DNA methylation of hepatic iron sensing genes and the regulation of hepcidin expression

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Abstract

Production of the iron regulatory peptide hepcidin is tightly controlled by a network of proteins in hepatocytes that sense levels of iron in the circulation (as diferric-transferrin) and in tissues (in ferritin). Human studies show high variability in the normal range of serum hepcidin levels. We have postulated that this may, in part, be related to inter-individual variability in the expression of genes in the iron sensing pathway, potentially governed by epigenetic factors. Here, we have investigated whether genes encoding hepatic iron sensing proteins and hepcidin are regulated by DNA methylation. Experiments were performed on two human hepatoma cell lines, HepG2 cells and Huh7 cells. Basal expression of TFR2 and HAMP was significantly lower in Huh7 cells compared with HepG2 cells. Analysis of bisulphite-converted DNA from Huh7 cells revealed partial methylation of TFR2 (α transcript), which could result in gene silencing. Demethylation using 5-aza-2’-deoxycytidine (AZA) increased TFR2 mRNA expression in Huh7. PCR analysis of bisulphite-converted HAMP promoter DNA, using methylation-specific primers, revealed no differences between cell lines. However, HAMP mRNA expression in Huh7 was increased by AZA treatment, suggesting that methylation of one or more iron sensing genes may indirectly influence HAMP expression. Our study provides evidence that DNA methylation might control expression of HAMP and other hepatic iron sensing genes, and indicates that epigenetic influences on iron homeostasis warrant further investigation.

Introduction

Iron homeostasis is maintained by a network of proteins in hepatocytes, which sense changes in circulating and cellular levels of iron. Downstream signalling from these sensors leads to the regulated production of hepcidin (reviewed in [1]). Once released from hepatocytes, serum hepcidin acts as a negative regulator of iron export from a number of cell types, including enterocytes and macrophages, by decreasing expression of the iron transport protein ferroportin [2–5]. In addition, in enterocytes, hepcidin limits iron absorption from diet through down-regulation of DMT1 [5–8].
In hepatocytes, increased cellular iron activates the bone morphogenetic protein (BMP) signalling pathway, which encompasses the ligand BMP6, BMP receptors and the co-receptor hemojuvelin (HJV), and induces the production of hepcidin via the SMAD signalling pathway [9,10]. The protein network which senses changes in serum iron levels (detected as changes in transferrin (Tf) saturation) and translates this information to produce appropriate levels of hepcidin is complex. At low Tf saturation levels, the hereditary haemochromatosis protein HFE and transferrin receptor 1 (TfR1) exist as a complex on the plasma membrane. However, at increased saturation levels, diferric-Tf competes with HFE for binding to TfR1. This causes HFE to dissociate from TfR1 and bind to a second transferrin receptor (TfR2). HFE/TfR2/diferric-Tf binding initiates an iron sensing pathway leading to increased production of hepcidin [11].

The TFR2 gene contains two promoter regions and encodes two receptor isoforms. TFR2-alpha transcript encodes the full length receptor and is expressed on the cell surface of hepatocytes, erythroid progenitors and peripheral blood mononuclear cells [12]. The TFR2-beta transcript is a truncated form which lacks the membrane spanning domain and is highly expressed in spleen, brain and heart [12]. Its physiological role is unclear, but may be involved in regulation of ferroportin in splenic macrophages [13]. The involvement of each of the TFR2 transcripts in iron sensing and the regulation of hepcidin production has not been studied previously and we have addressed this in our current work.

Mutations in TFR2 give rise to type III haemochromatosis [14], which is characterised by liver iron loading and inappropriately low hepcidin levels [15,16]. Furthermore, evidence from knockout studies in mice demonstrates that deletion of TFR2 [17,18], HFE [17,18], HFE2 (HJV) [19,20], or BMP6 [10] leads to inappropriately low HAMP (the gene encoding hepcidin) expression and liver iron loading. Taken together this suggests that each of these iron sensing elements has a specific role to play in the appropriate production of hepcidin. Interestingly, while the HFE/TfR2 and BMP signalling pathways regulating HAMP expression may operate independently of each other [21–23], there is also evidence for interaction between these sensing networks [17,24].

In addition to modulation by iron, HAMP expression is regulated by a variety of other signals including adipokines [25], pro-inflammatory cytokines [26–29], and hypoxia [30–34]. The normal range of serum hepcidin levels in the healthy population is highly heterogeneous [35–37]. While age and gender are significant factors in this distribution, there is still considerable variability (by as much as 50-fold) within population groups. The basis for this is unclear, but it could be related to the non-iron factors identified above (cytokine levels, etc.) or to inter-individual variability in the expression of genes in the iron sensing pathway. Gene expression at an individual level may be controlled by a number of genetic or epigenetic factors. One possible mechanism is through DNA methylation, which occurs at CG sites, resulting in the formation of 5-methylcytosine. CpG-rich regions, known as CpG islands, often occur within the promoter regions of genes, and when methylated result in the repression of gene expression [38]. In this study we have explored ENCODE DNA methylation data to identify putative methylation target sites in the promoter regions of iron sensing genes and HAMP, and assessed the effects of demethylation on mRNA expression of these genes in human hepatoma cell lines.

Materials & methods

Cell culture

HepG2 (ATCC) and Huh7 (gift from Prof. S. Srai, UCL) hepatoma cells were cultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 100 units/ml penicillin,
100 μg/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acid solution (all purchased from Thermo Fisher Scientific, UK). For experiments, cells were seeded into 6-well plates at a density of 1 x 10^5 cells/cm^2. In some experiments cells were treated with 5-aza-2'-deoxycytidine (AZA, 5 μM) for 72 hours; medium containing AZA was replaced every 24 hours.

