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

Supplementary information

Supplementary data-1: Creation of recombinant–TfR1 HepG2 cells

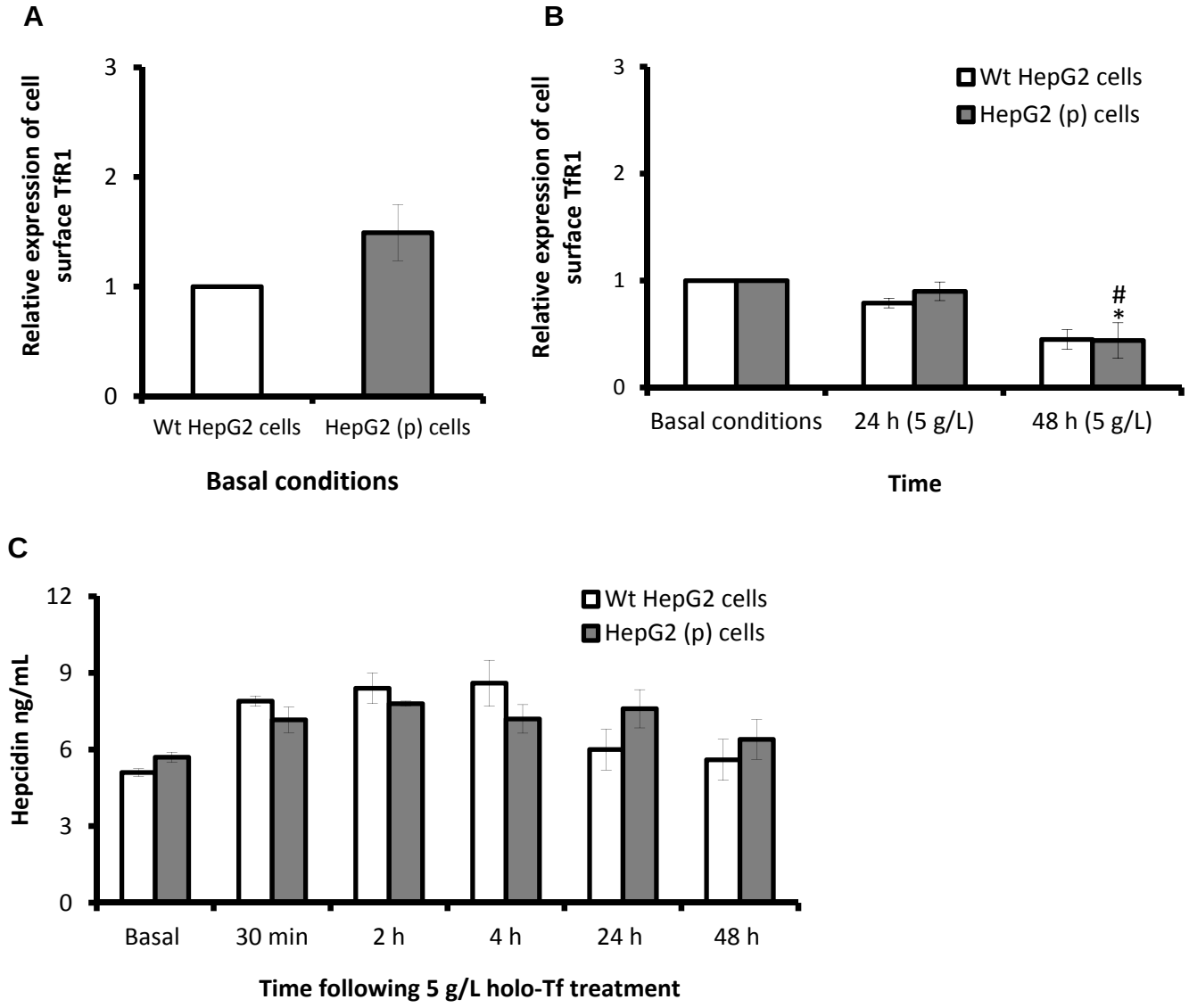
Principally, the protein coding region of human *TFRC* devoid of its *IRE* region was cloned into the plasmid vector pCEP4 (Invitrogen-Life technologies, UK) and transfected into Wt HepG2 cells, resultantly giving rise to the rec-TfR1 HepG2 cells. Briefly, the primers (Invitrogen-Life technologies, UK) TfR1F (TGATATATGCGGCCGCACCATGGATCAAGCTAGATCAGCATTCTC) incorporating a NotI site and a Kozak sequence and TfR1R (GCGCGCGGATCCTTATTAAAACTCATTGTC AATGTCCCAAACGTC) incorporating a BamHI site were used for PCR amplification of the specific section of full length *TFRC* gene from human MGC verified cDNA (clone ID 3354176, Invitrogen-Life technologies, UK). PCR was carried out with KOD DNA hot start polymerase mix (Novagen, UK) as follows: Initial denaturation at 95 ºC for 3 min, then 30 cycles of denaturation at 95 ºC for 30 sec, annealing at 58 ºC for 1 min and extension at 72 ºC for 1 min, followed by a final extension at 72 ºC for 5 min. The PCR product was gel extracted and purified by using PCR product purification kit (Qiagen, UK), then cut with HF-NotI/HF-BamH1 (New England Biolabs, UK) and ligated into HF-NotI/BamHI cut and phosphatased pCEP4. The ligation mixture was used to transform *Escherichia coli* KRX cells (Promega Ltd, UK) and colonies were selected on Luria-Bertani medium containing 100 µg/mL ampicillin (Sigma-Aldrich UK). Recombinant plasmid from one colony was sequenced to confirm the presence of the *TFRC* gene. HepG2 cells were transfected with recombinant pCEP4 by using X-tremeGENE-9 DNA transfection reagent (Roche,UK) as per manufacturer's protocol.

