# Overexpression of the hereditary hemochromatosis protein, HFE, in HeLa cells induces an iron-deficient phenotype

Barbara Corsi[a], Sonia Levi[a], Anna Cozzi[a], Angelo Corti[a], Domenico Altimare[a], Alberto Albertini[b], Paolo Arosio[a,b,*]

[a] *Dibit, Department of Biological and Technological Research, IRCCS H. San Raffaele, Via Olgettina 58, 20132 Milan, Italy*
[b] *Department of Biomedical Technologies, University of Brescia, Brescia, Italy*

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**Abstract A transfectant HeLa cell clone expressing HFE under the control of a tetracycline-repressible promoter was generated. HFE expression was fully repressed by the presence of doxycycline, while it was strongly induced by growth in the absence of doxycycline. HFE accumulation was accompanied by a large (~10-fold) decrease in H- and L-ferritin levels, by a ~3–4-fold increase in transferrin receptor, and a ~2-fold increase in iron regulatory protein activity. These indices of cellular iron deficiency were reversed by iron supplementation complexes. The overexpressed HFE immunoprecipitated together with transferrin receptor, indicating a physical association which is the likely cause for the observed ~30% decrease in $^{55}$Fe-transferrin incorporation after 18 h incubation. In the HFE-expressing cells the reduction in transferrin-mediated iron incorporation was partially compensated by a ~30% increase in non-transferrin iron incorporation from $^{55}$Fe-NTA, evident after prolonged, 18 h, incubations. The findings indicate that HFE binding to transferrin receptor reduces cellular iron availability and regulates the balance between transferrin-mediated and non-transferrin-mediated cellular iron incorporation.**



*Key words:* Hemochromatosis; Iron metabolism; Recombinant protein; HFE protein; Transferrin receptor

## 1. Introduction

Hereditary hemochromatosis (HHC) is a common autosomal recessive disorder characterized by an upregulated iron absorption [1]. The hemochromatosis gene encodes the HFE protein (formerly HLA-H) resembling major histocompatibility complex class I molecules [2]. The HFE Cys282→Tyr mutation, which is homozygous in >70% of HHC patients [3–5], results in the loss of a structural disulfide bond, prevents association with $\beta_2$-microglobulin (β2m) and proper presentation to cell surfaces [6,7]. Mice deleted for the HFE or the β2m gene show a fast accumulation of iron in the parenchyma cells of liver similar to that observed in HHC [8,9]. The artificially induced C282Y mutation in mice produces an HHC-like phenotype less severe than that of HFE null mice [10].

HFE mRNA is expressed in various tissues with the highest levels in liver and intestine [2], and immunohistochemical HFE was found in the syncytiotrophoblasts of human placenta [11], in the epithelial cells of the alimentary tract, in the crypt cells of the small intestine [12] and in Kupffer cells, in liver and brain sinusoidal cells, and in scattered epithelial cells in the crypts of the small and large intestine [13].

HFE associates with the transferrin receptor (TfR) more tightly at neutral (pH 7.5) than at acidic pH (pH 6) and the binding reduces TfR affinity for Fe-transferrin [14,15]. In transfected cells an association between HFE and TfR occurs in the endoplasmic reticulum/*cis*-Golgi compartment soon after synthesis, it stabilizes HFE protein from degradation [16], and reduces cellular iron uptake [17,18]. HeLa cell clones overexpressing HFE showed low levels of endogenous ferritins and a reduced capacity to incorporate $^{55}$Fe-transferrin, while they took up $^{55}$Fe-NTA at the same rate as control clones [17]. The pH-dependent association with TfR appears a major and important linkage of HFE with cellular iron metabolism, but its physiological role in the regulation of body iron absorption and tissue distribution is unclear.

We describe the production of a HeLa cell clone expressing HFE under the control of a tetracycline-inducible promoter. Analysis of the clone showed that HFE overexpression is accompanied by a large (~10-fold) decrease of H- and L-ferritin accumulation, by a ~3–4-fold increase in TfR protein and by a strong activation of iron responsive element (IRE) binding activity of the iron regulatory proteins (IRPs) which are the typical indices of cellular iron deficiency. The cells have a reduced capacity to incorporate $^{55}$Fe-transferrin, while they have an increased capacity to accumulate non-transferrin $^{55}$Fe-NTA.