**Quantitative real-time (qRT)-PCR**

RNA isolated from hepatic cells was converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Expression of TFR2 alpha transcript, TFR2 beta transcript, HAMP, HFE, BMP6, HFE2 (HJV) and B2M (reference gene) was analysed using Fast SYBR green Master Mix (Thermo Fisher Scientific) and a ABI Prism 7500 FAST sequence detection system (Applied Biosystems). Primer sequences are given in Table 1. Data were analysed using the 2^(-ΔΔCt) method [39].

**Methylation PCR**

DNA from HepG2 and Huh7 cells was subject to bisulphite conversion using EZ DNA Methylation-Gold™ kit (Zymo Research) according to the manufacturer’s instructions. For analysis of the TFR2 alpha promoter, a 246 bp fragment containing the whole of exon 1, plus the 5' flanking region and intron 1 (base pairs -154 to +92, relative to the translation start site), was amplified using bisulphite-specific primers (Table 1, designed using MethPrimer; [www.urogene.org/cgi-bin/methprimer/methprimer.cgi](http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi)). The amplicon contained a ClaI restriction digest site (ATCGAT) which allowed the PCR product to be cut into two fragments (138 and 108 bp, respectively) if the CpG within the restriction enzyme recognition site was methylated. Aliquots of ClaI-digested and undigested PCR products were resolved on 2% agarose-ethidium bromide gels. Further aliquots of the full amplicon were cloned into E. coli using a TOPO TA Cloning Kit (Invitrogen). Positive colonies were selected and DNA extracted and sequenced (ABI 3730x1 DNA Analyser).

For analysis of the HAMP promoter, no suitable bisulphite-specific primers could be designed. Instead we designed methylation-specific primers (Table 1) that would bind to either the predicted methylated or unmethylated DNA sequences within the bisulphite-converted HAMP promoter. Primers targeted identical regions in the HAMP promoter, and differed only

| Primer       | Forward sequence | Reverse Sequence |
|--------------|------------------|------------------|
| B2M RT       | CCA CTG AAA AAG ATG AGT ATG CCT | CCA ATC CAA ATG CGG CAT CTT CA |
| HAMP RT      | CTG CAA CCC CAG GAC AGA G | GGA ATA AAT AAG GAA GGG AGG GG |
| TFR2-α RT    | GTC AGT GAG GAT GTC AA | CCA CGT GTC CAG CTT CT |
| TFR2-β RT    | CCA GAA AAG TCC CCA CCA CCT C | TGC TCT CGG ACC TTC CC |
| HFE RT       | AGA ACA GGG CCT ACC TGG AG | TGT GTC ACC TCC ACC AAA GG |
| HFE2 (HJV) RT| GGA GCT TGG CCT CTA CTC GA | ATG GTG AGC TTC CGG GTG |
| BMP6 RT      | CGG TGT ATG ATG GGC CTC AGA | TCA CAA CCC ACA GAT TGC TAG T |
| TFR2-α BIS   | GGG GGG TGA GGG ATT AGA GAA | GGA AAA CTA TCA CCC CAC CCT TAA AA |
| HAMP Met     | TTT TGT TTT CGT TTA TTT TTT TCG T | AAA CTC ATG ACC ATC GTA CGC TC |
| HAMP Unmet   | TTT TGT TTT GAG TTA TTT TTT TGG T | AAA AAG TCA ATA CCA TCA TAC CAT |

RT = qRT-PCR primer; BIS = primers for bisulphite-converted DNA; Met = specific primers for methylated bisulphite-converted DNA; Unmet = specific primers for unmethylated bisulphite-converted DNA.

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at two C/T bases in each of the forward and reverse primers. Analysis was carried out using qRT-PCR.

Statistics
Data are presented as mean ± S.E.M. Data were analysed using Mann-Whitney tests and differences with p < 0.05 considered statistically significant. Linear regression analysis was performed using Sigmaplot (version 13, Systat Software Inc., UK).

Results
The relative basal RNA expression levels of iron sensing genes were measured in HepG2 and Huh7 cells, respectively (Fig 1). Expression of HAMP and TFR2 alpha was significantly greater in HepG2 cells compared with Huh7 cells (Fig 1A and 1B). There were no significant differences in levels of TFR2 beta (Fig 1C), HFE (Fig 1D) or BMP6 (Fig 1E) between the two cell lines. HFE2 (HJV) levels were significantly higher in Huh7 cells (Fig 1F).

Fig 1. Baseline expression of iron sensing genes in HepG2 and Huh7 cells. mRNA expression of (a) HAMP, (b) TFR2 alpha, transcript, (c) TFR2 beta transcript, (d) HFE, (e) BMP6 and (f) HFE2 (HJV) were measured by RT-PCR. Data are means ± SEM of 5–8 observations in each group and were normalised to expression in HepG2 cells. * P < 0.02 (Mann-Whitney test).

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We hypothesised that differences in expression may be related to cell-specific methylation of the gene promoters. To investigate this possibility, we interrogated data from the ENCODE project [40] on the University of California Santa Cruz Genome Browser. We restricted our search to CpGs within the 5' promoter for each gene of interest, since methylation in this region has been shown to repress gene expression [38]. At least one CpG was identified in the promoter region of each gene of interest and β-values for the degree of methylation are reported in Table 2. Data were available for experiments with human hepatocytes and with HepG2 cells. In the context of the differences observed in basal expression of TFR2α and HAMP in the hepatic cell lines, it was interesting to note that there was some variation in the degree of methylation of CpGs in both the TFR2α and HAMP gene promoters in hepatocytes and HepG2 cells (Table 2).