Supplementary data-2-Pre-iron supplementation studies

A HepG2 cell line with the unmodified plasmid vector was created for reference and referred to as HepG2 (p) cells. Study of the main iron-related parameters in the HepG2 (p) cells showed no difference from the Wt HepG2 cells (supplementary Fig.1). For example, similarities with the Wt HepG2 cells were observed in cell-surface TfR1 levels under basal conditions (supplementary Fig.1A) and following iron-supplementation over time (supplementary Fig.1B). Moreover, following holo-Tf treatment, hepcidin peptide levels in the HepG2 (p) cells matched levels in the Wt HepG2 cells (supplementary Fig.1C). In addition, intracellular iron levels in the HepG2 (p)

cells were similar to Wt HepG2 cells under basal conditions over time (4.7 to 6 nmoles/ mg protein) and upon holo-Tf treatment (6.5 nmoles/ mg protein). The relative mitochondrial activity was either 90-95 % similar or showed no difference from the Wt HepG2 cells (data not shown for brevity). Thus, further parameters in HepG2 (p) cells were not studied and subsequent studies were conducted using Wt HepG2 cells as reference to enable comparison with other groups that have also used Wt HepG2 cells for iron supplementation studies.

Supplementary Fig. 1



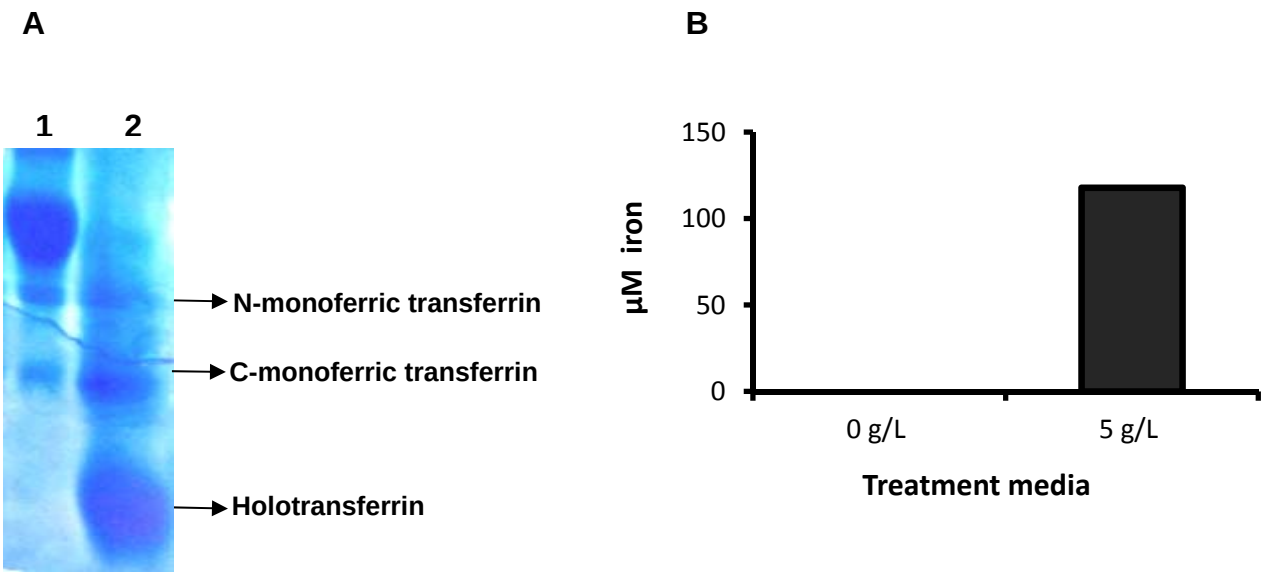
Supplementary Fig 1. Cell-surface TfR1 and hepcidin expression in HepG2 (p) cells. (A) Cell-surface TfR1 expression in HepG2 (p) cells in comparison to Wt HepG2 cells under basal conditions and (B) following 5 g/L holo-Tf treatment are shown. (C) Hepcidin peptide

