Expression of alternative transcripts of ferroportin-1 during human erythroid differentiation

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Background and Objectives. Ferroportin-1 (FPN1) is expressed in various types of cells that play critical roles in mammalian iron metabolism and appears to act as an iron exporter in these tissues. The aim of this study was to investigate whether erythroid cells possess specific mechanisms for iron export.

Design and Methods. The expression of FPN1 during human erythroid differentiation, the characterization of alternative transcripts, the modulation by iron and the subcellular localization of this protein were studied.

Results. FPN1 mRNA and protein are highly expressed during human erythroid differentiation. The iron-responsive element (IRE) in the 5'- untranslated region (UTR) of FPN1 mRNA is functional but, in spite of that, FPN1 protein expression, as well as mRNA level and half-life, seem not to be affected by iron. To explain these apparenthy discordant results we searched for alternative transcripts of FPN1 and found at least three different types of transcripts, displaying alternative 5' ends. Most of the FPN1 transcripts code for the canonical protein, but only half of them contain an IRE in the 5'-UTR and have the potential to be translationally regulated by iron. Expression analysis shows that alternative FPN1 transcripts are differentially expressed during erythroid differentiation. Finally, sustained expression of alternative FPN1 transcripts is apparently observed only in erythroid cells.

Interpretation and Conclusions. This is the first report describing the presence of FPN1 in erythroid cells at all stages of differentiation, providing evidence that erythroid cells possess specific mechanisms of iron export. The existence of multiple FPN1 transcripts indicates a complex regulation of the *FPN1* gene in erythroid cells.

Key words: ferroportin, iron, erythroid, differentiation.

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Iron is essential for life and is required for many specific functions in mammalian cells. However, iron excess is toxic, while iron deficiency leads to anemia; for this reason iron homeostasis necessitates a tight control of iron uptake, storage, export and appropriate management of intracellular iron distribution. Regulation of iron homeostasis occurs at the level of uptake and storage, but multicellular organisms have also evolved cellular iron export pathways to release iron out of the cells and transport it among tissues. Over the last few years the main pathways of iron uptake processes have been clarified and the principal membrane proteins involved have been identified, but the mechanisms of iron exit from the cells are still poorly defined.[1-4] Ferroportin-1 (FPN1, also known as Ireg 1 or MTP1), the product of the Slc40a1 gene, was independently identified by three groups, using different approaches.[5-7] It is expressed in various types of cells that play critical roles in mammalian iron metabolism, including duodenal enterocytes, hepatocytes, syncytiotrophoblasts and reticuloendothelial macrophages[8] and appears to act as an iron exporter in these tissues. This function has been demonstrated in *Xenopus* oocytes in which exogenous expression induces significant efflux of iron.[6,7] Moreover, FPN1 overexpression in tissue culture cells also depletes intracellular and cytosolic iron.[5] Finally, several different mutations of the human *FPN1* gene in patients with type 4 hemochromatosis have been associated with excess iron deposits in macrophages,[9,10] indicating an important role for FPN1 in iron recycling from phagocytosed red cells.[11] In line with this hypothesis, overexpression of FPN1 in the macrophage cell line J774 stimulates iron release after erythrophagocytosis.[12] The *FPN1* gene has been highly conserved during evolution[5] and encodes for a protein composed of 571 amino acids with a predicted mass of 62 KDa.[7] Sequence data showed that FPN1 has at least nine transmembrane α helices with the N-terminus on the intracellular side of the membrane.[5-7] In the 5'-untranslated region (UTR) of FPN1 mRNA a putative iron responsive element (IRE) was found[5] that could confer translational regulation by iron regulatory proteins (IRP) in a manner similar to other 5'-UTR-IRE-regulated genes, i.e. ferritin, erythroid-δ-aminolevulinate synthase (ALAS-E) and mitochondrial aconitase.[13]

However, the regulation of FPN1 expression by iron is currently poorly understood and direct proof of IRP-IRE control has not yet been provided. Both transcriptional and post-transcriptional mechanisms have been implicated in the regulation of FPN1 induced by changes in cellular iron status.[10,14] Indeed, in J774 macrophages, FPN1 mRNA[10] and protein[14] levels have been shown to decrease following iron depletion and to increase in response to iron overload, a regulation likely occurring at the level of gene transcription.[10] However, there is also evidence that iron could post-transcriptionally regulate FPN1 in macrophages, through modulation of IRP activities.[10] Most of the body's iron content is incorporated into hemoglobin during differentiation of erythroid precursors into mature red cells by the transferrin (Tf)-transferrin receptor (TfR) iron uptake pathway.[2-4] Erythroid cells also possess mechanisms for the uptake of non-transferrin bound iron[15] and for its storage in the cytosolic[16] and mitochondrial[17] compartments. The aim of this study was to investigate whether erythroid cells also possess specific mechanisms for iron export. We focused our attention on FPN1 expression during human erythroid differentiation and its regulation both at the mRNA and protein levels.

Design and Methods

Cell lines, culture conditions and treatments

The human erythroleukemic K562, TF-1 and HEL cell lines are grown in RPMI 1640 medium (Euro Clone, Milan, Italy) containing 10% (v/v) heat inactivated fetal calf serum (FCS). The human MonoMac-6 cell line was obtained from the German Collection of Microorganisms and Cell Cultures. NT2/D1 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (EuroClone, Milan, Italy), 1 mM sodium pyruvate, 10 mM Hepes pH 7.3, 10% FCS. NT2/D1 cells were induced to neural differentiation by treatment with 10 μM all-trans retinoic acid (Sigma) for 7 days. The human intestinal carcinoma $CaCo_2$ cells were grown in DMEM, 1 mM sodium pyruvate, 10 mM Hepes pH 7.3, 100 μM non-essential amino acids, and 10% FCS and the cells were collected after 7 and 21 days of culture. Cell cultures were maintained in an incubator at +37°C in a humidified atmosphere with 5% CO_2. To deplete K562 cells of iron, 100 μM desferrioxamine mesylate (Ciba-Geigy, Switzerland), an iron chelator, was added to the cell culture media for 24 hours. Iron loading was achieved by incubating cells for 24 hours in the presence of ferric ammonium citrate (FAC, Sigma) 100 μM or Fe-nitrilotriacetate (Fe-NTA) 200 μM. Fe-NTA (molar ratio 1:4) was prepared as a 20 mM stock solution from NTA and ferric chloride hexahydrate (Sigma-Aldrich). To mimic hypoxia or oxidative stress conditions cells were treated for 24 h with $CoCl_2$ 100 μM, or H_2O_2 100 μM, respectively. The concentration of $CuSO_4$ used was 50-200 μM. To inhibit transcription, cells were treated for various times with actinomycin D (1 μg/mL, Sigma-Aldrich) in the presence or absence of 100 μM desferrioxamine or 100 μM FAC.

Erythroid cell cultures

Human $CD34^+$ progenitor cells were purified from peripheral blood by positive selection using a midiMACS immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions (purified cells contained >90-95% $CD34^+$ cells, as evaluated by fluorescence activated cell sorter analysis). $CD34^+$ progenitors were grown in serum-free liquid suspension culture and induced to unilineage erythropoietic differentiation by an erythroid-specific hematopoietic growth factor cocktail (a saturating dose of erythropoietin and low doses of interleukin-3 and granulocyte-macrophage colony-stimulating factor), as previously described.[19,20] The differentiation stage of erythroid cells was evaluated by May-Grünwald-Giemsa staining and cytological analysis.