To determine whether the TFR2α and HAMP promoters were methylated in Huh7 hepatoma cells, and to confirm their methylation status in HepG2 cells, we carried out bisulfite conversion of DNA and used specific primers to interrogate the CpGs within each promoter that corresponded to the 450K probe sites (Illumina Infinium HumanMethylation450K BeadChip) from the ENCODE project. For TFR2α, the amplified promoter fragment contained a potential ClaI restriction digest site if the C of the relevant CpG site was retained following bisulfite treatment (i.e., methylated). Following ClaI treatment only the full-length TFR2α PCR product (246 bp) was identified in HepG2 cells, whereas three fragments (246; 138 and 108 bp) were detected in Huh7 cells (Fig 2A) indicating that the TFR2α promoter site was unmethylated in HepG2 cells, but partially methylated in Huh7 cells. K562 cells (ENCeDE β-values: cg10681065, 0.04; cg04423314, 0.06) and Jurkat cells (ENCeDE β-values: cg10681065, 0.88; cg04423314, 0.69) were used as positive and negative methylation controls, respectively. Selection of these cell lines as controls was based on previously published data showing high expression of TFR2α in K562 cells, but low expression levels in Jurkat cells [12]. Only the full-length TFR2α PCR product (246 bp) was present in K562 cells, whereas the ClaI digested fragments (138 and 108 bp) predominated in Jurkat cells (Fig 2A). To further investigate the cell-specific difference in TFR2α promoter methylation, we subjected amplicons from all cell lines to bisulfite sequencing. Five CpGs sites were present in the TFR2α amplicon. All sequenced CpGs in K562 cells and the majority of CpGs in HepG2 cells

### Table 2. Beta-values from ENCODE database showing degree of methylation of CpG sites in promoters of iron sensing genes.

| Gene of Interest | CpG identifier | HepG2 cells | Hepatocytes |
|------------------|----------------|-------------|-------------|
| TFR2α            | cg10681065     | 0.09        | 0.28        |
|                  | cg04423314     | 0.09        | 0.28        |
| TFR2β            | cg04499151     | 0.96        | 0.70        |
| HFE              | cg06892726     | 0.08        | 0.10        |
|                  | cg05569784     | 0.21        | 0.30        |
| HJV              | cg00953211     | 0.22        | 0.13        |
|                  | cg00987513     | 0.73        | 0.27        |
|                  | cg06589885     | 0.13        | 0.05        |
| BMP6             | cg22505205     | 0.50        | 0.13        |
|                  | cg22541378     | 0.24        | 0.03        |
|                  | cg03447931     | 0.32        | 0.02        |
| HAMP             | cg26283059     | 0.12        | 0.27        |
|                  | cg17907567     | 0.10        | 0.25        |
|                  | cg23677000     | 0.13        | 0.21        |

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a

DNA methylation

b

HAMP expression

Clamp

246 bp

TFR2-A

K562

Jurkat

HepG2

Huh7
were unmethylated (Fig 2B). In contrast, 95% of CpG dinucleotides in Jurkat cells and more than 50% of CpGs in Huh7 cells were methylated.

qRT-PCR analysis of the HAMP promoter using combinations of methylated and unmethylated sequence-specific primers revealed no significant differences between HepG2 cells and Huh7 cells for each primer combination (Fig 3). Melt curves indicated only a single PCR product was generated with each of the primer combinations. Lowest Ct values (and therefore highest levels) were observed using the methylated forward: unmethylated reverse primer combination (mean Ct values: 30.6, HepG2; 30.4, Huh7). There was no effect of the demethylating agent 5-deoxy-2′-azacytidine (AZA) on amplicon levels in either cell line using the methylated forward: unmethylated reverse primer combination (HepG2 cells (normalised to control values): control 1.0 ± 0.1; AZA 0.9 ± 0.1; n = 4 for both groups. Huh7 (normalised to control values): control 1.0 ± 0.1; AZA 1.0 ± 0.3; n = 4 for both groups). Since there were no differences in amplicon levels in the presence or absence of AZA in HepG2 and Huh7 cells, we concluded that there was no cell-specific methylation within this region of the HAMP promoter and therefore amplicons were not subjected to full bisulphite sequencing.

Next we investigated the effects of AZA on the expression of iron sensing genes in HepG2 cells and Huh7 cells. There was no effect of AZA on expression of HAMP and TFR2 alpha in HepG2 cells (Fig 4A and 4B); however, expression of both genes was significantly increased by AZA treatment in Huh7 cells (Fig 5A and 5B). TFR2 beta (Fig 4C; Fig 5C) and BMP6 (Fig 4E; Fig 5E) expression was significantly increased by AZA treatment in both cell lines. HFE2 (HJV) expression was increased in AZA-treated HepG2 cells only (Fig 4F; Fig 5F). There was no effect of AZA on HFE expression in either cell line (Fig 4D; Fig 5D).

Our data indicated that cellular expression and regulation of HAMP and TFR2 alpha followed a similar pattern; i.e., highest expression in HepG2 cells (Fig 1A and 1B) and up-regulation by AZA-treatment only in Huh7 cells (Fig 5A and 5B). However, while there were clear differences in methylation of TFR2 alpha in HepG2 and Huh7 cells (Fig 2), there was no evidence for cell-specific differences in HAMP promoter methylation at documented CpG sites (Fig 3, Table 2). We therefore investigated the possibility that changes in expression of one or more of the iron sensing genes might indirectly influence HAMP expression. In HepG2 cells there was significant positive correlation between HAMP and TFR2 alpha, and HFE (Fig 6). We carried out multiple linear regression to identify the variables which most significantly predict HAMP expression and found that HFE was the only significant predictor of HAMP mRNA levels (P < 0.005). In contrast, in Huh7 cells there was significant correlation between HAMP and all iron sensing genes (i.e., TFR2 alpha, TFR2 beta, HFE, BMP6 and HFE2 (HJV); Fig 7). Multivariate analysis revealed that BMP6 (P < 0.001) and HFE2 (HJV) (P < 0.03) were the only significant predictors of HAMP expression in Huh7 cells.