secreted by Wt HepG2 and HepG2 (p) cells under basal conditions and following holo-Tf treatment were measured. Data is presented as mean ± SEM (n=3). *p <0.03 compared to basal conditions and #p=0.05 compared to 24 h of 5 g/L treatment in HepG2 (p) cells.

Supplementary data-3- Assessment of iron saturation and iron content in treatment medium

Holo-Tf was prepared by iron–loading apo-Tf using 0.1 M stock solution of ferric citrate, at a final molar concentration equal to twice the transferrin molar concentration. The iron-saturation in 5 g/L holo-Tf was assessed by PAGE on 6 M urea gel, as per Evans et al., (1978). Data suggested that 90 % of the protein was diferric (supplementary Fig. 2A). Iron content in 5 g/L holo-Tf medium was determined by the ferrozine assay, as per Reimer et al., (2004), which was 93 % of the expected iron molarity in the holo-Tf preparation (supplementary Fig.2B).

Supplementary Fig. 2



Supplementary Fig. 2. Determination of iron saturation and iron concentration of treatment medium.

(A) Iron content in iron-loaded transferrin was determined by 6 M urea PAGE. Lane 1: apo-transferrin; lane 2: 5 g/L holotransferrin. The three species of iron-transferrin, C-and N-monoferric transferrin and holotransferrin can be observed with a major proportion of holotransferrin. (B) Measurement of iron concentration in treatment medium confirmed iron concentration of expected morality. 0 g/L corresponds to serum-free and holo-Tf-free

maintenance medium, whereas 5 g/L corresponds to serum-free maintenance medium supplemented with 5 g/L holo-Tf. Data is presented as mean ± SEM (n=3).

Supplementary data-4- Primers

The primers used for RTPCR were AAAATCCGGTGTAGGCACAG (F) and TTAAATGCAGGGACGAAAGG (R) for *TFRC* (Mehta et al., 2015), ACAGCCAGACAGACGGCACGA (F) and TTCGCCTCTGGAACATGGGCATC (R) for *HAMP*, GCCAAAAGGGTCATCATCTC (F) and GGTGCTAAGCAGTTGGTGGT (R) for *GAPDH*, TGTTTCTGGTAGAGCTCTAT (F) and GATATAGCAGGAAGTGAGAA (R) for *SLC40A1* (Jacolot et al., 2008), TGATCTGGGAGCCCTCACC (F) and GACGACAAAAACAGCAATTCC (R) for *HFE* (Jacolot et al., 2008), TATACTCCTGGAGCTGGTGC (F) and GCACGCTGAGGTAGCCCTCTA (R) for *TFR2* (Rapisarda et al., 2010). Prior to expression analysis, PCRs using cDNA templates were performed by using Taq PCR master mix (Qiagen, UK) as per manufacturer's instruction. The amplicons were sequenced at Wolfson Institute for Biomedical Research (University College London, UK) to confirm product identity.