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%),

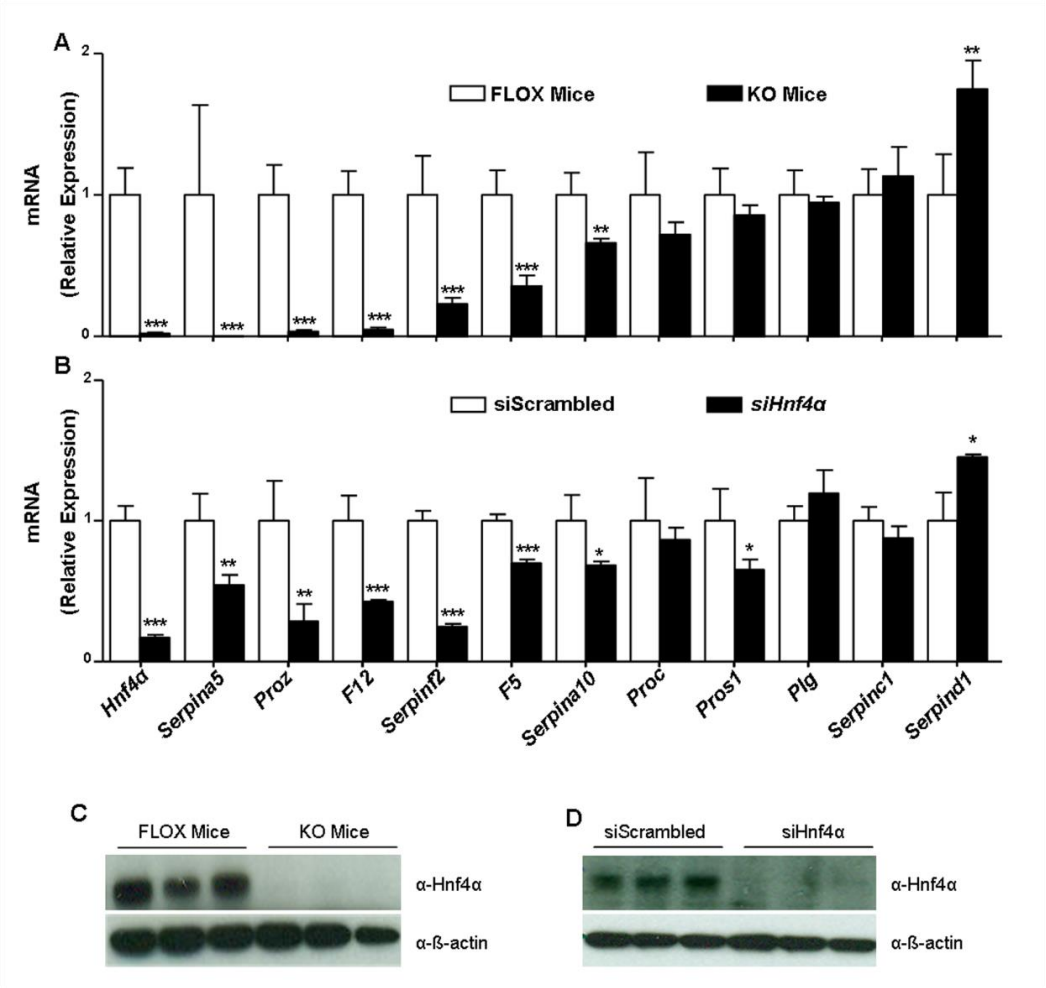

Figure 1: Anticoagulation and fibrinolysis gene transcript levels in livers of HNF4α-null mice and cultured mouse primary hepatocytes transfected with *Hnf4*α-specific siRNA.

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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