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Characterization of hepcidin response to holotransferrin in novel recombinant TfR1 HepG2 cells

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ABSTRACT

Hepcidin is the key regulator of systemic iron homeostasis. The iron-sensing mechanisms and the role of intracellular iron in modulating hepatic hepcidin secretion are unclear. Therefore, we created a novel cell line, recombinant-TfR1 HepG2, expressing iron-response-element-independent *TFRC* mRNA to promote cellular iron-overload and examined the effect of excess holotransferrin (5 g/L) on cell-surface TfR1, iron content, hepcidin secretion and mRNA expressions of *TFRC*, *HAMP*, *SLC40A1*, *HFE* and *TFR2*. Results showed that the recombinant cells exceeded levels of cell-surface TfR1 in wild-type cells under basal (2.8-fold; $p < 0.03$) and holotransferrin-supplemented conditions for 24 h and 48 h (4.4- and 7.5-fold, respectively; $p < 0.01$). Also, these cells showed higher intracellular iron content than wild-type cells under basal (3-fold; $p < 0.03$) and holotransferrin-supplemented conditions (6.6-fold at 4 h; $p < 0.01$). However, hepcidin secretion was not higher than wild-type cells. Moreover, holotransferrin treatment to recombinant cells did not elevate *HAMP* responses compared to untreated or wild-type cells. In conclusion, increased intracellular iron content in recombinant cells did not increase hepcidin responses compared to wild-type cells, resembling hemochromatosis. Furthermore, *TFR2* expression altered within 4 h of treatment, while *HFE* expression altered later at 24 h and 48 h, suggesting that TFR2 may function prior to HFE in *HAMP* regulation.

1. Introduction

Hepcidin, the 25-mer iron-hormone is secreted by the liver hepatocytes in response to increased systemic iron levels in the body [1,2]. It binds to ferroportin, the iron-exporter protein on hepatocytes, duodenal enterocytes and splenic and liver macrophages [3–5] and causes intracellular degradation of both hepcidin and ferroportin [6]. Hepcidin thus prevents iron efflux via ferroportin into the circulation and acts as the main regulator of systemic iron homeostasis. Accordingly, insufficient systemic hepcidin levels lead to elevated duodenal iron absorption and uncontrolled iron efflux into the circulation. This causes progressive iron accumulation in vital organs such as liver, pancreas and heart, eventually resulting in organ failure due to iron toxicity. These features are hallmarks of the iron-overload heredity hemochromatosis [7,8] and also observed in alcoholic liver disease [9,10].

The extracellular and intracellular iron-sensing mechanisms of hepatocytes that lead to hepcidin synthesis and secretion in response to circulatory iron are not clearly defined yet. In this context, previous studies suggested roles of plasma transferrin (Tf) and stored intracellular iron in the induction of *HAMP*, gene encoding hepcidin [11–13]. In addition, intracellular iron was proposed to induce the maturation of pro-hepcidin into secretable bioactive hepcidin [14]. However, its precise role in hepcidin peptide secretion has not been sufficiently studied. Alongside, although the proteins HFE and TFR2 on the hepatocytes are pivotal in *HAMP* induction [15], their mechanisms of action, whether independent or in conjunction, remain ambiguous [16–18]. Collectively, the complete picture of hepcidin regulation and secretion in response to iron is yet to be fully elucidated.

Hence, here, the effect of excess holo-Tf and the consequently altered intracellular iron content on hepcidin secretion (at peptide level) and synthesis (at mRNA level) was examined simultaneously. Since hepcidin is secreted predominantly by the hepatocytes, the liver-derived cell line, HepG2, was chosen for the study. Cellular iron-uptake is mediated by the cell-surface protein TfR1, which is regulated at the iron-response element (IRE) region on its mRNA via the IRE/iron-regulatory protein (IRP) system [19]. Here, using wild-type (Wt)

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HepG2 cells, a novel recombinant HepG2 cell line was created that expressed IRE-independent *TFRC* mRNA to diminish sensitivity towards increasing intracellular iron content and allow intracellular iron overload. It was hypothesized that upon holo-Tf treatment, these recombinant (rec)-TfR1 HepG2 cells would demonstrate higher intracellular iron content and consequently, alter hepcidin peptide secretion and *HAMP* mRNA responses compared to Wt cells. Previous ex vivo studies investigated *HAMP* regulation only at longer time-points such as 24 h (h), 48 h and 72 h [20–24]. Here, we studied the effects of holo-Tf on hepcidin secretion for short-term i.e. at 30 min, 2 h and 4 h, in addition to long-term i.e. 24 h and 48 h, in conjunction with mRNA expression studies of key iron-related genes such as *TFRC*, *HAMP*, *SCL40A1*, *HFE*, and *TFR2*.

2. Materials and methods

2.1. Cell culture

HepG2 cells (Health Protection Agency, UK) were maintained in Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich UK), 1% non-essential amino acids (NEAA) (Sigma-Aldrich UK), 2 mM glutamax (Fisher Scientific International Inc. UK), 1% antibiotic/antimycotic solution (Fisher Scientific International Inc. UK) and 10% fetal calf serum (FCS) (Biosera, UK). Rec-TfR1 HepG2 cells expressing IRE-independent human *TFRC* mRNA were created (supplementary data-1) and maintained in 200 μg/mL hygromycin B (Invitrogen, UK) as a selection for exclusive growth of recombinant cells. *TFRC-IRE* mRNA response was explored by probing the mRNA (cDNA) of rec-TfR1 HepG2 cells and Wt HepG2 cells (as control) with the *IRE*-specific primers (GGTTGCTA AGAAGCGAGCAC and TGCTCCCGATAATGTGTTAGG). As an additional reference, the cDNA was probed with *TFRC*-specific primers (Supplementary Fig. 1A–1B). Results showed diminished *TFRC-IRE*-specific amplification in the rec-TfR1 HepG2 cells (supplementary Fig. 1A), indicating diminished *IRE*-related responsiveness, as desired for the study. Also, a HepG2 cell line with the unmodified plasmid vector [HepG2 (p) cells] was created for pre-iron-supplementation studies [(supplementary data-2 and supplementary figures (2A-2C)].

2.2. Holo-Tf supplementation

The iron saturation of holo-Tf and the iron content of the treatment medium were assessed (supplementary data-3 and supplementary Fig. 3A–3B). Cells were seeded at a density of 5×10^5 per well in a 6-well plate in maintenance medium (with and without hygromycin B for rec-TfR1 HepG2 cells and Wt cells, respectively) for 72 h. For the next 24 h, all cells were incubated in fresh hygromycin-free maintenance medium. Then, the cells were treated with 5 g/L holo-Tf prepared in serum-free EMEM (0 g/L) for the desired period and assessed for various parameters.