**Discussion**

In this study we employed two well-characterised human hepatoma cell lines, HepG2 and Huh7 cells, to investigate the putative effects of methylation on expression of hepatic iron sensing genes.
sensing genes. HepG2 cells were originally derived from a well-differentiated hepatocellular carcinoma [41], which displays a high level of morphological and functional differentiation in vitro. Huh7 hepatoma cells were derived from a Japanese patient [42] and it has been shown that these cells contain a mutated form of \textit{HFE} [43]. Given that \textit{HFE} is required for iron sensing and appropriate control of \textit{HAMP} expression [17,18], we hypothesized that iron sensing and \textit{HAMP} expression would differ in the two model cell lines.

Basal expression of \textit{TFR2} alpha and \textit{HAMP} was significantly greater in HepG2 cells than Huh7 cells. Differential tissue expression of \textit{TFR2} isoforms has been well documented [12]. \textit{TFR2} alpha is the predominant isoform in liver and blood mononuclear cells, and is highly

Fig 3. Methylation of \textit{HAMP} promoter. Bisulphite-converted DNA from HepG2 and Huh7 was subjected to qRT-PCR using a combination of primers specific for methylated and unmethylated sequences. Data are presented as relative levels; ΔCt relative to the methylated forward: methylated reverse primer set (M-M) for HepG2 cells.

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expressed in associated tumour cell lines, e.g. HepG2 hepatoma cells and K562 erythroleukae-
mia cells. In contrast, highest expression of TFR2 beta was observed in spleen and T-cell leu-
kaemia cell lines, e.g., Jurkat cells; these cells also had low expression of TFR2 alpha [12]. One
explanation for the differential expression patterns of the TFR2 transcripts might be regulation
at the level of DNA methylation. To investigate this possibility, we used data from the
ENCODE project [40] including the methylation of specific CpG dinucleotides from experi-
ments using the Illumina Infinium HumanMethylation450K BeadChip array platform. We
focussed on CpGs in the 5’ promoter of TFR2, since methylation of CpGs in gene promoters is
associated with silencing of gene expression [38]. For TFR2 alpha, the methylation pattern
from ENCODE (Table 2) and our bisulphite sequencing analysis (Fig 2) showed that CpGs
within the TFR2 alpha promoter were unmethylated in K562 cells and HepG2 cells, but meth-
ylated in Jurkat cells. These findings are consistent with previously published mRNA expres-
sion data showing high expression of the TFR2 alpha transcript in K562 and HepG2 cells, but

Fig 4. Effect of AZA on iron sensing gene expression in HepG2 cells. Cells were grown in the presence or absence of the global demethylating agent AZA (5 μM, 72 h). Expression of (a) HAMP, (b) TFR2 alpha transcript, (c) TFR2 beta transcript, (d) HFE, (e) BMP6 and (f) HFE2 (HJV) were measured by RT-PCR. Data have been normalised to the control group and are presented as mean ± SEM of 7–8 observations in each group. Data were analysed using Mann-Whitney tests. * P < 0.005; ** P < 0.03.

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low expression in Jurkat cells [12]. Interestingly, our data demonstrated that the TFR2 alpha promoter was partially methylated in Huh7, and this was associated with significantly lower basal TFR2 alpha mRNA expression in Huh7 cells compared with HepG2 (Fig 1B). ENCODE data for HepG2 cells and hepatocytes (Table 2) and our own analysis in Huh7 cells (56% methylation of promoter CpG dinucleotides) found significant methylation of the TFR2 beta promoter, which may account for low expression of this transcript in both HepG2 and Huh7 cells. Together, we have evidence that differences in DNA methylation are strongly associated with tissue-specific expression of the TFR2 isoforms.

The tissue-specific epigenetic control of TFR2 observed here points to tissue-specific functions of the isoforms in the regulation of iron homeostasis. In addition to its well-documented role in hepatic iron sensing, TFR2 is a component of the erythropoietin receptor signalling complex and is required for efficient erythropoiesis [44]. Haematopoietic deletion of Tfr2 in mice results in impaired erythroid differentiation [45,46]. High expression of TFR2 in erythroid cells [47] and its role in erythroid differentiation have led to speculation of a clinically
relevant role in haematological disorders such as myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML). In support of this, a recent retrospective analysis of samples from patients with MDS found that expression of \textit{TFR2} alpha and \textit{TFR2} beta was significantly more variable in MDS samples than in non-malignant bone marrow samples. Furthermore, low expression of both \textit{TFR2} transcripts was associated with poorer survival rates in patients with myelodysplastic syndrome with excess blasts than for those with normal to high \textit{TFR2} levels [48]. Similarly, a study in patients with AML found higher expression of both \textit{TFR2} transcripts was associated with significantly longer survival rates [49]. Interestingly, aberrant DNA methylation is the dominant epigenetic alteration in MDS [50] and DNA methyltransferase inhibitors such as azacitidine are approved as treatment strategies in patients with MDS [51].

\textit{HAMP} expression is also known to be tissue specific with highest expression in liver [52] and lower levels present in other tissues including adipose and peripheral blood mononuclear cells [53,54]. ENCODE data suggest that \textit{HAMP} promoter CpG sequences adjacent to the translation start site are unmethylated in HepG2 cells (Table 2), but are partially methylated in both K562 and Jurkat cells (\textit{\beta}-scores: cg26283059, 0.31—K562, 0.72—Jurkat; cg17907567, 0.31—K562, 0.45—Jurkat). This is consistent with relatively low expression of \textit{HAMP} in peripheral blood mononuclear cells compared to hepatic cells [54], and may indicate a role for methylation in determining cell- and tissue-specific expression of \textit{HAMP}. To determine whether methylation accounted for the observed differences in basal expression of \textit{HAMP} between HepG2 and Huh7 cells we analysed the region of the promoter containing the CpG dinucleotides cg17907567, cg23677000 and cg26283059. These CpGs are unmethylated in HepG2 cells.

