action on *Hnf4a, Klkb1, Pck1* and *Proc* may involve interaction with estrogen bound estrogen receptors. Alternatively, effects of FOXA1 silencing may have been masked by upregulation of FOXA2, which has been documented previously.[36-38] We determined hepatic transcript levels of FOXA2, which remained unaffected upon FOXA1 silencing (Supplementary figure 4). Although this does not exclude that sufficient FOXA2 is available or FOXA2 protein levels were affected by FOXA1 knockdown and underlied the absence of effects of FOXA1 silencing on *Hnf4a, Klkb1, Pck1* and *Proc*. Despite the lack of confirmation for the latter genes, we report that a number of well-documented hepatic genes were responsive to FOXA1, thereby, suggesting that FOXA1 has a role in the constitutive expression of hepatic genes, which may extend beyond the genes analyzed. Genome- wide transcriptional analysis (RNAseq) may provide further insights in the role of FOXA1 in transcriptional control in normal liver.

Decreased ERα-chromatin binding upon hepatic FOXA1 silencing did not result in altered estrogen ERα-mediated hepatic gene transcription. So far,

we have no good explanation for this. Possibly extended estrogen exposure masks the role of FOXA1 in ERα-induced hepatic gene transcription. Normally, exogenous estrogen acts fast and rapidly (within few hours) modulates hepatic gene transcript levels.[25] We also examined whether FOXA1 silencing resulted in altered estrogen ERα-mediated hepatic gene transcription upon a short term estrogen exposure (5 hours after estrogen administration). However, FOXA1 silencing was also unable to modulate short-term effects of EE (data not shown). Alternatively, EE may impact the transcription via ways other than estrogen receptors, thereby being out of reach of FOXA1 effects.[39] However, previous studies demonstrated that the genes analysed are induced by EE via pathways that solely rely on estrogen receptor α.[25] Furthermore, we cannot exclude that exogenous estrogen alters the transcript levels of our genes of interest by modulating the stability of transcripts. This may be an ERα dependent process that is not regulated at the level of the gene promoter[39] and thereby insensitive to FOXA1-mediated modulation of ERα-binding to promoter regions. We concluded from the present study that FOXA1 modulate ERα-chromatin binding also in normal livers as it does in (human) cancer cell lines. Future studies should focus on the importance of this interesting interaction between hepatic FOXA1 and ERα.

Previous studies have shown that estrogen exposure causes a rapid down-regulation of the steady-state level of ERα expression, thereby providing a negative feedback mechanism to (down) regulate ERα itself and the genes regulated by ERα.[40;41] Here, we showed that estrogen treatment also induced a considerable (30%) down- regulation of hepatic FOXA1 levels (Figure 3A and Supplementary figure 1). This opens up the possibility that FOXA1 is involved in the negative feedback regulation in ERα signalling. Such a role may also be of therapeutic relevance in cancers where estrogen receptor expression is disregulated.

In summary, our data demonstrated that FOXA1 regulated hepatic (coagulation) gene transcription in mouse liver independent of endogenous estrogen. FOXA1 silencing interfered with the interaction of ERα-chromatin binding, for a panel of representative estrogen-responsive genes but this did not translate into altered response of ERα on hepatic transcription. We concluded that FOXA1 can facilitate estrogen ERα-chromatin binding in normal mouse liver, similar to what observed for estrogen-responsive (human) cancer cell lines.

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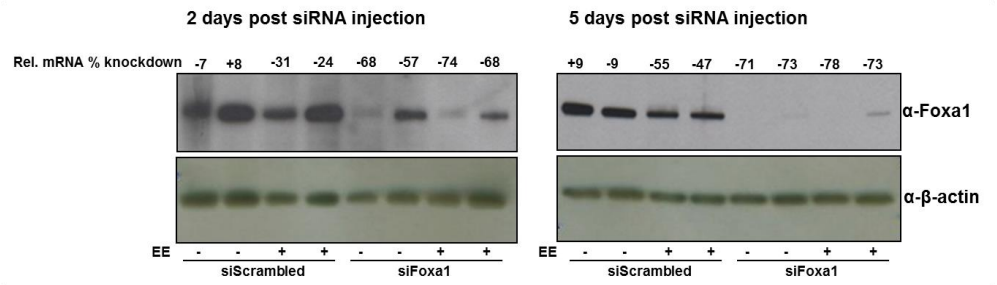
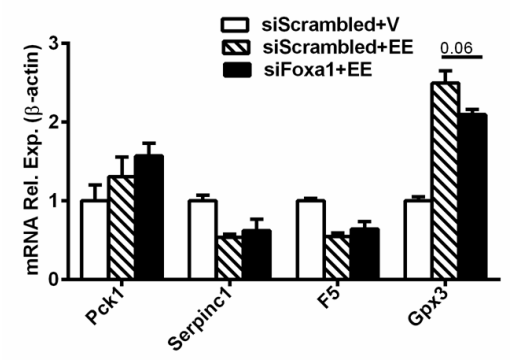
SUPPLEMENTAL FIGURES AND TABLE

Supplementary Figure 1. Uncropped protein blot.

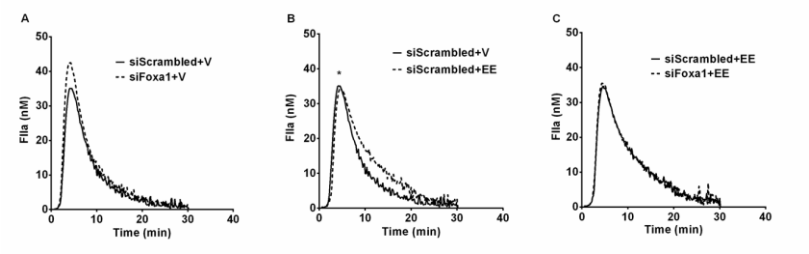


Supplementary figure 2. Sub-analysis of EE-bound ERα hepatic gene transcription.



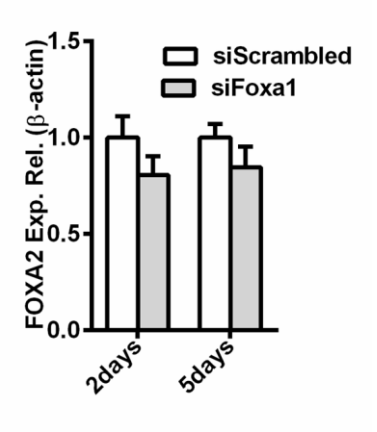
Sub-analysis of **Figure 4** was performed on samples which were used for ChIP assay (n=3 for each group). Data are presented for iScrambled+Vehicle (Open bars), siScrambled+EE (hatched bars) and siFoxa1+EE (Black bars). Data are expressed as means with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data were statistically analyzed using Student's t-test. *P*-values < 0.05 were regarded as statistically significant.

Supplementary figure 3. Thrombin generation assay



Thrombin generation were assessed in 1:6 diluted mouse plasma of five days post injection. (**A**) Effect of FOXA1 silencing on thrombin generation, siScrambled control siRNA (solid line) and siFoxa1 siRNA (dashed line) (**B**) Thrombin generation affected upon estrogen hormone treatment, siScrambled vehicle (solid line) and siScrambled EE (dashed line) treated animals. (**C**) In the presence of hormone FOXA1 silencing does not affect thrombin generation, siScrambled EE (solid line) and siFoxa1 EE (dashed line) treated animals. Data represented are of n=12 mice per group. siScrambled mice group set as a reference. *P*-values < 0.05 were regarded as statistically significant.

Supplementary figure 4: Gene expression of hepatic FOXA2



Data are presented for siFoxa1 (gray bars) with siScrambled injected animals as controls (open bars). β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the siScrambled group. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt (see figure 1 legends). Data were statistically analyzed using Mann Whitney Rank Sum test. *P*-values < 0.05 were regarded as statistically significant.

Supplemental table 1: QPCR primer sequences for ChIP

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
Shp	GGGCATCAATAGAAACAGCAG	TGCCCTTTATCGGATGACTC
Serpinc1	ATCCTTTGGTTGCCCTTACC	TAAGCATTGCGCCTTTCC
Pck1	CAACAGGCAGGGTCAAAGTT	GCACGGTTTGGAACTGACTT

Chapter 5

Acute and Severe Coagulopathy in Adult Mice Following Silencing of Hepatic Antithrombin and Protein C Production

Huma Safdar, Ka Lei Cheung, Daniela Salvatori, Henri H Versteeg, El Houari Laghmani, Gerry T. M. Wagenaar, Pieter H. Reitsma, Bart J.M. van Vlijmen

Blood. 2013;121(21):4413-4416

Summary

Mice deficient in the anticoagulants antithrombin (*Serpinc1*) or protein C (*Proc*) display early lethality due to thrombosis-related coagulopathy, thereby precluding their use in gene function studies or thrombosis models. Here, RNA interference was used to silence *Serpinc1* and/or *Proc* in normal adult mice, and the severe coagulopathy following combined 'knockdown' of these genes is reported. Two days after siRNA injection, (occlusive) thrombi were observed in (large and medium-sized) vessels in multiple tissues, and hemorrhages were prominent in the ocular, mandibular, and maxillary areas. Tissue fibrin deposition and plasma fibrinogen lowering accompanied this phenotype. The coagulopathy was prevented by dabigatran treatment. Silencing *Serpinc1* alone yielded a comparable but milder phenotype with later onset. The phenotype was absent when targeting *Proc* alone. We conclude that RNA interference of *Serpinc1* and/or *Proc* allows studying the function of these genes *in vivo*, and provides a novel, controlled mouse model for spontaneous venous thrombosis.

INTRODUCTION

Wild type mice do not spontaneously develop venous thrombosis. Knock-out mice for the natural anticoagulants antithrombin (*Serpinc1*) and protein C (*Proc*) feature spontaneous thrombosis-related coagulopathy[1;2], and succumb during embryogenesis and/or perinatally, precluding their use in studies on the function of these anticoagulants, or as a model for thrombosis. To overcome early lethality we employed RNA interference to silence antithrombin and protein C production, alone or in combination, in livers of wild type mice. Here, we report the spontaneous thrombotic phenotype observed shortly after this treatment.

METHODS

Effective synthetic siRNAs (Life Technologies, Carlsbad, USA) targeting *Serpinc1* and *Proc* were identified using mouse hepatocytes as described previously[3]. Selected si*Serpinc1*, si*Proc* (sequences, see supplemental methods) and control siNEG (Life Technologies), complexed with Invivofectamine® (Life Technologies), were tail vein-injected in 8-10 weeks old female C57Black/6J mice (Charles River, Maastricht, The Netherlands), alone or in combination. Mice were sacrificed at different time points and subjected to necropsy according to international pathology guidelines[4]. Liver *Serpinc1* and *Proc* transcript, plasma antithrombin, protein C, thrombin-antithrombin complexes, and fibrinogen levels, PT and aPTT, and tissue fibrin deposition were analyzed as described previously[3;5;6].