2.3. Determination of intracellular iron content and assessment of mitochondrial activity

Cellular iron content determined by the ferrozine assay [25] was expressed as nmoles of iron per mg of protein, as quantified by the Bradford method. Mitochondrial activity was assessed by using the MTT assay [26]. To compare the mitochondrial activity of cells in between different treatments, 2.5×10^4 cells were seeded per well of a 96 well plate in maintenance medium. The plate was incubated overnight at 37 °C in a humidified atmosphere with 5% CO_2. The following day, the

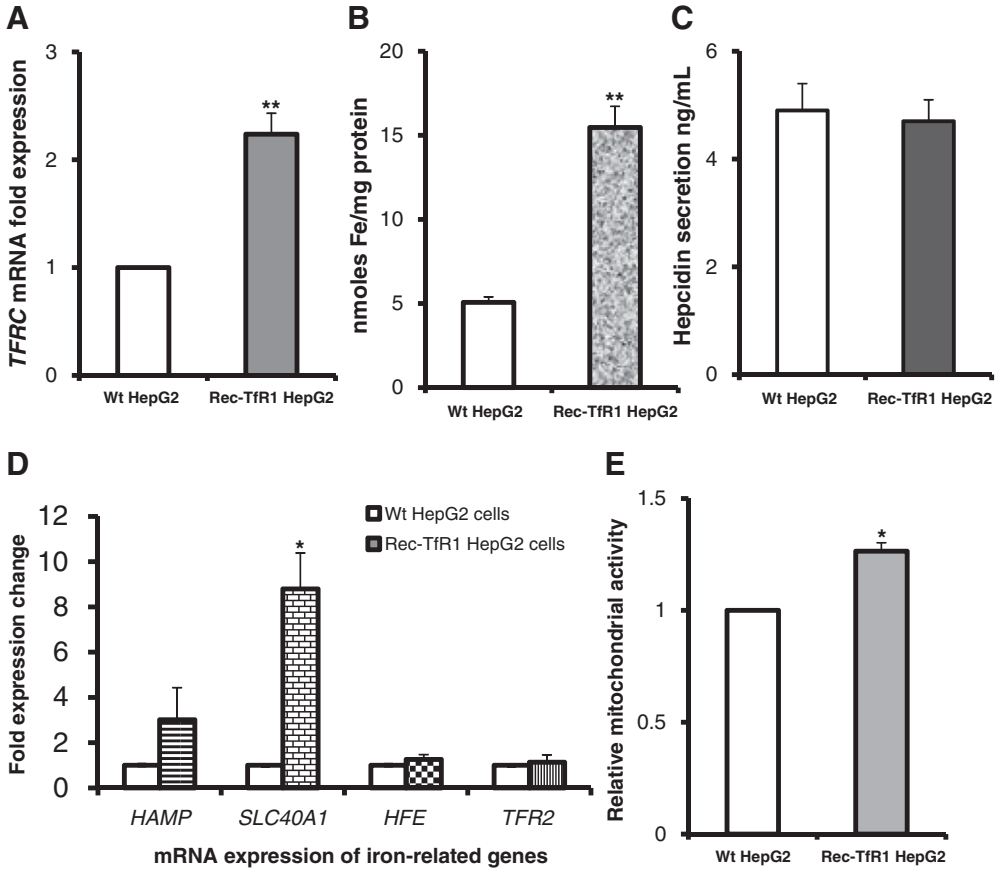

Fig. 1. Rec-TfR1 HepG2 cells in basal conditions (10% FCS). (A) *TFRC* mRNA expression, (B) Intracellular iron content, (C) hepcidin peptide secretion, (D) mRNA expression of iron-related genes in rec-TfR1 HepG2 cells in comparison with Wt HepG2 cells and (E) relative mitochondrial activity are shown. Data is presented as mean ± SEM ($n = 3$). $^*p < 0.05$ and $^{**}p < 0.03$ compared to Wt HepG2 cells.

maintenance medium was removed and the cells were washed twice with warm PBS to remove traces of the maintenance medium. Treatment media were then added to each well and the plates were again incubated at 37 °C in a humidified atmosphere with 5% CO_2. On completion of the treatment exposure period, the old media were replaced with 200 μL of fresh media per well. This was followed by addition of 5 μL per well of 5 mg/mL of MTT prepared in PBS. The plates were incubated at 37 °C for 2 h, the media were removed and 100 μL DMSO was added to each well. Plates were incubated at RT for 15 min, vortexed for 30 s and absorbance was read at 550 nm using a spectrophotometer. The absorbance values of blanks (medium only) were subtracted from controls and treated samples. This assay was appropriate for our study because it measures the mitochondrial activity [26] and the mitochondrion is a major organelle for ROS production [27], especially under excess iron conditions [28,29]. Thus, mitochondrial activity was chosen as a marker of cell/mitochondrial health over other markers.

2.4. Cell-surface TfR1 expression

Cells were harvested using TrypLE Express (ThermoFisher Scientific, UK), followed by centrifugation for 4 min at 89 ×*g*. Cells were suspended in PBC (PBS containing 0.15% BSA and 1 mM calcium chloride) and transferred to a 96-well plate (2×10^5 cells per well) and centrifuged at 1260 ×*g* for 30 s. To each well, 20 μL of 16 mg/mL blocking IgG was added. Human TfR1 antibody (Abcam, UK) [5 μg/mL in PBC)] was added to each test well and PBC was added to the control wells. Cells were incubated for 45 min on ice. Following washing, the cells were incubated with 10 μg/mL of FITC-labeled secondary antibody STAR9B rabbit F (ab′) 2 anti-mouse IgG (ABD Serotec, UK). After incubation for 30 min on ice, cells were washed with PBC again. Cell layer at the bottom of each well was suspended in 400 μL of PBS and TfR1 expression was analyzed by flow cytometry [30]. For the gating of cells for flow cytometry, first, live cells were selected based on the forward and side scatter. On the selected cell population, the positive threshold was determined based on the staining results obtained in the absence of the TfR1 antibody (cells treated with secondary antibody only). This selected a cell population of TfR1 positive cells. Then, the position of the gate was kept fixed while assessing samples from Wt and rec-TfR1 HepG2 cells and samples from different treatment conditions for both the cell lines. This enabled relative comparisons between the cell types and in between treatments.