Reverse transcription polymerase chain reaction analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Nilden, Germany) according to the manufacturer's protocol. About 0.8 μg of total RNA were reverse transcribed (SuperScript III RNase H-Reverse Transcriptase, Invitrogen) using oligo(dT) as a primer. After GAPDH normalization (20 PCR cycles), an aliquot of reverse transcribed-RNA was amplified for each sample. The primer pairs used are reported in Figure 4B. Reverse transcribed-RNA amplification within the linear range was obtained by 38 PCR cycles. The initial denaturation took place at 95° C for 2 minutes, followed by cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 s, with a final extension at 72°C for 5 min. The GAPDH amplification procedure involved denaturation at 95°C for 45 s, annealing at 56°C for 45 s and extension at 72°C for 45 s. PCR were performed in a total volume of 50 μL using a PTC-100 DNA Thermal Controller (MJ Research, Inc); 10 μL of each sample were separated in a 2% (w/v) agarose gel, transferred to a nylon filter and hybridized with a specific probe end-labeled with [^{32}P]-ATP and a polynucleotide kinase. A negative control lacking template RNA or reverse transcriptase was included in each experiment.

Northern blot analysis

Electrophoresis and northern blot analysis were performed as described elsewhere.[21] The following DNA probes were used: 1) a 1.7 kb PCR product from the complete coding region of human FPN1; 2) a 4.9 kb full-length cDNA fragment from human TfR (kindly provided by

Dr. L. Kuhn, Lausanne, Switzerland); 3) an 860 bp PstI full-length cDNA of human ferritin H; 4) a 1.9 kb full-length cDNA of human beta-actin; 5) a 1.4 kb full-length cDNA of mouse GAPDH; and 6) a 364 bp MspI-EcoRI fragment from the FPN1 *variant II(A+B)* specific 5'-UTR.

Immunoblotting

Whole cell extracts were prepared from cells by standard techniques as already described.[22] Subcellular fractionation was carried out according to a procedure reported previously.[22,23] Electrophoresis and western blotting analysis were performed as described in detail elsewhere.[22] Affinity-purified rabbit anti-FPN1 polyclonal antibody was kindly provided by Dr. David Haile, University of Texas Health Science Center, San Antonio, TX, USA. Anti-beta-actin monoclonal antibody was from Oncogene Research Products, Boston, MA, USA.

5' RACE

Cytoplasmic RNA was purified from K562 cells using the RNeasy Mini kit (Qiagen, Nilden, Germany) and 10 μg were used for the RACE procedure. 5'-RACE analysis was performed using the FirstChoice RLM-RACE Kit (Ambion), which is designed to amplify cDNA clones from full-length capped mRNA. The primers used for FPN1-specific outer and inner PCR are shown in Figure 4B. Products were cloned in pGEM-T Easy plasmid vector (Promega) and sequenced (MMedical).

RNA analysis by RNase protection

Twenty micrograms of total RNA were used in each sample for RNase protection analysis (RNase Protection kit, Roche). We used the following fragments, derived from 5'-RACE inner PCR products, as FPN1 *variant*-specific RNA probes: 1) a 425 bp for *variant I* (IRE); 2) a 502 bp for *variant IIA*; 3) a 299 bp for *variant IIB*; and 4) a 299 bp for *variant IIIA* and *IIIC*. Each probe was composed of a *variant*-specific 5'-region and a common 178 bp sequence containing the first 25 codons of the reported coding sequence of FPN1. An RNA probe containing a β-actin 93 bp RsaI 3' cDNA sequence was used for normalization. [^{32}P]-α-GTP-labeled antisense RNA probes were synthesized using the Riboprobe *in vitro* Transcription System (Promega). After electrophoresis the strength of each band was evaluated using an InstantImager (Packard) and normalized against β-actin. To effectively evaluate the relative amount of each alternative transcript in respect to overall FPN1 mRNA in K562 cells we corrected the value of intensity for each band by dividing it for the G-content of its protecting sequence and calculated the percentage in respect to the sum of corrected values of all FPN1 bands in the line.

Luciferase assays

We prepared three different plasmid constructs each producing, under control of the SV40 promoter, a luciferase reporter transcript with a 5'-UTR containing a different *variant*-specific 5' sequence from an alternative FPN1 transcript. That is, 5'-RACE clones were digested with the restriction enzyme BfaI (Biolabs), which cuts 4 bp 5' to the ATG canonical start codon, to obtain: (i) a 278 bp fragment, containing most part of 5'-UTR of the *variant I(IRE)* transcript, including the IRE sequence; (ii) a 414 bp fragment, containing the entire *variant IIA* specific 5'-UTR; 3) a 218 bp fragment, containing both the *variant IIIC* specific exons. DNA fragments were blunted and inserted in the HindIII site of a pGL3 promoter plasmid vector (Promega). K562 cells were co-transfected with one FPN1 *variant*-specific plasmid construct and Renilla phRL-TK control vector (Promega) using Lipofectamine 2000 (Invitrogen), according to the protocols suggested for K562 cells. Twenty-four hours after transfection cells were treated with FAC 50, 100 or 200 μg/mL; desferrioxamine 50, 100 or 200 μM; and $CuSO_4$ 50, 100 or 200μM. Forty-eight hours after transfection cells were lysed and extracts were assayed using the Dual-Luciferase Reporter Assay System (Promega). Luciferase expression values (LAR) were normalized using Renilla values (GLO) and comparatively analyzed. Each transfection experiment was repeated three times.

FPN1-GFP constructs and cellular localization of green fluorescent protein (GFP)-tagged FPN1

In order to determine the cellular localization of putative proteins encoded by the FPN1 alternative transcripts we generated three constructs producing FPN1-GFP fusion proteins. All constructs were prepared using Gateway technology-based primers (Invitrogen, Carlsbad, CA, USA). The full-length coding sequences of *variant I/II, IIIA and IIIC* were obtained by RT (reverse transcription)-PCR on K562 RNA (primers indicated in Figure 4B). Open reading frames were amplified using Pfu DNA polymerase (Promega), cloned into pENTR/D-TOPO vector using the bacterial strain TOP10' (Invitrogen) and verified by sequencing (MMedical). After that, the full-length open reading frames were transferred by clonase reaction into the pcDNA-DEST47 expression vector under the control of cytomegalovirus (CMV) immediate-early promoter/enhancer (Invitrogen), to generate p(*variant I/II*) FPN1-GFP, p(*variant IIIA*)FPN1-GFP and p(*variant IIIC*) FPN1-GFP fusion proteins. In order to determine the cellular localization of the fusion proteins the HEK293 cells were plated on poly-L-lysine-coated glass coverslips at a density of 1.5×10^4, while the K562 cells were plated in a 24-well plate at a density of 2.5×10^4 cells/well. HEK293 and K562 cells at 60-80% confluence were transfected with one of the different FPN1-GFP constructs using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the protocols suggested for HEK293 and K562 cells. Forty-eight hours after transfection HEK293

and K562 cells were tested for protein expression by measuring the native fluorescence of GFP. K562 cells were adhered to poly-L-lysine-coated microscope slides by incubation at +4°C for 30 minutes. Following three washes in PBS, HEK293 and K562 cells were fixed with 4% paraformaldehyde in PBS at +4°C for 20 minutes and analyzed by confocal laser-scanning microscopy, as described below.

Immunofluorescence and confocal laser-scanning microscopy analysis

Erythroid progenitors at various stages of differentiation were adhered to poly-L-lysine-coated microscope slides by incubation at +4°C for 30 minutes. Following three washes in PBS the cells were fixed in 4% paraformaldehyde in PBS for 15 min at +4°C and then permeabilized with saponin 0.1% in PBS containing 0.2% BSA (Sigma) for 10 minutes at +4°C. Incubation with primary polyclonal anti-FPN1 antibody was performed for 60 min at +4°C in PBS, 0.1% saponin, 0.2% BSA, then the cells were washed with five changes of PBS, 0.1% saponin, 0.2% BSA and goat anti-rabbit-Alexa Fluor 488 was added as the secondary antibody for 30 min at +4°C. Cells were washed in five changes of PBS, 0.1% saponin, 0.2% BSA and mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories Inc., Bulingame, CA, USA). Negative controls were treated identically except for omission of the primary antibody. Confocal laser-scanning microscopy was performed with Leica TCS SP2 apparatus. Several cells were analyzed for each labeling condition and representative results are presented.

