Chapter 4

The Role of FOXA1 in Mouse Hepatic Estrogen Receptor α Signaling

Huma Safdar, Peter E. Thijssen, Judit Balog, Hans L Vos, Ka Lei Cheung, Pieter H. Reitsma, Bart J.M. van Vlijmen

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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).

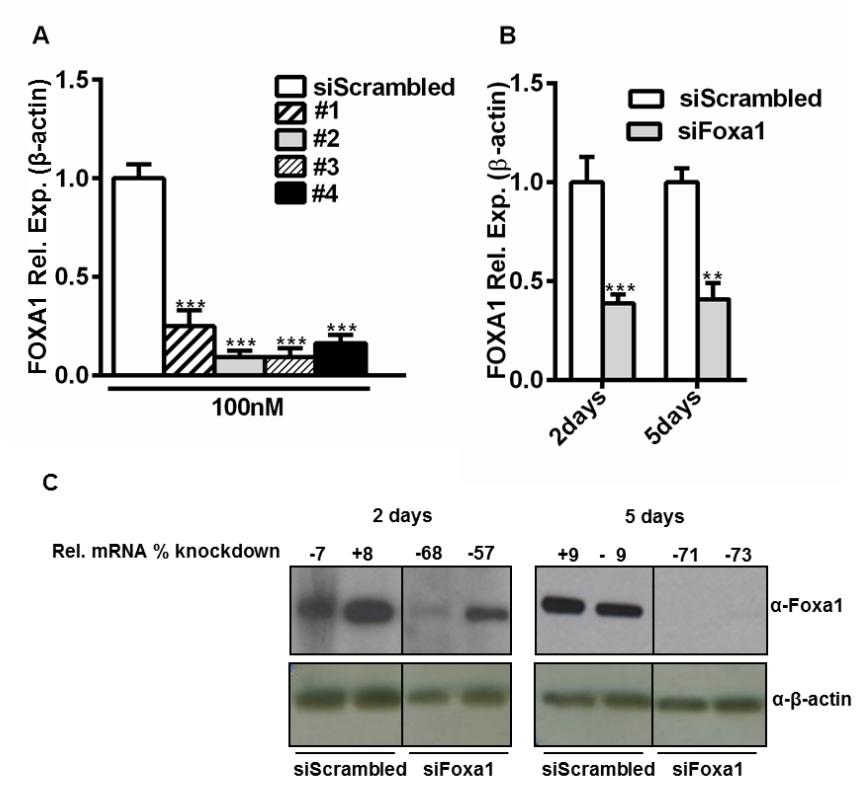

Figure 1. Screening and validation of siRNA in *in vitro* and *in vivo*. Screening of siRNA in mouse primary hepatocytes 24 hours after transfection with 100nM of four *FOXA1*-specific (#1-4), or a control siRNA (siScrambled, panel **A**). *FOXA1* transcript levels were determined by quantitative real-time 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**.

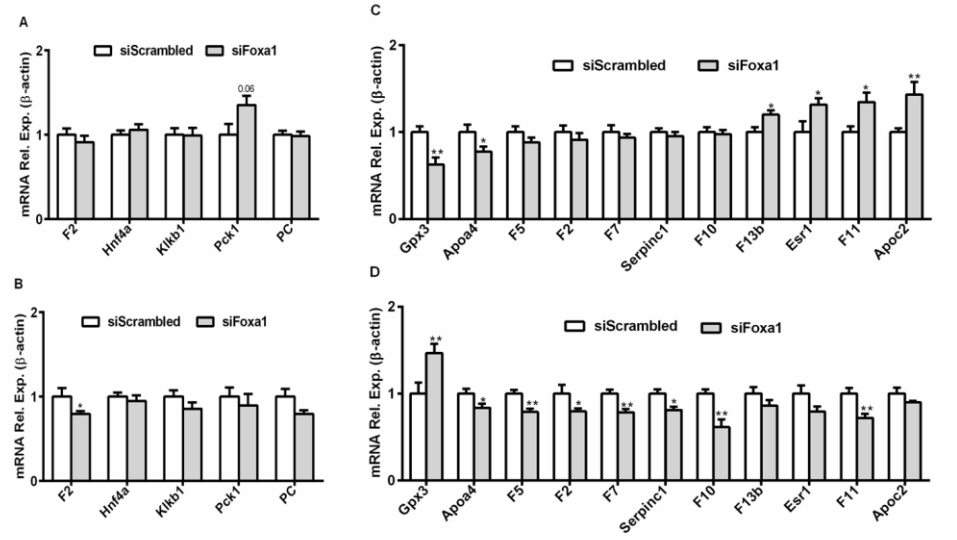

Figure 2. Hepatic gene transcription following FOXA1 knockdown in mouse liver. At two (upper panels, **A** and **C**) and five days (lower panels, **B** and **D**) after siRNA injection, mouse livers were subjected to gene transcript analyses by QPCR. Data are presented for siFoxa1 (gray bars) with siScrambled 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*,