RESULTS AND DISCUSSION

Intravenous injection of si*Serpinc1* or si*Proc* (7 mg siRNA/kg body weight) resulted in a strong reduction in *Serpinc1* or *Proc* hepatic transcript (4±0.3% and 11±0.5% of siNEG-treated animals, respectively (n=3)) and plasma antigen level (1±2.4% and 2±1.8% of siNEG, respectively (n=3)) after 2 days. In si*Serpinc1*-injected mice, plasma thrombin-antithrombin

complexes were not detectable (<2 ng/ml), indicating that low plasma antigen was not due to consumptive loss, at least for antithrombin. At 2 days the animals appeared fully normal (observation in 6 animals per siRNA). A similar reduction in transcript and plasma antigen level was observed when animals were treated with a combination of si*Serpinc1* and si*Proc* (7 mg/siRNA/kg; n=6, Figure 1A, B). However, here, 2 days after injection, one animal died and the remaining 5 animals displayed severe weight loss (-15.6±2.4%, *P*=0.0011 vs siNEG-treated animals (n=6)), lethargy, unresponsiveness to stimuli, and hypothermia. Four of the 5 surviving animals featured unilateral lesions involving the eye (exophthalmos), intraocular and periocular hemorrhages (Figure1C). Maxillary, mandibular and submandibular regions were severely swollen and showed focally extensive subcutaneous and intramuscular hemorrhages especially involving the masseter muscle (Figure1D). In 2 animals hind leg cyanosis was observed (Figure 1G). Because of the severe and irreversible clinical conditions animals were promptly sacrificed also allowing further pathological and biochemical analyses. In affected animals, collecting citrate-blood from the caval vein was difficult or not possible, likely because of circulatory failure and shock. At day 3 and 4, the surviving si*Serpinc1*/si*Proc* animal and si*Serpinc1*-alone animals also featured the clinical signs (hind leg cyanosis not observed). During 5 days of observation none of the si*Proc*-treated animals (n=6) featured abnormalities.

Because of the severe symptoms in the *Serpinc1*/si*Proc* group further experiments using the 7 mg/siRNA/kg dose were discontinued. Using half the dose of *Serpinc1*/si*Proc* (3.5 mg/siRNA/kg) reproduced most pathology findings (n=13), and all animals showed the described clinical signs within 72 hours after siRNA injection. However, weight loss was less severe (-12.1±5.1%, *P*<0.0001 vs siNEG), immediate death and hind leg cyanosis

were not observed, and appropriate collection of citrate-blood was possible for most animals. In total, 19 out of 19 si*Serpinc1*/si*Proc* (sum of animals for 3.5 and 7 mg/kg dose) versus 0 out of 11 siNEG-treated animals featured abnormalities (P<0.0001, Fisher's exact test).

Necropsy was performed on si*Serpinc1/siProc* (7 and 3.5 mg/siRNA/kg dose, n=5 and 12, respectively) and si*Serpinc1* animals (7 mg/siRNA/kg, n=6). Sagittal and coronal serial sections of the head showed severe multifocal hemorrhages within the eye (Figure 1E), surrounding muscles and harderian glands (Figure1E, F). The masseter and temporal muscles of affected eyes consistently displayed severe hemorrhages, mild muscle degeneration and necrosis. Hemorrhages and vascular thrombi were observed in the above-mentioned areas and submucosa of the palate, nasal turbinates, tongue and subdural spaces. Lesions were observed in all animals and were comparable between the si*Serpinc1/siProc* and si*Serpinc1*-alone groups. Hind leg cyanosis, if present, coincided with hemorrhages and thrombi in the subcutis and tibia-femoral muscular fibers (Figure1H). Regarding the liver, in at least six specimens (high and low dose of si*Serpinc1*/si*Proc*) multifocal areas of necrosis were grossly visible (Figure 1I). Microscopic abnormalities in the liver were found for all animals and ranged from minimal multifocal hepatic degeneration and presence of rare thrombi (4 out of 12 animals receiving the low dose of si*Serpinc1*/si*Proc*, 4 out of 6 animals receiving si*Serpinc1* alone*)* to multifocal, extensive areas of severe coagulative and lytic necrosis with (occlusive) thrombi in large and medium-sized vessels (Figure 1J, for 8 out of 12 animals receiving low dose si*Serpinc1*/si*Proc*, all animals receiving high dose si*Serpinc1*/si*Proc*, 2 out of 6 animals for si*Serpinc1*-alone). In head, leg and liver, thrombi were located in veins, characterized by organized fibrin layering, and surrounding tissues devoid of inflammatory cells, indicative for an acute process. Neither animal featured lesions,

thrombi and/or hemorrhages in kidney or gastrointestinal tract. Minor incidence was observed in heart, lung, and brain.

Biochemical analysis of livers of si*Serpinc1*/si*Proc*-treated animals at 3.5 mg/siRNA/kg demonstrated fibrin deposition (Figure 1K), coinciding with reduced plasma fibrinogen levels (Figure 1L). For the 7 mg/siRNA/kg dose, liver fibrin deposition was massive and plasma fibrinogen was virtually absent (Figure 1L), indicating a relation with the siRNA dose. Liver fibrin deposition was at background level in siNEG (5.4±2.7 ng/mg, n=11) and si*Proc*-treated animals (5.6±2.7 ng/mg, n=6). Remarkably, si*Serpinc1*-treated animals had low liver fibrin deposition (10.7±3.1 ng/mg, n=6). As si*Serpinc1* animals displayed a later onset of the phenotype (reproduced in additional experiments; both 3.5 and 7 mg/si*Serpinc1*/kg, n=5 per dose), and unaffected plasma fibrinogen (data not shown), this suggests that a combination of si*Serpinc1* and si*Proc* results in a more severe phenotype than the sum of si*Serpinc1* and *siProc* alone. Likely, combined loss of Serpinc1 and Proc pushes the animals over a thrombotic threshold that cannot be reached with diminution of either one.

Tissue fibrin deposition was not restricted to the liver; lungs of si*Serpinc1*/si*Proc*-treated mice demonstrated increased fibrin deposition (3.5 mg/siRNA/kg dose; 50±38 (n=13) vs 13±7.3 ng/mg (n=11) in siNEG, *P*=0.0010), despite minor incidence of microscopically visible thrombi in this tissue (1 animal out of 12).

The presence of (occlusive) thrombi and fibrin deposition in si*Serpinc1*/si*Proc*-treated animals indicates that the observed coagulopathy is thrombotic in nature and hemorrhages are likely secondary to consumption of fibrinogen and/or other coagulation factors as illustrated by prolonged PT and aPTT for 6 out of 10 si*Serpinc1*/si*Proc*-treated animals (>70 and >120 s, respectively vs 11±0.3 and 27±1.6 s for siNEG-treated animals, both *P*=0.0039, Fisher test). To demonstrate that thrombin forma-

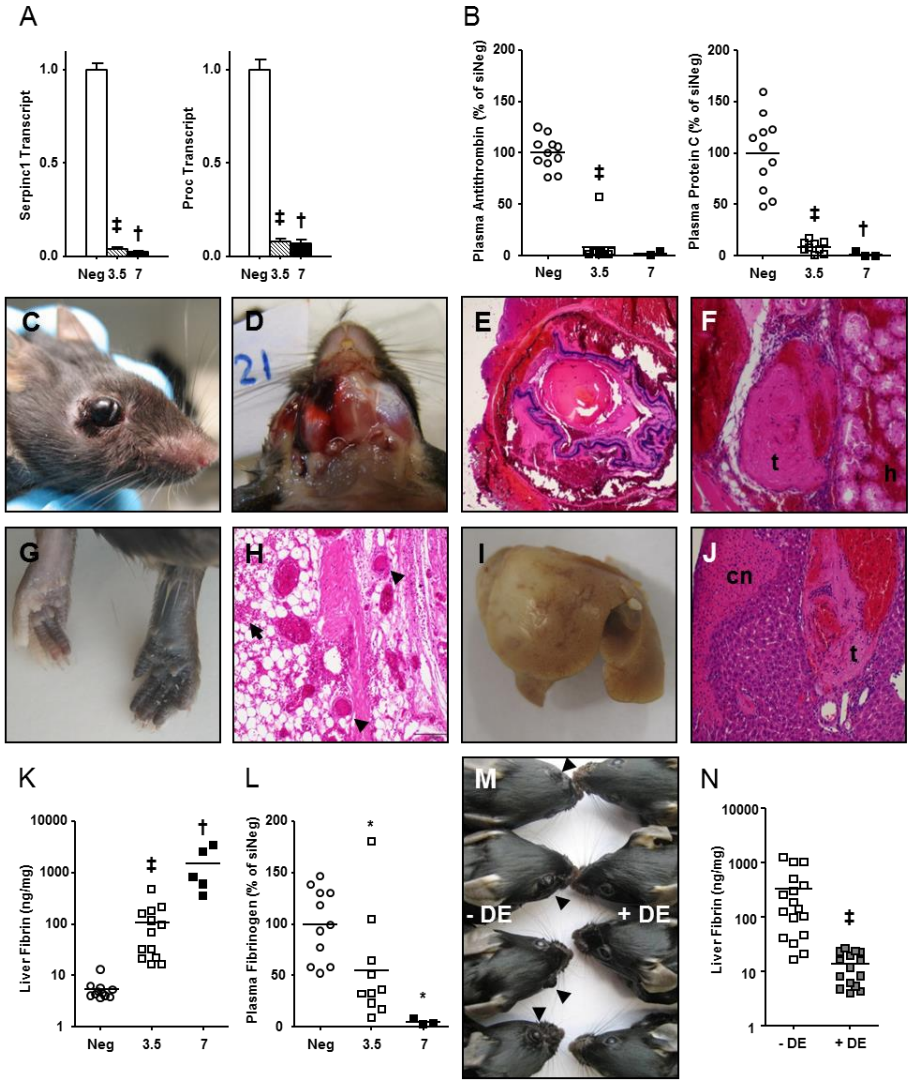


Figure 1: Phenotypic Appearance of Mice Following Silencing of Hepatic Antithrombin and Protein C Production. **A**. Effectiveness of silencing of *Serpinc1* and *Proc* in mouse liver. siRNAs targeting *Serpinc1* and *Proc* were complexed, mixed and intravenously injected in C57Black/6J mice at a dose of 3.5 (hatched bars, n=13) or 7 (black bars, n=6) mg per siRNA per kg mouse. At two days post siRNA injection mice were removed from the

