Regulation of divalent metal transporter 1 (DMT1) non-IRE isoform by the microRNA Let-7d in erythroid cells

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ABSTRACT

Background
Divalent metal transporter 1 (*DMT1*) is a widely expressed metal-iron transporter gene encoding four variant mRNA transcripts, differing for alternative promoter at 5' (DMT1 1A and 1B) and alternative splicing at 3' UTR, differing by a specific sequence either containing or lacking an iron regulatory element (+IRE and -IRE, respectively). DMT1-IRE might be the major DMT1 isoform expressed in erythroid cells, although its regulation pathways are still unknown.

Design and Methods
The microRNA (miRNA) Let-7d (miR-Let-7d) was selected by the analysis of four miRNAs, predicted to target the DMT1-IRE gene in $CD34^+$ hematopoietic progenitor cells, in K562 and in HEL cells induced to erythroid differentiation. Using a luciferase reporter assay we demonstrated the inhibition of DMT1-IRE by miR-Let-7d in K562 and HEL cells. The function of miR-Let-7d in erythroid cells was evaluated by the flow cytometry analysis of erythroid differentiation markers, by benzidine staining and by iron flame atomic absorption for the evaluation of iron concentration in the endosomes from K562 cells over-expressing miR-Let-7d.

Results
We show that in erythroid cells, DMT1-IRE expression is under the regulation of miR-Let-7d. DMT1-IRE and miR-Let-7d are inversely correlated with $CD34^+$ cells, K562 and HEL cells during erythroid differentiation. Moreover, overexpression of miR-Let-7d decreases the expression of DMT1-IRE at the mRNA and protein levels in K562 and HEL cells. MiR-Let-7d impairs erythroid differentiation of K562 cells by accumulation of iron in the endosomes.

Conclusions
Overall, these data suggest that miR-Let-7d participates in the finely tuned regulation of iron metabolism by targeting DMT1-IRE isoform in erythroid cells.

Key words: iron metabolism, DMT1-IRE, miRNAs.

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The online version of this article has a Supplementary Appendix.

Introduction

Divalent metal transporter 1 (*DMT1, Nramp2/DCT1*) is also known as *SLC11A2*, and its protein product is a widely expressed metal-iron transporter that is involved in iron absorption (in enterocytes) and use (in erythroid cells).[1-4] Transcription of the mammalian *SLC11A2* gene coding for DMT1 gives rise to four mRNA transcript variants that differ in their tissue distribution and regulation.[5] The 5'-end mRNA processing gives two isoforms: one starts from exon 1A, which is located upstream of the first identified exon ('1B'). Exon 1A (which contains a start codon) is followed by a consensus splice sequence, and it is spliced directly to exon 2.[5] Exon 1B is not translated, with translation beginning with a start codon in exon 2. While the 1B isoform is ubiquitous, the 1A isoform is tissue-specific, and is expressed predominantly in duodenum and kidney.[5] In addition, variable 3'-end mRNA processing yields two transcripts that differ in their 3'-translated and untranslated regions (UTRs).[3,6] One transcript contains an iron-responsive element (+IRE) in its 3'-UTR, which can alter the mRNA stability according to iron status.[7] The other transcript does not contain an IRE sequence (-IRE). Consequently, both forms are expected to differ mainly in their regulation by iron. Notably, both the exon 1A and IRE variants contribute to regulation according to iron status in duodenal and Caco-2 cells.[8-10] The C-terminus of the DMT1+IRE protein contains 18 amino-acid residues that substitute for the final 25 of the DMT1-IRE protein.

The DMT1 N- and C-termini might contain signal sequences that direct subcellular targeting of each of these four isoforms. Functional studies in *Xenopus* oocyte plasma membranes have demonstrated that they are equally efficient iron transporters, since for each isoform, iron transport activity correlates with abundance of the protein in oocyte plasma membranes.[11,12] Several studies have reported that tetracycline-inducible expression of the 1B/-IRE isoform is detected predominantly in intracellular compartments in HEK-293 cells and in reticulocytes and erythrocytes from normal mice and anemic mk/mk mice (with DMT1 mutation). The 1A/+IRE isoform is instead detected predominantly at the plasma membrane, and it is expressed in the proximal portion of the duodenum, where it is dramatically up-regulated by dietary iron deprivation.[10,13,14] Expression of the DMT1-IRE isoform in young red blood cells of normal mice and mk/mk mice suggests that the DMT1-IRE protein is involved in iron transport across the membranes of acidified endosomes into the cytoplasm of erythroid precursor cells, so this might be the major DMT1 isoform expressed in erythroid precursor cells.[10,13,14]

The discovery of a new class of small non-coding RNAs, the so-called microRNAs (miRNAs), has opened up other possible mechanisms for regulation of gene expression. MiRNAs are single-stranded RNAs of ~22 nucleotides, and in animals they exert their regulatory effects by binding to imperfect complementary sites within the 3'-UTRs of their mRNA targets, affecting the mRNA degradation or translation.[15-21] These miRNAs are involved in several important biological processes, such as developmental timing and patterning, apoptosis, hematopoietic differentiation, cell proliferation, organ development, and tumorigenesis.[15-21]

We have focused our attention on the possible miRNA regulation of iron metabolism. In particular, we have analyzed the regulation mechanism of DMT1-IRE by miRNAs. Our approach started with a search of the miRNA registry of the Sanger Institute and the Probability of Interaction by Target Accessibility (PITA) tool for miRNAs that target genes involved in iron metabolism.[22-24] Although many miRNAs have been implicated in hematopoiesis,[25] there are no previous studies on miRNA regulation of genes involved in iron metabolism. Here we have investigated DMT1-IRE in erythroid cells because the mechanism underlying regulation of its expression is still unknown. Moreover, we focused our attention on this isoform because we demonstrated the higher expression of DMT1-IRE than DMT1+IRE during erythroid differentiation. We have thus identified miRNA Let-7d (miR-Let-7d) as a regulator of DMT1-IRE expression in erythroid cells.