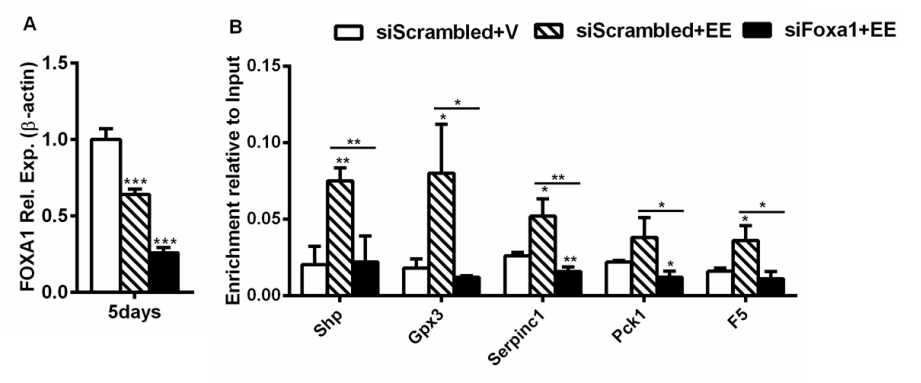

Figure 3. Binding of ERα to chromatin is influenced by expression levels of FOXA1 in mouse liver. Ovariectomized C57Black/6J mice were injected with siScrambled or siFoxa1 (7 mg siRNA per kg mouse) and two days post siRNA injection mice were treated for three days with 1µg EE d^{-1} or vehicle per mouse. (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).

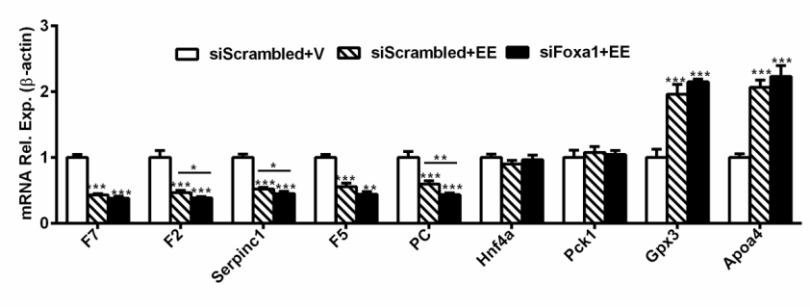

Figure 4. EE-bound ERα hepatic gene transcription is dependent on FOXA1 expression. Livers from ovariectomized mice, five days post siRNA and after three days of EE/vehicle treatment (as mentioned in the legends of Figure 3) were subjected to transcript analysis by performing QPCR (see figure 1 legends). 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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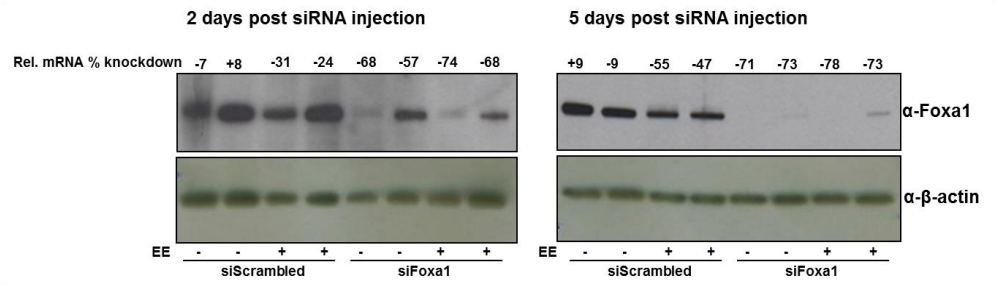
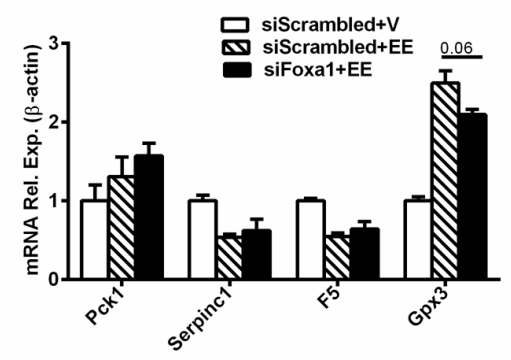
SUPPLEMENTAL FIGURES AND TABLE

Supplementary Figure 1. Uncropped protein blot.

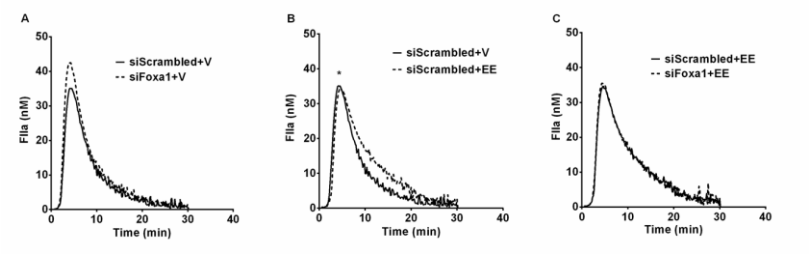


Supplementary figure 2. Sub-analysis of EE-bound ERα hepatic gene transcription.



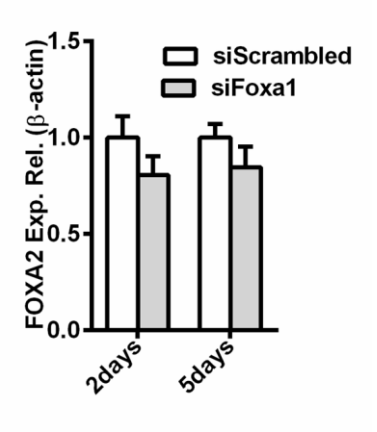
Sub-analysis of **Figure 4** was performed on samples which were used for ChIP assay (n=3 for each group). Data are presented for iScrambled+Vehicle (Open bars), siScrambled+EE (hatched bars) and siFoxa1+EE (Black bars). Data are expressed as means with error bars representing the difference between 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