Results

FPN1 is expressed in erythroid cells

In order to investigate whether erythroid cells possess specific mechanisms for iron export we studied the expression of the *FPN1* gene by semiquantitative reverse transcription-PCR analysis on early hematopoietic progenitor cells, purified from human adult peripheral blood and induced in liquid suspension culture to unilineage erythroid differentiation, as previously described in detail.[18,20] In our system, purified hematopoietic progenitor cells undergo a gradual wave of differentiation/maturation selectively along the erythroid lineage. Briefly, during the first week of culture the early erythroid progenitors (burst-forming units-erythroid) differentiate into late colony-forming units erythroid and proerythroblasts; the second week is characterized by a progressive wave of erythroblast maturation (>90% of polychromatic and orthochromatic erythroblasts), coupled with a gradual increase of erythroid-specific markers (at day 12-14, >95% of the cells clearly express glycophorin-A) (*data not shown*). We observed that FPN1 mRNA was expressed in quiescent hematopoietic progenitor cells and persisted at sustained levels during all stages of erythroid differentiation (Figure 1A). To validate these results, polyA+ RNA from mature erythroblasts (day 11 of culture) was analyzed by northern blot. We observed a single mRNA species, about 3.7 kb in size, expressed in erythroblasts at a higher level than in K562 cells (Figure 1B). Moreover, we performed western blot analysis to determine whether the observed high levels of FPN1 mRNA correlated with protein expression. We used an affinity purified rabbit anti-FPN1 antibody (a generous gift from Dr. David Haile, University of Texas Health Science Center, San Antonio, TX, USA) and whole cell extracts derived from erythroid cells at different stages of differentiation. We observed a single immunoreactive species with a molecular mass of ~62 kDa, in accordance with the predicted mass of FPN1 protein, constantly expressed during all stages of erythroid differentiation, including in quiescent hematopoietic progenitor cells (Figure 1C, on the left). An immunoreactive protein of the same mass was detected in whole cell extracts from CaCo2 and MonoMac 6 cells at a level comparable to that observed in mature erythroid cells (Figure 1C, on the right); moreover, in HEK293 cells transfected with a FPN1-GFP construct generating a FPN1-GFP fusion protein, an immunoreactive species with a molecular mass of ≈98 kDa, corresponding to FPN1-GFP fusion protein (*results not shown*), was detected. These positive control samples supported the identification of the erythroid protein, immunoreactive with anti-FPN1 antiserum, as FPN1. To determine the intracellular distribution of FPN1 in mature erythroblasts at day 10-11 of culture and in K562 cells we employed conventional cell fractionation, followed by immunoblot analysis.[22,23] We obtained two fractions: a cytosolic (free from membrane compartments) and a membranous fraction. This subcellular fractionation was not able to separate plasma membrane from microsomal membranes. Our results indicated that FPN1 protein was present in both the membrane-enriched and cytosolic fractions (Figure 1D). The subcellular localization of FPN1 expression was also examined during different stages of erythroid differentiation by immunofluorescence and confocal laser-scanning microscopy, as described in the Design and Methods. We observed the presence of FPN1 in all stages, beginning from undifferentiated $CD34^+$ cells (Figure 1E) and blasts (day 2 of culture, Figure 1F), to proerythroblasts (day 7 of culture, Figure 1G) and mature erythroblasts (day 11-13 of culture, Figure 1H). Fluorescence was particularly intense in regions corresponding to the cytoplasm; visualization of the nuclei in erythroblasts at day 10 of culture, using the DNA dye DAPI, indicated the presence of a large nucleus with no FPN1 protein expression (Figure 1I). The analysis of immunofluorescence pictures at 1000X enlargement

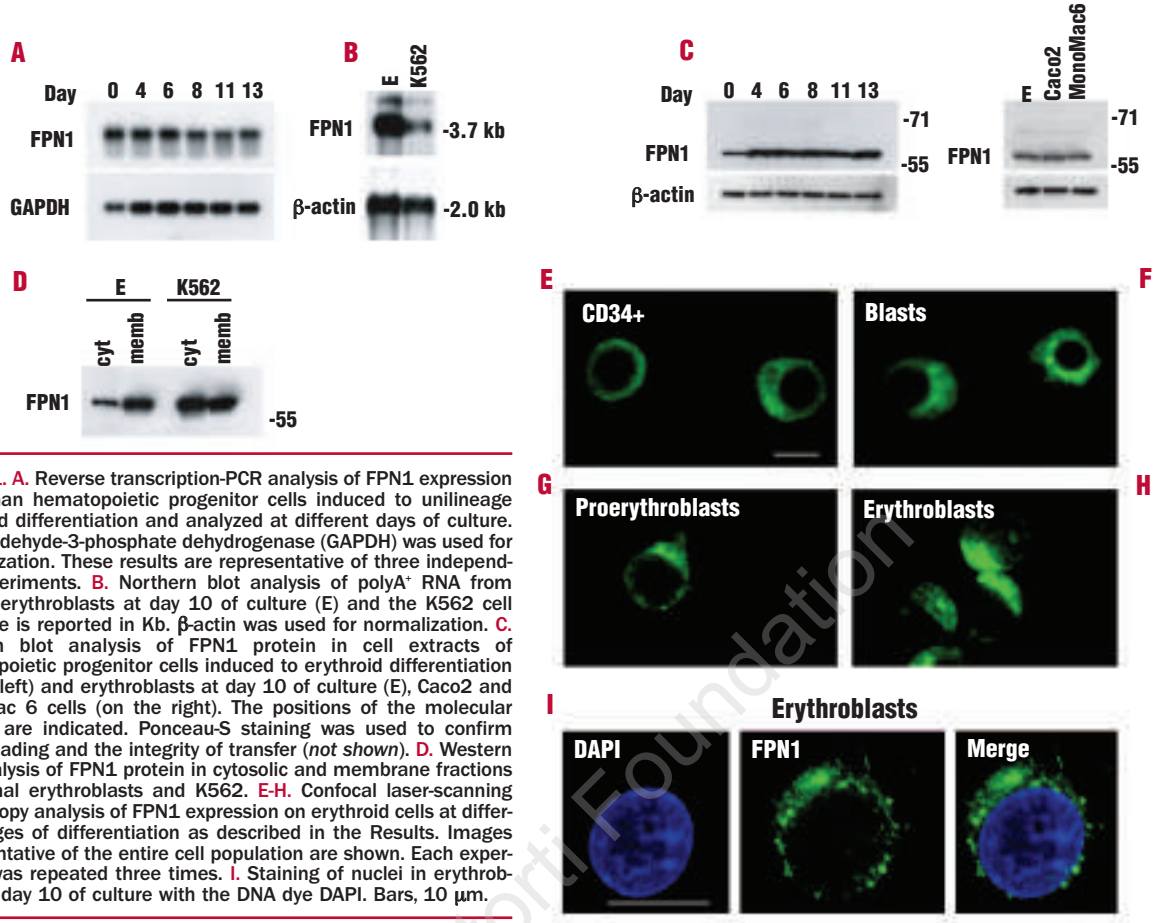

Figure 1. A. Reverse transcription-PCR analysis of FPN1 expression on human hematopoietic progenitor cells induced to unilineage erythroid differentiation and analyzed at different days of culture. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. These results are representative of three independent experiments. B. Northern blot analysis of polyA+ RNA from human erythroblasts at day 10 of culture (E) and the K562 cell line. Size is reported in Kb. β-actin was used for normalization. C. Western blot analysis of FPN1 protein in cell extracts of hematopoietic progenitor cells induced to erythroid differentiation (on the left) and erythroblasts at day 10 of culture (E), Caco2 and MonoMac 6 cells (on the right). The positions of the molecular marker are indicated. Ponceau-S staining was used to confirm equal loading and the integrity of transfer (*not shown*). D. Western blot analysis of FPN1 protein in cytosolic and membrane fractions of normal erythroblasts and K562. E-H. Confocal laser-scanning microscopy analysis of FPN1 expression on erythroid cells at different stages of differentiation as described in the Results. Images representative of the entire cell population are shown. Each experiment was repeated three times. I. Staining of nuclei in erythroblasts at day 10 of culture with the DNA dye DAPI. Bars, 10 μm.

clearly showed that FPN1 immunoreactivity was mainly concentrated in vesicles localized in the cytoplasm (Figure 1I). In conclusion, we show that FPN1 mRNA and protein are highly expressed during normal human erythroid differentiation, and that FPN1 protein is localized in the cytoplasm and/or membranous compartments.