![Fig 6. Correlation between iron sensing genes and \textit{HAMP} in HepG2 cells. \(\Delta C_t\) values (using \textit{B2M} as the reference gene) from control and AZA-treated cells were plotted for each gene of interest vs \textit{HAMP}. Data were analysed using linear regression.](https://doi.org/10.1371/journal.pone.0197863.g006)
(Table 2) and our studies suggest that there is no difference in methylation between HepG2 and Huh7 cells. In summary, our analysis does not support the hypothesis that differential methylation of this region of the HAMP promoter explains the differences in basal expression of HAMP between HepG2 and Huh7 cells.

To assess the effects of methylation on HAMP and TFR2 alpha mRNA expression we treated cells with the 5-aza-2’deoxycytidine (AZA), which inhibits DNA methyltransferase activity and results in global DNA demethylation [55]. AZA treatment increased TFR2 alpha mRNA expression in Huh7 cells, but not in HepG2 cells. Taken together, our data and those from ENCODE suggest that differential expression of TFR2 alpha between HepG2 cells and Huh7 cells is mediated by methylation of promoter CpG dinucleotides. Furthermore, this is also a likely explanation for tissue-specific expression of TfR2 isoforms demonstrated previously [12]. Expression of TFR2 beta and BMP6 was increased in both HepG2 and Huh7 cells, and HFE2 (HJV) was elevated in HepG2 cells following AZA treatment indicating that methylation plays an important role in regulating the expression of a number of iron sensing genes.

AZA treatment significantly increased HAMP expression in Huh7 cells, but did not alter HAMP levels in HepG2 cells. Interestingly, a recent report has shown that HAMP expression is suppressed in hepatocellular carcinoma through hypermethylation of CpGs within the gene promoter [56]. These findings are in contrast to the data presented here. However, it is important to note that the HAMP promoter region analysed by Udali et al. (-940 to -398 bp relative to the translation start site) [56], did not overlap with the promoter amplicon studied in our analysis (-186 to +14 bp relative to the translation start site). It is possible therefore that
hypermethylation within the distal promoter modulates HAMP expression. Moreover, it is possible that epigenetic silencing of elements within one or more of the iron signalling pathways may contribute to decreased HAMP expression. For example, SOSTDC1, an inhibitor of BMP 2, 4 and 7 activity, decreases SMAD-signalling and subsequent hepcidin secretion in prostate epithelial cells [57]. Interestingly in prostate cancer the SOSTDC1 gene promoter is highly methylated leading to suppression of gene transcription, and this is associated with increased HAMP expression and poorer prognosis in patients with prostate tumours [57].

A further possibility is that differential methylation of one or more of the hepatic iron sensing genes could have an indirect effect on HAMP expression. Our previous work shows that TFR2 mRNA correlates significantly with HAMP expression levels in human primary hepatocytes [58], while others have shown strong correlation between HFE and HAMP in HepG2 cells [59]. Here we found a significant correlation between levels of both TFR2 alpha and HFE, and HAMP in HepG2 cells, with HFE being the most significant predictor of HAMP expression. Interestingly, while there is significant positive correlation between HAMP and each individual iron sensing gene in Huh7 cells, multivariate analysis showed HFE2 (HJV) and BMP6 to be the only significant predictors of HAMP. This divergence between the iron sensing pathways that correlate with HAMP expression in HepG2 and Huh7 cells is perhaps not surprising given that there is a mutation in the HFE gene in Huh7 cells [43], which alters the conformation of the α3 domain of the mature protein. TFR2 interacts with the HFE α3 domain [60], and therefore this mutation might silence downstream signalling pathways in Huh7 cells which regulate HAMP expression. Similarly, the very low basal expression of HFE2 (HJV) in HepG2 cells, due at least in part to DNA methylation, is likely to dampen BMP/SMAD signalling in these cells. Interestingly, in patients with MDS, the HFE2 promoter in bone marrow cells was found to be hypermethylated. Treatment with AZA increased HFE2 expression and this was associated with elevated serum hepcidin levels [61]. Taken together, our data suggest that HFE/TFR2 signalling predominates as the iron sensing pathway in HepG2 cells, while the BMP/HJV/SMAD pathway is dominant in Huh7 cells. Key iron sensing genes in both cell lines may be silenced by DNA methylation and this may have an important bearing on HAMP expression.

In summary, our study provides evidence for a role of DNA methylation in controlling hepatic iron sensing and the production of hepcidin. Serum hepcidin levels in the healthy population are highly heterogeneous [35–37] and differential patterns of DNA methylation may be important in determining the variability in iron status and hepcidin production at a population level. This possibility remains to be explored. It has been demonstrated recently that there is widespread inter-individual epigenetic variation, including DNA methylation, in human neutrophils from healthy individuals, which might relate to phenotypic differences and potentially susceptibility to a range of diseases [62]. The epigenetic influences on iron homeostasis and the risk of developing iron metabolism disorders may be similarly linked and this possibility warrants further investigation.