Design and Methods

The *Online Supplementary Appendix* provides information and data concerning: cell morphology, histological staining, and determination of hemoglobin content; RNA isolation and cDNA synthesis from $CD34^+$ cells; endosome extraction; flame atomic iron absorption; reticulocyte, granulocyte and lymphocyte separation; PITA tool; vector cloning; site-directed mutagenesis; cell transfections and luciferase assays; RNA isolation, cDNA preparation and quantitative real-time PCR; cell transfections and luciferase assays; creation of the stable clones in K562 cells and their erythroid differentiation; Western blotting.

Standard cell culture

The K562 human myeloid leukemia cell line and the HEL erythroleukemia cell line were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). K562 cells were maintained in Iscove's medium (Sigma Aldrich, Milan, Italy), supplemented with 10% fetal bovine serum (FBS) (Celbio Pero, Milan, Italy), 10 U/mL penicillin and 0.1 mg/mL streptomycin (Pen/Step) (Celbio Pero). HEL cells were maintained in RPMI medium (Sigma, Aldrich), supplemented with 10% FBS (Celbio Pero), and Pen/Strep (Celbio Pero). The two cell lines were grown in a humidified 5% CO_2 atmosphere at 37 °C.

K562 cells treatment with miRNA inhibitor

Chemically modified, single-stranded RNAs, modified antisense oligonucleotides were used to inhibit miRNA expression in K562 cells. Anti-miR Let-7d oligonucleotides (Quiagen) were transfected (100 μM) using the TransIT-LT1 transfecting reagent (Mirus, Bio Corporation, Madison, USA) according to the manufacturer's procedures. Scrambled anti-miR (100 μM) were transfected as negative control. The cells were collected 48h after the transfection to perform RNA and protein extractions.

TaqMan miRNAs assay

Reverse transcriptase reactions contained 40 ng RNA sample, 50 nM stem–loop reverse transcriptase primer, 1x reverse transcriptase buffer (P/N:4319981, Applied Biosystems, Branchberg, NJ, USA), 0.25 mM each of the dNTPs, 3.33 U/mL MultiScribe reverse transcriptase (P/N: 4319983, Applied Biosystems) and 0.25 U/mL RNase inhibitor (P/N: N8080119; Applied Biosystems). The 15-μL reactions were incubated in an Applied Biosystems 9700 Thermocycler for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C, and then held at 4°C.

Real-time (RT)-PCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7900HT Sequence

Detection System. The 20-μL PCRs included 2 μL reverse transcriptase product, 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems), 0.2 mM TaqMan probe (for miR-Let-7d and mU6). The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min.

Results

DMT1 expression during erythroid differentiation

To examine the role of DMT1 in human erythroid cells, we analyzed DMT1 mRNA expression by qRT-PCR, as the DMT1-IRE and DMT1+IRE isoforms in lymphocytes, granulocytes and reticulocytes extracted from the peripheral blood of healthy volunteers. As shown in *Online Supplementary Figure S1A*, reticulocytes showed significantly greater relative expression of DMT1-IRE compared to DMT1+IRE (P=0.0003). The same relative trends were seen at the protein level (*Online Supplementary Figure S1C*). These data confirmed that the mRNA and protein of DMT1-IRE is specifically expressed in the erythroid lineage. Furthermore, we isolated CD34$^+$ cells from the peripheral blood of healthy volunteers and we induced these cells to erythroid differentiation with erythropoietin for 14 days, as detailed in Design and Methods (see *Online Supplementary Figure S2A* for the quality controls of the erythroid maturation). We analyzed DMT1-IRE mRNA expression during erythroid differentiation on day 7, when the proerythroblasts expand and undergo maturation, and on day 14, when most of the cells in culture resemble late stage erythroblasts. As shown in *Online Supplementary Figure S1B*, DMT1-IRE mRNA levels rose rapidly with seven and 14 days of differentiation. Although the same trend was seen for DMT1+IRE, the increase in DMT1-IRE expression was significantly greater with 14 days of erythropoietin treatment (P=0.002). This trend was also seen at the protein level by Western blotting (*Online Supplementary Figure S1D*).

Furthermore, we analyzed the expression of DMT1 mRNA and protein in two *in vitro* cellular models: K562 cells, a model widely used to study hematopoietic cell growth and differentiation, and HEL cells. The K562 and HEL cells were differentiated through the erythroid lineage with hemin for six days, as confirmed by FACS analysis (*Online Supplementary Figure S2B and C*). After four and six days of differentiation, there was a gradual increase in protein levels of DMT1-IRE and DMT1+IRE, as seen for the CD34$^+$ cells at seven and 14 days, for both cell lines (*Online Supplementary Figure S2D*).

Expression analysis of microRNA Let-7d during erythroid differentiation

Several prediction programs have been developed to identify potential miRNA targets.[24] Our *in silico* analysis using the mirBase targets database (version 4)[22] was directed towards identification of miRNAs that can potentially target the DMT1-IRE. We identified four potential miRNAs: miR-15a, miR-15b, miR-223 and miR-Let-7d, and we analyzed their expression during erythroid differentiation of these CD34$^+$ cells (Figure 1A). MiR-15a and miR-15b were seen to be up-regulated at days 7 and 14 of erythroid maturation, whereas the levels of miR223 decreased gradually, but significantly, to days 7 and 14 of differentiation (day 14 *vs.* day 7; P=0.0004); miR-Let-7d was strongly down-regulated by day 7 (P=0.02) and day 14 (P=0.02) of erythropoietin (EPO) treatment (Figure 1A). Only miR-Let-7d and miR-223 showed an opposite trend with respect to DMT1-IRE mRNA and protein, which was up-regulated during erythroid differentiation (*Online Supplementary Figure S1B*). Furthermore, using the PITA tool (see *Online Supplementary Materials, and Design and Methods*), we analyzed the ΔΔG (energy-based score for miRNA–target interactions) for the binding of the four miRNAs to the 3'-UTR of DMT1-IRE mRNA.[23] The values of ΔΔG for miR-15a, miR-15b and miR-223 were very high (-8.80, -8.50, -7.22, respectively), and only the ΔΔG of miR-Let-7d was very low (-14.93), indicating good accessibility for miR-Let-7d to the 3'-UTR of DMT1-IRE.