## 2. Materials and methods

### 2.1. DNA, expression and purification of HFE protein

Full length human HFE cDNA was generated by RT-PCR from mRNA extracted from human pulmonary cells. The cDNA was subcloned into pCDNA3.1 vector (Invitrogen) and pUDH10-3 vector (Clontech) fused to the myc-tag peptide at the C-terminus. The cDNA sequence encoding HFE ectodomains (residues 26–304) was subcloned into vector pET12b for expression in *Escherichia coli*, fused to a polyhistidine tag. DNA sequencing confirmed that the plasmids encoded the correct protein sequence reported in [2]. Transformation of *E. coli* strain BL21(DE3)pLysS and protein expression were performed essentially as described [19]. HFE was purified by affinity chromatography on Ni-NTA Sepharose, and protein purification was confirmed by SDS-PAGE and Coomassie blue staining. Protein concentration was determined with the BCA assay (Pierce) calibrated on bovine serum albumin.

### 2.2. Antibodies, metabolic labeling and SDS-PAGE

Mouse anti-HFE antibodies were elicited by the purified and denatured recombinant HFE protein from *E. coli*. Antibody specificity was assessed by Western blotting and immunoprecipitation. To increase antibody production we induced antibody-containing ascitic fluid in some mice by intraperitoneal injection of pristane [20]. Anti-myc-tag

*Corresponding author. Fax: (39) (2) 2643-4844.
E-mail: arosio.paolo@hsr.it

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antibody 9E10 was purchased from Sigma and anti-transferrin receptor antibody from Zimed, anti-ferritin H- and L-chain monoclonal antibodies and ELISA assays for H- and L-ferritins calibrated with recombinant H- and L-ferritin homopolymers are described in [21]. Metabolic labeling with [$^{35}$S]methionine, immunoprecipitations, SDS-PAGE and fluorography were performed as described in [19]. Immunocytochemistry was performed on fixed cells permeabilized with 0.5% Triton X-100 using anti-myc-tag antibody (2 μg/ml) followed by TRITC anti-mouse IgG rabbit antibodies (Dako) and the stain visualized by fluorescence microscopy.

*2.3. Cell culture*

HeLa-Tet-Off cells (Clontech) were co-transfected with plasmid pUDH10-3-HFE and with plasmid pTK-Hyg (Clontech) using the calcium phosphate method [19]. The colonies selected with hygromycin D (150 μg/ml) and doxycycline (2 μg/ml) were screened for the HFE gene by PCR and for HFE expression by immunoprecipitation of metabolically labeled cells with anti-myc antibody 9E10. The selected cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Clontech), 100 μg/ml G418 (Geneticin, Sigma), 150 μg/ml hygromycin D (Clontech) 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine and with (Dox+) or without (Dox−) 2 μg/ml doxycycline (Sigma).

*2.4. Western blotting*

Subconfluent HFE-HeLa-Tet-Off cells were lysed and 30 μg of soluble protein loaded on SDS-PAGE. For HFE and TfR analysis, the samples were denatured by heating at 100°C for 10 min in the presence of 2% 2-mercaptoethanol, and run in 12% polyacrylamide gels. For ferritin analysis, the samples were not heated to avoid protein disassembly and loaded on 7.5% polyacrylamide gels. After transfer, the nitrocellulose filters were incubated with anti-HFE antibody (dilution 1:4000), with anti-TfR antibody (dilution 1:1000), or with anti-H ferritin rHO2 monoclonal antibody (concentration 7 μg/ml) followed by secondary, peroxidase-labeled antibody (Envision, Dako). Bound activity was revealed by ECL (Amersham).

*2.5. IRP activity*

Bandshift experiments for IRP activity were performed as in [22]. Cells ($2\times10^5$) subjected to various treatments were lysed and 2 μg samples of soluble protein were incubated with a molar excess of $^{32}$P-labeled H-ferritin IRE probe, RNase T1 and heparin in the presence or absence of 2% 2-mercaptoethanol [22]. RNA protein complexes were separated on 6% non-denaturing PAGE and exposed to autoradiography.

*2.6. Cellular $^{55}$Fe incorporation*

Cells ($2\times10^5$) were grown for various lengths of time in the presence of 2 μCi/ml of $^{55}$Fe-NTA (0.4 μM Fe(III), 4 μM NTA) in DMEM, 0.5% fetal calf serum, 0.5% bovine serum albumin, 150 μM ascorbate. The cells were washed and lysed in 0.3 ml lysis buffer (20 mM Tris-HCl pH 8.0, 200 mM LiCl, 1 mM EDTA, 0.5% NP40), centrifuged and 10 μl samples of the soluble fraction were mixed with 0.5 ml of Ultima Gold (Packard) and counted for 3 min in a scintillator counter (Packard). In other experiments the cells were incubated with 0.5 μM $^{55}$Fe-transferrin for 18 h in serum-free medium, and treated and counted as above.

