STAT-3 mediates hepatic hepcidin expression and its inflammatory stimulation

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ABSTRACT

Hepcidin is a key iron-regulatory hormone produced by the liver. Inappropriately low hepcidin levels cause iron overload, while increased hepcidin expression plays an important role in the anaemia of inflammation (AI) by restricting intestinal iron absorption and macrophage iron release. Its expression is modulated in response to body iron stores, hypoxia, inflammatory and infectious stimuli, involving at least in part cytokines secreted by macrophages. In this study we established and characterized IL-6-mediated hepcidin activation in the human liver cell line Huh7. We show that the proximal 165bp of the hepcidin promoter are critical for hepcidin activation in response to exogenously administered IL-6 or to conditioned medium from the monocyte/macrophage cell line THP-1. Importantly, we show that hepcidin activation by these stimuli requires a STAT-3 binding motif located at position -64/-72 of the promoter. The same STAT binding site is also required for high basal level hepcidin mRNA expression under control culture conditions, and siRNA-mediated RNA knock-down of STAT-3 strongly reduces hepcidin mRNA expression. These results identify a missing link in the acute phase activation of hepcidin and establish STAT-3 as a key effector of baseline hepcidin expression and during inflammatory conditions.
INTRODUCTION

The iron-regulatory hormone and hepatic acute phase protein hepcidin is strongly implicated in the anaemia of inflammation (AI), a common clinical disorder that affects patients with acute and chronic infections, trauma, inflammatory disorders and malignancies. The disease is characterized by hypoferremia, low serum iron-binding capacity and normal to elevated ferritin levels. Several cytokines that participate in the pathogenesis of AI also modulate iron metabolism. For example, IL-1 and TNF-α induce hypoferremia in mice, and IL-6 increases hepatic ferritin synthesis and transferrin uptake in rats. Importantly, hypoferremia is induced within few hours after injection of hepcidin into mice. Hepcidin binds to the iron export protein ferroportin (also known as SLC11A3, IREG1 and MTP) and triggers its degradation to decrease iron egress from duodenal enterocytes, macrophages and hepatocytes, thus contributing to the hypoferremia that hallmarks AI. Hepcidin may hence be considered to be a principal iron regulatory hormone, a key mediator of AI, and a bridge between innate immunity and iron metabolism.

IL-6 induces hepcidin expression both in cell culture and in vivo. Conditioned medium from LPS-treated macrophages activates hepcidin mRNA expression in hepatocytes, a response that can be blocked by anti-IL-6 antibodies. However, additional factors seem to regulate hepcidin expression, because mice with a targeted disruption of the IL-6 gene (IL-6 -/-) still activate hepcidin expression in response to endotoxin injection. Interestingly, the treatment of primary mouse hepatocytes with IL-1α and IL-1β strongly increases hepcidin mRNA expression. Moreover, a previous report suggests that mice lacking the protein HFE fail to mount an appropriate hepcidin response following LPS injection in spite of a normal increase in IL-6 expression, although other investigators have reached different conclusions using different experimental systems. Taken together, these results reflect the complexity of hepcidin regulation by inflammatory stimuli.

HFE, TfR2 and hemojuvelin (HJ), three genes mutated in a group of frequent iron overload disorders, the hereditary hemochromatosis (HH), control appropriate hepcidin expression. Recent work demonstrates that HJ can function as a bone morphogenetic protein (BMP) 2 coreceptor, that mediates BMP signalling. BMP positively regulates hepcidin expression at the transcriptional level, a response that is enhanced by HJ expression. HJ-deficient mice show reduced levels of phosphorylated Smad 1/5/8, suggesting that BMP-dependent Smad activation is important for hepcidin activation in the mouse. Further work supports a role for the TGF-beta pathway and SMAD4 in the transcriptional activation of hepcidin in response to iron overload or IL-6. The hepcidin response to TGF-β was abrogated in SMAD4-deficient hepatocytes, and mice with a liver-specific SMAD4 deficiency develop iron overload. Hepcidin expression is further affected by the CCAAT/enhancer-
binding protein (C/EBP), because mice with a liver-specific disruption of C/EBPalpha show decreased hepcidin mRNA levels and periportal hepatic iron overload.\cite{23}