FPN1 is not responsive to iron overload or depletion

Since the FPN1 mRNA contains an iron responsive element (IRE) in its 5'- UTR we explored its modulation by iron. K562 cells were grown in conditions of iron loading (with FAC or FeNTA) or iron deprivation (with desferrioxamine) for 20 hours, then total RNA was collected and analyzed by northern blot. As iron homeostasis is reported to be affected by hypoxia and oxidative stress conditions,[13] K562 cells were also grown in the presence of $CoCl_2$ or H_2O_2, to simulate these two conditions, respectively. Our results showed that, when normalized to β-actin, FPN1 mRNA levels were not greatly modified by iron loading nor by $CoCl_2$ and H_2O_2 treatment (Figure 2A). Desferrioxamine treatment caused a slight decrease in FPN1 mRNA (70%) and also β-actin mRNA levels: the latter finding has been previously reported,[24] but its mechanism is unknown. We

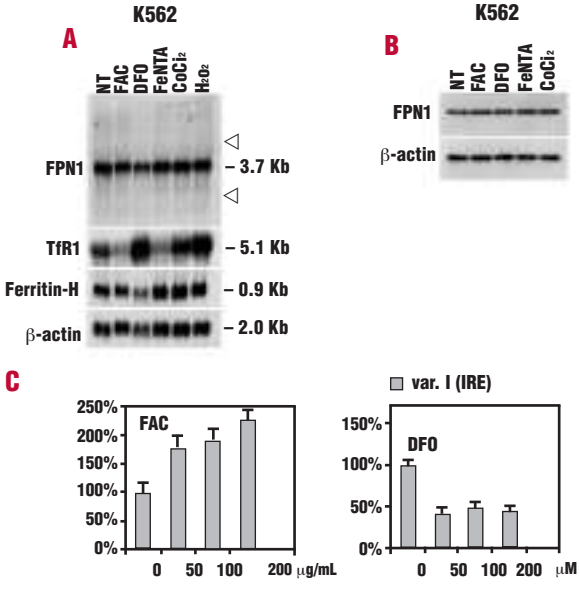

Figure 2. A. Northern blot analysis of total RNA samples from K562 cells untreated (NT) and treated with ferric ammonium citrate (FAC), desferrioxamine (DFO), iron nitrilotriacetic acid (FeNTA), cobalt chloride ($CoCl_2$) and hydrogen peroxide (H_2O_2). Bands from hybridization with FPN1, TfR1, ferritin H and β-actin probes are reported, with size in kb on the right. Open triangles indicate 28S and 18S ribosomal RNA. B. Western blot analysis of K562 treated as above. Ponceau-S staining was used to confirm equal loading and the integrity of transfer (*not shown*). C. Graphical representation of luciferase expression from K562 cells transfected with *variant I(IRE)* –5' UTR-FPN1-luciferase construct and treated with FAC and desferrioxamine (DPO). The SD is indicated.

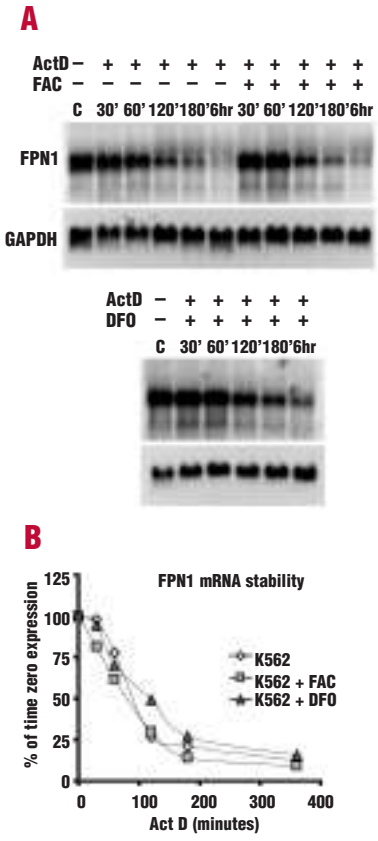

Figure 3. Northern blot analysis (A) and densitometric analysis (B) of FPN1 mRNA decay in K562 cells untreated or treated with ferric ammonium citrate (FAC) or desferrioxamine (DFO) for 24 hours and then incubated for 0-6 hr in the presence of 1 μg/mL actinomycin D. (A) At different times after drug addition, the culture medium was removed and cells were immediately processed for RNA extraction and northern blot analysis. Data were normalized by hybridization to GAPDH, whose mRNA decay is negligible at the times considered here, i.e. GAPDH mRNA half-life is >8h. Representative results from three independent experiments are shown (B). Results are normalized to the GAPDH control and are expressed as percentages of time zero expression.

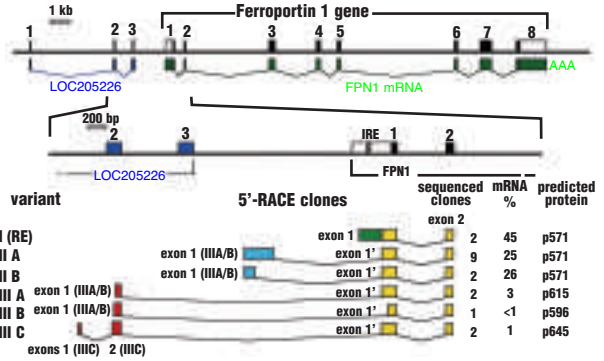

Figure 4 A. *FPN1* gene structure and transcripts. Top: genomic organization and exon distribution of LOC205226 locus and *FPN1* gene, with (below) reported LOC205226 predicted mRNA and *FPN1* mRNA (GenBank accession XM_047592). Middle: enlarged genomic region with exons 2-3 of LOC205226 locus and 1-2 of *FPN1* mRNA. Non-coding regions are reported as open boxes, coding sequences are indicated as black boxes and the IRE element is indicated as a dashed box. Bottom: structure of clones from 5'-RACE. Alternative 5' regions are indicated in different colors; sequences shared by all transcripts are in yellow. The number of sequenced clones for each transcript type, relative amounts (%) of each alternative transcript and the sizes of putative proteins coded by the respective transcripts are reported on the right.

also analyzed transferrin receptor1 (TfR1) mRNA, which contains IRE elements in the 3'-UTR, and ferritin H mRNA that, like FPN1, contains an IRE in the 5'-UTR, on the same samples. As expected, we observed TfR1 mRNA down-regulation by FAC and FeNTA treatments and strong up-regulation by desferrioxamine, $CoCl_2$ and H_2O_2 treatment, as previously reported,[25,26] whereas Ferritin H mRNA level was not modified by desferrioxamine treatment (the slight decrease was mainly related to a concomitant decline of β-actin mRNA level). Moreover, western blot analysis of K562 cells, grown under conditions of either iron loading or deprivation, showed that FPN1 protein levels did not appear to be affected by either iron treatment or hypoxia (Figure 2B). These results showed that FPN1 mRNA and protein levels are not modulated by iron in erythroid cells. In contrast, the luciferase assay of K562 cells transfected with constructs carrying the FPN1-IRE element upstream to the luciferase reporter gene and grown in the presence of FAC or desferrioxamine for 24 hours showed a clear translational modulation by iron levels (Figure 2C), as previously reported for other cell types.[6,7,27,28] We also investigated the effect of iron levels on the mRNA half-life of FPN1 by northern blot analysis on K562 grown in the presence of FAC or desferrioxamine for 24 hours and then treated with actinomycin D and harvested after different times of treatment. Although baseline FPN1 mRNA levels were increased in desferrioxamine treated cells as compared to those treated with iron, no change in mRNA half-life was found (Figure 3). In conclusion, we report that the IRE element in the 5'-UTR of FPN1 mRNA is functional in erythroid cells, but, in spite of that, FPN1 protein expression, as well as mRNA level and half-life, seem not to be affected by iron conditions.

