DUODENAL CYTOCHROME B AND HEPHAESTIN EXPRESSION IS REGULATED BY THE SOLUBLE HFE ISOFORM

Bruno Silva, Rute Martins, Daniela Proença and Paula Faustino

Departamento de Genética Humana, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal

Introduction

Hereditary Hemochromatosis is an autosomal recessive disorder characterized by excessive intestinal iron absorption and pathological iron deposition in organs such as liver, heart and pancreas. The disease is predominantly caused by homozygosity for the p.C282Y mutation in *HFE*, which impairs protein association with its chaperone beta-2 microglobulin (B2M) for correct folding and traffic to the cell surface.[1]

The role of full length HFE on iron homeostasis remains unclear. It has been postulated that it may contribute to iron metabolism regulation by acting in hepatocytes, activating hepcidin synthesis and in duodenum, regulating the expression of iron metabolism related genes, thus preventing iron overload.

***HFE* transcripts are widely expressed and the predominant (full length) mRNA identified has ≈4.2 kb in length. In addition, several alternative splicing *HFE* transcripts have been reported but their functional significance remains elusive. [1,2,3]**

Since we have identified a novel alternative *HFE* transcript due to the intron 4 inclusion[4] (Fig. 1), we aimed to investigate its tissue-specific expression level, the corresponding protein structure and cellular location, as well as its putative physiological function at duodenal level.

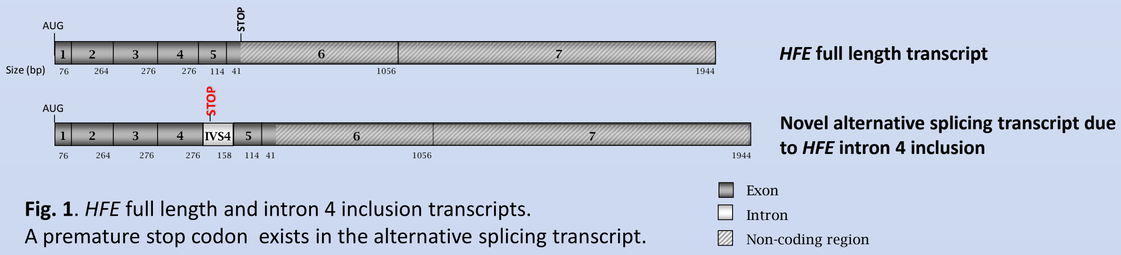


Fig. 1. *HFE* full length and intron 4 inclusion transcripts.
A premature stop codon exists in the alternative splicing transcript.

Methods

✓ RT-qPCRs using RNAs from 8 human tissues were performed to quantify the *HFE* intron 4 inclusion splicing transcript as well as the total amount of *HFE* transcripts.

✓ *HFE* cDNA was tagged to the GFP gene in the pEGFP-N1 vector. The hepatocarcinoma cell line HepG2 was transfected with the full length or the intron 4 inclusion construct, in order to obtain the corresponding GFP-tagged protein variants. Protein cellular localization was detected by immunofluorescence assays raising antibodies against B2M, Calnexin (an endoplasmic reticulum marker; ER) and Transferrin Receptor 1 (TfR1).

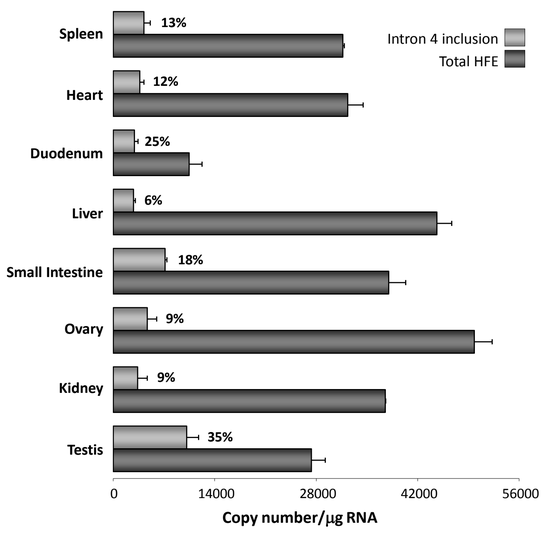
✓ HuTu-80 cells (human duodenum adenocarcinoma) were transfected with pcDNA3 constructs expressing HFE_full length (HFE) and HFE_ivs4 (sHFE) tagged to Flag, followed by an iron stimulus (holo-transferrin 20µM; holo). Cell lysates and cell culture supernatants were submitted to immunoprecipitation assays using mouse anti-Flag antibody. These were subjected to a 12% SDS-PAGE, followed by transfer to a nitrocellulose membrane. Immunodetections were performed with mouse antibody anti-Flag, mouse anti-TfR1 and rabbit anti-B2M.