experiment, sacrificed and livers were subjected to *Serpinc1* (left) or *Proc* (right) transcript analysis by qPCR. β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group (siNEG, 7 mg per kg, open bars, n=11); **B**. Plasma antithrombin (left) and protein C levels (right) in siNEG (open circles), si*Serpinc1*/si*Proc*-injected animals at 3.5 (open squares) or 7 (black squares) mg siRNA per kg mouse; **C**. Right eye of a si*Serpinc1*/si*Proc* injected animal. Unilateral severe exophthalmos and periocular hemorrhages; **D**. Multifocal hemorrhages in the mandibular, submandibular area, and masseter muscle; **E**. Severe multifocal hemorrhages within the eye (HE-stained 5-µm section, magnification 40x); **F**. Eye region: harderian glands with multifocal hemorrhages (h) and thrombus (t) presence (HE-stained 5-µm section, magnification 100x); **G**. Cyanosis of the right hind leg; **H**. Hemorrhages (arrow) and thrombi (arrow heads) were present in the subcutis but also among muscular fibers of the tibial and femoral areas (HE-stained 5-µm section, magnification 200x); **I**. Liver (formalin-fixed specimen) presenting focally extensive areas of necrosis (asterisk); **J**. Liver section presenting severe multifocal to coalescing coagulative necrosis (cn) and thrombosis (t) in hepatic vein (HE-stained 5-µm section, magnification 100x); **K**. Liver fibrin, and **L**. plasma fibrinogen in siNEG (open circles), si*Serpinc1*/si*Proc* injected animals at 3.5 (open squares), or 7 (black squares) mg siRNA per kg mouse; **M**. Treatment of si*Serpinc1*/si*Proc*-injected treated mice (3.5 mg/kg dose) with dabigatran etexilate (DE). DE was administered by oral gavage of 3 mg per mouse at 7 am, 3 and 11 pm for five days, starting the day before siRNA injection. This results in an aPTT of 66.9±6.8 (s) to 36.2±4.4 (s) as determined in a parallel treated control group at two hours after dosing and one hour before the next dose (vehicle treated animals aPTT of 26.7±5.1 and 24.5±0.8 (s), respectively). Presence of periocular contusion (arrow heads) for vehicle treated animals (left) and not DE-treated animals (right); **N**. Liver fibrin deposition in si*Serpinc1*/si*Proc* (3.5 mg per siRNA per kg) treated with DE (open squares) or vehicle (filled squares). Data were analyzed with the Instat software (GraphPad, San Diego, USA). Statistical differences between control siNEG and si*Serpinc1*, si*Proc*, si*Serpinc1*/si*Proc* were evaluated using a Mann-Whitney Rank sum test. P-values<0.05 were regarded as statistically significant. *P<0.05, †P<0.01, ‡P<0.001

tion underlied this phenotype, si*Serpinc1*/si*Proc*-treated mice (3.5 mg/siRNA/kg) were administered the thrombin inhibitor dabigatran etexilate. Dabigatran prevented the clinical signs of si*Serpinc1*/si*Proc*-treated mice, including weight loss (-5.7±5.2 vs -11.2±3.9% in vehicle-

treated si*Serpinc1*/si*Proc* animals, n=16-17, *P*=0.0011), exophthalmos and periocular contusion (Figure 1M, 0/17 versus 16/ 16, *P*<0.0001, Fisher test), and largely suppressed liver fibrin deposition (Figure 1N).

We conclude that silencing hepatic *Serpinc1*/*Proc* or *Serpinc1* alone acutely induces thrombotic coagulopathy. This study a) highlights the importance of protein C and antithrombin in animals under challenge-free conditions, b) points to synergism between these anticoagulant systems c) will help to further unravel the *in vivo* function of these anticoagulants and d) provides a novel, controlled model for venous thrombosis research.

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SUPPLEMENTAL METHODS

Structural information on reagents and negative control

We used pre-designed siRNAs for mouse Serpinc1 and Proc mRNAs that were purchased from Life Technologies, Carlsbad, California, USA (Ambion Silencer® Select Pre-designed siRNA) catalogue number 4404014. For mouse Serpinc1 with sense sequence UCCUGGUUCUUAUAAGGGATT and antisense sequence UCCCUUAUAAGAACCAGGAAG and for mouse Proc with sense sequence GCAAGAUCCUCAAACGAGATT and antisense sequence UCUCGUUUGAGGAUCUUGCTGT. Ambion's siNEG (catalogue number 4404020) was used as control siRNA. This negative control siRNA was selected using a modified blast to account for short sequence length and demonstrated to exclude significant homology to any known gene targets in RefSeq and MirBase (more detailed documentation on this negative control siRNA is available on the manufacturer's website;http://products.invitrogen.com/ivgn/product/4457289?ICID==%3D%3D%3Dsearch-product).

Chapter 6

Regulation of the *F11, Klkb1, Cyp4v3* Gene Cluster in Livers of Metabolically Challenged Mice

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SUMMARY

Single nucleotide polymorphisms (SNPs) in a 4q35.2 locus that harbors the coagulation factor XI (*F11*), prekallikrein (*KLKB1*), and a cytochrome P450 family member (*CYP4V2*) genes are associated with deep venous thrombosis (DVT). These SNPs exert their effect on DVT by modifying the circulating levels of FXI. However, SNPs associated with DVT were not necessarily all in *F11,* but also in *KLKB1* and *CYP4V2*. Here, we searched for evidence for common regulatory elements within the 4q35.2 locus, outside the *F11* gene, that might control FXI plasma levels and/or DVT risk. To this end, we investigated the regulation of the orthologous mouse gene cluster under several metabolic conditions that impact mouse hepatic *F11* transcription. In livers of mice in which HNF4α, a key transcription factor controlling *F11*, was ablated, or reduced by siRNA, a strong decrease in hepatic *F11* transcript levels was observed that correlated with *Cyp4v3* (mouse orthologue of *CYP4V2*), but not by *Klkb1* levels. Estrogens induced hepatic *F11* and *Cyp4v3*, but not *Klkb1* transcript levels, whereas thyroid hormone strongly induced hepatic *F11* transcript levels, and reduced *Cyp4v3*, leaving *Klkb1* levels unaffected. Mice fed a high-fat diet also had elevated *F11* transcription, markedly paralleled by an induction of *Klkb1 and Cyp4v3* expression. We conclude that within the mouse *F11*, *Klkb1*, *Cyp4v3* gene cluster, *F11* and *Cyp4v3* frequently display striking parallel transcriptional responses suggesting the presence of shared regulatory elements.

INTRODUCTION

Blood coagulation serine protease factor XI (FXI) contributes to hemostasis by activating coagulation factor IX [1]. Although bleeding associated with FXI deficiency is relatively mild, there has been a resurgence of interest in FXI, because candidate gene studies revealed a role of high FXI levels as a risk factor for venous thrombosis [2;3]. Factor XI, like the blood coagulation protease factors II, VII, IX, X, XII and XIIIb, is produced primarily in hepatocytes. FXI is a dimeric serine protease that is structurally closely related to prekallikrein, a serine protease that is also involved in the intrinsic blood coagulation pathway [4]. Both factor XI and prekallikrein are activated by active coagulation factor XII [5-7].

The genes encoding FXI (*F11*) and prekallikrein (*KLKB1*) are located in tandem on the long arm of chromosome 4 (4q35.2) directly downstream from the cytochrome P450 family member (*CYP4V2*) gene and family with sequence similarity 149 A (*FAM149A,* Figure 1). The size of this 4q35.2 locus is approximately 200 kb (197.715 bp; position 187297059—187494774). It is assumed that CYP4V2, which has a role in fatty acid metabolism [8], is not involved in coagulation. The function of FAM149A is unknown. Recent genetic studies of deep vein thrombosis (DVT) reported that several common single nucleotide polymorphisms (SNPs) in the *F11*, *KLKB1*, and *CYP4V2* region (not including *FAM149A*) were associated with DVT and plasma FXI antigen levels (individuals with FXI antigen in 90 percentile having a 2-fold increased risk for DVT) [3]. The two SNPs that were independently associated with DVT and high plasma FXI antigen levels were located within *F11* gene (rs2036914 and rs2289252) [9]. SNP rs13146272 within *CYP4V2* was associated with DVT and FXI levels also after adjustment for rs2036914 and rs2289252 [9]. Of note, these three SNPs remain associated with DVT after adjustment for FXI levels. Possibly average levels are not so much affected by these SNPs, but rather that peak levels (e.g. as a result of hormone stimuli) are affected. It could also

be that qualitative changes in the FXI protein not detected by quantitative effects are responsible (e.g. missense or splice changes). Alternatively, SNPs in *F11* and *CYP4V2* may also affect the expression of KLKB1, which is clearly also a strong candidate DVT risk gene. The above suggests that the DVT risk associated with variation in the 4q35.2 region may not necessarily be solely attributed to *F11* gene variation and/or FXI protein levels. Furthermore, it opens the possibility that *KLKB1* and *CYP4V2* may be more than just neighbors of *F11,* but may also share regulatory elements.

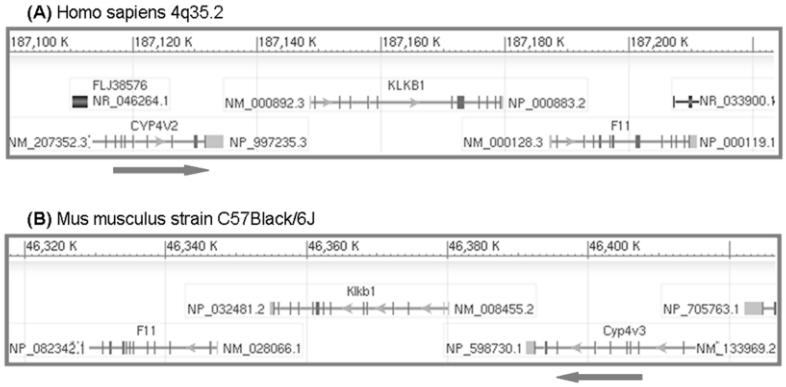

Figure 1: F11 gene locus organization in human (A) and mice (B). Vertical bars representing exons, and lines representing introns. Arrows show the orientation of transcription. Source; http://www.ncbi.nlm.nih.gov/

Experimental evidence for the possible regulatory interaction of the genes within 4q35.2 region should follow from a detailed *in vitro* analysis of this cluster, but is complicated as this requires expression analysis of the entire cluster with (the as yet unidentified) regulatory elements in their natural positions. Such analysis is limited by the size of the cluster (~100 kb). In mice, *F11*, *Klkb1* and *Cyp4v3* (the mouse orthologue of *CYP4V2*) are also located together (Figure 1). Furthermore, in mice, hepatic *F11* transcription, unlike many other coagulation proteases, is clearly responsive to changes

in estrogen status [10], thyroid hormone status [unpublished observation] or dietary fat intake [11]. Here, we took advantage of mouse hepatic *F11* transcriptional responsiveness to obtain additional evidence for possible regulatory interaction of the genes within the 4q35.2 locus. To this end, we studied variation in hepatic *F11*, *Klkb1* and *Cyp4v3* transcript levels under a number of conditions that affect mouse hepatic *F11* gene transcription. We observed parallel responses in *F11* and *Cyp4v3* transcript levels when hepatocyte nuclear factor 4α (HNF4α), estrogen, and dietary status were varied. In contrast, *Klkb1* only showed an *F11*-like response for the fat feeding condition.