2.5. Gene expression analysis

Primers (Invitrogen, UK) for expression analyses of the genes *TFRC* (NCBI accession no.NM_001128148.1), *HAMP* (NCBI accession no.AF309489.1) and *GAPDH* (NCBI accession no. NM_002046.4) were designed [*Primer 3* (*http://frodo.wi.mit.edu/*)] (supplementary data-4). RNA was extracted using TRI reagent (Sigma-Aldrich, UK) and converted to cDNA using QuantiTect reverse transcription kit (Qiagen, UK), as per manufacturer's protocol. Gene expression was analyzed through real-time PCR by using Quantifast SYBR green kit (Qiagen, UK) according to the manufacturer's protocol. Real-time PCRs were carried out in the Rotor-gene Q machine (Qiagen, UK) and the results were analyzed through Rotor-gene software series 1.7. Data was analyzed by the relative quantification method, Delta-Delta C_t (ΔΔCt) and expressed as $2^{-\Delta\Delta Ct}$ [31].

2.6. Hepcidin peptide levels

The concentration of hepcidin peptide secreted in the cell media was measured by using a previously well-described immunoassay by Busbridge et al. [32].

2.7. Statistical analysis

Data analysis was performed using one-way or two-way ANOVA, with post-hoc analysis by Tukey's test using the software SPSS, version 22. The level of significance was set at $p < 0.05$. Data was presented as mean ± SEM.

3. Results

3.1. Characterization of novel rec-TfR1-HepG2 cells in basal conditions

The rec-TfR1 HepG2 cells showed significantly higher *TFRC* mRNA expression (2.2-fold; $p < 0.03$) and iron content than Wt cells (3-fold; $p < 0.03$) (Fig. 1A and 1B). However, hepcidin secretion levels were similar to the Wt cells (Fig. 1C). These cells expressed substantially higher levels of *SLC40A1* (8.8-fold; $p < 0.05$), but did not significantly differ from the Wt HepG2 cells in mRNA expressions of *HAMP*, *HFE* and *TFR2* (Fig. 1D), whilst mitochondrial activity increased by 30% ($p < 0.05$) (Fig. 1E). Under basal conditions, the rec-TfR1 HepG2 cells displayed significantly higher cell-surface TfR1 levels than Wt cells (2.8-fold; $p < 0.03$) (Fig. 2).

3.2. Rec-TfR1-HepG2 cells under holo-Tf-supplemented conditions

3.2.1. Cell-surface TfR1 and TFRC mRNA expression

Following holo-Tf treatment, the Wt cells displayed a significant decrease in cell-surface TfR1 over time at 24 h ($p < 0.04$), which further declined at 48 h ($p < 0.01$) (Fig. 2 and supplementary Fig. 4). Conversely, the rec-TfR1 HepG2 cells maintained high levels following 24 h and 48 h of holo-Tf treatment and showed significantly higher levels than Wt cells at these times ($p < 0.01$) (Fig. 2 and supplementary Fig. 4A). The rec-TfR1 HepG2 cells showed no statistically significant difference in TfR1 levels under holo-Tf-treated and basal conditions (Fig. 2). In addition, *TFRC* mRNA expression was assessed and compared to respective untreated (0 g/L) cells at each corresponding time-point. The Wt cells showed significantly decreased *TFRC* mRNA expression at 2 h ($p < 0.05$), 4 h ($p < 0.05$) and 24 h ($p < 0.05$) (Fig. 3). In contrast, rec-TfR1 HepG2 cells showed significant 3-fold elevations in *TFRC* mRNA expression at 2 h ($p < 0.05$) and 24 h ($p < 0.02$) (Fig. 3). In the Wt HepG2 cells, decreased *TFRC* mRNA expression following holo-Tf treatment for 24 h and 48 h (Fig. 3) correlated with decreased cell-surface TfR1 levels observed at these time-points (Fig. 2). However, in rec-TfR1 HepG2 cells, the correlation was less clear.

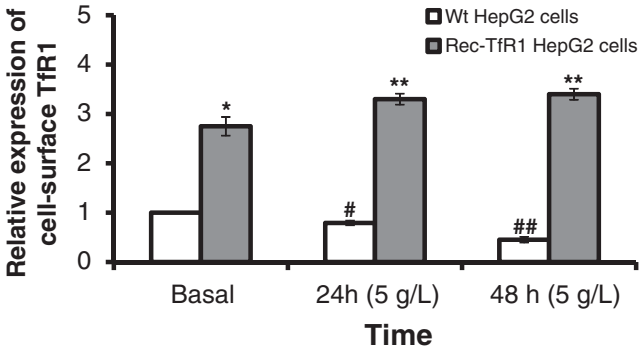

Fig. 2. Effect of holo-Tf treatment on TfR1 cell-surface expression. HepG2 cells were treated with 5 g/L holo-Tf up to 48 h and total cell-surface expression of TfR1 before treatment (basal conditions) and after treatment was studied by flow cytometry. Representative images for each treatment for Wt HepG2 cells and rec-TfR1 HepG2 cells have been shown in supplementary Fig. 4 and the effect of treatment has been summarized in the above figure. Data is presented as mean ± SEM ($n = 3$–6). $^{*}p < 0.03$ and $^{**}p < 0.01$ compared to Wt HepG2 cells at the corresponding time-point; $^{\#}p < 0.04$ compared to Wt HepG2 cells in basal conditions; $^{\#\#}p < 0.01$ compared to Wt HepG2 cells following treatment at 24 h.

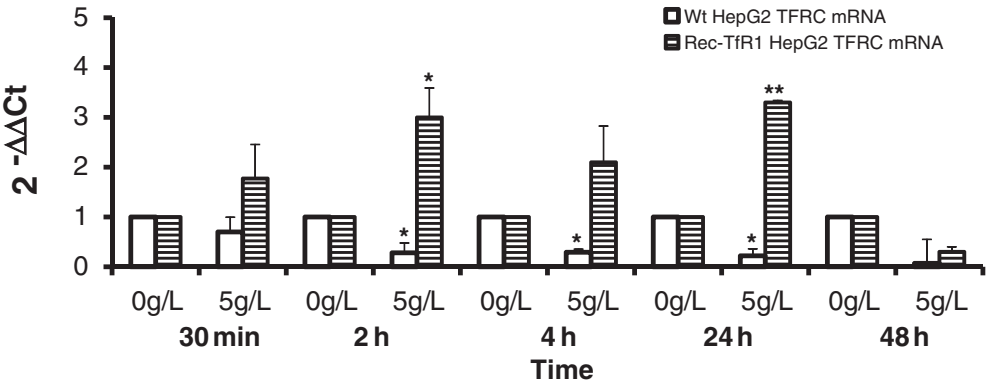

Fig. 3. Effect of holo-Tf treatment on *TFRC* mRNA expression. Upon 5 g/L holo-Tf treatment, mRNA expression of *TFRC* was assessed and expressed relative to respective untreated (0 g/L) cells at the corresponding time-point. Data is presented as mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.02$ compared to untreated (0 g/L) cells at the corresponding time-point.