✓RNA was extracted from these cells and the expression of iron-related genes *TFR1*, *DMT1*, *B2M*, *SLC40A1*, *CYBRD1* and *HEPH* were assessed by RT-qPCR.

Results

1) *HFE*_intron 4 inclusion transcript expression in different human tissues

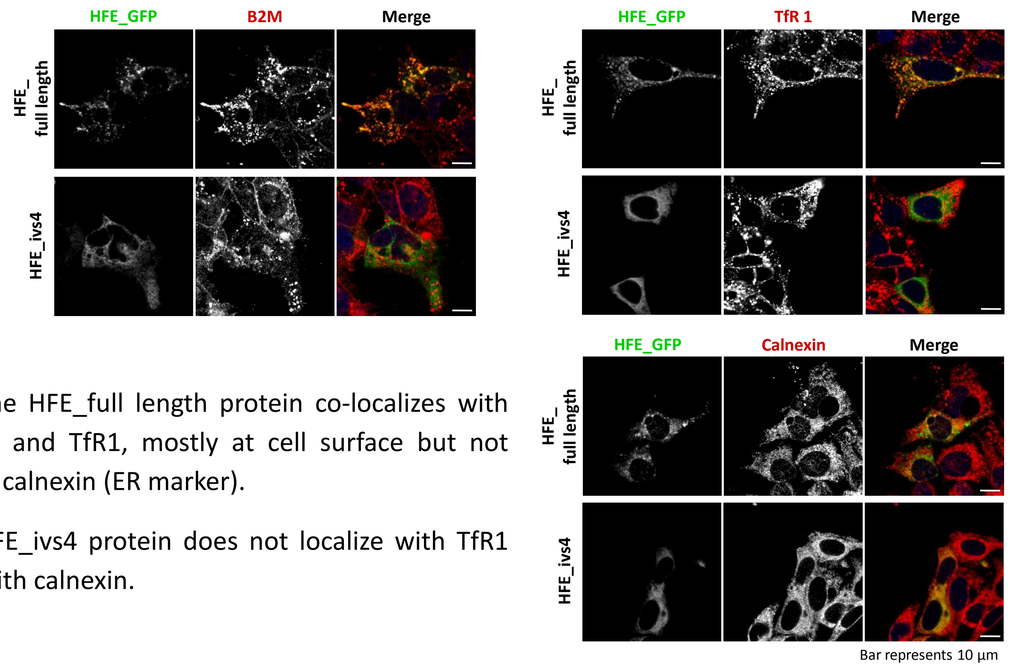
RT-qPCR



✓ HFE_intron 4 inclusion transcript has an ubiquitous expression, being its relative expression higher in duodenum and testis and lower in liver.

2) HFE_ivs4 protein variant cellular co-localization in hepatic cells

Immunofluorescence (HepG2)



✓ The HFE_full length protein co-localizes with B2M and TfR1, mostly at cell surface but not with calnexin (ER marker).

✓ HFE_ivs4 protein does not localize with TfR1 or with calnexin.

Bar represents 10 µm

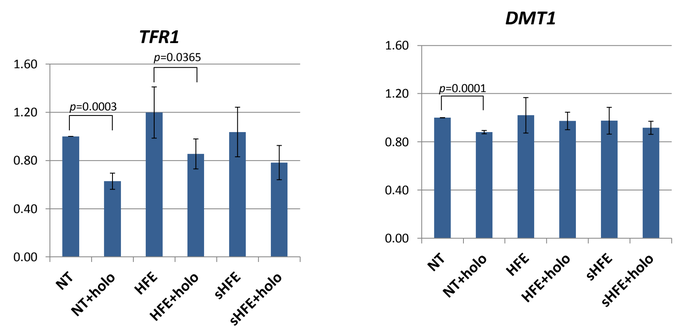
3) HFE_ivs4 protein variant cellular traffic

Immunoprecipitation (HuTu-80)

✓ Both HFE_full length and HFE_ivs4 proteins are associated with B2M in cell lysates, however, only HFE_full length protein is found associated with TfR1 in the cell lysates without holo-Tf stimulus.