We also analyzed miR-Let-7d expression in the K562 and HEL cells. As shown in Figure 2B, in the K562 cells, the expression of miR-Let-7d significantly and rapidly decreased by day 4 (P=0.02) and day 6 (P=0.03) of hemin treatment. The same trend was seen in the HEL cells, in which the expression of miR-Let-7d rapidly decreased by day 6 (P=0.04) of hemin treatment (Figure 1C).

Furthermore, we analyzed the phylogenetic conservation of miR-Let-7d binding site in DMT1-IRE 3'UTR in four species (see *Online Supplementary Figure S3* for a schematic representation that shows the location of the +IRE and –IRE sequences).

On the basis of these data described above, we then further studied the potential regulation by miR-Let-7d of the expression of DMT1-IRE in erythroid cells by miR-Let-7d.

MicroRNA Let-7D binds to the 3'-UTR of DMT1-IRE

To verify the bioinformatic predictions, a renilla luciferase reporter vector was constructed that contained the full-length 3'-UTR of human DMT1-IRE mRNA, the expression of which was driven by the thymidine kinase (Tk) promoter (Tk-ren/DMT1-IRE) (Figure 2A). Pre-miR-Let-7d was cloned into a mammalian expressing vector (pcDNA3.1). The reporter construct (Tk-ren/DMT1-IRE) and miR-Let-7d were co-transfected in the K562 cells, with the pGL3-CMV-firefly luciferase vector to normalize for transfection efficiency. Interestingly, the relative luciferase activity was markedly decreased (-40%) in cells co-transfected with the Tk-ren/DMT1-IRE construct and miR-Let-7d (P=0.005) which indicates miR-Let-7d binding to the 3'-UTR of the DMT1-IRE, resulting in decreasing luciferase protein expression. As a control, the Tk-ren/DMT1-IRE construct was mutated in the miR-Let-7d binding site (Tk-ren/DMT1-IRE-Mut). Here, co-transfection of Tk-ren/DMT1-IRE-Mut and miR-Let7d increased the luciferase activity which suggests that this absence of the miR-Let-7d binding site abrogates the inhibitory effect on luciferase translation (Figure 2A). As further control, a mutated miR-Let-7d in seed region was co-transfected with Tk-ren/DMT1-IRE demonstrating an increase in luciferase activity (Figure 2A). The equivalent data were obtained in the HEL and SHSY5Y cells (*data not shown*).

From analysis of a PITA algorithm, we found that also DMT1 +IRE isoform could be a target of miR-let7d. To investigate miR-Let-7d binding specificity for DMT1-IRE mRNA in comparison with the DMT1+IRE mRNA, a renilla luciferase reporter was also constructed that contained the full-length 3'-UTR of human DMT1+IRE (Tk-ren/DMT1+IRE). Under the same experimental conditions as for DMT1-IRE above, and as shown in Figure 2B, the co-transfection of Tk-ren/DMT1+IRE and miR-Let-7d did not decrease the luciferase activity, which indicates that

miR-Let7d does not bind the 3'-UTR of the DMT1+IRE mRNA. As a control here, the miR-Let-7d muated was co-transfected with Tk-ren/DMT1+IRE, demonstrating the absence of change in the luciferase activity. Thus we can conclude here that miR-Let-7d binds the 3'-UTR of DMT1-IRE mRNA in a manner that is specific for this DMT1 isoform, whereas it cannot bind to the 3'-UTR of DMT1+IRE mRNA.

MicroRNA Let-7d overexpression decreases human DMT1-IRE expression at the mRNA and protein levels in K562 and HEL cells

We then analyzed miR-Let-7d and DMT1 expression in the two erythroid cell lines, K562 and HEL cells. To determine whether miR-Let-7d targeted the endogenous DMT1-IRE mRNA for degradation, we carried out qRT-PCR on RNA isolated from the miRNA-transfected cells. As suggested by the classical miRNA activity, DMT1-IRE mRNA levels decreased in cells transfected with miR-Let-7d as compared to control cells at steady-state, for both K562 and HEL cells (Figure 3A; $^{**}P$=0.005, $^{*}P$=0.02). All of these experiments also had the negative control of miR-Let-7d mutated in the seed region.

The protein levels were analyzed for DMT1-IRE before and after miR-Let-7d transfection, using Western blotting. MiR-Let-7d provided a significant reduction in DMT1-IRE protein levels, as compared to the empty-vector controls, in both the K562 (P=0.000002) and HEL (P=0.01) cells (Figure 3B). Figure 3C shows the densitometry analysis of the relevant Western blotting. Taken together, these data demonstrate that miR-Let-7d decreases DMT1-IRE expression at mRNA and protein levels.

Moreover, we inhibited the endogenous levels of miR-Let-7d in K562 cells by an miRNA inhibitor, single-stranded, modified RNAs which, after transfection, specifically inhibited miRNA function. After 48h of transfection, we observed an increased expression of the target DMT1-IRE at mRNA and protein level (Figures 3D).

MiR-Let-7d belongs to a conserved cluster of miRNAs that maps to chromosome 9, which also includes miR-Let-7a and miR-Let-7f.[26] Such an miRNA cluster is defined as a group of miRNAs that is expressed from within one locus. The expression of this miR-Let-7d cluster (miR-Let-7d/ miR-Let-7a/ miR-Let-7f) was analyzed in the K562 cells that over-express the transgenic mir-let-7d, where the levels of mir-let-7a and mir-let-7f did not increase (*Online Supplementary Figure S4A*). Moreover, during erythroid differentiation of the K562 cells, miR-Let-7a and miR-Let-7f were up-regulated while miR-Let-7d was down-regulated, as shown above (*Online Supplementary Figure S4B*). These data demonstrate that individual mature miRNAs within this miRNAs cluster have differential transcriptional regulation in erythroid cells, as previously demonstrated in mammals during embryonic development.[26]

Inhibition of erythroid differentiation by microRNA Let-7d in K562 cells due to iron accumulation in endosomes