Little information is presently available about promoter elements and transcription factors that control hepcidin expression. To address this issue, we established a cell-based assay system to investigate the cis-acting elements and trans-acting factors for basal hepcidin expression and in response to inflammatory stimuli. We show that a STAT-binding motif at position -64/-72 of the hepcidin promoter and STAT-3 are critical for the control of baseline hepcidin mRNA expression and under inflammatory conditions.
MATERIAL AND METHODS

Cell culture
Human hepatocarcinoma Huh7 and monocyte/macrophage THP-1 cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; high glucose; Invitrogen). Medium was supplemented with 10% heat-inactivated low endotoxin fetal bovine serum (FBS, Invitrogen) and 100 U/ml penicillin, and 100 µg/ml streptomycin. All cultures were maintained at 37°C under 5% CO₂. Cell-free conditioned medium (CM) from THP-1 cells growing at an initial cell confluence of 70% was collected 12h, 24h, 48h and 72h after plating of the cells and used undiluted to treat Huh7 cells. Human recombinant IL-6 (Roche) was used at final concentrations of 1ng/ml.

RNA isolation
Total RNA was isolated using the Qiagen RNAeasy kit according to the manufacturer’s instruction (Qiagen). The concentration and purity of the RNA was determined by OD260/280 reading. The quality of the RNA was assessed by gel electrophoresis and ethidium bromide staining.

Reverse transcription and quantitative Real-Time PCR analysis
2 µg of total RNA was reverse transcribed using 10 µM each dCTP, dGTP, dATP, dTTP, 100 ng random primers, 1x first-strand buffer (Gibco, BRL), 0.01 M DTT and 200 U of SuperScript II reverse transcriptase (Gibco, BRL) in a 20 µl reaction for 90min at 42°C. Real time PCR was performed using the ABI Prism 7500 Applied Biosystems (Applera Deutschland GmbH). Amplification reactions were carried out in 25 µl volume using SYBR Green I dye and the following amplification conditions: 50°C 2min, 95°C 10min, (95°C 15sec, 60°C 15sec) x 45 cycles. Primers were designed to specifically amplify 123 bp of hepcidin cDNA (forward 5´-CTCTGTTTTCCCCACAACAGAC-3’, reverse 5´-TAGGGGAAGTGTTGTCTC-3’), 113 bp of GAPDH cDNA (forward 5´-CATGAGAAGTATGACAACAGCCT-3’, reverse 5´-AGTCCTTCCACGATACCAAAGT-3’); 152 bp of STAT-3 cDNA (forward 5´-CATATGCGGCCAGCAAAGAA-3’, reverse 5´-ATACCTGCTCTGAAGAAACT-3’); 127 bp of C/EBPα cDNA (forward 5´-AAGAACAGCAACGAGTACCGG-3’, reverse 5´-CATTGTCACTGGTGCTGCTCCA-3’).

The mRNA/cDNA abundance of each gene was calculated relative to the expression of a housekeeping gene, GAPDH (glyceraldehyde-3-phosphate-dehydrogenase).

Promoter analysis
-942-bp and -165-bp nucleotide fragments of the 5´-flanking genomic region plus the 5´UTR of the human hepcidin gene were obtained from human genomic DNA by PCR amplifications using the following forward primers: 5´-GGCTCGAGGTACTCATCGGACTGTAGAT-3’ and 5´-GGCTCGAGTAACACACCTCTGCGGCTGA-3’ respectively and 5´-
GGAAGCTT CGTGCCGTCTGTCTGGCTGT-3´ reverse primer; the incorporated XhoI and HindIII sites are underlined. PCR fragments were digested with XhoI / HindIII and inserted in the promoter-less luciferase reporter vector pGL3-Basic (Promega). The -385-bp fragment was obtained by XhoI / Bsu36I digestion of the -942-bp construct.