MATERIALS AND METHODS

Materials

Livers from 45-day-old female liver-specific *Hnf4a*-null mice with a liver-specific deletion of exons 4 and 5 of the *Hnf4a* gene (*Hnf4a*-floxed/floxed with albumin-Cre; KO) or control mice (*Hnf4a*-floxed/floxed without albumin-Cre; FLOX) were described previously [12]. Liver from 10-week-old ovariectomized female C57BL/6J mice treated orally with synthetic estrogen hormone (17α-ethinylestradiol) (1µg) once for 'single doses' or daily for 10 days for 'multiple doses' and respective vehicle-treated controls have been described previously [10].

Animal experiments.

C57BL/6J males and females were purchased from Charles River Laboratories (Maastricht, the Netherlands) and housed under a 12-h light/dark cycle, with standard chow diet and drinking water provided ad libitum. For studying the impact of the natural estrogen 17ß-estradiol, 8-week-old female mice were bilaterally ovariectomized under isoflurane anesthesia, and after a 2 week recovery period, they were randomly assigned to either the experimental group or vehicle treatment group. 17ß-

estradiol (Sigma Aldrich, Steinheim, Germany) stock was prepared in ethanol and diluted in arachid oil at a final concentration of 1% ethanol prior to injection. 17ß-estradiol was injected subcutaneously once or for 5 days, at a (daily) dose of 2 µg per mouse. For the vehicle treatment, mice were subcutaneously injected with 100 µL of arachid oil with an ethanol concentration of 1%.

For study the effect of thyroid hormone, male C57BL/6J mice, 8 weeks of age, were fed a low iodine diet (ICN Biomedicals, Inc., Aurora, OH) and drinking water supplemented with 1% (wt/vol) potassium perchlorate (Sigma). 3,3′,5-triiodo-L-thyronine sodium salt (T_3) (Sigma Aldrich) stocks of 1 mg/ml were prepared in 4 mM sodium hydroxide and stored at 4 degrees Celsius. For injection, a T_3 stock was diluted to 2.5 µg T_3/ml in phosphate buffered saline supplemented with 0.02% bovine serum albumin with a final concentration of sodium hydroxide of 0.2 mM. Mice received a daily intraperitoneal injection of (0.5 µg) 200 µl thyroid hormone solution for 14 days.

For studying the effect of fat feeding, 8-week-old male C57BL/6J mice were fed a low-fat control diet (LFD; 10% energy in the form of fat, D12450B, Research Diet Services, The Netherlands) as a run-in for a period of 2 weeks. Subsequently, they were randomly assigned to either the experimental group fed a high-fat diet (HFD; 45% energy in the form of fat, D12451, Research Diet Services, The Netherlands) or maintained on the control LFD for 1 or 7 days.

For small interfering (si) RNA-mediated knockdown of HNF4α in 8-week-old female mice, a control siRNA (siNEG; cat. # 4404020, Ambion, Life Technologies Corporation, USA) and a siRNA tested in mouse primary hepatocytes to be effective to reduce HNF4α transcript and protein levels by 90% at a concentration of 3 nM were used as described previously [13]. The sequences of the two siHNF4α RNA-strands were, sense: 5´-AGA GGU CCA UGG UGU UUA AUU-3´ and antisense: 5´-UUA AAC ACC AUG

GAC CUC UUG-3´ (siHNF4α, cat. # 67635). Control siNEG (catalogue number 4404020) and siHNF4α were complexed with Invivofectamine® 2.0 (Invitrogen, Life technologies Corporation, USA) exactly according to the manufacturer's protocol. Subsequently, C57BL/6J mice were intravenously injected via the tail vein with 200µl complexed siRNA, a dose of 7 mg of siRNA per kg body weight.

At the indicated time points, 17ß-estradiol-treated, T_3-treated, fat-fed, or siHNF4α injected animals and the respective controls were anesthetized by a subcutaneous injection with a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg) and atropine (125 µg/kg) after which the abdomen was opened by a midline incision and a blood sample on sodium citrate (final concentration 0.32%) was drawn from the inferior caval vein. Plasma was obtained by centrifugation and stored at -80°C until use [10]. Liver was isolated and weighed, and part of a liver lobule was snap-frozen for mRNA analyses and stored at -80°C until use. All experimental procedures were approved by the animal welfare committee of the Leiden University (under registration # 10244, 10032 and 11005).

Hepatic transcript and plasma protein analyses

RNA was isolated from mouse livers and subsequently analyzed for transcripts by quantitative real-time PCR as described previously [10]. The gene-specific quantitative primers used are presented in Table S1. ß-actin was used as an internal control for normalization and quantification. The ΔC_t values of the individual samples were related to the mean ΔC_t of the reference group (i.e. $\Delta\Delta C_t$). Values are expressed as mean (2 POWER of mean $\Delta\Delta C_t$) with a lower range (2 POWER of mean $\Delta\Delta C_t$+SEM) and an upper range (2 POWER of mean $\Delta\Delta C_t$-SEM).

Plasma FXI activity levels were determined as described previously [10]. Respective control groups were used as a reference.

Statistical analyses

Data were analyzed with 'GraphPad Instant' software and statistical differences were assessed using the Student's t-test (plasma analysis) or the Mann-Whitney Rank sum test (transcript levels). The Pearson correlation coefficient (r) was used to evaluate whether hepatic transcript levels were correlated. A *p*-value <0.05 was considered to be significant.

RESULTS

To find support for the hypothesis of common regulatory elements in the 4q35.2 locus, we examined whether *F11*, *Klkb1* and *Cyp4v3* transcription in mice responds in a concerted manner to stimuli affecting *F11* transcript levels i.e. estrogen hormone [10] and thyroid hormone treatments (unpublished observation) and fat feeding [10;11]. Before doing so, we first investigated the regulatory role of hepatocyte nuclear factor 4α (HNF4α) for the genes located in this locus, as this transcription factor controls the *F11* gene [14]. In livers of 45-day-old mice lacking HNF4α in liver (KO), the *F11* transcript levels and *Cyp4v3* levels were strongly reduced or even absent when compared to their control littermates (FLOX) (-96 and -90%, respectively; Table 1). For *Klkb1*, located in between the *F11* and *Cyp4v3,* transcript levels were also clearly affected by HNF4α status, but to a lesser extent (-48%, Table 1). As these reducing effects may be secondary to changes in liver physiology due to prolonged (45 days) ablation of hepatic HNF4α, transcript levels of the gene cluster in adult C57BL/6J mice, in which hepatic *Hnf4a* was rapidly reduced by means of a liver-targeted specific small interfering (si) RNA were also examined. Two days after intravenous injection of a double-stranded siRNA known to be effective for *Hnf4a* knockdown, a 61% reduction of hepatic *Hnf4a* transcript levels was observed that correlated with the reduction in *F11* and *Cyp4v3* transcript levels (-52% and -61% respectively, Table 1). *Klkb1* transcript levels were not affected by the HNF4α siRNA. At reduced hepatic HNF4α RNA levels,

F11 and *Cyp4v3* transcript levels in the individual mice strongly and significantly correlated (Figure 2A; Table 2), whereas *F11* and *Klkb1* were not significantly correlated (Table 2). Furthermore, *Hnf4a* transcript levels significantly correlated with *F11* and *Cyp4v3*, but not with *Klkb1* (Figure S1). The specificity of the effects of HNF4α modulation (KO or in siRNA-mediated knockdown) on *F11*, *Cyp4v3* and to a lesser extent *Klkb1* transcription are illustrated by the absence of effects for hepatic transcript levels of coagulation factor genes *F2*, *F7*, and *F10*, while that of the established HNF4α target *F12* was clearly reduced (Table 3).

Subsequently, the effect of estrogen on transcription in the locus harboring *F11*, *Klkb1* and *Cyp4v3* was determined. In mice, estrogens have an overall downregulatory effect on liver coagulation gene transcription, but upregulated *F11*. Both oral administration of the synthetic estrogen 17α-ethinylestradiol (EE) for 10 days (1 µg/mouse/day) and subcutaneous injection of the natural estrogen 17ß-estradiol (E2) for 5 days (2 µg/mouse/day) to ovariectomized C57BL/6J mice induced moderate reductions of hepatic transcript for coagulation factor genes F2, F7, F10 and F12 as compared to the respective vehicle control treated animals (-38, -45, -48 and -24% for EE and -16, -17, -11 and -15% for E2, respectively, Table 3). However, the reduction in *F10* and *F12* transcript levels under E2 did not reach statistical significance. Although more subtle, for EE, but not for E2, these reducing effects were already apparent at 5 hours after a single dose of hormone. As expected [10], the opposite was observed for *F11*, with significant increased hepatic transcript levels upon prolonged exposure to both EE and E2 (+59% and +62%, respectively; Table 1). These changes were apparent at 5 hours after a single dose of EE (+35%) and E2 (+31%) (Table 1). For EE, but not for E2, the transcriptional response of *Cyp4v3*, but not that of *Klkb1*, largely resembled the response of *F11*. Consequently, also under EE significant correlations were only

Table 1: Transcript levels of *F11*, *Klkb1* and *Cyp4v3* in livers of challenged mice.

	n	F11	Klkb1	Cyp4v3
Hepatic HNF4α status				
siNEG mice	6	1 (0.95 - 1.05)	1 (0.95 - 1.05)	1 (0.92 - 1.08)
siHNF4α mice	6	0.48 (0.45 - 0.52)†	0.92 (0.88 - 0.97)	0.39 (0.34 - 0.45)†
FLOX mice	8	1 (0.89 - 1.13)	1 (0.91 - 1.11)	1 (0.94 - 1.06)
KO mice	8	0.04 (0.04 - 0.05)‡	0.52 (0.50 - 0.54)‡	0.10 (0.09 - 0.11)‡
Estrogen hormone				
Vehicle control	5	1 (0.91 - 1.10)	1 (0.96 - 1.04)	1 (0.95 - 1.05)
EE (1µg, 5 hours)	5	1.35 (1.29 - 1.42)*	1.20 (1.14 - 1.27)*	1.23 (1.17 - 1.29)*
Vehicle control	10	1 (0.94 - 1.07)	1 (0.93 - 1.07)	1 (0.93 - 1.08)
EE (1µg/day, 10days)	10	1.59 (1.49 - 1.69)‡	1.10 (1.04 - 1.16)	1.65 (1.59 - 1.72)‡
Vehicle control	7	1 (0.95 - 1.05)	1 (0.93 - 1.07)	1 (0.93 - 1.08)
E2 (2µg/day, 24h)	7	1.31 (1.20 - 1.43)*	1.04 (0.98 - 1.11)	1.09 (1.04 - 1.14)
Vehicle control	7	1 (0.92 - 1.09)	1 (0.92 - 1.08)	1 (0.94 - 1.07)
E2 (2µg/day, 5days)	7	1.62 (1.46 - 1.80)‡	1.02 (0.97 - 1.08)	1.00 (0.96 - 1.05)
Thyroid hormone				
Vehicle control	13	1 (0.94 - 1.07)	1 (0.97 - 1.03)	1 (0.97 - 1.03)
T_3 (0.5µg/day, 14days)	13	1.18 (1.10 - 1.26)*	0.92 (0.89 - 0.95)	0.55 (0.54 - 0.57)‡
Feeding condition				
Low fat control	11	1 (0.89 - 1.13)	1 (0.95 - 1.05)	1 (0.90 - 1.11)
High fat (1 day)	10	1.70 (1.61 - 1.76)‡	1.49 (1.44 - 1.53)‡	1.67 (1.60 - 1.76)†
High fat (7 days)	8	1.58 (1.42 - 1.76)‡	1.32 (1.25 - 1.38)†	1.73 (1.66 - 1.80)†

Data are expressed as 2 POWER of mean ΔΔCt with lower and upper range. β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group. *p<0.05, $^{\dagger}p$<0.01, $^{\ddagger}p$<0.001. siNEG/siHNF4α mice; mice injected with control (negative) or HNF4α siRNA respectively, KO/FLOX mice; HNF4α conditional liver knockout mice and control littermates, respectively, EE; ethinylestradiol, E2; 17-ß estradiol, T_3; 3,3′,5-Triiodo-L-thyronine.