## 3. Results

To study the effect of HFE expression on cellular iron metabolism we generated stable HeLa transfectants with the HFE gene under the control of a tetracycline-responsive promoter. The screening for HFE expression was based on immunoprecipitation of [$^{35}$S]methionine-labeled cells with the 9E10 antibody specific for the myc-tag peptide attached at the C-terminus of the protein. From a clone grown in the absence of doxycycline (Dox−) we immunoprecipitated a double band of 45–47 kDa attributed to the glycosylated and unglycosylated form of HFE, and a band of ~94 kDa corresponding to TfR. No detectable immunoprecipitate was obtained with the same antibody when the clone was maintained in the presence of 2 μg/ml doxycycline (Dox+) (not shown). Immunocytochemistry staining with the anti-myc antibody produced a strong, mainly intracellular decoration in the Dox− transfected cells and no significant background in Dox+ cells (not shown). These findings indicated that the HeLa clone (HFE-HeLa-Tet) expresses a myc-tagged HFE functional in binding TfR and that the expression is fully repressed by 2 μg/ml doxycycline.

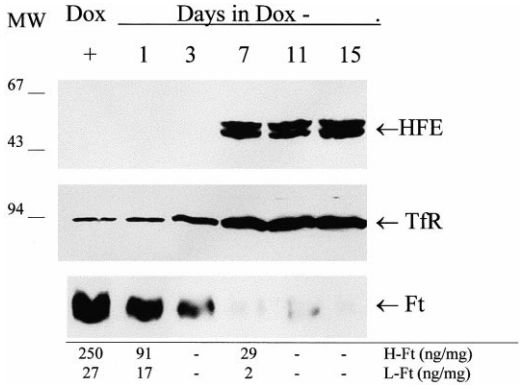


Fig. 1. Time course of HFE expression in HeLa transfected cells. Soluble lysates (30 μg) of transfected cells grown in 2 μg/ml doxycycline (+) or in the absence of doxycycline for the indicated days were subjected to SDS-PAGE, transferred to nitrocellulose and detected with mouse anti-HFE antibody (1:4000), anti-transferrin receptor antibody (1:1000) or anti-H-ferritin monoclonal antibody rHO2 (7 μg/ml) and the appropriate peroxidase-labeled secondary antibody (1:100). HFE and TfR were separated on 12% polyacrylamide gels under reducing conditions, while ferritins were separated on 7.5% polyacrylamide gels under non-denaturing conditions. The mobility of the 45–47 kDa bands of HFE, of the ~94 kDa band of TfR and of the ferritin bands is indicated by arrows. The ELISA-derived cellular concentrations of H- and L-ferritins on the indicated days of induction are shown at the bottom. MW: molecular weight standards. Representative data from two independent experiments.

The time course of HFE accumulation after doxycycline removal was analyzed by Western blotting. We used mouse antisera elicited by the denatured recombinant HFE produced in *E. coli*, which is specific for HFE in immunoblotting and does not cross-react with HLA molecules (not shown). The antibody did not detect HFE in the homogenate of Dox− cells at days 1–3 and recognized the 45–47 kDa HFE doublet from day 7 on (Fig. 1). From densitometry of the blots in comparison with those of known amounts of recombinant HFE from *E. coli* we estimated an HFE level of about 500 ng/mg of total soluble proteins which remained constant from day 7 to 15. Staining of the blots with an anti-TfR antibody which recognizes denatured TfR from SDS-PAGE [17] revealed the expected ~94 kDa band in all cell homogenates with an intensity that increased with the time of growth in Dox− to about 3–4-fold over Dox+ at day 7 (Fig. 1). An anti-H-ferritin monoclonal antibody was used to stain blottings from non-denaturing PAGE and it detected the typical slow moving band of assembled ferritin (~500 kDa) the intensity of which steadily declined in the Dox− cells to become virtually undetectable from day 7 on (Fig. 1). For quantitative data, the H- and L-ferritin concentrations were evaluated with ELISA assays based on specific monoclonal antibodies and

recombinant H- and L-ferritin homopolymers, they showed an about 10-fold decrease of both H- and L-ferritin in the Dox− cells at day 7 (Fig. 1, bottom). The ferritin concentration of Dox+ cells (250 and 27 μg/mg for H- and L-ferritins, respectively) was remarkably similar to that of untransfected parent cells (250 ± 5 and 25 ± 3 μg/mg for H- and L-ferritins, respectively). In addition, as a control, we analyzed a different transfected HeLa cell clone which did not express detectable HFE protein to find that the ferritin levels were unaffected by growth in the presence or the absence of 2 μg/ml doxycycline (not shown).