The inverse relationship between miR-Let-7d and DMT1-IRE levels in our expression experiments suggested a more complex relationship under physiological conditions. Therefore, to investigate a potential functional relationship between miR-Let-7d expression, iron metabolism and physiological erythropoiesis, several stable clones were created that over-expressed miR-Let-7d in the K562 cell line to improve the DMT1-IRE downregulation obtained by the transient transfection assay. The K562 cells were used here as they showed a better downregulation of DMT1-IRE at the protein level in the transient miR-let-7d transfection than Hel cells. Furthermore, K562

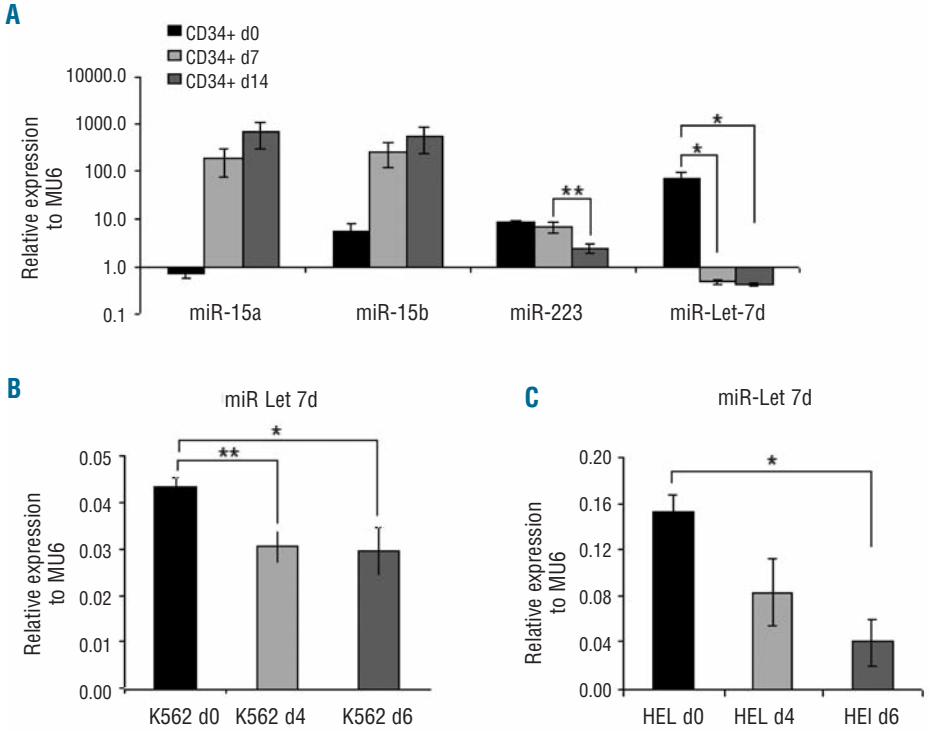

Figure 1. MiR-Let-7d during erythroid differentiation. (A) Relative expression of miRNAs 15a, 15b, 223 and Let-7d during erythroid differentiation of CD34^{+} cells induced by erythropoietin, on days 0 (d0), 7 (d7) and 14 (d14), from two cell pools, each from 4 healthy volunteers. $^{*}P$=0.02, $^{**}P$=0.0004. Data are representative of four independent experiments. (B) Relative expression of miR-Let-7d during erythroid differentiation of K562 cells induced by hemin, on days 0 (d0), 4 (d4) and 6 (d6). Data are representative of three independent experiments. $^{*}P$=0.013, $^{**}P$=0.005. (C) Relative expression of miR-Let-7d during erythroid differentiation of HEL cells induced by hemin, on days 0 (d0), 4 (d4) and 6 (d6). $^{*}P$=0.04. Data are representative of three independent experiments. P value >0.05 (not significant) are not shown.

cells represent the best cell line for use in studies of hematopoietic cell growth and differentiation. Four clones were obtained in which we analyzed the expression of mature miRNAs using a miRNA TaqMan assay (Figure 4A) determined the DMT1-IRE endogenous mRNA levels by qRT-PCR (Figure 4B). The data show that in the four K562 clones over-expressing miR-Let-7d, the levels of DMT1-IRE decreased, as compared to the empty-vector clone control. Furthermore, an analysis of DMT1 protein expression by Western blotting and relative densitometry (Figure 4C and D) confirmed the downregulation seen for the mRNA. The K562 cell clones 2 (C2), 3 (C3) and 4 (C4) showed significant downregulation of DMT1-IRE levels as compared to the K562 cell empty-vector clone (**P*=0.03, ***P*=0.004, ****P*=0.005, respectively); we selected these K562 cell C2 and C4 clones for the functional study because of the superior downregulation of DMT1-IRE protein levels.

The iron status of the K562 cell C2 and C4 clones was thus analyzed. Significant downregulation of ferritin heavy chain 1 (FTH1) (*P*=0.02) and significant upregulation of transferrin receptor C (TFRC) (*P*=0.0001) were seen in these miR-Let-7d over-expressing stable clones, as compared to the empty-vector clone. These data thus demonstrate the iron depletion that arises from this downregulation of DMT1-IRE (Figure 4E). The same trend was observed by FTH1 and FTL1 protein expression analysis (Figure 4F).