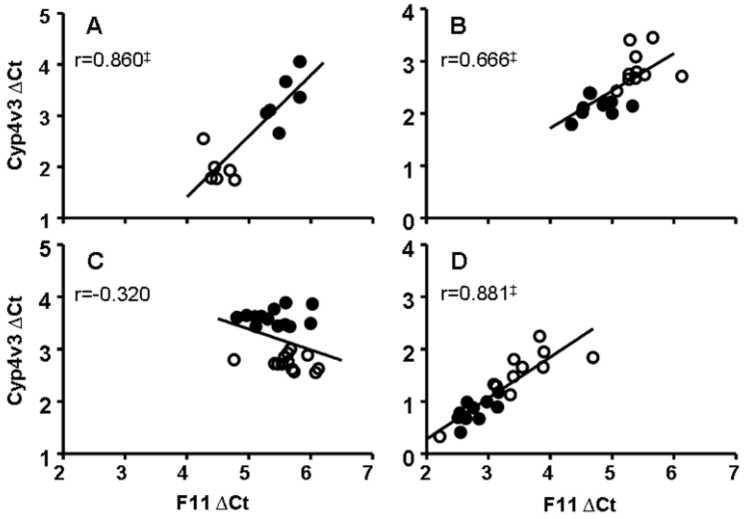

Figure 2: **Correlation between *F11* hepatic transcript levels and *Cyp4v3* under different metabolic conditions.** (A) siRNA-mediated HNF4α (●) depletion or control siRNA (○) in mouse liver, (B) 10 days of oral EE (●) or vehicle (○) treatment in ovariectomized mice, (C) 14 days of T_3 (●) or vehicle treatment (○) and (D) mice fed with high (●) or low (○) fat diet for one day. Correlations were determined using Pearson correlation coefficient (r). *p*-values < 0.05 were regarded as statistically significant. $^{‡}p < 0.001$

found for *F11* and *Cyp4v3* transcript levels. Although *Klkb1* and *Cyp4v3* were not affected by E2, we found significant correlations with *F11* at 24 hours (Figure 2B, Table 2).

Next, the modulation of the *F11*, *Klkb1* and *Cyp4v3* harboring locus by thyroid hormone was determined. C57BL/6J mice with suppressed endogenous thyroid hormone production were treated with triiodothyroxine (T_3; 0.5 µg T_3 per mouse for 14 days). T_3 results in a decrease in most hepatic coagulation gene transcript levels, with downregulatory effects of -35 and -27% observed for *F2* and *F10* respectively, while *F7* levels remained unchanged (Table 3). In contrast, *F11 and F12* displayed a statistically significant increase in transcription (+18 and +24% as

compared to vehicle, Tables 1 and 3). These upregulatory effects required sustained elevation of T_3 as none of the transcripts analyzed demonstrated an immediate response at 4 hours after a single injection of T_3 (data not shown). Increased hepatic *F11* transcript levels at 14 days of T_3 treatment coincided with a 45% decrease in *Cyp4v3* transcript levels, while *Klkb1* levels were not significantly affected. Consequently, under changing in T_3 levels, significant correlations between hepatic *F11*, *Klkb1* and *Cyp4v3* transcript levels were not observed (Figure 2C, Table 2).

Table 2: Correlation between transcript levels of *F11*, *Klkb1* and *Cyp4v3* in livers of challenged mice.

	F11-Cyp4v3		***F11-Klkb1***	
	r	***p*-values**	***r***	***p*-values**
Hepatic Hnf4α status				
siNEG/siHNF4α mice	0.860	0.0003	0.307	0.332
FLOX/KO mice	0.994	< 0.0001	0.886	< 0.0001
Estrogen hormone				
Vehicle/EE (1µg, 5hours)	0.703	0.0233	0.475	0.165
Vehicle/EE (1µg/day, 10days)	0.666	0.0001	0.277	0.2518
Vehicle/E2 (2µg, 24hours)	0.541	0.0457	0.640	0.014
Vehicle/E2 (2µg/day, 5days)	0.333	0.2450	0.254	0.4032
Thyroid hormone (T3)				
T_3 (0.5µg/day, 14days)	-0.320	0.1110	0.281	0.164
Feeding condition				
High fat (1 day)	0.881	< 0.0001	0.716	0.0003
High fat (7 days)	0.778	< 0.0001	0.546	0.019

Correlation between hepatic *F11* transcript levels and *Cyp4v3* (*F11-Cyp4v3*) or *Klkb1* (*F11-Klkb1*) under different metabolic conditions. Data was statistically analyzed with Pearson correlation coefficient (r). *p*-values < 0.05 were regarded as statistically significant.

siNEG/siHNF4α mice; mice injected with control (negative) or HNF4α siRNA respectively, KO/FLOX mice; HNF4α conditional liver knockout mice and control littermates, respectively, EE; ethinylestradiol, E2; 17-ß estradiol, T_3; 3,3′,5-Triiodo-L-thyronine.

Table 3: Transcript levels of *F2, F7, F10* and *F12* in livers of challenged mice.

	n	F2	F7	F10	F12
Hepatic HNF4α status					
siNEG mice	6	1 (0.96 - 1.05)	1 (0.96 - 1.05)	1 (0.97 - 1.04)	1 (0.97 - 1.04)
siHNF4α mice	6	0.90 (0.88 - 0.93)	1.02 (0.97 - 1.06)	1.04 (0.96 - 1.13)	0.65 (0.58 - 0.72)†
FLOX mice	8	1 (0.93 - 1.08)	1 (0.89 - 1.13)	1 (0.90 - 1.11)	1 (0.92 - 1.09)
KO mice	8	1.17 (1.11 - 1.22)	0.97 (0.93 - 1.01)	0.84 (0.79 - 0.89)	0.05 (0.04 - 0.06)‡
Estrogen hormone					
Vehicle control	5	1 (0.90 - 1.11)	1 (0.96 - 1.05)	1 (0.89 - 1.13)	1 (0.94 - 1.07)
EE (1µg, 5 hours)	5	0.75 (0.72 - 0.78)*	0.75 (0.72 - 0.77)‡	0.75 (0.71 - 0.79)	0.72 (0.67 - 0.78)*
Vehicle control	10	1 (0.95 - 1.05)	1 (0.89 - 1.13)	1 (0.95 - 1.05)	1 (0.96 - 1.04)
EE (1µg/day, 10days)	10	0.62 (0.58 - 0.66)‡	0.55 (0.53 - 0.56)‡	0.52 (0.50 - 0.55)‡	0.76 (0.73 - 0.80)†
Vehicle control	7	1 (0.95 - 1.06)	1 (0.92 - 1.09)	1 (0.94 - 1.06)	1 (0.93 - 1.08)
E2 (2µg/day, 24h)	7	1.03 (0.98 - 1.08)	1.08 (1.04 - 1.12)	1.15 (1.07 - 1.23)	1.09 (1.04 - 1.15)
Vehicle control	7	1 (0.97 - 1.04)	1 (0.96 - 1.04)	1 (0.96 - 1.04)	1 (0.92 - 1.08)
E2 (2µg/day, 5days)	7	0.84 (0.79 - 0.89)†	0.83 (0.79 - 0.88)†	0.89 (0.84 - 0.94)	0.85 (0.81 - 0.90)
Thyroid hormone					
Vehicle control	13	1 (0.96 - 1.05)	1 (0.97 - 1.03)	1 (0.95 - 1.05)	1 (0.95 - 1.05)
T_3 (0.5µg/day, 14days)	13	0.65 (0.61 - 0.68)‡	0.96 (0.90 - 1.02)	0.73 (0.70 - 0.77)‡	1.24 (1.17 - 1.31)†
Feeding condition					
Low fat control	12	1 (0.95 - 1.05)	1 (0.96 - 1.04)	1 (0.97 - 1.04)	1 (0.97 - 1.03)
High fat (1 day)	10	1.17 (1.11 - 1.23)*	1.13 (1.07 - 1.19)	1.11 (1.05 - 1.18)	1.13 (1.08 - 1.19)
High fat (7 days)	8	1.03 (1.00 - 1.06)	1.00 (0.97 - 1.03)	1.02 (0.99 - 1.04)	1.03 (0.98 - 1.08)

Table 3: Data are expressed as 2 POWER of mean ΔΔCt with lower and upper range. β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group. *p*-values < 0.05 were regarded as statistically significant. **p*<0.05, †*p*<0.01, ‡*p*<0.001.
siNEG/siHNF4α mice; mice injected with control (negative) or HNF4α siRNA respectively, KO/FLOX mice; HNF4α conditional liver knockout mice and control littermates, respectively, EE; ethinylestradiol, E2; 17-ß estradiol, T_3; 3,3′,5-Triiodo-L-thyronine.