3.2.2. Intracellular iron levels

Upon holo-Tf treatment, the rec-TfR1 HepG2 cells showed higher intracellular iron content than Wt cells at 30 min (3.3-fold, $p < 0.03$), 2 h (3.7-fold, $p < 0.01$), 4 h (6.6-fold, $p < 0.01$), 24 h (5.7-fold, $p < 0.01$) and 48 h (2.2-fold, $p < 0.01$) (Fig. 4). Even under holo-Tf deprivation (0 g/L), the rec-TfR1 HepG2 cells showed higher intracellular iron content than Wt cells at 30 min (3.6-fold, $p < 0.01$), 2 h (3.2-fold, $p < 0.01$), 4 h (2.8-fold, $p < 0.01$), 24 h (2.5-fold, $p < 0.01$) and 48 h (1.9-fold, $p < 0.03$) (Fig. 4).

Over time, the Wt cells showed no major variation in intracellular iron content upon holo-Tf deprivation and treatment (Fig. 4). In contrast, during short-term treatment, the rec-TfR1 HepG2 cells showed a pattern of increasing iron-uptake compared to respective untreated (0 g/L) cells at the same time-point i.e. no significant uptake at 30 min, a 1.4-fold increase at 2 h ($p < 0.02$) and a 3.1-fold increase at 4 h ($p < 0.01$) (Fig. 4). Also, a 2.4-fold increase ($p < 0.01$) in iron-uptake was observed at 24 h of holo-Tf treatment (Fig. 4).

3.2.3. Hepcidin peptide levels

Despite the substantially higher intracellular iron content in rec-TfR1 HepG2 cells (Fig. 4), hepcidin secretion in these cells was not higher than Wt cells (Fig. 5A and 5B). However, both, Wt and rec-TfR1 HepG2 cells secreted significantly higher levels of hepcidin upon holo-Tf supplementation in comparison to their respective untreated (0 g/L) cells (Fig. 5). For example, the Wt HepG2 cells showed significantly elevated hepcidin secretion at 2 h ($p < 0.02$), 4 h ($p < 0.02$), 24 h ($p < 0.02$) and 48 h ($p < 0.02$) (Fig. 5A). Likewise, the rec-TfR1 HepG2 cells showed elevated hepcidin secretion at 30 min ($p < 0.05$), 2 h ($p < 0.02$), 4 h ($p < 0.02$), 24 h ($p < 0.02$) and 48 h ($p < 0.02$) (Fig. 5B).

Upon holo-Tf deprivation (0 g/L), hepcidin peptide secretion gradually decreased over time in both, Wt and rec-TfR1 HepG2 cells (Fig. 5); from 2 h to 24 h (1.6-fold; $p < 0.05$), and further decreased at 48 h (2.2-fold; $p < 0.02$) in Wt cells (Fig. 5A) and from 30 min to 24 h (3.1-fold; $p < 0.04$), and further decreased at 48 h (3.4-fold; $p < 0.03$) in recombinant cells (Fig. 5B).

3.2.4. Gene expression analyses

The mRNA expression of core iron-related genes were assessed and compared to respective untreated (0 g/L) cells at each corresponding time-point. In Wt cells, *HAMP* mRNA expression significantly increased at 4 h (2.2 fold; $p < 0.05$) and 24 h (1.6 fold; $p < 0.02$), whereas in rec-TfR1 HepG2 cells, it significantly increased only at 2 h (1.5-fold; $p < 0.02$) (Fig. 6A). While *SLC40A1* mRNA expression remained unaltered in Wt cells (Fig. 6B), in rec-TfR1 HepG2 cells, an unaltered response at 30 min, a 1.8-fold increase at 2 h ($p < 0.05$) and a 3-fold increase at 4 h ($p < 0.05$) was observed, followed by unchanged expression at 24 h and 48 h of holo-Tf treatment (Fig. 6B).

Both, Wt and rec-TfR1 HepG2 cells showed no change in *HFE* mRNA expression during short-term treatment (Fig. 7A). However, it was down-regulated at 48 h (2.7-fold, $p < 0.05$) in Wt cells and at 24 h (2.7-fold, $p < 0.05$) in rec-TfR1 HepG2 cells (Fig. 7A). In contrast, *TFR2* mRNA expression remained unaltered at 24 h and 48 h and altered earlier during short-term treatment in both, Wt and rec-TfR1 HepG2 cells (Fig. 7B). However, the two cell types exhibited different *TFR2* mRNA responses to treatment. In Wt cells, *TFR2* mRNA expression was up-regulated at 30 min (2.3-fold, $p < 0.05$), and later again at 4 h (2.9-fold, $p < 0.05$), whereas in rec-TfR1 HepG2 cells, *TFR2* mRNA expression was significantly reduced at 4 h ($p < 0.01$), compared to untreated (0 g/L) cells (Fig. 7B). Following 5 g/L treatment, there was no major

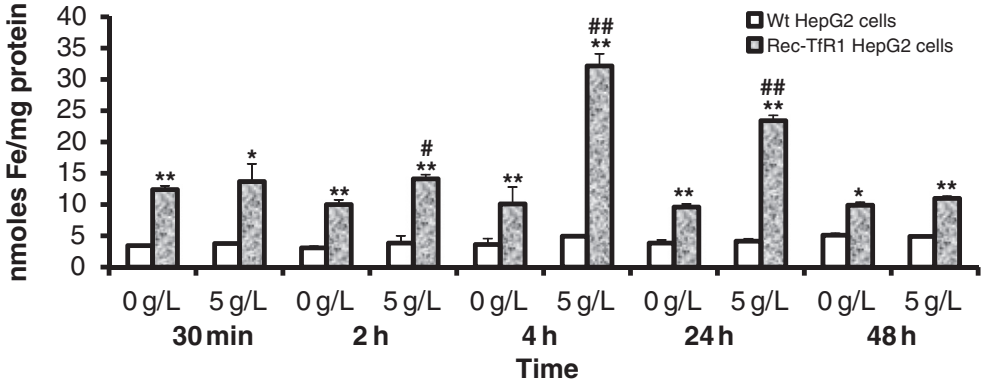

Fig. 4. Effect of holo-Tf treatment on iron-uptake. Intracellular iron levels were measured following holo-Tf deprivation and treatment. Data is presented as mean ± SEM ($n = 3$–6). $^{*}p < 0.03$, $^{**}p < 0.01$ compared to Wt HepG2 cells at the corresponding time-point and $^{\#}p < 0.02$, $^{\#\#}p < 0.01$ compared to untreated (0 g/L) rec-TfR1 cells at the corresponding time-point.