Late stages of erythroid maturation are characterized by heme synthesis, where the final step is the loading of a ferrous iron into the protoporphyrin IX group. To perform

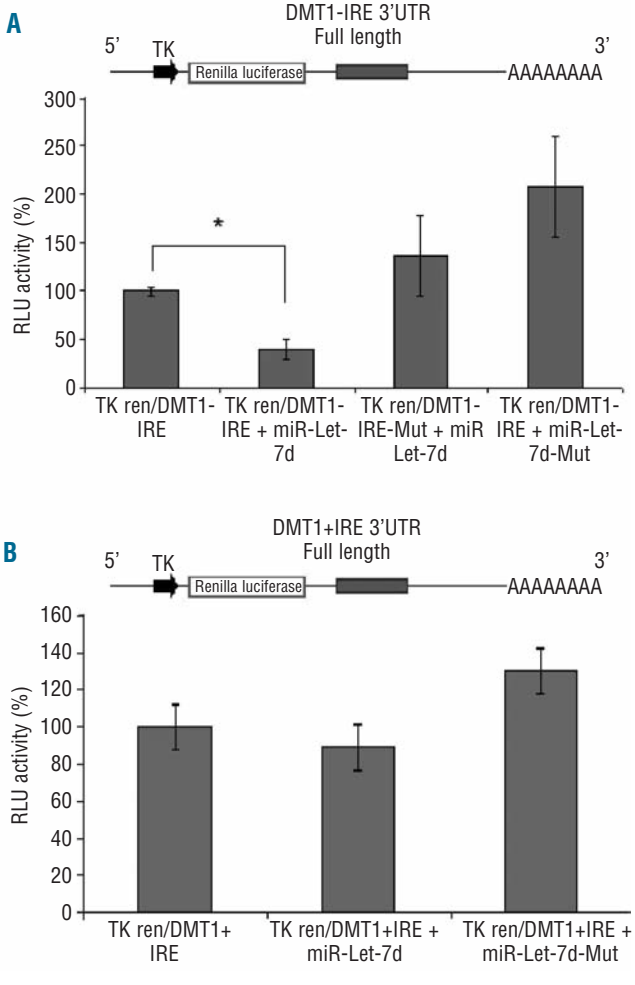

Figure 2. MiR-Let-7d specifically binds to the 3'-UTR of DMT1-IRE. (A) MiR-Let-7d regulates luciferase activity of a report vector containing full-length 3'-UTR of DMT1-IRE mRNA. Relative luciferase activity was assayed in K562 cells 48 h after co-transfection with a luciferase report plasmid and miR-Let-7d. As a control, the Tk-ren/DMT1-IRE construct was mutated in the miR-Let-7d binding site (Tk-ren/DMT1-IRE-Mut) and the mutated miR-Let-7d-Mut was used as further control. *P*=0.005. Data are representative of three independent experiments. (B) MiR-Let-7d does not regulate luciferase activity of a report vector containing full-length 3'-UTR of DMT1+IRE mRNA. Relative luciferase activity was assayed in K562 cells 48 h after co-transfection with a luciferase report plasmid and miR-Let-7d, with the mutated miR-Let-7d-Mut control. Data are representative of three independent experiments. *P* value >0.05 (not significant) are not shown.

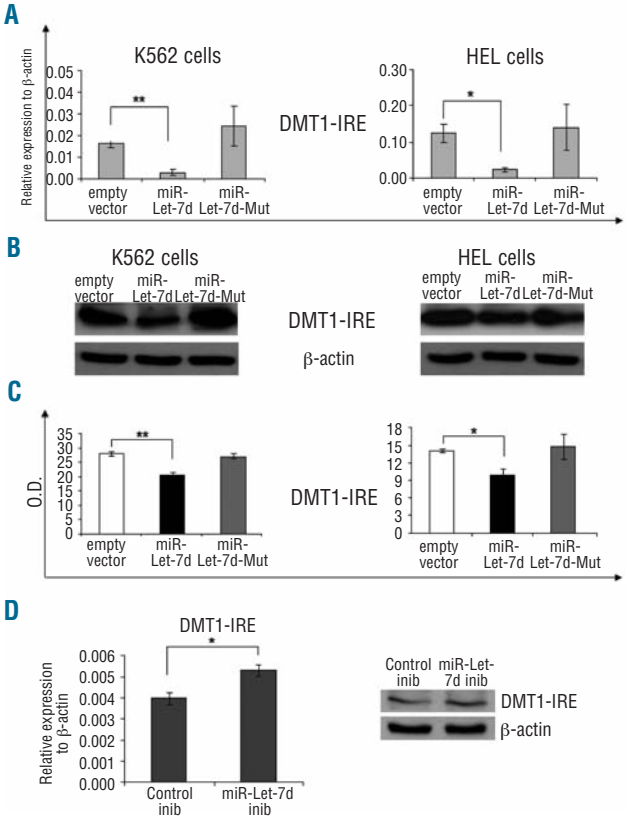

Figure 3. MicroRNA Let-7d overexpression decreases human DMT1-IRE expression. (A) Relative mRNA expression of DMT1-IRE in K562 and HEL cells 48 h after overexpression of miR-Let-7d. Data are representative of three independent experiments. ***P*=0.005, **P*=0.02. (B) Representative Western blots showing DMT1-IRE protein expression in K562 and HEL cells 48 h after overexpression of miR-Let-7d. (C) Protein expression relative to β-actin was quantified by densitometry analysis of Western blots as shown in (B). ***P*= 0.003, **P*=0.02. Data are representative of three independent experiments. (D) Relative mRNA expression of DMT1-IRE in K562 cells (on the left) 48 h after inhibition of miR-Let-7d with the relative negative control (CTR inhibitor). Data are representative of three independent experiments. **P*=0.01. Representative Western blots (on the right) showing DMT1-IRE protein expression in K562 cells 48 h after inhibition of miR-Let-7d with the relative negative control (CTR inhibitor). Data are representative of three independent experiments. *P* value >0.05 (not significant) are not shown.

this step, the DMT1-IRE protein is required, as an iron transporter in the cytosol. Our hypothesis is that erythroid differentiation is impaired if miR-Let-7d binds to the DMT1-IRE mRNA, thus repressing its expression. To investigate this hypothesis, the K562 cell C2 and C4 stable clones were assayed for differentiation following their treatment with hemin. After four and six days of differentiation, these C2 and C4 clones showed lower levels of glycophorin (a marker of erythroid differentiation) as compared to the empty-vector clone (Figure 5A). Moreover, the C2 and C4 clones showed lower levels of benzidine-positive cells, as compared to the empty-vector clone (Figure 5B). This test for the peroxidase activity of the cells reflects their hemoglobin content, and thus the C2 and C4 clones over-expressing the miR-Let-7d had less hemoglobin. Furthermore, when we analyzed the expression of the *HBG* gene (which encodes the γ-globin chain of hemoglobin) as another marker of erythroid differentiation, *HBG* expression was down-regulated in the C2 and C4 clones as compared to the empty-vector clone (Figure 5C).