FPN1 is expressed in multiple alternative transcripts

In order to elucidate our apparently conflicting results on FPN1 modulation by iron and to verify the possibility of a more complex and specific FPN1 gene regulation in the erythroid lineage, we searched for alternative FPN1 transcripts. To isolate new FPN1 5'-alternative transcripts from erythroid cells, we performed a full-length, cap-specific 5'-RACE analysis using cytoplasmic RNA from K562 cells. Eighteen clones were isolated and sequenced (Figures 4A-C). Among these, only two clones matched the canonical IRE-containing mRNA sequence (*variant I(IRE)* transcript, GenBank accession DQ065759). All the other clones were spliced at acceptor splice sites located 103 bp (15 clones) or 65 bp (only one clone) 5' to the ATG start codon, did not have the IRE element and contained upstream exons. The great majority of them (11 clones) contained an exon, 324 bp in the longest clone, that was 1.3 kb 5' to the ATG on genomic sequencing, and did not contain an IRE element. We named these transcripts as *variant IIA* (GenBank accession DQ065760) or *variant IIB* (GenBank accession DQ065761), according to which of two different (downstream or upstream, respectively) donor splice sites were used. *Variant II*-specific exon does not contain open reading frames, so *variant I* (*IRE*) and *variant II* transcripts potentially code for the same protein (p571,

B

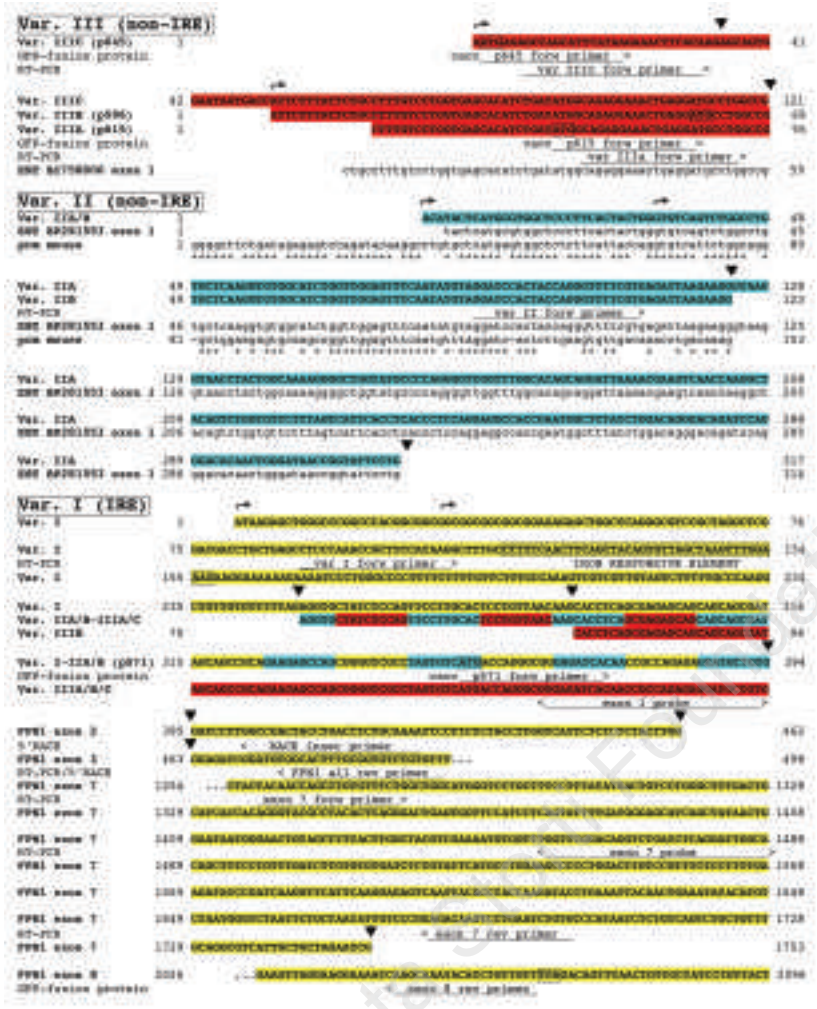

Figure 4B. Sequences of alternative FPN1 exons. Only relevant regions are reported. Exon sequences included in 5'RACE clones are in capital letters and boxed using different colors for different FPN1 variants. Black triangles and bent arrows above a sequence indicate splicing sites and putative transcription starts, respectively. Start codons are boxed. The primers and probes used in different experiments reported in the text, and sequences of EST clones from GenBank and non-IRE clones from pcm mice[32] are reported below the sequences.

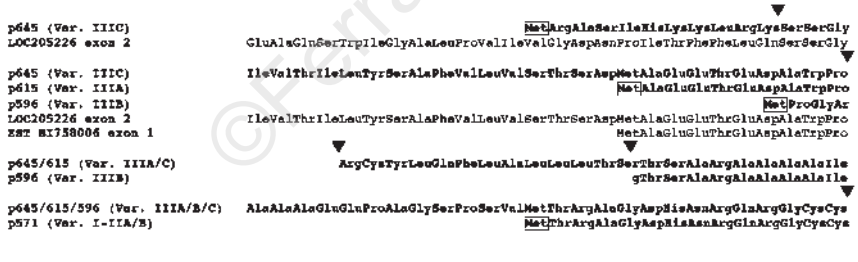

Figure 4C. Comparisons of amino acid sequences of 5' regions from putative proteins encoded by different variants of FPN1 transcripts. The predicted protein sizes are indicated. Start Met are boxed. Black triangles above a sequence indicate splicing sites. The sequences of the exon 2 from LOC205226 and of the exon 1 from EST BI758006 clone from GenBank are reported below the sequences.

GenBank protein id. AAY78556.1). An EST sequence (GenBank accession BP281553) from K562 cells is identical to our *variant IIA* transcript. The sequences of FPN1 non-IRE transcripts, originated in polycythemic mice by a microdeletion in the promoter region,[32] strictly correspond to our *variant IIB* (sequences have been kindly provided by Dr. Armin Schumacher, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA) (Figure 4B). The five other clones contained a different upstream exon, 87 bp in the longest clone, which localizes in the genomic sequence (genomic contig NT_005403) 2.9 kb 5' to the FPN1 ATG codon. This exon coincides in part with the second exon of the upstream LOC205226 predicted mRNA (GenBank accession XM_119528), but we did not find evidence for inclusion of LOC205226 exons 1 or 3 in FPN1 transcripts. Two of these clones (*variant IIIA* transcript, GenBank accession DQ065762) had the ability to code for a longer protein with 44 additional N-terminal amino acids (p615, GenBank protein id. AAY78559.1) in frame with the canonical open reading frame (Figure 4C). Amino acid analysis using the

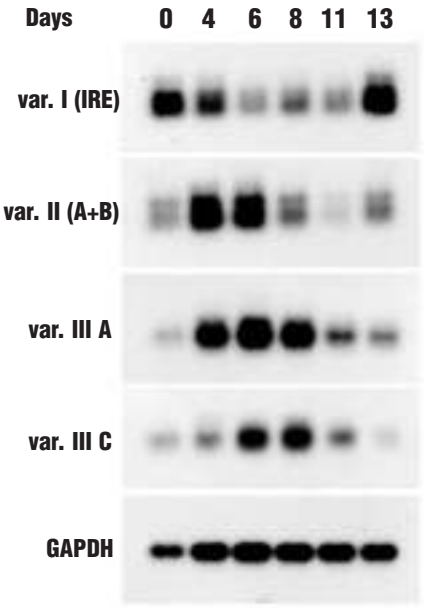

Figure 5. RT-PCR analysis of FPN1 alternative transcript expression in hematopoietic progenitor cells induced to undergo unilineage erythroid differentiation and analyzed at different days of culture. GAPDH was used for normalization. These results are representative of three independent experiments.