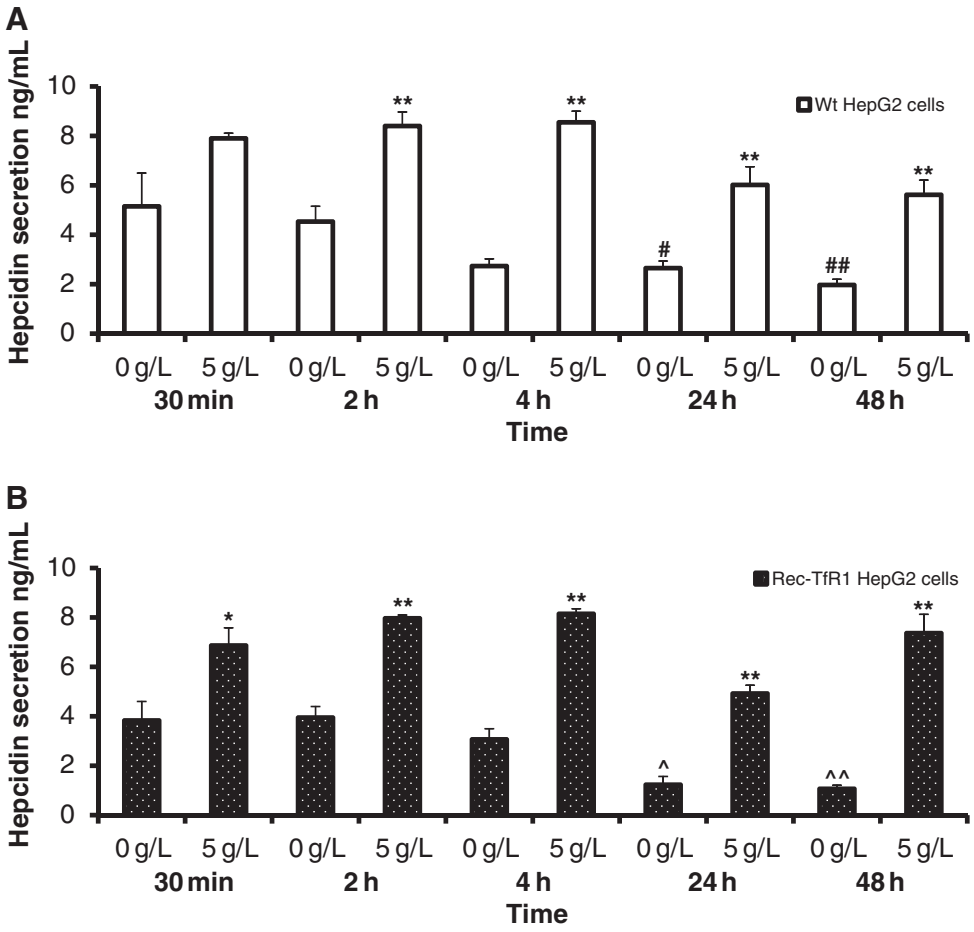

Fig. 5. Effect of holo-Tf treatment on hepcidin peptide secretion. Levels of secreted hepcidin were measured following holo-Tf treatment and deprivation in wt HepG2 cells (A) and rec-TfR1HepG2 cells (B). Data is presented as mean ± SEM ($n = 3$–6). $^{*}p < 0.05$, $^{**}p < 0.02$ compared to respective untreated (0 g/L) cells at the corresponding time-point; $^{\#}p < 0.05$, $^{\#\#}p < 0.02$ compared to 2 h of 0 g/L treatment in Wt HepG2 cells; $^{\wedge}p < 0.04$, $^{\wedge\wedge}p < 0.03$ compared to 30 min of 0 g/L treatment in rec-TfR1 HepG2 cells.

alteration in mitochondrial activity except the 40% increase in Wt cells at 48 h ($p < 0.05$), compared to untreated (0 g/L) cells at the same time-point (Fig. 8).

4. Discussion

The liver-secreted iron-hormone hepcidin is the master regulator of systemic iron homeostasis in the body. However, the iron-sensing mechanisms in the hepatocytes are not fully understood. A role of intracellular iron in modulating hepcidin secretion [14] and *HAMP* mRNA expression [11–13] was suggested, but not fully elucidated. Accordingly, here, for the first time, novel rec-TfR1 HepG2 cells were created that expressed IRE-independent *TFRC* mRNA to diminish sensitivity towards high intracellular iron and to allow continuous iron-uptake upon holo-Tf supplementation. In these cells, the combined effect of excess extracellular holo-Tf and the subsequently increased intracellular iron on hepcidin secretion was examined over time for the first time, in conjunction with mRNA expression studies of key iron-related genes like *TFRC*, *HAMP*, *SLC40A1*, *HFE* and *TFR2*. The responses of this novel cell line were compared with those of the Wt cells.

4.1. Deregulated cell-surface TfR1 and high iron content in recombinant cells

Upon holo-Tf treatment, the Wt cells down-regulated cell-surface TfR1 levels over time (Fig. 2). This was an anticipated response because the IRE-replete *TFRC* transcripts would sense increments in intracellular iron content via the IRE/IRP system and eventually decrease TfR1 protein expression on cell-surface to prevent further iron-uptake [33]. Accordingly, and as expected, the Wt cells showed no major variation in intracellular iron content following holo-Tf treatment (Fig. 4), resembling physiological conditions and other studies where 4.5 g/L of holo-Tf treatment for 48 h did not significantly increase iron content [34].