Next, we analyzed IRP activity by bandshift experiments on cell homogenates using $^{32}$P-labeled H-ferritin IRE. In transfected control HeLa cells non-expressing HFE the IRP activity was analogous in the Dox+ or Dox− conditions (Fig. 2, lanes 1 and 2), while in the transfected cells expressing HFE, ∼2-fold higher activity was observed at day 7 in Dox− conditions (compare lanes 3 and 6 of Fig. 2). Addition of iron salts reduced IRP activity in Dox− and Dox+ cells, while treatment with desferrioxamine (DFO) resulted in an evident IRP activation in Dox+ cells, but not in Dox− cells. The total IRP activity detected after incubation with 2% 2-mercaptoethanol remained unchanged in the Dox+ and Dox− cells. Parallel ELISA analysis showed that FAC supplementation increased H-ferritin levels to 470 ng/mg in Dox+ and to 440 ng/mg in Dox− cells, while DFO treatment reduced H-ferritin levels to < 50 ng/mg in Dox+ and Dox− cells.

Finally, we studied cellular iron incorporation by incubating the cells with 0.5 μM $^{55}$Fe-transferrin or with 0.4 μM $^{55}$Fe-NTA and analyzing $^{55}$Fe in the soluble extracts of the cell homogenates. Dox− cells, at day 7, incorporated ∼30% less $^{55}$Fe-transferrin than the Dox+ cells, after 18 h incubation (Fig. 3), while they incorporated about 30% more iron from $^{55}$Fe-NTA at 18 h incubation. It should be noted that iron incorporation from $^{55}$Fe-NTA in the first 60 min of incubation was analogous in the Dox+ and Dox− cells, suggesting similar rates of iron incorporation.

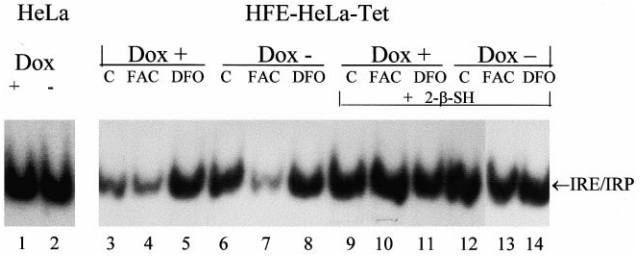


Fig. 2. IRP activity in the induced and non-induced cells. Autoradiograms of representative RNA bandshift assays. Lanes 1 and 2: a transfected HeLa cell control clone non-expressing HFE was grown for 7 days in the presence (Dox+) or the absence (Dox−) of 2 μg/ml doxycycline. Lanes 3–14: the HFE-HeLa-Tet clone was grown for 7 days in the presence (Dox+) of the absence (Dox−) of 2 μg/ml doxycycline, as in Fig. 1; cells were untreated (lanes C), supplemented with 100 μM ferric ammonium citrate (lanes FAC) or 100 μM desferrioxamine for 18 h (lanes DFO). The soluble lysates (2 μg protein per lane) were incubated with a $^{32}$P riboprobe spanning the IRE of the ferritin H-chain in the absence or presence of 2% 2-mercaptoethanol (β-SH) and the RNA-protein complexes separated on non-denaturing gel electrophoresis and exposed to autoradiography. Representative data of three independent experiments.