AP-1 (position -242/-233) and STAT (position -69/72) binding sites were deleted by site-directed mutagenesis (Invitrogen) using the following primer pairs: 5´- ACTTTTTTCCCGATCAGCAGTGATGGGGAAA-3´ and 5´- GTTTTCCCCCCTTGAAAAAGGGCTAGTCGTC-3´; GCCTTTTCGGCGCCACCACCTGGAAATGAGA-3´ and 5´- GGTGGGTGGCAGCCAAAAGGCCGGAGAGATA-3´, respectively.

Details of the constructs are available upon request. All constructs were confirmed by DNA sequencing. Search for putative transcription factor binding sites was performed using MatInspector 24 and TFsearch version 1.3.

**Cell transfection and Luciferase Assay**

Huh7 cells were seeded at 30% confluency in 35-mm-diameter dishes and grown over night. 500ng of pGL3-hepcidin-promoter vectors were co-transfected with a control plasmid containing the Renilla gene under the control of the CMV promoter (details of the control plasmid are available upon request). Transfections were performed using lipofectamine 2000 (Invitrogen) according to the manufacturers instructions. 24 hours after transfection the cells were harvested or treated with the indicated stimuli. At the indicated time points cells were lysed in passive lysis buffer (Promega) and cellular extracts were analysed for luciferase activity using the Dual-Luciferase-Reporter assay system (Promega) and a Centro LB 960 luminometer (Berthold Technologies).

**siRNA-mediated Knock-down of C/EBPa and STAT-3**

Huh7 cells were seeded at 30% confluency in DMEM supplemented with 10% low endotoxin FBS without antibiotics. After 24h, cells were transfected using Oligofectamine Reagent (Invitrogen) and 100nM of siRNA directed against C/EBPa (Dharmacon) or STAT-3 (Dharmacon). After further 24h a second round of transfection was performed and the cells were harvested 24 later. As a control siRNA directed against Luciferase (Dharmacon) was transfected. The efficiency of the knock-down was analysed at the mRNA level by quantitative real-time PCR.

**Statistics**

Results were expressed as mean ± S.D. Student´s t test was used for estimation of statistical significance.
RESULTS

IL-6-dependent hepcidin activation is mediated by a STAT binding motif at position -64/-72 of the hepcidin promoter.

To investigate how the inflammatory cytokine IL-6 activates hepcidin mRNA expression we generated luciferase reporter vectors containing the 942 bp upstream of the transcription start site of the human hepcidin promoter, or different truncations/mutations thereof (Figure 1A and 1B). Promoter truncations were designed such that putative transcription factor binding sites, as defined by bioinformatic analyses (e.g. an AP-1 binding motif at position -242/-233 or a STAT-binding motif at position -64/-72), were contained within the promoter fragments analysed. The luciferase reporter constructs were transfected into the human hepatocyte cell line Huh7. Huh7 cells were selected for their high level of endogenous hepcidin mRNA expression compared to other hepatocyte cell lines (e.g. HepG2, Hepa1-6, AML12) that we tested (data not shown). Hepcidin mRNA is also expressed in the human and mouse liver 25. Transfected Huh7 cells were treated with IL-6 (1 ng/ml) and luciferase activity was measured 24 hours later. Luciferase activity increased 2-3 fold in response to IL-6 treatment when Huh7 cells were transfected with the -942-bp, -385-bp and -165-bp hepcidin promoter constructs (Figure 2A). By comparison, luciferase activity remained almost unaltered when expressed from the pGL3 control vector (Figure 2A). Importantly, endogenous hepcidin mRNA expression as an internal control also increased approximately 3-fold upon treatment with 1 ng/ml IL-6 (Figure 2B). These results suggest that the -165 promoter fragment harbours the essential element(s) for IL-6 induction.

Interestingly, bioinformatic analysis 24 identified a highly conserved, putative STAT binding motif within the first 165bp of the hepcidin promoter at position -64/-72. The deletion of this STAT binding motif both in the context of the complete -942-bp or the shortened -165-bp construct completely abolished the IL-6-dependent increase in luciferase activity (Figure 2A). By contrast, the deletion of the AP-1 binding motif in the -385-bp construct did not affect IL-6 activation. These data show that a functional STAT binding motif at position -69/72 within the hepcidin promoter mediates the hepcidin response to exogenously administered IL-6.