PROSITE database (*http://www. expasy.org/prosite/*) found a protein kinase C phosphorylation site (amino acids 25-27) and no potential N-glycosylation sites in the additional sequence. The PSORT II tool (*http://psort.nibb.ac.jp /cgi-bin/runpsort.pl*) predicted no additional transmembrane domains, the N-terminal side inside and a 69.9% probability of plasma membrane localization. An EST sequence (GenBank accession BI758006) from human brain is identical to our *variant IIIA* transcript. One clone had the same upstream exon, but used, in its second exon, an alternative acceptor splice site located 65 bp 5' to the ATG (*variant IIIB* transcript, GenBank accession DQ065763), so potentially producing a different protein with 25 additional N-terminal amino acids (p596, GenBank protein id. AAY78560.1) (Figure 4C). Finally, two clones had a 34 bp additional short upstream exon (*variant IIIC* transcript, GenBank accession DQ065764), and potentially coded for a long protein with 74 additional amino acids (p645, GenBank protein id. AAY78561.1), N-terminal to and in frame with the canonical open reading frame (Figure 4C). Analysis of additional amino acids using the PROSITE database found a casein kinase II (amino acids 27-30) and a protein kinase C (amino acids 55-57) phosphorylation sites in the N-terminal sequence. The PSORT II tool predicted a possible cleavable signal peptide (1 to 58) and a 52.2% probability of plasma membrane localization. Our results show that human FPN1 is expressed in K562 cells in at least six variants of alternative transcripts, which have different 5' ends and are able to code for multiple proteins differing in their N-region. Only *variant I(IRE)* transcript has an IRE sequence in the 5'-UTR, whereas all the other transcript types do not. Alternative transcripts differ by 100-200 bp in length and cannot be easily detected as distinct bands in northern analysis.

FPN1 alternative transcripts are differentially expressed during erythroid differentiation

To analyze the expression of alternative FPN1 transcripts during erythroid differentiation a semiquantitative reverse transcription-PCR analysis was performed. A set of forward primers, each one specific for the 5' sequence of a variant of human FPN1 mRNA, and a common reverse primer (Figure 4B) were used on total RNA isolated from human erythroid cells at different stages of differentiation. The primers spanned across introns such that genomic amplification would not yield the product observed. After GAPDH normalization we observed that the *variant I(IRE)* transcript was highly expressed in quiescent hematopoietic progenitor cells, rapidly declined during early phases of erythroid differentiation and increased during terminal stages of maturation (i.e., when the majority of cells became polychromatic and orthochromatic erythroblasts) (Figure 5). Conversely, *variant II, variant IIIA* and *variant IIIC* transcripts were expressed at very low levels in quiescent progenitor cells, increased in the early phases of erythroid differentiation and declined to a nearly undetectable level during terminal stages (Figure 5). Our results show that alternative FPN1 transcripts are differentially expressed during erythroid differentiation, in particular indicating a sequential and specific activation pathway, with an apparently mutual exclusion between *variant I(IRE)* and *variant II/III* (not containing the IRE) transcripts.

Cellular localization of proteins coded by FPN1 alternative transcripts

In order to analyze the subcellular localization of the proteins predicted to be coded by FPN1 alternative transcripts, we performed transient transfection of HEK293 cells with plasmid constructs producing FPN1(p571)-, FPN1(p615)- or FPN1(p645)-GFP fusion proteins. Forty-eight hours after transfection HEK293 cells were tested for specific protein expression by native fluorescence of GFP. Confocal laser-scanning microscopy, analysis indicated a predominantly cytoplasmic intense localization for p571 canonical protein (Figure 6B), according to the data reported above; a similar pattern of labeling was also observed for p615 and p645 FPN1 proteins (Figure 6 C-E). The confocal microscopy analysis revealed a more spotlike distribution of p615 (Figure 6C,D), in comparison with p571 and p645 (Figure 6B,E), which showed a more widespread intracellular distribution. Similar results were obtained in K562 cells transiently transfected with the FPN1(p571)-GFP construct (Figure 6A) and FPN1(p615)- or FPN1 (p645)-GFP construct (*data not shown*).

mRNA for alternative FPN1 transcripts was not responsive to iron levels

As our results indicated that FPN1 expression did not change at the overall mRNA level after cell treatments,

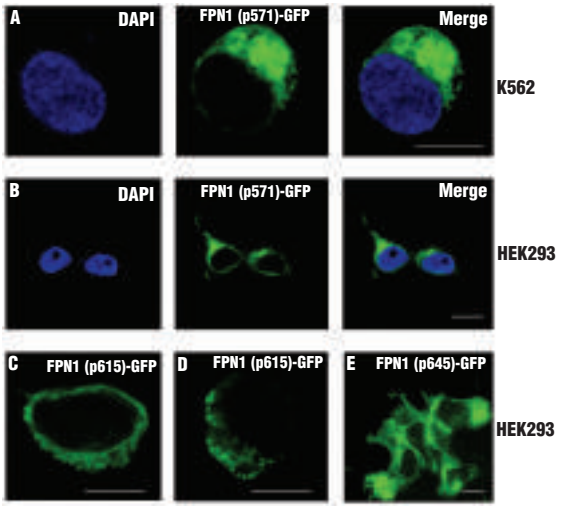

Figure 6. Confocal laser-scanning microscopy analysis of HEK293 (B-C-D-E) and K562 (A) cells transiently transfected with p(variant I)-, p(variant IIIA)-, and p (variant IIIC)-FPN1-GFP plasmid constructs producing fusion proteins containing the sequences from the p571, p615 or p645 FPN1 protein, respectively. Images obtained by confocal microscopy are: (A) FPN1(p571)-GFP; (B) FPN1(p571)-GFP; (C-D) FPN1(p615)-GFP; (E) FPN1(p645)-GFP. Images representative of the entire cell population are shown. Each experiment was repeated three times. Bars: about 10 μm.

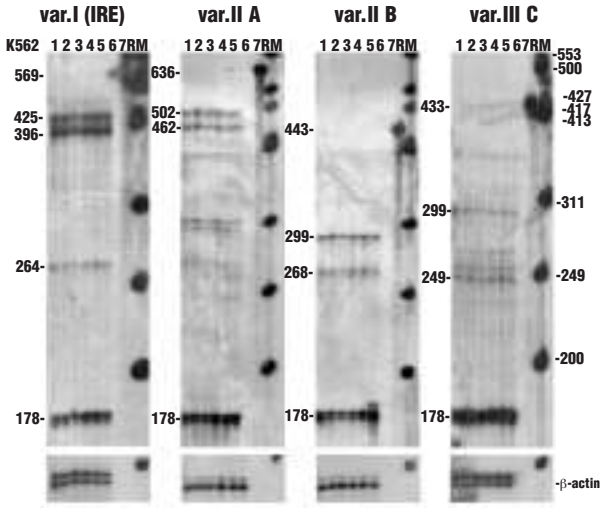

Figure 7. RNase protection analysis of total RNA samples from K562 cells untreated (1) and treated with ferric ammonium citrate (2), desferrioxamine (3), iron nitrilotriacetic acid (4), cobalt chloride (5) and hydrogen peroxide (6). A tRNA sample was used as a negative control (7). β-actin was used for normalization and the bands are reported below. In each experiment a different variant-specific riboprobe (whose name is reported above) was used and an aliquot was run on the gel (R). The ϕX174 size marker is indicated (M) with band sizes on the right. The sizes of the main bands are indicated in bp.

we re-analyzed total RNA samples from K562 cells treated with FAC, desferrioxamine, FeNTA, $CoCl_2$ and H_2O_2, in RNase Protection experiments using four *variant*-specific RNA probes derived from cap-based 5'-RACE products (Figure 7). In all cases we observed at least two bands possibly corresponding to multiple transcription start sites (see bent arrows over sequence in Figure 4B). In the *variant I(IRE)* experiment the 396 bp band corresponds to the reported murine FPN1 transcription start site.[27] Quantifying the relative abundance of the alternative transcripts, with respect to overall FPN1 mRNA in untreated K562 cells, showed that: (i) the canonical *variant* I(IRE) transcript represents 45% of total; (ii) the *variant IIA*– and *variant IIB* – transcripts account for 25 and 26% respectively, and together represent half of the FPN1 transcripts in K562 cells; (iii) the *variant III* are rare transcripts, in that *variant IIIA* accounts for 3%, variant IIIB is undetectable, and *variant IIIC* forms approximately 1% of the total transcripts. These results show that most (96%) of the FPN1 transcripts in K562 cells code for the canonical p571 protein, but only half contain an IRE element in the 5'-UTR and have the potential to undergo translational regulation by iron. Finally, only a small amount of heterogeneous transcripts (4%) could code for longer proteins.