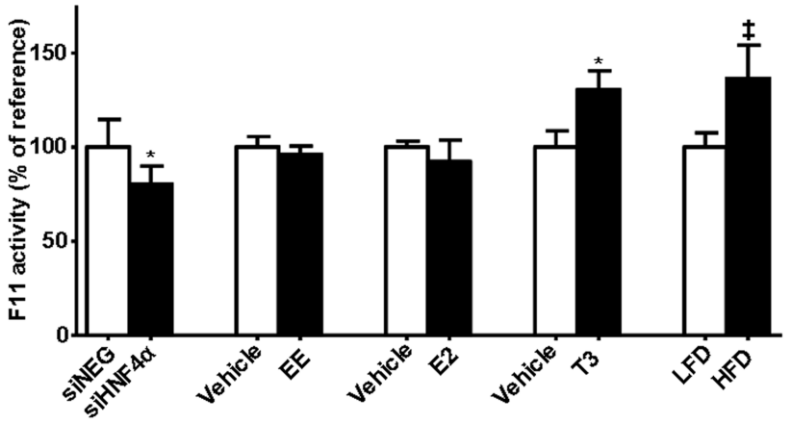

Figure 3: Plasma FXI activity under several metabolic conditions. Plasma FXI activity was measured under different metabolic conditions i.e.; siRNA-mediated depletion of HNF4α (siHNF4α) / siNEG (control siRNA), ovariectomized mice treated with vehicle / EE for 10 days or with E2 for 5 days, mice treated with vehicle / T_3 for 14 days or mice were fed with low (control) / high fat diet for 1 day. Same mice were used for plasma and liver transcript analysis. For number of animals 'n' see Table 1. Data are represented as percentage of reference group ± standard deviation; data were statistically analyzed using the Student's t-test. *p*-values < 0.05 were regarded as statistically significant. **p* < 0.05, ‡*p* < 0.001 vs reference group.

High fat feeding of mice also affects transcription of *F11* [11]. Thus transcription of our genes of interest was assessed in livers of C57BL/6J mice that were fed a low-fat diet for 2 weeks (10 kcal% fat; LFD) as a run-in followed by a switch to a high-fat diet (45 kcal% fat; HFD) known to induce obesity when administered long-term (16 weeks [11]). Controls were

maintained on the LFD control diet. Short exposure to the fat-rich diet (1 or 7 days) strongly induced *F11* transcript levels (+70 and +58% for 1 or 7 days, respectively), without significantly altering the hepatic transcript levels of *F2* (except at 1 day HFD), *F7*, *F10* or *F12* (Table 3). For both time points, increased *F11* transcript levels coincided with increased *Cyp4v3* and *Klkb1* transcript levels with strong correlations (Figure 2D, Table 2).

The above study conditions affecting mouse hepatic *F11* (*Klkb1* and *Cyp4v3)* transcription also affected the mouse FXI protein activity at the level of the plasma. This resulted in statistically significant correlations between hepatic *F11* transcript levels and plasma FXI activity, with the exception of synthetic and natural estrogen treatments (Figure 3, Table S2).

DISCUSSION

In the present study, we searched for evidence for the existence of common regulatory elements within a locus that has been linked to deep vein thrombosis and harbors *F11, KLKB1* and *CYP4V2* i.e. the 4q35.2 locus. The size of this gene cluster hampers *in vitro* analysis of the interaction of the genes within the 4q35.2 region. Therefore as an alternative, we used mice to study the regulation of the orthologous genes in mice under conditions that modulate transcription of the *F11* gene. Upon modulating hepatic HNF4α levels, estrogen status, thyroid hormone status or dietary fat intake, a significant effect on hepatic *F11* transcription was observed in a setting where transcription of other coagulation proteases was not, or differentially, affected. In contrast, parallel responses in *F11* and *Cyp4v3* transcript levels were produced when HNF4α, estrogen, and dietary status were varied. *Klkb1* showed an *F11*-like response for the fat feeding condition only.

Thus, we conclude that in mice, hepatic *F11* and *Cyp4v3* display parallel transcriptional responses suggesting the presence of shared regulatory

elements. Possible concerted regulation does not include the *Klkb1* gene, despite the fact that it is located in between these two genes.

Co-regulation of the transcription of genes in close proximity of one another, as the *F11* and *Cyp4v3* genes, may be the result of two different mechanisms that are not mutually exclusive. On one extreme, the genes may be under the control of a common enhancer that stimulates transcription of those nearby promoters with which it can form productive transcription factor complexes. On the other end of the spectrum, co-regulation may be the result of the presence of binding sites of comparable importance and activity for the same transcription factor in the various promoters. A combination of both scenarios is also possible. In the case of a common enhancer, the genes need to be in close proximity of one another, in case of the presence of similar transcription factor binding sites in their promoters, their relative position is not necessarily important; the genes might have been on different chromosomes and the same effect would have been observed. The present mouse study shows that sufficient HNF4α levels are crucial to the regulation of *F11* and *Cyp4v3* (Figure S1, Table 1). This suggests that HNF4α, at ~50% levels of normal, is the limiting factor in the transcription complexes regulating the expression of *F11* and *Cyp4v3.* On the basis of these data, one would predict that HNF4α regulatory element(s) are present near the murine promoters and/or enhancers of the *F11* and *Cyp4v3* genes. Indeed, the mouse and human *F11* loci are predicted to carry multiple functional HNF4α binding sites [15;16]. Hence, such HNF4α binding sites are candidate for the common regulatory sequence within 4q35.2 locus.

Using Genomatix MatInspector and JASPAR programs, we identified two putative HNF4α binding sites in the enhancer (~5kb upstream of the start-site) and one in the promoter region (located just upstream of the transcription start site) of *F11* gene. No binding sites for estrogen receptor α and thyroid hormone receptor were identified using these softwares. In

addition, direct comparison of the human and mouse (enhancer and promoter) sequences did not show very convincing conservation of the putative binding sites. This exemplifies that a reliable identification of functional binding sites requires, perhaps unsurprisingly, functional data. These were beyond the scope of the present manuscript.

It was proposed that the human SNPs associated with thrombotic risk may modulate FXI expression in response to changing age and hormonal levels [9]. Our mouse data demonstrate that the cluster harboring *F11*, *Klkb1* and *Cyp4v3* is highly responsive to changes in hormones and metabolism. Of note, these changes are much larger than, and in a different direction from the alterations in the expression of coagulation genes *F2*, *F9*, *F10*, *F12*, which were included as controls in the analysis. Both single/multiple estrogen doses were able to modulate *F11* hepatic transcription. Studies indicated that there are ERα binding sites near the *F11* gene [17] which explains immediate transcriptional response of *F11*. Alternatively, prolonged thyroid hormone exposure was required to evoke a clear *F11* transcriptional response. This could be explained by an indirect modulation involving an intermediate transcription factor (e.g. HNF4α) additional to thyroid hormone receptor. A small number of studies reported that estrogens, thyroid hormone and dietary fat may affect hepatic HNF4α [18-20]. Although hepatic HNF4α transcript and protein levels were not affected (both) in the conditions mentioned above (Figure S2), this does not exclude that HNF4α transcriptional activity is affected by hormones and diet thereby contributing to the observed possible concerted regulation of *F11* and *Cyp4v3*. Alternatively, dietary fat can induce acute hepatic stress and inflammation [11;21] that involving many transcription factors. FOXA1 is one of such transcription factor [22] and ChIP-seq data from the ENCODE consortium [23] indicates that there are FOXA1 binding sites near the *F11* gene. Whether stress-related FOXA1 or HNF4α contribute to possible concerted regulation in the human 4q35.2 locus has not been studied.

It was also suggested that human SNPs in *F11* and *CYP4V2* may impact the expression of KLKB1, and thereby increase risk for DVT [18-20]. The mouse data do not provide additional support for regulatory interaction between *F11* and *CYP4V2* on one hand and KLKB1 on the other hand, and make modulation of DVT risk through impact on *KLKB1* by SNPs in *F11* and *CYP4V2* less likely.

The mouse data encouraged us to investigate whether SNP rs2036914, rs2289252 and rs13146272 within the 4q35.2 locus are related to (common) regulatory sequences for this locus. SNPs rs2036914 and rs2289252 are located in introns of the *F11* gene and ChIP-seq analysis for HNF4α or FOXA1 binding sites, among others, does not provide any evidence for transcription factor binding sites in these regions. Also, the sequences around the SNPs are not strongly conserved in mammals. rs13146272 is a missense mutation in *CYP4V2* and this position is relatively well conserved in other species and in other CYPs. It might be speculated that this SNP alters sensitivity or specificity of *CYP4V2* and thereby affects plasma lipid levels, as some mutations in *CYP4V2* have been shown to do [24]. Thus, SNP rs2036914, rs2289252 and rs13146272 do not seem to be present in regulatory sequences in the 4q35.2 locus. However, it should be stressed that for all three SNPs, it is still entirely possible that they are merely genetically linked to other causal/functional SNPs which do impact regulation of the locus.

Parallel responses in *F11* and *Cyp4v3* transcript levels were produced when HNF4α and dietary status were varied. Such responses were also produced upon variation in estrogen, however minimal or not when using 17ß-estradiol (E2) (Table 1 and 2). Overall ethinylestradiol (EE) treatment had stronger impact on hepatic transcription as compared to 17ß-estradiol (compare also the effects of the two compounds on *F2*, *F7*, *F10* and *F12* transcript levels). In contrast to 17ß-estradiol, ethinylestradiol is orally effective and thereby likely results in more effective exposure of the liver to

the estrogenic compound as compared to a subcutaneous administration (17ß-estradiol). This is in line with our earlier observations using these compounds [10]. For thyroid hormone *F11* and *Cyp4v3* transcript levels were affected in opposite direction. We have no explanation for these opposite effects, but these observations highlight that, although mouse *F11* and *Cyp4v3* may share regulatory elements, the transcription of these two genes is clearly not under control of one single shared regulatory region.

Remarkably, only for the estrogen treatment conditions, changes in *F11* transcript levels did not translate into increased FXI plasma protein levels, at least at the level of protein activity (Table 1, Figure 3). This phenomenon was observed in multiple experiments [10]. Whether estrogen possibly induces increased plasma FXI antigen, but not FXI protein activity, or whether estrogen increases FXI protein clearance and degradation thereby masking the effects on transcription on protein levels, has not been studied. Alternatively, estrogen may affect other (unknown) mechanism that influence FXI activity such as the post-translational modifications. Thus, whether changes in hepatic *F11* transcript levels truly were not translated into changes in plasma FXI (protein and activity) is at present subject to speculation. Although not determined, it would also be interesting to determine whether effects on hepatic *Cyp4v3* transcription translate to effects on hepatic Cyp4v3 protein (activity) level. This would shine a light on whether parallel transcriptional responses of *F11* and *Cyp4v3* extends to protein level.

In conclusion, our mouse data demonstrate that within the mouse *F11*, *Klkb1*, *Cyp4v3* gene cluster, in particular *F11* and *Cyp4v3,* frequently display a striking parallel transcriptional response suggesting the presence of regulatory elements. We speculate that, if present, SNPs within the human orthologues of these unidentified elements could causally influence DVT risk.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

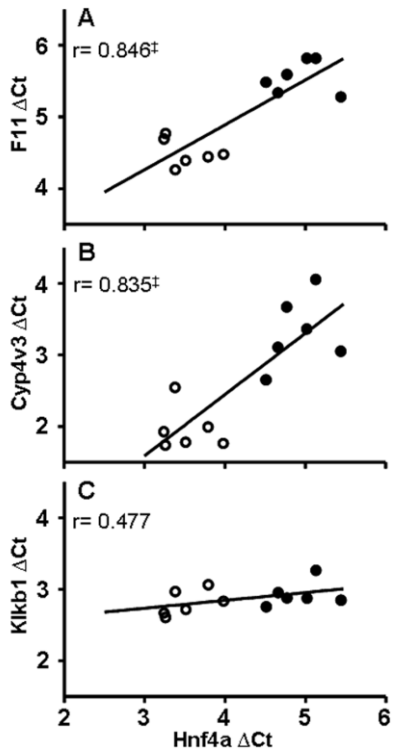

Figure S1: Correlation between hepatic *Hnf4a* and *F11* (A), *Hnf4a* and *Klkb1* (B) and *Hnf4a* and *Cyp4v3* (C) under siRNA-mediated HNF4α knockdown (●) and control siRNA (○) in mouse liver. Data were statistically analyzed with Pearson correlation coefficient (r). *p*-values <0.05 were regarded as statistically significant. ‡$p < 0.001$.