STAT-3 mediates hepcidin expression

A previous report identified a putative C/EBPα binding site at position -231/-222 of the hepcidin promoter and suggested that C/EBPα is required for high level hepcidin mRNA expression both in Huh7 cells and mice 23. Here we show that a truncation of the hepcidin promoter to -385-bp does not affect the luciferase read out in unstimulated control Huh7 cells. A further truncation to -165-bp, however, reduces luciferase activity more than 3-fold after transfection (Figure 3A). Because the C/EBPα binding site is located within this deleted promoter region, we assessed the effect of siRNA-mediated knockdown of C/EBPα on endogenous hepcidin expression in Huh7 cells (Figure 3B). As
expected, the knock-down of C/EBPα decreased hepcidin mRNA expression, confirming previous reports (Figure 3B).

To investigate whether the STAT binding motif at position -64/-72, which mediates the IL-6 activation of the hepcidin promoter (Figure 2A), is also required to maintain high level luciferase expression in untreated Huh7 cells, we analyzed the -942 ΔSTAT construct. Interestingly, we observed a 9.3 fold reduction of luciferase activity compared to the 942-bp construct, suggesting a role for this putative STAT binding site in maintaining high level hepcidin mRNA expression (Figure 4A).

To determine whether the STAT-3 transcription factor that is predicted to interact with this binding motif controls hepcidin mRNA expression, we performed siRNA-mediated knock-down of STAT-3. A knock-down of STAT-3 mRNA levels to 90% caused a 9-fold decrease in endogenous hepcidin mRNA expression as measured by quantitative real-time PCR (Figure 4B).

Taken together, these results confirm the role of C/EBPα in promoting hepcidin expression. They identify STAT-3 as a critical transcription factor for the basal expression of hepcidin mRNA under control culture conditions, as well as for the IL-6-mediated inflammatory response via the STAT binding motif located at position -64/-72 of the hepcidin promoter.

**Hepcidin activation by conditioned medium from macrophages is also mediated by the -64/-72 STAT binding motif**

In whole organisms, cytokine release by one cell type (e.g. macrophages) can trigger changes in gene expression in other cell types (e.g. hepatocytes). Previous reports demonstrated that medium supernatant from cultured macrophages or macrophage cell lines can activate hepcidin mRNA expression in hepatocytes.\(^8,9\)

We therefore investigated hepcidin mRNA expression following treatment of Huh7 cells with cell-free conditioned medium (CM) from a monocyte/macrophage cell line, THP-1. Hepcidin mRNA expression strongly increases under these conditions. The time course of hepcidin activation depends on the time that THP-1 cells were cultured to generate the CM (12h, 24h, 48h or 72h). CM from THP-1 cells cultured for 12h [CM(12h)] and 24h [CM(24h)] activates hepcidin mRNA expression 6.3- and 13.9-fold, respectively, 24h after the addition of CM to Huh7 cells (Figure 5A). Interestingly, CM(48h) and CM(72h) causes a fast (≤ 4h) 8-9 fold increase in hepcidin mRNA levels (Figure 5A). These data indicate that the longer THP-1 cells were maintained in culture (e.g. 48h or 72h) before collection of the CM, the stronger and earlier is the increase in hepcidin mRNA expression in Huh7 cells.

To identify promoter element(s) responsible for the CM-mediated induction, we transfected Huh7 cells with the luciferase reporter constructs and added CM (48h) 24h after transfection for a further 24h. Luciferase activity increases 2.9-, 4.5- and 2.3-fold if luciferase is expressed from vectors containing 942-bp, -385-bp and -165bp hepcidin promoter fragments, respectively (Figure 5B). This
shows that the hepcidin response (approx. 10-fold after addition of CM(48h) for 24h) is at least partially mediated via the hepcidin promoter. This induction is retained in the absence of the AP-1 binding site in the –385bp construct (data not shown), indicating that the AP-1 site does not contribute to this activation. However, the deletion of the putative STAT binding site in the -165bp hepcidin promoter construct completely abrogates the responsiveness of the promoter construct to the CM from macrophages, demonstrating that the STAT binding motif is critically important for the transcriptional activation of the hepcidin promoter both in response to IL-6 and CM.
DISCUSSION