In contrast, the rec-TfR1 HepG2 cells displayed several fold higher cell-surface TfR1 than Wt cells under basal and prolonged holo-Tf-supplemented conditions (Fig. 2), despite their several fold higher intracellular iron content than Wt cells (Figs. 1B and 4). Such an unexpected response could be because the recombinant *TFRC* construct in rec-TfR1 HepG2 cells was devoid of the IRE region and therefore independent from the IRE-mediated iron-regulatory mechanisms [35]. This iron-insensitive feature was also reflected through up-regulated *TFRC* mRNA expression following holo-Tf treatment, unlike the Wt cells that showed down-regulated expression (Fig. 3). In Wt cells, the reduced *TFRC* mRNA expression at 24 h (Fig. 3) is consistent with the previously observed decrease following 2.5 g/L of holo-Tf treatment for 24 h [24]. Thus, contrasting the Wt cells, rec-TfR1 HepG2 cells demonstrated constitutively elevated cell-surface TfR1regardless of excess extracellular holo-Tf and high intracellular iron content, as required for this study.

Both, Wt and rec-TfR1 HepG2 cells exceeded basal levels of hepcidin secretion upon holo-Tf treatment and decreased hepcidin levels over time upon holo-Tf deprivation (0 g/L) (Figs. 5 and 1C). This is similar to the human response where increased circulatory iron levels lead to increased circulatory hepcidin levels [1,36–38] and reduced hepcidin response prevails under iron deficiency [37,39]. Interestingly, upon holo-Tf treatment, the Wt cells exceeded the basal level by 1.7-fold at 4 h and markedly reduced hepcidin secretion at 24 h (Figs. 1C and 5).

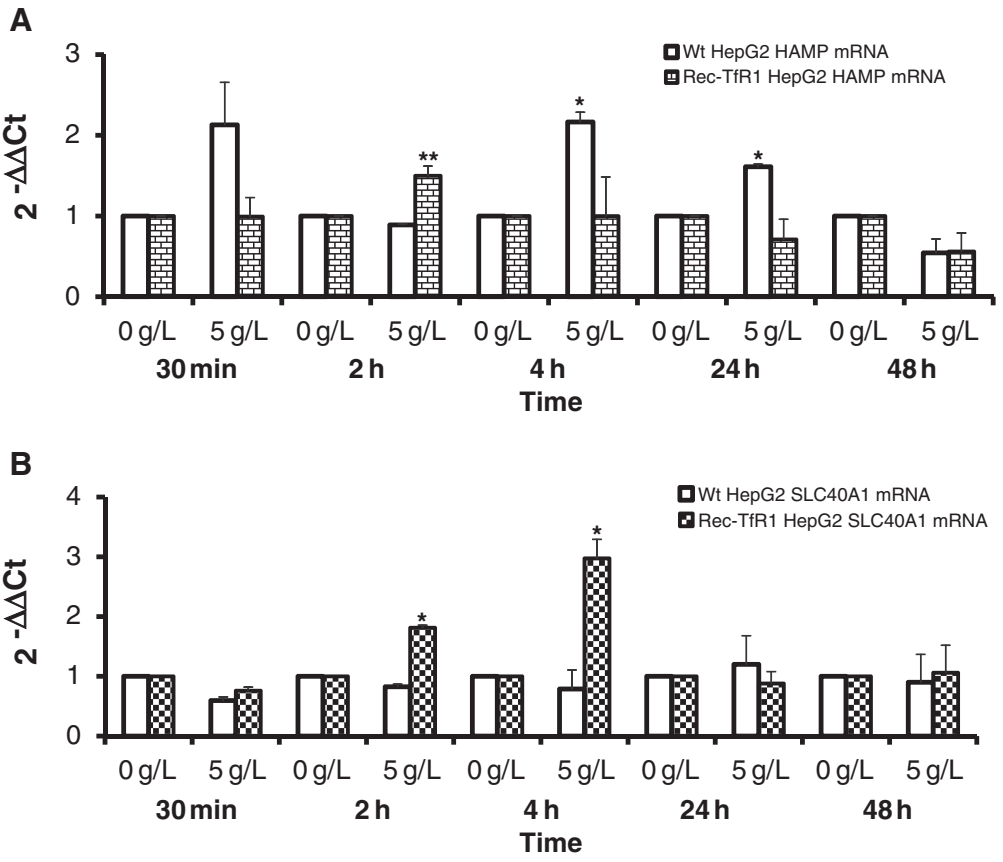

Fig. 6. Effect of holo-Tf treatment on *HAMP* and *SLC40A1* mRNA expression. Upon 5 g/L holo-Tf treatment, mRNA expression of (A) *HAMP* and (B) *SLC40A1* in the HepG2 cells was assessed and expressed relative to respective untreated (0 g/L) cells at the corresponding time-point. Data is presented as mean ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.02$ compared to untreated (0 g/L) cells at the corresponding time-point.

A similar pattern has been reported in humans where an oral dose of 65 mg of iron led to a 2-fold increase in hepcidin levels after 4 h and returned to baseline levels after 24 h [38].

The intracellular iron status of the cells is not only related to iron-uptake via TfR1 but also related to cellular iron efflux mediated by regulations in the iron-efflux gene *SLC40A1*. Here, *SLC40A1* expression remained unaltered in Wt HepG2 cells at all time points (Fig. 6B). In the rec-TfR1 HepG2 cells, it was upregulated at the early contiguous time points (2 h and 4 h), but remained unaltered at the longer contiguous time points (24 h and 48 h). This difference in the responses between short-term and long-term treatments could be due to the presence of 5′ IREs on the *SLC40A1* mRNA. These sense intracellular iron-increments in the rec-TfR1 HepG2 cells at a particular treatment time-point and resultantly increase expression to promote cellular iron-efflux [19]. Thus, its expression would be determined by the intracellular iron status at a particular treatment time-point and therefore vary.

4.2. Hepcidin levels and intracellular iron content

The rec-TfR1 HepG2 cells demonstrated higher iron content than Wt cells under basal and holo-Tf-supplemented conditions (Figs. 1B and 4). However, hepcidin secretion in the rec-TfR1 HepG2 cells did not exceed the levels secreted by the Wt cells, in neither basal nor holo-Tf-supplemented conditions (Figs. 1C and 5).