Author Contributions

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References

1. Ganz T. Systemic iron homeostasis. Physiol Rev. 2013 Oct; 93(4):1721–41. https://doi.org/10.1152/physrev.00008.2013 PMID: 24137020

2. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004 Dec 17; 306(5704):2096–9. https://doi.org/10.1126/science.1104742 PMID: 15151116

3. Rivera S, Nemeth E, Gabayan V, Lopez MA, Farshidi D, Ganz T. Synthetic hepcidin causes rapid dose-dependent hypoferremia and is concentrated in ferroportin-containing organs. Blood. 2005 Sep 15; 106(6):2196–9. https://doi.org/10.1182/blood-2005-04-1766 PMID: 15933050

4. Chaston T, Chung B, Mascarenhas M, Marks J, Patel B, Sra KL, et al. Evidence for differential effects of hepcidin in macrophages and intestinal epithelial cells. Gut. 2008 Mar; 57(3):374–82. https://doi.org/10.1136/gut.2007.131722 PMID: 15933050

5. Mena NP, Esparza A, Tapia V, Valdés P, Núñez MT. Hepcidin inhibits apical iron uptake in intestinal cells. Am J Physiol Gastrointest Liver Physiol. 2008 Jan; 294(1):G192–8. https://doi.org/10.1152/ajpgi.00122.2007 PMID: 17962361

6. Brasse-Lag nel C, Karim Z, Letteron P, Bekri S, Bado A, Beaumo nt C. Intestinal DMT1 cotransporter is down-regulat ed by hepcidin via proteasom e internalization and degradat ion. Gastroenter ology. 2011 Apr; 140(4):1261–1271. https://doi.org/10.1053/j.gastro.2010.12.037 PMID: 21199652

7. Andriopou los B Jr, Corradi ni E, Xia Y, Faasse SA, Chen S, Grgurevic L, et al. BMP6 is a key endoge nous regulator of hepcidin expression and iron metabolism. Nat Genet. 2009 Apr; 41(4):482–7. https://doi.org/10.1038/ng.335 PMID: 19252486

8. Meynard D, Kautz L, Darnaud V, Canonne- Hergaux F, Coppin H, Roth MP. Lack of the bone morphoge netic protein BMP6 induces massive iron overload. Nat Genet. 2009 Apr; 41(4):482–8. https://doi.org/10.1038/ng.320 PMID: 19252488

9. Goswami T, Andrews NC. Hereditary hemochromatosis protein, HFE, interaction with transferrin recep tor 2 suggests a molecular mechanism for mammalian iron sensing. J Biol Chem. 2006 Sep 29; 281(39):28494–8. https://doi.org/10.1074/jbc.C600197200 PMID: 16833896

10. Kawabata H, Yang R, Hirama T, Vuong PT, Kawano S, Gomba rt AF, et al. Molecular cloning of transfer rin receptor 2. A new member of the transferrin receptor-lik e family. J Biol Chem. 1999 Jul 23; 274(30):20826–32. PMID: 10409623

11. Gelovani T, Andrews NC. Hereditary hemochromatosis protein, HFE, interaction with transferrin recep tor 2 suggests a molecular mechanism for mammalian iron sensing. J Biol Chem. 2008 Sep 29; 283(39):28494–8. https://doi.org/10.1074/jbc.C600197200 PMID: 16833896

12. Kawabata H, Yang R, Hirama T, Vuong PT, Kawano S, Gomba rt AF, et al. Molecular cloning of transfer rin receptor 2. A new member of the transferrin receptor-like family. J Biol Chem. 1999 Jul 23; 274(30):20826–32. PMID: 10409623

13. Roetto A, Di Cunto F, Pellegrino RM, Hirsch E, Azzolino O, Bondi A, et al. Comparison of 3 Tfr2-defi cient murine models suggests distinct functions for Tfr2-alpha and Tfr2-beta isoforms in different tis- sues. Blood. 2010 Apr 22; 115(16):3382–9. https://doi.org/10.1182/blood-2009-09-240660 PMID: 20179178

14. Campassella C, Roetto A, Ciali A, De Gobbi M, Garozzo G, Carella M, et al. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. Nat Genet. 2000 May; 25(1):14–5. https://doi.org/10.1038/ng.335 PMID: 10802645

15. Nemet E, Roetto A, Garozzo G, Ganz T, Camassella C. Hepcidin is decreased in TFR2 hemochromatosis. Blood. 2005 Feb 15; 105(4):1803–6. https://doi.org/10.1182/blood-2004-08-3042 PMID: 15486069

16. Pelucchi S, Mariani R, Trombini P, Coletti S, Pozzi M, Paolini V, et al. Expression of hepcidin and other iron-related genes in type 3 hemochromatosis due to a novel mutation in transferrin receptor-2. Haematologica. 2009 Feb; 94(2):276–9. https://doi.org/10.3324/haematol.13576 PMID: 19144662

17. Wallace DF, Summerville L, Crampton EM, Frazer DM, Anderson GJ, Subramaniam VN. Combined deletion of Hfe and transferrin receptor 2 in mice leads to marked dysregulation of hepcidin and iron overload. Hepatology. 2009 Dec; 50(6):1992–2000. https://doi.org/10.1002/hep.23198 PMID: 19824072

18. Corradini E, Rozier M, Meynard D, Odhiambo A, Lin HY, Feng Q, et al. Iron regulation of hepcidin despite attenuated Smad1,5,8 signaling in mice without transferrin receptor 2 or Hfe. Gastroenterology. 2011 Nov; 141(5):1907–14. https://doi.org/10.1053/j.gastro.2011.06.077 PMID: 21745449

19. Niederkofler V, Saile R, Arber S. Hemojuvelin is essential for dietary iron sensing, and its mutation leads to severe iron overload. J Clin Invest. 2005 Aug; 115(8):2180–6. https://doi.org/10.1172/JCI25683 PMID: 16075058
20. Gkouvatsos K, Fillebeen C, Daba A, Wagner J, Sebastiani G, Pantopoulos K. Iron-dependent regulation of hepcidin in Hjv−/− mice: evidence that hemojuvelin is dispensable for sensing body iron levels. PLoS One. 2014 Jan 7; 9(1):e85530. https://doi.org/10.1371/journal.pone.0085530 PMID: 24409331