Hepcidin is a key regulatory hormone that plays a central role in frequent clinical disorders like hereditary hemochromatosis (HH) and the anaemia of inflammation (AI). Hepcidin expression increases in response to iron overload and inflammatory stimuli to reduce duodenal iron absorption and to increase iron retention in the reticuloendothelial system (RES). In the setting of AI, these responses result in hypoferreraemia, a common hallmark of this disease. On the contrary, inappropriately low hepcidin expression in the iron overload disorder HH causes increased iron absorption and iron accumulation in the liver and other parenchymal organs.

To understand the control of hepcidin expression, we investigated the regulatory, cis-acting elements in the hepcidin promoter and trans-acting factors required for baseline hepcidin mRNA expression and in response to inflammatory stimuli. We established experimental conditions in which hepcidin mRNA expression in Huh7 cells is activated by treatment with IL-6 or following administration of conditioned medium from the macrophage/monocyte THP-1 cell line. Using luciferase reporter vectors, we show that the -942bp hepcidin promoter is activated by IL-6. Only the immediate 165 base pairs of the promoter suffice to retain IL6 activation (Figure 2A). Importantly, the deletion of a highly conserved STAT binding motif within this 165 bp or the -942 bp fragment at position -64/-72 completely abrogates the IL-6 effect (Figure 2A). Transcriptional inflammatory responses via IL-6 are classically mediated via IL-6 binding to the IL-6 receptor (p130) to activate the JAK kinases which in turn switch on latent transcription factors – the signal transducers and activators of transcription (STAT) proteins. Our data show that hepcidin activation via IL-6 follows this classical pathway and identifies the critical regulatory element. Together with the STAT-3 ablation experiments (Figure 4B), our data unravel a missing link in the acute phase activation of hepcidin in which IL-6 activates the transcription of hepcidin via the STAT binding motif at position -64/-72 of the hepcidin promoter.

In addition to exogenously added IL-6, conditioned medium from THP-1 cells also activates luciferase activity via the same STAT binding motif (Figure 5B). It is therefore possible that IL-6 secreted from the THP-1 cells or IL6 endogenously produced in Huh7 cells in response to cytokines contained within the CM mediate hepcidin activation by CM. Notably, hepcidin activation is accelerated and more pronounced when conditioned medium from THP-1 cells maintained in culture for 48h or 72h is used, compared to CM(12h) and CM(24h). Thus, such differences in cytokine composition seem to alter the kinetics of hepcidin activation (Figure 5A).

Surprisingly, the STAT binding site at position -64/-72 of the hepcidin promoter not only controls its IL-6-dependent transcriptional activation, but also transcriptional activity under control culture conditions (Figure 4A). Knock down of STAT-3 by RNAi significantly reduces endogenous hepcidin mRNA expression in Huh7 cells (Figure 4B), suggesting that STAT-3 controls hepcidin transcription both in the absence and presence of experimental inflammatory stimuli. It is possible, however, that
agents contained in the control culture medium stimulate a low level ‘inflammatory response’, although we use high purity, low endotoxin culture media and FBS. Nonetheless, the finding of STAT-3-mediated hepcidin transcription even in the absence of experimental inflammatory stimuli implies that stimuli which mediate hepatic STAT-3 activation may also enhance hepcidin mRNA expression.

A previous report suggested that C/EBP\(\alpha\) also controls hepcidin transcription \(^{23}\). In Huh7 cells, the deletion of the putative C/EBP\(\alpha\) binding site at position -231/-222 from our reporter constructs reduced luciferase expression (Figure 3A), and siRNA-mediated knock down of C/EBP\(\alpha\) mRNA significantly diminished endogenous hepcidin mRNA expression (Figure 3B), confirming and extending these earlier data. In contrast to STAT-3, however, C/EBP\(\alpha\) does not seem to be involved in the activation of the hepcidin promoter in response to inflammatory stimuli (Figure 2A and 5B). Whether and how STAT3 and C/EBP\(\alpha\) cooperate to maintain high level hepcidin transcription requires further experimentation.