Taken together, these data demonstrate a reduction in erythroid differentiation in these K562 cell C2 and C4 clones.

We also hypothesized that impaired differentiation of the clones over-expressing miR-Let-7d was due to iron accumulation in endosomes. Here, we isolated endosomes from the K562 cell C2 and C4 clones and the empty-vector clones. We verified that the preparations were enriched in endosomes by analysis of the endosome marker EEA1; furthermore, the purity of the extraction was evaluated by using GRP78 protein, a marker of endoplasmic reticulum (*Online Supplementary Figure S5*). We then quantified the iron content of these endosomes: in these C2 and C4 clones, the iron concentrations were significantly increased as compared to the empty-vector clone (P=0.02, P=0.01, respectively) (Figure 5D). This thus demonstrates that the downregulation of DMT1-IRE (by the miR-Let-7d overexpression) impedes the transport of iron from the endosome to the mitochondria for heme synthesis (Figure 5D). The overexpression of miR-Let-7d thus decreased the level of DMT1-IRE, causing iron accumulation in the endosomes which affects the iron available for use by the erythroid cells for heme synthesis.

Discussion

Tightly regulated iron homeostasis is essential for maintenance of life in eukaryotic organisms. Stable plasma concentrations and adequate levels of cellular iron are maintained through a balance of multiple processes that regulate its absorption, transport, storage and recycling.[27,28] DMT1 has a crucial role in this iron homeostasis because it is an H^+/divalent metal symporter that is involved in intestinal non-heme iron uptake, as well as in the peripheral iron cycle, as seen in erythroblasts.[1-3] DMT1 expression is finely regulated by different mechanisms. One involves the iron-responsive element (+IRE) in the 3'-UTR of the DMT1+IRE which can alter mRNA stability according to iron status.[3,6,7] The other concerned the two motifs (CCAAAGTGCTGGG) in its 5'-regulatory region that are similar to hypoxia-inducible factor 1 (HIF-1) binding sites.[29] Lis *et al.* demonstrated that hypoxia selectively increases the expression of exon 1A of DMT1, with lesser increases seen in both the +IRE and –IRE isoforms in rat pheochromocytoma (PC12) cells.[30] Recently, intestinal HIF-2 signaling was shown to be critical in the regulation

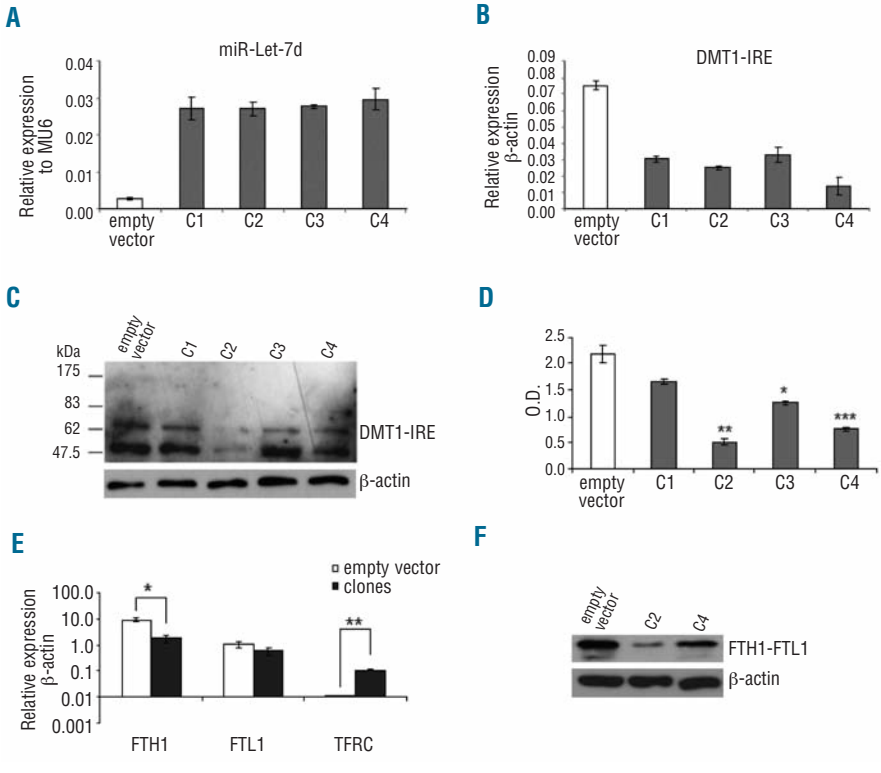

Figure 4. MiR-Let-7d stable clones (A) Relative expression of miR-Let-7d in the four stable K562 clones, and in the empty-vector clone. (B) Overexpression of miR-Let-7d in the four stable K562 clones down-regulates DMT1-IRE mRNA expression, as compared to the empty-vector clone. (C) Representative Western blot showing DMT1+IRE and DMT1-IRE protein expression in the four K562 stable clones over-expressing miR-Let-7d, and in the empty-vector clone. (D) Protein expression relative to β-actin quantified by densitometry analysis of Western blots, as shown in (C). **P*= 0.03, ***P*=0.004, ****P*=0.005. Data are representative of three independent experiments. (E) Relative expression of the FTH1, FTL1, and TFRC genes related to iron status in the two K562 stable clones (C2 and C4), and in the empty-vector clone. **P*=0.02, ***P*=0.0001. (F) Representative Western blot showing FTH1 and FTL1 protein expression in K562 stable clones C2 and C4 over-expressing miR-Let-7d, and in the empty-vector clone. Data are representative of three independent experiments. *P* value >0.05 (not significant) are not shown.

of DMT1 expression following iron deprivation in a mouse model.[31]