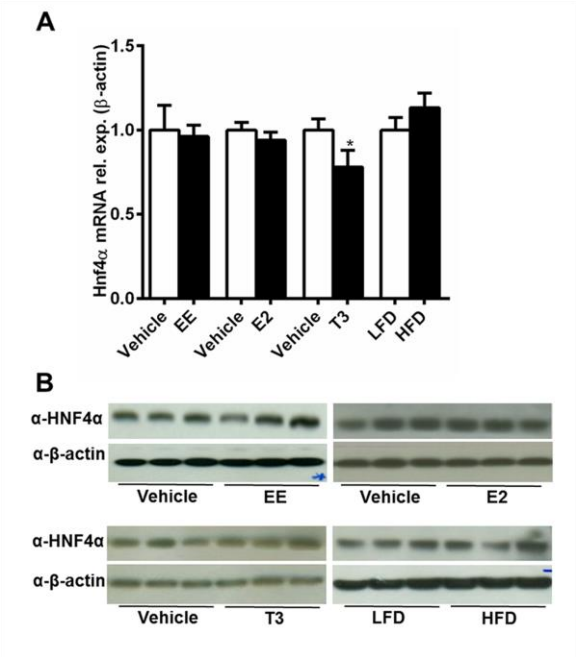

Figure S2: Hepatic transcript and protein levels of HNF4α under metabolically challenged conditions in mice. (A) Hepatic transcript levels were determined by quantitative real-time PCR. Data are expressed as mean with error bars representing the difference between 2 POWER of upper and lower range of the mean ΔΔCt. β-actin was used as internal control for quantification and normalization. The ΔCt values of the individual samples were related to the mean ΔCt of the reference group. On the x-axis the metabolic conditions are depicted. Data were statistically analysed using Mann Whitney Rank Sum Test. *P*-values less than 0.05 were regarded as statistically significant. *p<0.05. (B) Immunoblotting for HNF4α was performed for liver homogenates that were prepared for three randomly selected mice per condition. 15µg total protein lysate was loaded in each lane and HNF4α was detected using anti-HNF4α antibody (C-19, sc-6556, Santa Cruz Biotech., Santa Cruz, CA, USA). β-actin was used as a protein loading control.

siNEG/siHNF4α mice; mice injected with control (negative) or HNF4α siRNA respectively, EE; ethinylestradiol, E2; 17-β estradiol, T_3; 3,3′,5-Triiodo-L-thyronine.

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.

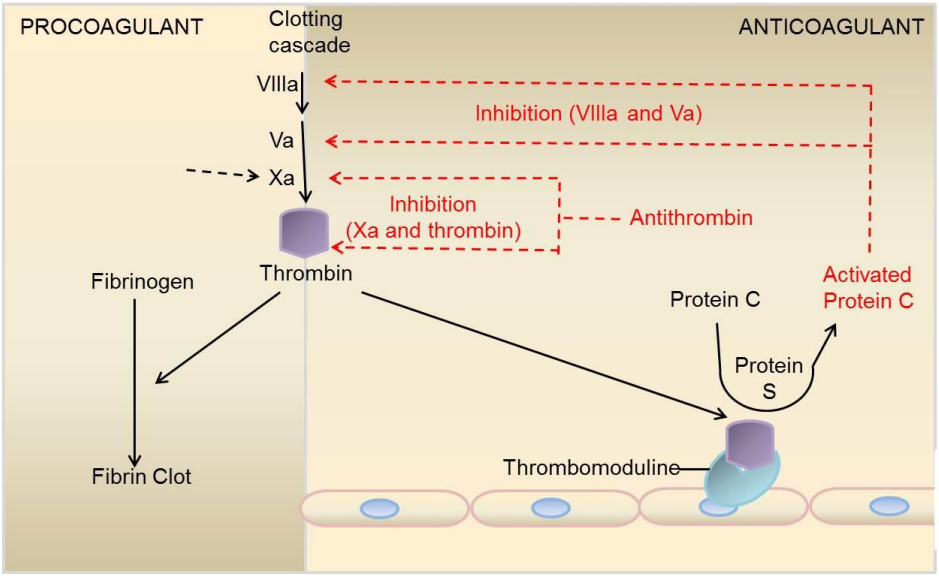

Figure 1: Anticoagulation by Protein C/Protein S and antithrombin pathway. Adapted from http://what-when-how.com/acp-medicine/hemostasis-and-its-regulation-part-1/

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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Chapter 8

Summary

Summary in Dutch

(Nederlandse samenvatting)

Over the past years epidemiological studies have revealed a number of acquired risk factors - including estrogen and thyroid hormones, obesity, immobility, pregnancy, malignancies, surgery and metabolic syndrome - that increase the risk of venous thrombosis. Several of these acquired risk factors coincide with changes in the plasma coagulation profile and lead to a hypercoagulable state. Since the liver is the major organ for the production of coagulation factors, this hypercoagulable state may occur when altered transcription of coagulation genes in the liver leads to an imbalance in the ratio of pro- and anti-coagulant proteins in the plasma.

Mechanisms by which acquired risk factors modulate transcription of coagulation genes and lead to the hypercoagulable state are not completely elucidated. Therefore, we aimed to study the mechanisms underlying alterations in hepatic coagulation gene transcription in more detail in an attempt to increase our understanding of why a thrombotic risk condition coincides with abnormal coagulation profiles. RNA interference (via synthetic small interfering RNA; siRNA) was used as a tool to study genes involved in coagulation and coagulation control in mice.

General background on venous thrombosis, risk factors and the possible mechanisms by which these factors increased the risk of venous thrombosis are discussed in **chapter 1**. Alterations in coagulation profile by thrombotic risk factors occur probably by modulation of gene transcription in the liver. Gene transcription can be modulated at several levels, ranging from transcription factors, hormone-mediated nuclear hormone receptors, co-regulators or intermediate factors. Furthermore, to study the mechanisms underlying alterations in hepatic coagulation gene transcription, several experimental models and methodologies are reviewed. Mice and siRNA-induced gene silencing are discussed as a potential suitable model system and approach for studies described in this dissertation.

As part of **chapter 2**, we investigated the contribution of hepatocyte nuclear factor 4α (HNF4α) to controlling hepatic transcription of anticoagulant and fibrinolytic factors *in vitro* and *in vivo*. To study the contribution of HNF4α, a dual approach was used; conditional liver-specific *Hnf4α*-null mouse (*in vivo)* and siRNA-mediated fast and acute silencing of HNF4α in mouse primary hepatocytes (*in vitro)*. This latter approach was used to exclude delayed/indirect effects of HNF4α deletion (in *Hnf4α*-null mouse) on transcript levels of anticoagulant and fibrinolytic factors. We showed that HNF4α is critical in regulating hepatic transcription of anticoagulant and fibrinolytic factors *in vivo* and *in vitro*.

In **Chapter 3** we established the use of siRNA-mediated gene silencing *in vivo*. Efficient, fast, acute and strong knockdown of transcription factors HNF4α and C/EBPα was achieved with sequence specific synthetic siRNA in mouse liver. Hepatic gene expression levels of a number of procoagulant, anticoagulant and fibrinolytic factors were affected upon acute and fast knockdown of HNF4α and C/EBPα in mouse liver, which demonstrated a direct role of these factors (HNF4α and C/EBPα) in controlling hepatic gene transcription. Moreover, this study showed that synthetic siRNA forms a powerful and relatively simple tool to determine the role of hepatic transcription factors involvement *in vivo*.

Estrogen hormones are able to modulate gene expression of coagulation factors via estrogen receptor α (ERα). Moreover, a factor like forkhead protein (FOXA1), an intermediate protein which acts as a pioneer factor, is a major determinant of estrogen-ERα function in breast and non-breast cancer cells, whereas knowledge of the function of hepatic FOXA1 and interaction with hepatic ERα is missing. In **Chapter 4** we investigated the role of FOXA1 in absence of estrogen and as an intermediate factor in estrogen response in mouse liver. siRNA-mediated knockdown of FOXA1 in mouse liver was associated with altered coagulation gene expression in absence of estrogen, which showed the role of FOXA1 in regulation of

coagulation gene transcription. Additionally, we showed that hepatic FOXA1 is required for estrogen-bound-ERα chromatin binding in mouse liver, as it is in (human) estrogen responsive cancer cells.

Deletion of anticoagulant factors (antithrombin and protein C) is embryonic lethal in mice therefore precluding the use of these knockout mice in gene function studies or as thrombosis model. siRNA-mediated gene silencing provided a relatively simple tool to study the function of such proteins in adult mice, which is described in **chapter 5.** Combined gene silencing of both of these factors resulted in thrombosis-related severe coagulopathy in adult mice, showing the vital anticoagulant function of these proteins in adult mouse. Moreover, silencing of antithrombin and protein C provided a relatively easy, controlled, and novel mouse thrombosis model.

Single nucleotide polymorphism (SNP) studies demonstrated the association of the human chromosome 4q35.2 locus with increased risk for deep venous thrombosis. In **chapter 6**, the aim was to search for experimental evidence for putative common regulatory elements in the orthologous mouse gene cluster of the human 4q35.2 locus that contains the coagulation factor XI (*F11*), prekallikrein (*Klkb1*) and cytochrome P450 family member (*Cyp4v3*) genes. We analyzed transcript levels of *F11*, *Klkb1* and *Cyp4v3* in several metabolically challenged mice in which hepatic *F11* transcript levels were impacted. Metabolic conditions included HNF4α deletion, (natural and synthetic) estrogen hormone treatment, thyroid hormone, and high fat diet in mice. We observed parallel hepatic transcriptional response of *F11* and *Klkb1* for high fat feeding condition. In contrast, strikingly parallel hepatic transcript response of *F11* and *Cyp4v3* were observed in all studied conditions except for thyroid hormone suggesting the presence of shared regulatory elements.

In **chapter 7**, findings presented in this dissertation and possibilities for future research are discussed on basis of available recent literature.