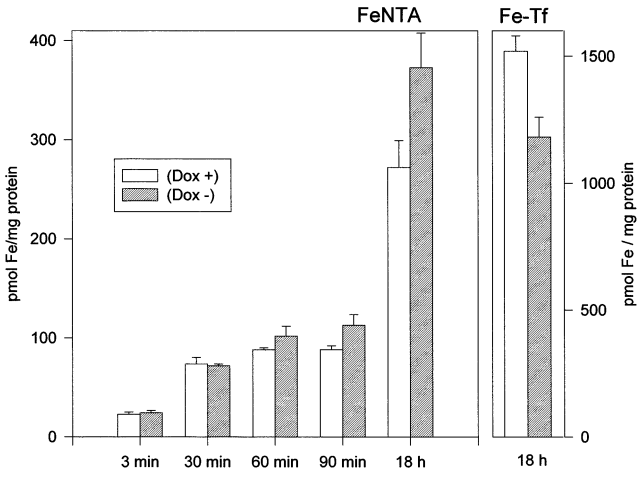


Fig. 3. HFE alters transferrin-mediated and non-transferrin-mediated iron incorporation after prolonged incubations. Uninduced (Dox+, empty bars) and induced transfectant cells grown 7 days in the absence of doxycycline (Dox−, dashed bars) were incubated for the indicated time with 0.4 μM $^{55}$Fe-NTA (Fe:NTA 1:10 molar ratio, 2 μCi/ml) or for 18 h with 0.5 μM $^{55}$Fe-transferrin. Cells were washed, lysed and the soluble extracts counted for radioactivity and analyzed for protein concentration. Data are means+S.D. of an experiment in triplicate representative of four experiments without significant variations.

## 4. Discussion

HFE accumulation in our HeLa transfectant clone became evident after 7 days of growth in the absence of doxycycline, probably the time needed to eliminate the excess of the drug which represses the Tet promoter. The amount of accumulated HFE remained constant for at least a week and was rather high, comparable to that of the endogenous transferrin receptor and of H-ferritin. The HFE accumulation was accompanied by a ∼3–4-fold increase of TfR levels and by a ∼10-fold decrease in H- and L-ferritin levels, in agreement with recent data [17]. IRP proteins are considered the major sensors of cellular iron availability: their IRE binding activity is activated by iron deficiency and repressed by iron availability, although other factors such as cellular redox state and reactive oxygen species affect their functionality [23]. We observed that in the conditions of maximum expression of HFE, i.e. at day 7 of induction, IRE binding activity of IRPs was about 2-fold higher in the HFE+ than in HFE− cells. This was not due to a direct effect of the drug, since IRP activity in control cells non-expressing HFE was unaffected by doxycycline (Fig. 2). Iron supplementation decreased IRP activity on HFE+ as in HFE− cells and increased H-ferritin content to analogous levels. DFO treatment increased IRP activity in HFE−, but not in the HFE+ cells in which the activity was already high, consistent with the observation that DFO treatment and HFE overexpression induced a similar decrease in ferritin accumulation. These findings support the hypothesis that the HFE−-induced ferritin downregulation and TfR upregulation are caused by an iron-mediated effect on IRP activity. Thus, HFE overexpression limits cellular iron availability and causes an iron-deficient phenotype.

The upregulation of TfR in response to iron deficiency is expected to increase transferrin iron uptake, while we observed a significant ∼30% decrease, in agreement with reported data [17]. This is likely caused by the physical associ-

ation of HFE with TfR which was shown to reduce TfR capacity to bind Fe-transferrin [15–18]. The effect is probably stronger with bovine transferrin which has a lower affinity for human TfR [24], and the impaired uptake of iron from bovine transferrin, which is the major source of iron in the medium, should be the cause of cellular iron deficiency.

A novel finding of this work is that HFE overexpression in HeLa cells increases iron accumulation from non-transferrin-bound iron (Fe-NTA) after long incubations. Previous studies showed that Fe-NTA incorporation in HeLa cells is unaffected by cellular growth rate and DFO-induced iron depletion, while it is increased by pretreatment with iron salts [25]. However, cell lines with defective transferrin-mediated iron incorporation showed higher accumulation of $^{59}$Fe-NTA than control cells [26]. Thus, the observed higher $^{55}$Fe-NTA incorporation in HFE+ cells is probably secondary to the partial inactivation of TfR activity.

In conclusion, the present data demonstrate that HFE overexpression in HeLa cells reduces cellular iron availability and support a direct involvement of HFE in cellular iron metabolism. However, their physiological significance in HHC remains unclear, since they suggest that the inactivation of natural HFE (e.g. in C282Y homozygous) should favor Tf-mediated and decrease non-Tf-mediated tissue iron incorporation. However, in HHC patients and in HFE knockout mice the Tf-mediated basolateral iron uptake in duodenal crypt enterocytes is more likely reduced than increased [12,27], while the non-Tf-mediated iron incorporation in absorptive duodenal enterocytes and probably also in parenchymal cells of the liver and other organs is increased [1,27]. Possibly the unphysiological high overexpression of HFE in the transfectant cells may produce a paradoxical effect, or HFE's role in iron metabolism may be more complex than the only association with TfR.

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