Another step in DMT1 regulation involves protein modifications by ubiquitination and proteasome degradation. Foot *et al.* recently demonstrated that Ndfips regulates DMT1 by acting as an adaptor for the recruitment of the ubiquitin ligase WWP2, and by enhancing the ubiquitination of DMT1 and its subsequent degradation via the lysosome and proteasome.[32]

In the present study, we have defined and investigated a new level of regulation of DMT1-IRE that is mediated by an miRNA. MiRNAs are now well recognized as important posttranscriptional regulators of gene expression.[15-21] Their mechanisms of action have been intensively studied more recently, and their effects are known to be exerted primarily through suppression of mRNA translation or by promotion of the physical destruction of mRNA after the miRNA hybridizes with the 3'-UTR of their mRNA target(s).[15-21]

We have demonstrated that in erythroid cells, DMT1-IRE is expressed to greater levels than DMT1+IRE, and that expression of DMT1-IRE is increased during erythroid differentiation of $CD34^+$ cells, and of K562 and HEL cells. This trend of expression is the opposite to that of miR-Let-7d, a putative regulator of DMT1-IRE. In this context, we first examined whether miR-Let-7d can mediate the repression of a luciferase/DMT1-IRE 3'-UTR reporter construct in K562 and HEL cells. Interestingly, miR-Let-7d produced a 60% decrease in the luciferase activity, indicating that it binds to the 3'-UTR of the DMT1-IRE mRNA and decreases the luciferase protein translation. Then we evaluated the regulation of DMT1-IRE in K562 and HEL cells, our two models of erythroid differentiation, after overexpression of miR-Let-7d. Here, we have shown that the endogenous DMT1-IRE is downregulated by miR-Let-7d in both of these cell lines, at the mRNA levels, as previously described for other members of the miR-Let-7 family.[26] Furthermore, the inhibition of the endogenous levels of miR-Let-7d is able to increase the expression of DMT1-IRE, showing the direct relationship of the miRNA with its target under physiological conditions.

To study the regulation of DMT1-IRE by miR-Let-7d, we created several stable clones in the K562 cells that over-expressed miR-Let-7d. The four stable K562 clones showed reductions in DMT1-IRE at both the mRNA and protein levels. Two of these stable K562 clones were induced to erythroid differentiation to determine whether DMT1-IRE downregulation by miR-Let-7d can induce iron depletion, and thus impair erythroid differentiation. Indeed, in the stable K562 cell C2 and C4 clones induced to erythroid differentiation, expression of the marker antigen of erythroid cells, glycophorin, was seen to decrease as compared to the empty-vector clone. Furthermore, the C2 and C4 clones for miR-Let-7d showed downregulation of γ-globin expression as compared to the control, empty-vector clone. They also showed decreased levels of benzidine-positive cells, thus showing a reduction in their hemoglobin content, confirming that they differentiate poorly towards the erythroid lineage.

To validate our hypothesis, we analyzed the iron concentrations in the endosomes derived from the empty-vector and these miR-Let-7d-over-expressing K562 cell C2 and C4 clones. As expected, there was a significant increase in iron concentration in the endosomes from the C2 and C4 clones as compared to the empty-vector clones. These data demonstrate that downregulation of DMT1-IRE by miR-Let-7d impairs erythroid differentiation of the K562 cells because of diminished effectiveness of the transferrin cycle at releasing iron from the endosomes. As a result, there would be decreased iron availability to the mitochondria, where iron is essential for heme synthesis, a fundamental step in erythroid differentiation. Indeed, it is known that developing erythroid cells take up vast amounts of iron, there is some evidence that it is then delivered directly from transferrin-containing endosomes to the mitochondria.[33]

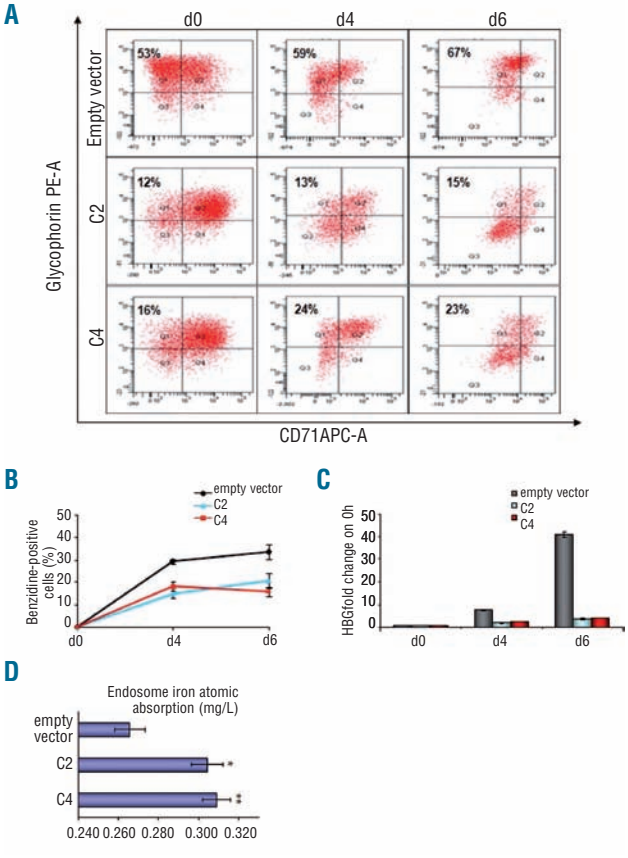

Figure 5. MiR-Let-7d inhibits erythroid differentiation of K562 cells. (A) FACS analysis for the CD71 and CD325 (glycophorin) markers of the K562 C2 and C4 stable clones over-expressing miR-Let-7d and the empty-vector clone during erythroid differentiation induced by hemin, on days 0 (d0), 4 (d4) and 6 (d6), with the relative percentages of positive cells as indicated. Data are representative of three independent experiments. (B) Time courses for benzidine-positive cells on days 0 (d0), 4 (d4) and 6 (d6) after hemin treatment, in the K562 C2 and C4 stable clones over-expressing miR-Let-7d and the empty-vector clone. At least 200 cells were counted in triplicate in two experiments. Stable clones showed a lower percentage of benzidine-positive cells during erythroid differentiation when compared to empty-vector clone (at d4: C1 *vs.* empty vector, P=0.00009; C4 *vs.* empty vector, P=0.0003. At d6: C1 *vs.* empty vector, P=0.02; C4 *vs.* empty vector, P=0.0005). (C) Expression analysis of the *HBG* gene during erythroid differentiation induced by hemin in the K562 C2 and C4 stable clones over-expressing miR-Let-7d and the empty-vector clone, on days 0 (d0), 4 (d4) and 6 (d6). Data are representative of three independent experiments. (C) Concentrations of iron in the endosomes of K562 C2 and C4 stable clones over-expressing miR-Let-7d and the empty-vector clone, determined by flame atomic absorption. *P=0.02. **P 0.01). Data are representative of three independent experiments. P value >0.05 (not significant) are not shown.