Thus, here, hepcidin secretion did not alter in proportion to the increased intracellular iron levels in the rec-TfR1 HepG2 cells, compared to Wt cells. This suggests that possibly, the proposed post-translation maturation of pro-hepcidin to bioactive hepcidin [14] may well be mediated by intracellular iron levels, requiring only subtle amounts of iron-uptake to trigger the maturation of pro-hepcidin to secretable hepcidin. This was reflected in Wt cells, which, following holo-Tf treatment, exceeded basal hepcidin secretion levels (Figs. 1C and 5) without any major iron-uptake (Fig. 4).

4.3. HAMP mRNA response to holo-Tf treatment in recombinant cells

Following holo-Tf supplementation, although intracellular iron levels in rec-TfR1 HepG2 cells were substantially higher than Wt cells (Fig. 4), *HAMP* mRNA responses did not proportionately exceed the responses of Wt cells (Fig. 6A). In fact, the rec-TfR1 HepG2 cells showed an elevated response only at 2 h while the Wt cells showed elevated expression at 4 h and 24 h (Fig. 7A). This could be due to several reasons. Firstly, excess cell-surface TfR1on rec-TfR1 HepG2 cells would have changed the native ratios of TfR1 with its hepcidin-inducing binding partners on the cell-surface, HFE and TFR2 [40,41]. This may have caused the majority of the HFE to bind to TfR1, aiming to regulate iron-uptake [40,42], thereby limiting the availability of free HFE for *HAMP* induction [43], with or without TFR2 [16,17]. Secondly, the down-regulated expression of *TFR2* following holo-Tf treatment (Fig. 7B) may have prevented *HAMP* up-regulation in rec-TfR1 HepG2 cells (Fig. 6A). In contrast, the Wt cells showed significantly elevated *TFR2* mRNA responses at 30 min and 4 h (Fig. 7B) that matched the pattern of *HAMP* mRNA expression in these cells (Fig. 6A). This reemphasizes and supports the role of TFR2 in modulating *HAMP* induction following holo-Tf stimulus [20,44].

Unlike Tf, which is known to influence hepcidin expression [45], the relationship between TfR1 (membrane-bound or soluble) and hepcidin expression remains unclear. In this study, the presence of high levels of membrane-bound TfR1 on rec-TfR1 HepG2 cells did not correlate with

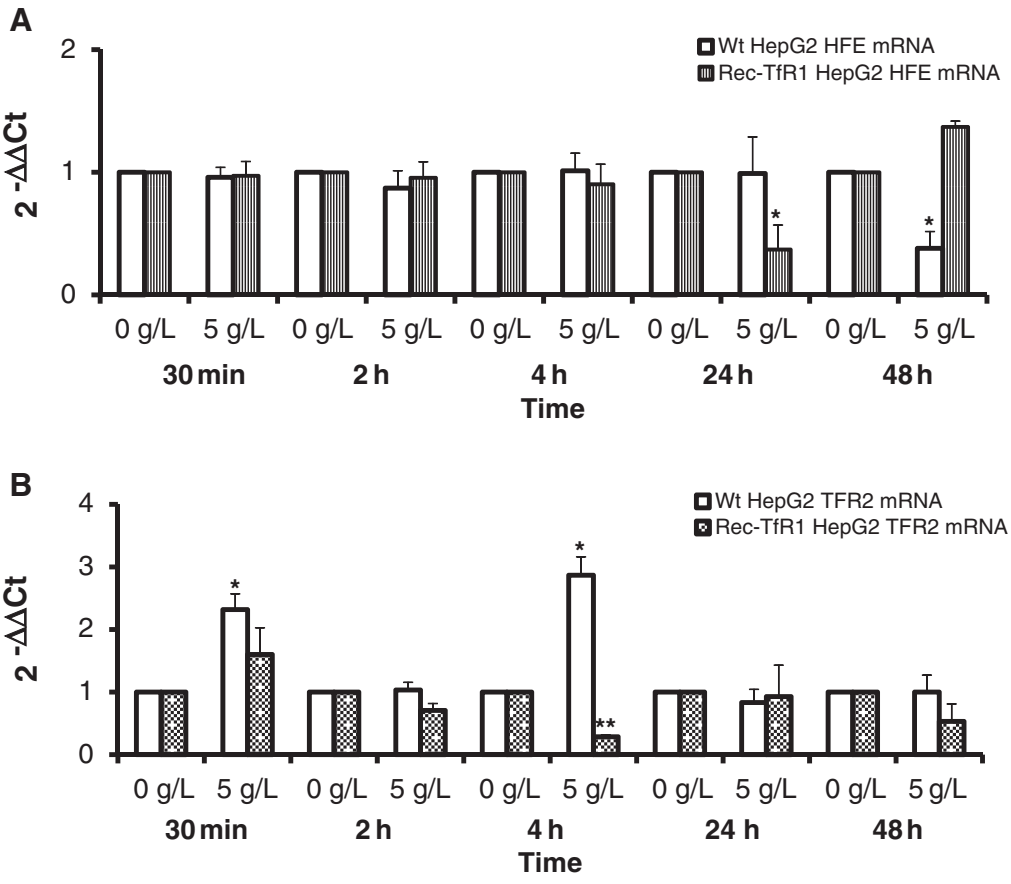

Fig. 7. Effect of holo-Tf treatment on *HFE and TFR2* mRNA expression. Upon 5 g/L holo-Tf treatment, mRNA expression of (A) *HFE* and (B) *TFR2* in the HepG2 cells was assessed and expressed relative to respective untreated (0 g/L) cells at the corresponding time-point. Data is presented as mean ± SEM ($n = 3$). $^*p < 0.05$ compared to untreated (0 g/L) cells at the corresponding time-point.

HAMP mRNA expression or secretion (Figs. 2, 6A and 5B). The high holo-Tf concentration (5 g/L) utilized provided the conditions for holo-Tf – TfR1 binding to occur, as demonstrated by the excess cellular iron-uptake by the rec-TfR1 HepG2 cells (Fig. 4). Following holo-Tf treatment, these cells demonstrated up to 6.6-fold higher intracellular iron content than the Wt HepG2 cells and showed increased iron-uptake over a 4 h period (Fig. 4). This suggests that any soluble TfR1 in the medium had not significantly affected the binding of holo-Tf with TfR1, else we would not expect to see increased iron uptake as observed in this study over the 4 h time period (Fig. 4). The binding of holo-Tf with TfR1 would also lead to potential *HAMP* induction via the holo-Tf-TfR1 pathway. This reduced the probability that the diminished *HAMP* induction could be due to limited availability of holo-Tf, resulting from the probable binding of soluble TfR1 to holo-Tf in the medium. Furthermore, increased circulating soluble TfR1 generally occurs in Iron Deficiency Anemia reviewed in [46,47].