21. Corradini E, Meynard D, Wu Q, Chen S, Ventura P, Pietrangelo A, et al. Serum and liver iron differently regulate the bone morphogenetic protein 6 (BMP6)-SMAD signaling pathway in mice. Hepatology. 2011 Jul; 54(1):273–84. https://doi.org/10.1002/hep.24359 PMID: 21488083

22. Ramos E, Kautz L, Rodriguez R, Hansen M, Gabayan V, Ginzburg Y, et al. Evidence for distinct pathways of hepcidin regulation by acute and chronic iron loading in mice. Hepatology. 2011 Apr; 53(4):1333–41. https://doi.org/10.1002/hep.24178 PMID: 21480335

23. Feng Q, Migas MC, Waheed A, Britton RS, Fleming RE. Ferritin upregulates hepatic expression of iron regulatory hormone HAMP expression in mice. J. Am. Physiol. 2012 Jun 15; 302(12):G1397–404. https://doi.org/10.1152/ajpgi.00200.2012 PMID: 22517766

24. Ramey G, Deschemin JC, Vaulont S. Cross-talk between the mitogen activated protein kinase and bone morphogenetic protein/hemojuvelin pathways is required for the induction of hepcidin by holo-transferrin in primary mouse hepatocytes. Haematologica. 2009 Jun; 94(6):765–72. https://doi.org/10.3324/haematol.2008.003541 PMID: 19454495

25. Chung B, Matak P, McKie AT, Sharp P. Leptin increases the expression of the iron regulatory hormone HAMP expression in primary human hepatocytes. J. Nutr. 2007 Nov; 137(11):2366–70. https://doi.org/10.1093/jn/137.11.2366 PMID: 17951471

26. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood. 2003 Apr 1; 101(7):2461–3. https://doi.org/10.1182/blood.v101.7.2461 PMID: 12433676

27. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, et al. IL-6 mediates hypoferremia in HuH7 liver cells via decreased SMAD4 signaling and its inflammatory stimulation. Blood. 2007 Jan 1; 109(1):353–8. https://doi.org/10.1182/blood-2006-07-033969 PMID: 16946298

28. Chaston TB, Matak P, Chaston TB, Matak P, Britton RS, Fleming RE. Ferritin upregulates hepatic expression of iron regulatory hormone HAMP expression in HuH7 liver cells. Haematologica. 2009 Jun; 94(6):765–72. https://doi.org/10.1093/jn/137.11.2366 PMID: 17951471

29. Chaston TB, Matak P, Pourvali K, Srai SK, McKie AT, Sharp PA. Activated macrophages induce hepcidin expression in HuH7 liver cells. Haematologica. 2009 Jun; 94(6):773–80. https://doi.org/10.3324/haematol.2008.003400 PMID: 19454498

30. Choi SO, Cho YS, Kim HL, Park JW. ROS mediate the hypoxic repression of the hepcidin gene by inhibiting C/EBPalpha and STAT-3. Biochem Biophys Res Commun. 2007 Apr 27; 356(1):312–7. https://doi.org/10.1016/j.bbrc.2007.02.137 PMID: 17349976

31. Braliou GG, Verga Falzacappa MV, Chachami G, Casanovas G, Muckenther MU, Simos G. 2-Oxoglutarate-dependent oxygenases control hepcidin gene expression. J. Hepatol. 2008 May; 48(5):801–10. https://doi.org/10.1016/j.jhep.2007.12.021 PMID: 18313788

32. Chaston TB, Matak P, Pourvali K, Srai SK, McKie AT, Sharp PA. Hypoxia inhibits hepcidin expression in HuH7 hepatoma cells via decreased SMAD4 signaling. Am J Physiol Cell Physiol. 2011 Apr; 300(4):C888–95. https://doi.org/10.1152/ajpcell.00121.2010 PMID: 21289291

33. Lakhal S, Schödel J, Townsend AR, Pugh CW, Ratcliffe PJ, Mole DR. Regulation of type II transmembrane serine proteinase TMPRSS6 by hypoxia-inducible factors: new link between hypoxia signaling and iron homeostasis. J Biol Chem. 2011 Feb 11; 286(6):4090–7. https://doi.org/10.1074/jbc.M110.173096 PMID: 20968077

34. Sonnweber T, Nachbaur D, Schroll A, Nairz M, Seifert M, Dernetz E, et al. Hypoxia induced downregulation of hepcidin is mediated by platelet derived growth factor BB. Gut. 2014 Dec; 63(12):1951–9. https://doi.org/10.1136/gutjnl-2013-305317 PMID: 24598129

35. Kroot JJ, Hendriks JC, Laarakkers CM, Klaver SM, Kemna EH, Tjalsma H, et al. (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: implications for clinical studies. Anal Biochem. 2009 Jun 15; 389(2):124–9. https://doi.org/10.1016/j.ab.2009.03.036 PMID: 19341701

36. Galesloot TE, Vermeulen SH, Geurts-Moespot AJ, Klaver SM, Kroot JJ, van Tienoven D, et al. Serum hepcidin: reference ranges and biochemical correlates in the general population. Blood. 2011 Jun 23; 117(25):e218–25. https://doi.org/10.1182/blood-2011-02-337907 PMID: 21527524

37. Handley S, Couchman L, Sharp P, Macdougall I, Moniz C. Measurement of hepcidin isoforms in human serum by liquid chromatography with high resolution mass spectrometry. Bioanalysis. 2017 Mar; 9(6):541–553. https://doi.org/10.4155/bio-2016-0286 PMID: 28229619

38. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002 Jan 1; 16(1):6–21. https://doi.org/10.1101/gad.947102 PMID: 11782440
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods. 2001 Dec; 25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012 Sep 6; 489(7414):57–74. https://doi.org/10.1038/nature11247 PMID: 22955616

Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science. 1980 Jul 25; 209(4455):497–9. PMID: 6248960

Nakabayashi H, Taketa K, Miyano K, Yamane T, Sato J. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. 1982 Sep; 42(9):3858–63. PMID: 6286115

Vecchi C, Montosi G, Pietrangeli A. Huh-7: a human “hemochromatotic” cell line. Hepatology. 2010 Feb; 51(2):654–9. https://doi.org/10.1002/hep.23410 PMID: 20017200

Forejtnikova H, Vieillevoye M, Zernati Y, Lambert M, Pellegrino RM, Guizard S, et al. Transferrin receptor 2 is a component of the erythropoietin receptor complex and is required for efficient erythropoiesis. Blood. 2010 Dec 9; 116(24):5357–67. https://doi.org/10.1182/blood-2010-04-281360 PMID: 20826723

Nai A, Lidoncini MR, Rausa M, Mandelli G, Pagani A, Silvestri L, et al. The second transferrin receptor regulates red blood cell production in mice. Blood. 2015 Feb 12; 125(7):1170–9. https://doi.org/10.1182/blood-2014-08-596254 PMID: 25499454

Rishi G, Secondes ES, Wallace DF, Subramaniam VN. Hematopoietic deletion of transferrin receptor 2 in mice leads to a block in erythropoiesis during iron-deficient anemia. Am J Hematol. 2016 Aug; 91(8):812–8. https://doi.org/10.1002/ajh.24417 PMID: 27169626

Kawabata H, Nakamaki T, Ikonomi P, Smith RD, Germain RS, Koeffler HP. Expression of transferrin receptor 2 in normal and neoplastic hematopoietic cells. Blood. 2001 Nov 1; 98(9):2714–9. PMID: 11675342

Di Savino A, Gaidano V, Palmieri A, Crasto F, Volpengo A, Lorenzatti R, et al. Clinical significance of TFR2 and EPOR expression in bone marrow cells in myelodysplastic syndromes. Br J Haematol. 2017 Feb; 176(3):491–495. https://doi.org/10.1111/bjh.13968 PMID: 26914246

Nakamaki T, Kawabata H, Saito B, Matsuura M, Suzuki J, Adachi D, et al. Elevated levels of transferrin receptor 2 mRNA, not transferrin receptor 1 mRNA, are associated with increased survival in acute myeloid leukaemia. Br J Haematol. 2004 Apr; 125(1):42–9. PMID: 15015967

Jiang Y, Dunbar A, Gondek LP, Mohan S, Rataul M, O’Keefe C, et al. Aberrant DNA methylation is a dominant mechanism in MDS progression to AML. Blood. 2009 Feb 5; 113(6):1315–25. https://doi.org/10.1182/blood-2008-06-163246 PMID: 18832655

Khan C, Pathe N, Fazal S, Lister J, Rossetti JM. Azacitidine in the management of patients with myelodysplastic syndromes, Ther Adv Hematol. 2012 Dec; 3(6):355–373. https://doi.org/10.1177/2040620712468820 PMID: 23609382

Armitage AE, Eddowes LA, Gileadi U, Cole S, Spottiswoode N, Selvakumar TA, et al. Hepcidin regulation in prostate and its disruption in prostate cancer. Cancer Res. 2015 Jun 1; 75(11):2254–63. https://doi.org/10.1158/0008-5472.CAN-14-2465 PMID: 25858146

Tesfay L, Clausen KA, Kim JW, Hegde P, Wang X, Miller LD, et al. Hepcidin regulation in prostate and its disruption in prostate cancer. Cancer Res. 2015 Jun 1; 75(11):2254–63. https://doi.org/10.1158/0008-5472.CAN-14-2465 PMID: 25858146

Tesfay L, Clausen KA, Kim JW, Hegde P, Wang X, Miller LD, et al. Hepcidin regulation in prostate and its disruption in prostate cancer. Cancer Res. 2015 Jun 1; 75(11):2254–63. https://doi.org/10.1158/0008-5472.CAN-14-2465 PMID: 25858146

Rapisarda C, Puppi J, Hughes RD, Dhawan A, Farnaud S, Evans RW, et al. Transferrin receptor 2 is crucial for iron sensing in human hepatocytes. Am J Physiol Gastrointest Liver Physiol. 2010 Sep; 299(3):G778–83. https://doi.org/10.1152/ajpgi.00157.2010 PMID: 20576915

Gao J, Chen J, Kramer M, Tsukamoto H, Zhang AS, Enns CA. Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. Cell Metab. 2009 Mar; 9(3):217–27. https://doi.org/10.1016/j.cmet.2009.01.010 PMID: 19254674
60. Chen J, Chloupková M, Gao J, Chapman-Arvedson TL, Enns CA. HFE modulates transferrin receptor 2 levels in hepatoma cells via interactions that differ from transferrin receptor 1-HFE interactions. J Biol Chem. 2007 Dec 21; 282(51):36862–70. https://doi.org/10.1074/jbc.M706720200 PMID: 17956864

61. Shucheng G, Chunkang C, Youshan Z, Juan G, Chengming F, Xi Z, et al. Decitabine treatment could ameliorate primary iron-overload in myelodysplastic syndrome patients. Cancer Invest. 2015 Apr; 33 (4):98–106. https://doi.org/10.3109/07357907.2014.1001895 PMID: 25699651

62. Chatterjee A, Stockwell PA, Rodger EJ, Duncan EJ, Parry MF, Weeks RJ, et al. Genome-wide DNA methylation map of human neutrophils reveals widespread inter-individual epigenetic variation. Sci Rep. 2015 Nov 27; 5:17328. https://doi.org/10.1038/srep17328 PMID: 26612583