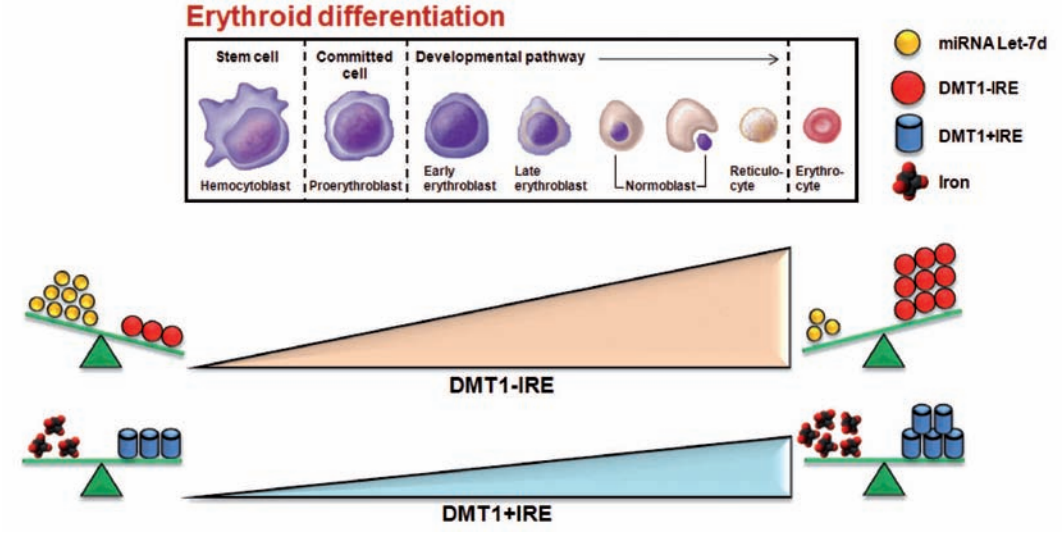

Figure 6. Schematic model of the regulation of DMT1-IRE and +IRE isoforms during erythroid differentiation The model represents both DMT1 isoforms and their increased expression during erythroid differentiation. DMT1-IRE isoform expression increases significantly more at the last stage of erythroid differentiation in respect to DMT1+IRE. The first observation is that DMT1-IRE has a markedly higher expression to than DMT1+IRE as shown by the bigger arrow in pink in respect to the blue one. We assume the existence of two different types of regulation that can specifically determine the levels of the two isoforms. DMT1-IRE expression levels are regulated by miR-Let-7d. MiR-Let-7d shows an opposite expression trend compared to DMT1-IRE; when the levels of miRNA increases, the expression of DMT1-IRE is inhibited and vice versa, as shown by the balance. On the other hand, the expression of DMT1+IRE is instead regulated by the iron concentration. When the cellular concentration of the iron increases, the DMT1+IRE mRNA is stabilized by the iron binding to IRE sequence and as a consequence the levels of DMT1+IRE rise. The panel showing the erythroid differentiation is modified from a picture covered by Copyright © 2001 Benjamin Cummings, an imprint of Addison Wesley Longman, Inc.

The phenotype seen in the clones that over-express miR-Let-7d resembles that of microcytic anemia (mk) mice[4] and Belgrade rats,[34] with both of these models carrying the same mutation in DMT1.[4,6] Indeed, these rodents show severe microcytic, hypochromic anemia due to a defect in iron uptake in the intestine, and also in iron acquisition and use in red blood cell precursors.[2,6] The mouse model with a completely inactivated murine DMT1 gene (DMT1 knock-out mice) shows that erythrocytes are the specific blood lineage involved in this defect.[35] Peripheral blood smears from these DMT1 knock-out mice show an abnormal erythropoiesis morphology that is consistent with iron deficiency erythropoiesis.[35]

Even in humans, DMT1 deficiency results in microcytic anemia, with three affected families described to date.[14,36,37] Priwitzerova *et al.* analyzed isolated erythroid progenitors from peripheral blood and bone marrow of a patient with a DMT1 mutation, using *in vitro* colony-forming assays.[38] The numbers and the sizes of the erythroid colonies from this patient where smaller compared to those of the healthy volunteers, and they showed low cell contents and abnormal morphology. Addition of iron chelaters to these cell cultures might correct the poor growth of the erythroid colonies from this patient.[38]

MiR-Let-7d adds a new step in the fine regulation of iron metabolism by DMT1-IRE in erythroid cells (see schematic model in Figure 6).

Further studies are needed to determine whether miR-Let-7d is another regulator of DMT1 in neurons where it plays a crucial role; indeed, failure of DMT1 regulation has been linked to human brain pathologies, such as Parkinson's disease.[39,40] The objectives of future studies should include verification of its implication in several pathologies linked to disorders in iron metabolism.

Authorship and Disclosures

IA designed and conducted most of the experiments, and prepared the manuscript; LDF designed and performed all the erythroid differentiation experiments and the creation of the stable clones; RA performed all the vector clonings, the endosome extraction, and the Western blotting experiments; RR performed all the statistical analysis and redefined the figures; SC contributed to the Western blotting experiments; MG performed the FACS analysis; MZ contributed to the preparation of the manuscript; AI designed and conducted the study, and prepared the manuscript.

The authors reported no potential conflicts of financial interests.

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