Hereditary hemochromatosis (HH) is a group of frequent iron overload disorders caused by mutations in the HFE, transferrin receptor 2 (TfR2) or hemojuvelin genes \(^{28}\). Interestingly, each of these gene defects results in inappropriately low expression of hepcidin, triggering increased intestinal iron absorption and hepatic iron overload. Signalling pathways that regulate gene expression through transcription factors, like C/EBP\(\alpha\) and STAT-3 may thus be of interest in the context of HH. Of note, are several reports that describe links between STAT and TGF\(\beta\)/SMAD signalling \(^{29-31}\). While, such a link up to now was not described in the liver, SMAD proteins are implicated in the transcriptional control of hepcidin in HH \(^{21,22}\) as well as in the response to iron overload or IL-6 \(^{22}\). Thus, the investigation of this link may warrant further study.

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REFERENCES


FIGURE LEGENDS

Figure 1. Luciferase reporter vectors containing the human hepcidin promoter

A. Nucleotide sequence of the 5´-flanking region of human hepcidin. The sequence of the 300bp of the transcription start site of the human hepcidin promoter is indicated in small letters. The 5´untranslated region of the hepcidin gene is indicated in capital letters starting at position +1. Underlined sequences indicate transcription factor binding motifs. The AP-1 and STAT binding motifs are phylogenetically conserved (100% identity of the core sequences) in human, mouse and rat. The transcription factor binding motifs were identified by MatInspector\(^{24}\) and TFsearch vs 1.3.

B. Luciferase (firefly) reporter vectors: The -942 construct contains the longest promoter region analysed in this study; the -385 construct contains the AP-1, C/EBP\(\alpha\) and STAT binding motifs; in the -385 \(\Delta\)AP-1 construct the AP-1 binding motif is deleted; the -165 construct contains the STAT binding motif whereas the AP-1 and C/EBP\(\alpha\) binding motifs are deleted, in the -165 \(\Delta\)STAT construct the STAT binding motif is deleted; in the -942 \(\Delta\)STAT construct the STAT binding motif is deleted, while the AP-1 and C/EBP\(\alpha\) binding motifs are preserved; SV40 driven pGL3 vector, expressing luciferase (renilla) was used as a control. Stars indicate the deletion of transcription factor binding motifs. A black box indicates the 5´end of the promoter region.

Figure 2. IL-6 dependent hepcidin activation is mediated by STAT binding motif at position -64/-72 of the hepcidin promoter

A. Luciferase reporter assays. Huh7 cells were transfected with luciferase reporter vectors and 24h later treated with IL-6 (1ng/ml; 24h). Transfections were performed in triplicates and results are presented as a fold change \(\pm\) SD of Firefly/Renilla (F/R).

B. Hepcidin mRNA expression. Huh7 cells were treated with IL-6 (1ng/ml) and endogenous hepcidin mRNA expression was analysed by quantitative real-time PCR. Data are normalised to mRNA expression of a house-keeping gene, Gapdh, and shown as fold change in comparison to untreated cells.

Significant expression changes are marked by an asterix (*) whereby * represents p-values <0.05 and ** represents p-values<0.005.

Figure 3. C/EBP\(\alpha\) mediates high level of basal hepcidin mRNA expression

A. Luciferase reporter assays. Huh7 cells were transfected with luciferase reporter vectors and luciferase activity was measured after 72 hours. Transfections were performed in triplicates and results are presented as fold change \(\pm\) SD of Firefly/Renilla (F/R). The -165-bp promoter construct (lacking the C/EBP\(\alpha\) binding motif) shows >3-fold reduced luciferase activity compared to the -942-bp and -385 constructs. Luciferase activity of the -942-bp construct was set to 100%.