Only the FPN1 variant I(IRE) transcript is translationally regulated by iron

The IRE element in the 5'-UTR of FPN1 mRNA has been reported to mediate translational up-regulation after iron treatment and down-regulation after desferrioxamine treatment in several cell types.[27,28] We assayed the effect of treatment with FAC, desferrioxamine, and $CuSO_4$ on luciferase constructs carrying the different 5' sequences from FPN1 alternative transcripts (*variant I(IRE)*, *variant IIA* and *variant IIIC*) transfected in K562 cells (Figure 8). FAC treatment produced a strong dose-related increase of the IRE construct expression, up to 228% in the FAC 200 μM sample, and only a slight decrease in cells containing *variant IIA* and *IIIC* constructs. Desferrioxamine treatment produced a clear down-regulation of luciferase expression with the *variant I(IRE)* construct (46%) and no effect with the other constructs. Moreover, treatment with $CuSO_4$ also produced a dose-related luciferase up-regulation only in cells containing *variant I(IRE)* construct, in accordance with to studies in CaCo2 cells suggesting FPN1 regulation by other metals in addition to iron.[29,31] In conclusion, only the transcript containing an IRE element is translationally modulated by iron and $CuSO_4$ in K562 cells, whereas 5' regions from FPN1 *variant II* and *III* alternative transcripts do not respond to these treatments.

FPN1 variant II alternative transcript is prevalently expressed in erythroid cells.

A non-IRE FPN1 alternative transcript has been reported in polycythemic mice,[32] as a result of a genomic microdeletion, whereas in human cells, only two EST sequences from human brain and K562 (see above) have been reported. We excluded the presence of a relevant deletion in the *variant I(IRE)* promoter in K562 cells by PCR analysis on genomic DNA (*data not shown*). In order

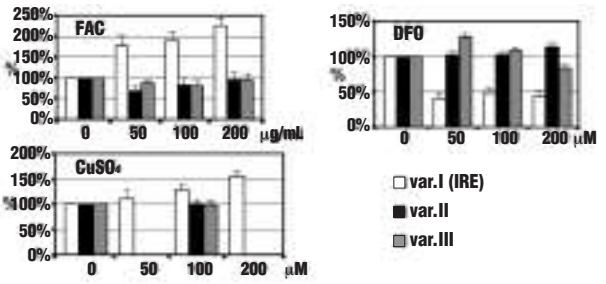

Figure 8. Graphical representation of luciferase expression from K562 cells transfected with different (*variant I(IRE), variant II* and *variant III*) 5'UTR-FPN1-luciferase constructs and treated with ferric ammonium citrate (FAC), desferrioxamine (DFO), and copper sulphate ($CuSO_4$).

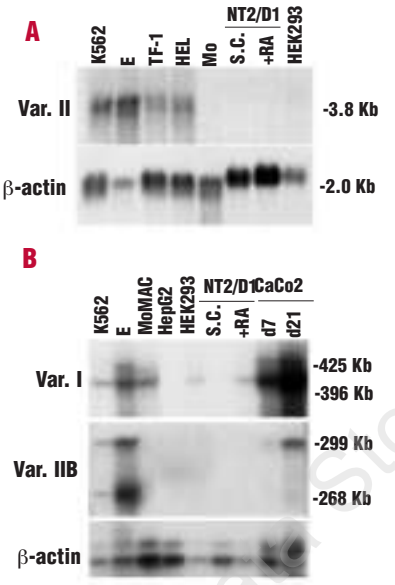

Figure 9. Northern blot analysis of *variant II (A+B)* (A) and RNase protection analysis of *variant I(IRE)* and *variant IIB* (B) FPN1 mRNA expression in total RNA (20 µg) from erythroleukemia K562, TF1 and HEL cell lines; normal erythroblasts (E); resting monocytes from peripheral blood (Mo); hepatoma HepG2; pluripotent embryonal carcinoma NT2/D1 stem (S.C.) and differentiating to neurons with retinoic acid (RA); human embryonic kidney (HEK 293; monocytic leukemia MonoMac-6); and intestinal carcinoma (Caco2) cells. Sizes are reported in Kb. β-actin was used for normalization.

to determine whether *variant II* transcripts were expressed in human tissues other than erythroid cells we used northern blot and RNase Protection to analyze their expression in resting monocytes from human peripheral blood and in various types of human cell lines: erythroleukemia K562, TF1 and HEL; monocytic leukemia MonoMac-6; pluripotent embryonal carcinoma NT2/D1 stem uninduced and induced to differentiate into neurons with retinoic acid treatment; embryonic kidney HEK293; intestinal carcinoma CaCo2 after 7 and 21 days of differentiation to absorptive epithelium; and human hepatoma HepG2 cells. Our results showed that *variant I (IRE)* transcript was expressed at variable levels in the majority of samples, except for NT2/D1 stem cells and HepG2 cells (Figure 9B). In the CaCo2 cells *variant I (IRE)* transcript expression increased along with the differentiation stage, and at day 21, was at least 50 times stronger than in K562 cells (Figure 9B). In contrast, the expression of *variant II* transcripts was undetectable in normal resting monocytes and in all non-erythroid cell lines (Figure 9A), except in differentiated CaCo2 cells (Figure 9B). RNase protection experiments performed with *variant IIA* showed the same results (*data not shown*). In particular, *variant II* transcripts were highly expressed in normal erythroblasts and erythroleukemic K562, TF1 and HEL cell lines (Figure 9A). Analysis on purified hematopoietic progenitor cells induced in liquid suspension culture to unilineage monocytic differentiation and on mature macrophages obtained from *in vitro* spontaneous maturing monocytes, indicated the absence of expression of non-IRE FPN1 transcripts (*data not shown*). Our results showed that *variant II* FPN1 transcripts were expressed at comparable levels to *variant I (IRE)* only in erythroblasts and erythroleukemic cell lines. The level of *variant II* FPN1 transcripts in differentiated CaCo2 cells was very low compared to the levels of *variant I (IRE)* expression.