In summary, we utilized a siRNA strategy throughout this dissertation, which proved to be a valuable tool to study genes in mice that are suspected to play a role in the pathophysiology of venous thrombosis. We showed that transcription factors like HNF4α, C/EBPα and FOXA1 (directly) regulated the expression levels of a number of hepatic coagulation genes. We suggest that these transcription factors are potentially relevant for the etiology of venous thrombosis. Depletion of antithrombin and protein C leads to the formation of thrombi and fibrin deposition, showing the importance of these factors in adult mouse hemostasis and provided a novel mouse model featuring a spontaneous (venous) thrombotic phenotype. Studies described in this dissertation may contribute to a better understanding of which genes are involved in coagulation (control) and how thrombotic risk factors result in a hypercoagulable state.

Summary in Dutch - Nederlandse samenvatting

In de afgelopen jaren hebben verschillende epidemiologische studies aangetoond dat een aantal verworven factoren, waaronder anticonceptiepil gebruik (oestrogeen hormoon), schildklierhormonen, obesitas, immobiliteit, zwangerschap, (bepaalde) tumoren, chirurgie en metabool syndroom, het risico op veneuze trombose verhogen. Sommige verworven risicofactoren gaan samen met veranderingen in het plasma stollingsprofiel en leiden tot een protrombotische verschuiving van de hemostase balans (hypercoagulatie). Omdat de lever van groot belang is voor de aanmaak van plasma stollingsfactoren kan de verstoring van deze balans o.a. een gevolg zijn van veranderingen in transcriptie regulatie van stollingsgenen in de lever. Afwijkende transcriptie van stollingsgenen kan gevolgen hebben voor de balans van pro- en antistolling eiwitten in het plasma. Echter, mechanismen waarbij verworven risicofactoren de gentranscriptie van stollingsfactoren zodanig beïnvloeden dat een protrombotische verschuiving van de hemostase balans ontstaat zijn niet volledig bekend. Daarom was het doel van het onderzoek om een gedetailleerde studie te verrichten naar veranderingen in de gentranscriptie van stollingsfactoren in de lever van muizen. Door de moleculaire mechanismen die ten grondslag liggen aan transciptionele regulatie van stollingsfactorgenen beter in kaart te brengen verschaffen we meer inzicht in de samenhang tussen trombotische risicocondities en abnormale bloedstollingsprofielen. We hebben voornamelijk gebruik gemaakt van RNA-interferentie (m.b.v. synthetische korte interfererende RNA, siRNA) technieken om genen te bestuderen die betrokken zijn bij transcriptie regulatie van bloedstolling factoren in de muis.

Achtergrondinformatie over veneuze trombose, risicofactoren en de mogelijke mechanismen waardoor deze factoren het risico op veneuze trombose verhogen worden besproken in **hoofdstuk 1**. Door het

moduleren van gentranscriptie in de lever, kunnen trombotische risicofactoren mogelijk veranderingen in het coagulatieprofiel veroorzaken. Deze gentranscriptie kan op verschillende niveaus gemoduleerd worden, variërend van transcriptie factoren, hormoon-gerelateerde nucleaire hormoon receptoren, co-regulatoren of intermediaire factoren. Bovendien worden een aantal experimentele modellen en methodieken bediscussieerd die gebruikt kunnen worden om de onderliggende mechanismen van transcriptionele veranderingen van stollingsgenen in de lever te bestuderen. Muizen en siRNA-geïnduceerde reductie (knockdown) van genexpressie worden besproken als een mogelijke geschikte modelsysteem en aanpak voor de studies beschreven in dit proefschrift.

Als onderdeel van **hoofdstuk 2** onderzochten we de bijdrage van hepatocyte nuclear factor 4α (HNF4α) in de lever op de transcriptionele regulatie van antistollings- en fibrinolytische factoren *in vitro* en *in vivo*. Er werd een tweeledige aanpak gebruikt om de bijdrage van HNF4α te bestuderen: gebruik van conditionele lever-specifieke Hnf4α-null muizen (*in vivo*) en door siRNA-gemedieerde snelle en acute knockdown van HNF4α expressie in primaire hepatocyten van muizen (*in vitro*). Deze laatste benadering is gebruikt om latere/indirecte effecten van HNF4α deletie (in Hnf4α-null muizen) op transcriptie niveaus van antistollings- en fibrinolytische factoren uit te sluiten. We hebben aangetoond dat HNF4α essentieel is in de lever voor de transcriptionele regulatie van antistollings- en fibrinolytische factoren *in vivo* en *in vitro*.

In **hoofdstuk 3** hebben we siRNA-gemedieerde *in vivo* knockdown toegepast om de expressie van specifieke genen te reduceren. Efficiënte, snelle, acute en sterke knockdown van twee verschillende transcriptiefactoren HNF4α en C/EBPα in muizenlever werd bereikt met sequentie-specifieke synthetische siRNA. Genexpressie niveaus van een aantal pro-stolling, antistolling en fibrinolytische factoren werden beïnvloed door acute en snelle knockdown van HNF4α en C/EBPα in muizenlever wat

aanduid op een directe rol van deze eiwitten (HNF4α en C/EBPα) in de transcriptionele regulatie van lever genen. Bovendien toonde deze studie aan dat synthetische siRNA een krachtige en relatief eenvoudig hulpmiddel is om de rol van lever transcriptiefactoren *in vivo* te bepalen.

Oestrogeen hormonen zijn in staat om de genexpressie van stollingsfactoren te moduleren via oestrogeenreceptor α (ERα). Bovendien is er een factor zoals het forkhead eiwit (FOXA1), een intermediair eiwit dat functioneert als een pionier factor, FOXA1 heeft een belangrijk rol in hormoongevoelige borstkankercellen. De kennis rondom de functie van de lever FOXA1 en interactie van FOXA1 met ERα in lever ontbreekt. In **hoofdstuk 4** onderzochten wij de rol van FOXA1 bij afwezigheid van oestrogeen en als intermediair factor in oestrogeenresponse in muizenlever. siRNA-gemedieerde knockdown van FOXA1 in muizenlever werd geassocieerd met een veranderde expressie van stollingsgenen in de afwezigheid van oestrogeen. Hieruit concluderen wij ten eerste dat FOXA1 een sleutelrol vervult in de regulatie van transcriptie van bloedstollingsgenen. Daarnaast toonden we aan dat hepatische FOXA1 nodig is voor oestrogeen-ERα gebonden chromatine binding in muizenlever, zoals in (humane) oestrogeen reagerende kankercellen.

Volledige deletie oftewel ‘knockout muizen’ voor genen betrokken bij de natuurlijke antistolling (zoals antitrombine en proteïne C) zijn niet levensvatbaar en daarom is dit een belangrijk obstakel voor het gebruik van deze methode in gen-functie studies of als diermodel voor trombose studies. Echter, siRNA-gemedieerde knockdown van gen expressie verstrekt een relatief eenvoudig hulpmiddel om de functie van dergelijke eiwitten bij volwassen muizen te bestuderen, dit wordt beschreven in **hoofdstuk 5**. Gecombineerd knockdown van beide factoren leidt tot trombose gerelateerde ernstige coagulopathie bij volwassen muizen hetgeen de vitale anti-coagulerende werking van deze eiwitten bij volwassen muizen aantoonde. Bovendien het knockdown van antitrombine

en proteïne C genexpressie verstrekt een relatief eenvoudige, gecontroleerde en nieuwe muis trombose model.

Single nucleotide polymorfisme (SNP) studies hebben een verband aangetoond tussen de menselijke 4q35.2 gen gebied met een verhoogd risico op diepe veneuze trombose. In **hoofdstuk 6** was het doel om te zoeken naar het experimentele bewijs voor de mogelijk gemeenschappelijk regulerende elementen in de orthologe muizen gen cluster van de menselijke 4q35.2 regio. Dit cluster bestaat uit stollingsfactor XI (*F11*), prekallikreïne (*Klkb1*) en cytochroom P450 familielid (*Cyp4v3*) genen. We hebben het transcript niveaus van *F11*, *Klkb1* en *Cyp4v3* geanalyseerd in diverse muizen met een afwijking in het metabolisme waarin *F11* transcriptie niveaus werden beïnvloed in lever. De geteste metabole defecten zijn HNF4α deletie, oestrogeen (natuurlijke en synthetische) hormoon, schildklierhormoon en een hoog vet dieet in muizen. De transcriptie van *F11* en *Klkb1* veranderde op een gelijke wijze wanneer de muizen een vetrijk dieet gevoerd werden. Daarentegen transcriptie van *F11* en *Cyp4v3* veranderde op aanzienlijke gelijke wijze in lever in alle onderzochte studie condities behalve in het schildklierhormoon; dit suggereert de aanwezigheid van gedeelde regulerende elementen.

In **hoofdstuk 7** worden de bevindingen in dit proefschrift nader bediscussieerd en mogelijkheden voor toekomstig onderzoek besproken op basis van de beschikbare recente literatuur.

Samenvattend hebben we in dit proefschrift gebruik gemaakt van de siRNA strategie en dat bleek een waardevolle techniek om de functie van genen te bestuderen die vermoedelijk een rol spelen in de pathofysiologie van veneuze trombose. We hebben aangetoond dat transcriptie factoren zoals HNF4α, C/EBPα en FOXA1 (rechtstreeks) de expressie van een aantal lever stollingsgenen reguleren. We stellen dat deze transcriptie factoren potentieel relevant zijn voor de etiologie van veneuze trombose. Reductie van antitrombine en proteïne C gen expressie leidt tot de vorming van

trombi- en fibrinedepositie in weefsels, wat ten eerste het belang van deze factoren laten zien in hemostase bij volwassen muizen. Ten tweede het verstrekt een nieuw muismodel die een spontane (veneuze) trombotische fenotype aantoont. Studies beschreven in dit proefschrift dragen bij aan een beter begrip van de rol van genen bij de bloedstolling (controle) en hoe risicofactoren van trombose leiden tot een protrombotische verschuiving van de hemostase balans.

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Curriculum Vitae

Publications

Huma Safdar was born in Lahore, Pakistan on 19 July 1979. In 2000, she graduated as a Bachelor of Science with Chemistry, Zoology and Botany as major subjects, at Govt. Jinnah Degree College, Lahore, Pakistan. In 2002, she got her Master in Science degree in Botany from the University of The Punjab, Lahore, Pakistan, standing seconding in her department. After getting married she moved to Netherlands and started her second Masters in Molecular and Cellular Biology at the University of Leiden (2006-2008). As a part of her master's internship, she studied the subcellular localization of zebrafish glucocorticoid receptors in *in vitro* and *in vivo* at the Department of Molecular and Cellular Biology of Leiden University under the supervision of Dr. Marcel Schaaf.

In 2009, she started her PhD study on characterization of mouse coagulation (regulatory) genes with use of RNAi, at the Department of Thrombosis and Hemostasis in the Einthoven Laboratory for Experimental Vascular Medicines under the mentorship of Prof. Dr. Pieter Reitsma and Dr. Bart van Vlijmen. The results of this research are presented in this dissertation.

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