✓**The HFE_ivs4 protein is a truncated soluble HFE protein variant (sHFE)** that is secreted by cells maintaining its interaction with B2M.

4) Effect of the sHFE variant in iron-metabolism related genes expression in a duodenal cell line (HuTu-80)

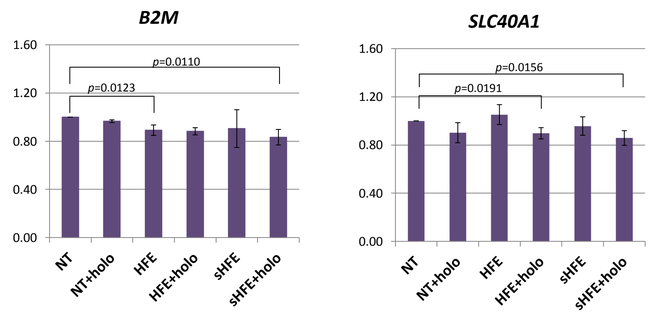


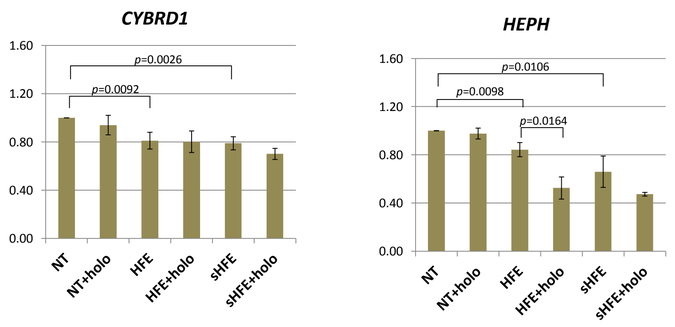
✓ As expected, *TFR1* expression is inhibited by holo-Tf.

✓ The over expression of HFE_full length (HFE) and HFE_ivs4 (sHFE) seem to slightly increase the expression of *TfR1*.

✓ *DMT1* does not vary with HFE or sHFE over expression.

✓ The expression of both *B2M* and *SLC40A1* (genes coding for B2M and ferroportin-1 proteins) seems to be slightly down-regulated by HFE and iron stimulus, respectively.





✓ ***CYBRD1*** **(duodenal cytochrome b) expression is reduced in about 20% after over expression of both HFE and sHFE, independently of iron stimulus.**

✓ ***HEPH*** **(hephaestin) expression is highly down regulated by HFE and sHFE, independently of iron status.**

NT – not transfected
Standard deviations are a representation of three independent experiments
p-values were calculated by the bicaudal student's t-test

Conclusions

➢ **Here we show a soluble isoform of the HFE protein (sHFE), resulting from a HFE alternative splicing transcript including the intron 4. Due to the presence of a premature termination codon within this intron, it originates a truncated protein which lacks the transmembrane and cytoplasmic domains. However, it seems to be correctly processed, since it surpasses the ER barrier and is secreted to the cell supernatant in association with B2M.**

➢ **The over expression of the sHFE in a duodenal cell line is able to modify de expression of some iron metabolism related genes . We found that it acts by repressing the expression of the duodenal cytochrome b and hephastin, independently of the iron status, as it happens with the full-length HFE. Since those are proteins presenting well known functions in iron absorption, we can hypothesize that their decrease expression modulated by the sHFE will be translated to a reduction of dietary iron absorption in the duodenum.**

➢ **Through this study we might have unveiled the contribution of the sHFE splice variant to iron homeostasis. In fact, sHFE may be secreted by several tissues into the bloodstream and may act in the regulation of dietary iron absorption in duodenum, reducing dietary iron absorption, preventing iron overload and contributing to iron metabolism regulation.**

References

1. Feder JN et al (1996). Nat Genet 13: 399-408.
2. Thenie A et al (2000). Blood Cells Mol Dis 26: 155-162.
3. Jeffrey GP et al (1999). Blood Cells Mol Dis 25: 61-67.
4. Martins R and Silva B et al (2011) Plos One 6(3):e17542.

Partially funded by FCT: PTDC/SAU-GMG/64494/2006; Pest-OE/SAU/UI0009/2011; SFRH/BD/21340/2005 and SFRH/BD/60718/2009