Discussion

FPN1 has been reported to be expressed and to play a critical role in several different tissues involved in mammalian iron homeostasis, including duodenal enterocytes (iron uptake and export into the circulation); hepatocytes (storage); syncytiotrophoblasts (transfer to embryo) and reticuloendothelial macrophages (iron recycling from senescent red blood cells). Several lines of evidence suggested that FPN1 expression in these tissues is specifically regulated according to body iron requirements.[5,8,9,33-36] Moreover, the presence of a well-conserved IRE in the 5'-UTR of FPN1 mRNA indicated the possibility of post-transcriptional control through the IRP-IRE systems: FPN1 promoter/IRE was responsive to iron in HepG2 and CaCo2 cells;[28] *in vitro* conditions of iron deprivation inhibited translational efficiency of FPN1 mRNA.[6,8,27] Iron homeostasis depends on a co-ordinated regulation of the expression of molecules involved in the import of this element and those exporting it out of the cells. In some cell types, such as erythroid cells, iron import mechanisms are highly expressed, thus allowing massive iron uptake.[3,4,21] Excessive iron many, however, be toxic for these cells, particularly in view of this metal's capacity to generate superoxide radicals and H_2O_2, which may freely diffuse into the nucleus resulting in cell damage.[37] It therefore seemed of interest to investigate whether erythroid cells possess specific mechanisms for iron export. To our knowledge, this is the first report showing the presence of FPN1 mRNA and protein in normal human erythroid cells at all stages of differentiation. This finding is surprising, because the presence of a transporter, which in the other tissues is delegated to export iron, was not expected for red blood cells, where all iron is believed to be retained in the cell, committed to heme synthesis and to exit only after death of these cells by macrophage phago-

cytosis. Indeed, a block in heme synthesis by ALAS2 gene knockout results in iron accumulation in the cytoplasm of erythroblasts[38] and in this condition there is no indication of a cellular system able to mediate the exit of large amounts of iron from the cell. This observation strongly suggests that in erythroid cells the iron import mechanisms are much more efficient than the iron export pathways.

The IRE element in the 5'-UTR of FPN1 mRNA is functional and is able to mediate translational modulation by cellular iron levels. Nonetheless, FPN1 protein expression appears to remain at a constant level during different steps of erythroid differentiation and after iron-manipulating treatments. This paradox would be difficult to explain, except supposing the existence of a non-IRE FPN1 transcript in erythroid cells. Previous studies had already indicated the possibility of an IRE-independent regulation of FPN1 in different tissues and cell types: iron deficiency induces a significant increase of FPN1 mRNA expression in mouse, human and rat duodenum, both *in vivo* and *in vitro*,.[7,34,36,39] A non-IRE FPN1 transcript has been only described in polycythemic mice as an aberrant mRNA resulting from a microdeletion in the FPN1 gene promoter.[32,33] Interestingly, the elimination of the IRE in the FPN1 mRNA results in increased FPN1 protein levels in duodenum and liver.[32] We describe two alternative FPN1 transcripts (*variant II and III*), other than the IRE-containing canonical one , that do not contain the IRE element in their 5'-UT region, do not respond to iron treatment and together account for more than half of the total FPN1 mRNA present in erythroid cells. Interestingly, these transcripts are expressed mainly during the middle steps (4-11 days) of *in vitro* erythroid differentiation, corresponding to the maturation from late erythroid progenitors to polychromatophilic erythroblasts. At these stages of erythroid differentiation TfR1, the receptor responsible for iron entering erythroid cells, is strongly and increasingly expressed.[21] Therefore, the non-IRE (*variant II and III*) FPN1 transcripts are expressed when erythroid progenitor/precursor cells need to accumulate iron within the cells. The expression of the non-IRE FPN1 transcripts could produce a constant level of the transporter, unresponsive to the very high iron levels present in maturing erythroid cells. In contrast, IRE-containing FPN1 transcript is mainly expressed in undifferentiated erythroid progenitors and in mature terminal erythroblasts, suggesting its potential role selectively at these stages of erythroid differentiation.To explain the surprising finding that FPN1 protein expression is not responsive to iron conditions although about 50% of FPN1 is encoded by the IRE transcript, we speculated that the IRE of erythroid FPN1 mRNA might not be really iron-responsive, suggesting that FPN1 expression in different cell types might be quite different in response to iron status. A first hypothesis to explain FPN1 expression in erythroid cells concerns a possible role for non-IRE-produced FPN1 membrane-bound transporter as an iron exit mechanism having a great tolerance for iron levels. A possible reason for this could be the need to remove iron not bound to heme from the cells in order to avoid its toxic effects. The FPN1 transporter has been reported to be regulated by copper[30,31] (*and our results*) as well and other metal transporters have been indicated to have promiscuous substrate specificity.[40] Therefore, a second hypothesis would be a possible role for FPN1 in erythroid cells as a transporter of heterogeneous divalent metals or other substrates. A third hypothesis could be based on our observation that FPN1 protein appears to be localized at the level of the cytoplasm both in vesicles and in the cytosol, suggesting that FPN1 may be involved in the intracellular trafficking of iron between the cytosol and organelles. Additional detailed studies of FPN1 subcellular localization will be necessary to determine its potential role in iron trafficking in the cytoplasm of erythroid cells. FPN1 has also been reported to be localized in the cytoplasm of other cell types, such as Küpffer cells and enterocytes.[5] At present we are unable to determine whether the putative longer p615 and p645 FPN1 proteins have the same functions as canonical FPN1 because specific antibodies for these isoforms are not available. Furthermore, the overexpression of FPN1 seems to be toxic to cells *in vitro*,[8] making it difficult to generate stable cell lines constitutively expressing the protein. Our observation that non-IRE FPN1 transcripts seemed nearly exclusively expressed in erythroid cells could explain why previous studies were unsuccessful at detecting alternative transcripts in other cell types.[5,27] Finally, given that several lines of evidence suggest that iron has an important role in the control of cell cycle progression,[41] we speculate that the expression of alternative FPN1 transcripts might be cell cycle-dependent in human erythroid cells. In fact, we observed IRE-FPN1 transcripts in slowly proliferating cells, i.e., quiescent progenitor cells and erythroblasts at the end stages of maturation, while only non-IRE FPN1 transcripts were expressed in highly proliferating erythroid cells.

Recently, some authors demonstrated that hepcidin, a major regulator of iron metabolism, binds to FPN1 in tissue culture cells resulting in internalization and degradation of FPN1 and in decreased export of cellular iron.[42] The post-translational regulation of FPN1 by hepcidin may thus complete a homeostatic loop: iron regulates secretion of hepcidin, which in turn controls expression of FPN1 on the cell surface, which then reduces export of cellular iron.[42] Recent studies[14] have shown that hepcidin modulates FPN1 on macrophagic cells and it is conceivable that it could similarly control FPN1 expression in erythroid cells.

In conclusion, we would like to emphasize that the control of FPN1 expression by iron conditions in different cell types might be complex. In erythroid cells the regulation of FPN1 mRNA expression through the 5'-UTR IRE

mechanism might be silenced because in this cell type a high level of iron uptake is needed to accumulate large amounts of iron for optimal heme synthesis. A solution to this problem could be to use an upstream alternative promoter to produce mRNA species in which the 5'-UTR IRE could be spliced out or made non-functional. A comparison of the sequence of our additional 324 bp *variant II* mRNA and pcm mRNA previously reported in mice[32] showed a strong homology between the two thus strengthening our hypothesis. However, as suggested by the authors,[32] it would be interesting to investigate the possibility of regulatory mutations in various iron disorders, in particular when type IV hemochromatosis is present in the absence of coding region mutations.[32] Recently a new mutation located at the 5' non-coding region has been reported in a case of hemochromatosis.[43]

NMS planned the study together with LC. NMS and LC performed most of the experiments and wrote the manuscript. PS performed the molecular experiments. AC performed the protein analyses. FF performed the molecular experiments immunofluorescence and confocal analyses. CR performed immunofluorescence and confocal analyses. OM and MG performed in vitro unilineage cell cultures. UT critically reviewed the article for important intellectual content. NMS takes primary responsibility for the paper. All the figures were created by LC and NMS.

All the authors gave final approval for submission of the paper and are listed according to a criterion of decreasing individual contribution to the work. The authors declare that they have no potential conflicts of interest, and also wish to thank Dr. David Haile for the generous gift of the FPN1 antibody; Dr. A. Schumacher for providing and authorizing the publication of sequences of ferroportin-1 non-IRE transcripts from polycythemic mice; Giuseppe Loreto for his excellent assistance with the the graphs; Annalisa Rossini and Ernestina Saulle for their contribution during the beginning of the work; Ginevra Galbiati, Luca Pasquini and Eleonora Petrucci for their help during preparation of the degree thesis.

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