B. siRNA mediated knock-down of C/EBP\(\alpha\). Hepcidin mRNA expression in Huh7 cells was assayed after transfection with specific siRNA directed against C/EBP\(\alpha\) or luciferase (Luc siRNA) as a control.
Hepcidin and C/EBPα mRNA expression was analysed by quantitative real-time PCR and data were normalised to mRNA expression of a house-keeping gene, Gapdh. Data are presented as fold change, whereby Huh7 cells transfected with the luciferase control siRNA were set to 100%. Significant expression changes are marked by an asterix (*) whereby * represents p-values <0.05 and ** represents p-values<0.005.

**Figure 4. STAT-3 mediate hepcidin expression**

A. **Luciferase reporter assays.** Huh7 cells were transfected with the -942-bp or the -942-bp ΔSTAT luciferase reporter vectors and luciferase activity was measured after 48 hours. Transfections were performed in triplicates and results are presented as a fold change ± SD of Firefly/Renilla (F/R).

B. **siRNA mediated knock-down of STAT-3.** Hepcidin mRNA expression in Huh7 cells was assayed after transfection with specific siRNAs directed against STAT-3 or luciferase (Luc siRNA) as a control. Hepcidin and STAT-3 mRNA expression was analysed by quantitative real-time PCR and data normalised to mRNA expression of a house-keeping gene, Gapdh. Data are presented as fold change, whereby Huh7 cells transfected with the luciferase control siRNA were set to 100%. STAT-3 mRNA expression was reduced to 12%. Significant expression changes are marked by an asterix (*) whereby * represents p-values <0.05 and ** represents p-values<0.005.

**Figure 5. Cell-free conditioned medium (CM) -dependent hepcidin activation**

A. Conditioned medium (CM) from THP-1 cells was collected after 12, 24, 48 and 72 hours [CM(12h); CM(24h); CM(48h); CM(72h), respectively] in culture. The undiluted CM was used to stimulate Huh7 cells for the indicated time periods. Endogenous hepcidin mRNA expression in Huh7 cells was analysed by quantitative real-time PCR and data were normalised to mRNA expression of a house-keeping gene, Gapdh. Data are presented as fold change ± SD.

B. **Increased luciferase activity after treatment with CM(48h) depends on a functional STAT binding site.** Huh7 cells were transfected with the luciferase reporter vectors. 24h after transfection cells were treated with CM(48h) and luciferase activity was assessed 24 hours later. Transfections were performed in triplicates and results are presented as fold change ± SD of Firefly/Renilla (F/R). Significant expression changes are marked by an asterix (*) whereby * represents p-values <0.05 and ** represents p-values<0.005.
Figure 1.
A.

-942 
-300 gctcatcaaactgtcagacaggaaggggaactttttcctg
-250 atcagcagaatgacagctagctggtg
-200 agcagtgtgtgtctgtgaccccgtctgccccaccccctgtaacacctct
-150 gccggctgagggtgacacaaccctgttccctgtcgctctgttcccgctta
-100 tctctcccgccttttcggcgccaccaccttcttggaa
+50 aaggggagggggctcagaccaccgcctcccctggcaggccccataaagc
+1   GACTGTCACTCGGTCCCAGACACCAGAGCAAGCTCAAGACCCAGCAGTGG

B.

-942 
-385 gctcatcaaactgtcagacaggaaggggaactttttcctg
-165 gctcatcaaactgtcagacaggaaggggaactttttcctg
-165 gctcatcaaactgtcagacaggaaggggaactttttcctg

Figure 2.
A.

-942 
-385 
-385 ΔAP-1 
-165 
-165 ΔSTAT 
-942 ΔSTAT

B.

Luciferase activity (-fold increase)

0 1 2 3 4 5

IL6 treatment (hours)

Figure 3.
A.

Luciferase activity (%) 

-942 -385 -165

B.

Hepcidin mRNA levels (%) 

siRNA Luc C/EBPα 

C/EBPα mRNA levels (%) 

siRNA Luc C/EBPα
Figure 4.

A.

B.

Figure 5A.
Figure 5B.

Luciferase activity (-fold increase)

- pGL3 ctrl
- -942
- -385
- -165
- -165 ΔSTAT

0 1 2 3 4 5 6
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