The BMP/SMAD, MAPK, IL6-JAK/STAT3, Matripase-2/HJV and hypoxia pathways are important in regulating *HAMP* mRNA expression [7,8] and investigation into these pathways would certainly lead to improved insights into hepcidin signaling. However, these pathways would highlight the transcriptional regulation of hepcidin and not hepcidin peptide secretion. The latter was the focus of this study, which was based on the previously proposed role of intracellular iron in the maturation of pro-hepcidin to release the bioactive 25-mer hepcidin [14]. Accordingly, it

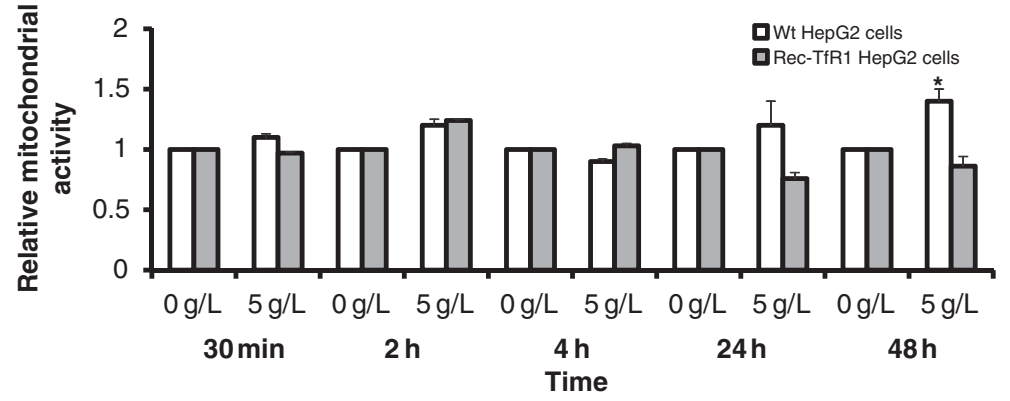

Fig. 8. Effect of holo-Tf treatment on mitochondrial activity. Following 5 g/L holo-Tf treatment, mitochondrial activity of HepG2 cells was assessed and expressed in comparison to untreated (0 g/L) cells at the corresponding time-point. Data is presented as mean ± SEM ($n = 3$–6). $^*p < 0.05$ compared to Wt HepG2 cells.

was hypothesized that increased intracellular iron levels would cause more maturation of pro-hepcidin to secrete more hepcidin. Thus, the effect of excess intracellular iron (as in these iron-overloaded recombinant cells) on hepcidin peptide secretion was evaluated, which has not been sufficiently studied so far, rather than *HAMP* mRNA regulation, which has been extensively studied. Moreover, each of these signal transduction pathways is dynamic, involves several proteins and therefore each pathway accounts for an independent study on its own. This was beyond the scope of the present study, which prioritized hepcidin peptide secretion over regulation of *HAMP* transcription.

4.4. TFR2 and HFE mRNA expression in recombinant cells

In both, Wt and rec-TfR1 HepG2 cells, *HFE* mRNA responses to holo-Tf treatment remained unchanged during short-term treatment and altered later at 24 h and 48 h (Fig. 7A). The down-regulation in *HFE* mRNA expression at 24 h in the Wt cells is similar to that observed previously upon 4.5 g/L holo-Tf treatment [34]. Unlike the *HFE* mRNA responses, *TFR2* mRNA responses significantly altered during short-term treatment and remained unaltered at 24 h and 48 h in both the cell lines (Fig. 7B). In the Wt cells, this was also observed in earlier studies where *TFR2* mRNA expression remained unchanged after 24 h of 4 g/L holo-Tf treatment [20]. Together, the data suggests that TFR2 may act prior to HFE in iron and hepcidin regulation, as also proposed earlier [20,38].

4.5. Virtual hemochromatosis

The rec-TfR1 HepG2 cells displayed several "hemochromatotic" characteristics such as hepcidin secretion disproportionate to iron-overload (Figs. 5 and 4), insufficient *HAMP* mRNA response upon holo-Tf stimulus (Fig. 6A) [48], up-regulated *SLC40A1* mRNA expression (Fig. 6B) [38,49] and putative digression from the normal physiological ratio between TfR1 and HFE on the cell-surface (Fig. 2). Thus, these cells may be described as "virtually hemochromatotic" as the cells displayed several "hemochromatotic" characteristics despite possessing the Wt alleles of the hepcidin-inducing genes [16,17,20], mutations in which cause hemochromatosis [7].

5. Conclusion

The novel rec-TfR1 HepG2 cells showed constitutively over-expressed cell-surface TfR1 and higher intracellular iron content than Wt cells under basal and holo-Tf supplemented conditions. However, under the conditions studied here this did not lead to higher hepcidin secretion levels compared to Wt cells, suggesting other regulatory mechanisms are involved.

Abbreviations

EMEM	Eagle's minimal essential medium
h	hours
IRE	iron responsive element
IRP	iron regulatory protein
min	minutes
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAGE	polyacrylamide gel electrophoresis
sec	seconds
Tf	transferrin
TfR	transferrin receptor

Gene annotations

HAMP	encoding the peptide hepcidin
HFE (High iron)	gene encoding the hepcidin-inducing hemochromatosis protein HFE
SLC40A1	gene encoding the cellular iron-exporter protein ferroportin
TFR2	gene encoding the hepcidin-inducing protein transferrin receptor 2 (TFR2)
TFRC	gene encoding the cellular iron-uptake protein transferrin receptor 1 (TfR1)

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Conflict of interest

Kosha Mehta, Mark Busbridge, Derek Renshaw, Robert W. Evans, Sebastien Farnaud and Vinood B. Patel declare that they have no conflict of interest.

Compliance with ethical requirements

This article does not contain any studies conducted on human or animal subjects.

Author contributions

Dr. Kosha Mehta: Key researcher; carried out experimental work and wrote the article.

Dr. Mark Busbridge: Measurement of hepcidin peptides.

Prof. Derek Renshaw: Provided protocol for analysis of TfR1 by flow cytometry and provided cell culture facility.

Prof. Robert W. Evans: Helped with the assessment of iron-saturation of holo-Tf.

Dr. Sebastien Farnaud: Concept of the research.

Dr. Vinood B. Patel: Director of studies and final approver for the version of the article to be published.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bcmd.2016.06.008.

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