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

Immacolata Andolfo,[1] Luigia De Falco,[1] Roberta Asci,[1] Roberta Russo,[1] Simona Colucci,[1] Marisa Gorrese,[1] Massimo Zollo,[1,2] and Achille Iolascon[1,2]

[1]CEINGE, Biotecnologie Avanzate, Naples, and [2]Department of Biochemistry and Medical Biotechnologies, "Federico II" University of Naples, Naples, Italy

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Supplemental Design and Methods

RNA isolation and cDNA synthesis from CD34+ cells

Total RNA was isolated from CD34+ cells during erythroid differentiation on days 0, 7 and 14. RNA isolation was performed according to Chomczynski and Sacchi.[1] cDNA was synthesized using 2 μg RNA as template, 2.5 units VILO reverse transcriptase (Invitrogen, La Jolla, CA, USA), and 2.5 μM oligo-dT as primer, in a total final volume of 20 μL.

Cell morphology, histological staining, and determination of hemoglobin content

For histological analysis, erythroblasts were cytocentrifuged at various stages of maturation onto glass slides and stained with histological dyes (May-Grunwald-Giemsa) and neutral benzidine for hemoglobin as previously described.[2] The cell antigen profile was analyzed by flow cytometry through evaluation of CD34, CD71 (proerythroblasts) and CD235a (proerythroblasts and orthochromatic erythroblasts). Monoparametric forward scatter (FSC) analysis was performed in order to evaluate the cell size during erythroid differentiation by flow cytometry. Analyses were performed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) with CELL Quest software, version 3.3, after gating for viable cells.

Endosome extraction

Endosome extractions were performed as previously described by Tjelle *et al.*[3] Briefly, approximately 8×10^6 cells (K562 empty vector, K562 C2 and C4 clones) were washed twice in cold phosphate-buffered saline and centrifuged at 1,000x g for 10 min at 4°C. The pellets were resuspended in 600 μL homogenization buffer (HB-EDTA: 250 mM sucrose, 3 mM imidazole, 0.5 mM EDTA, pH 7.4) and lysed by passing 10 times through a G22 needle. Lysates were monitored at each step by optical microscopy. These lysates were centrifugated at 1,000x g for 10 min at 4°C. The post-nuclear supernatants were recovered and taken to a final sucrose concentration of 40.6% using 62% sucrose in HB-EDTA. The samples were subsequently introduced to the bottom of an ultracentrifuge tube; 1.5 mL 35% sucrose in HB-EDTA was layered into the tubes, followed by 1 mL 25% sucrose in HB-EDTA. The tubes were finally filled with HB without EDTA (pH 7.4) and centrifuged for 2,000x g for 1h at 4°C (SW-41 rotor, Beckman Coulter, Optimal L-80 XP ultracentrifuge). Late endosomes were recovered at the interface between 25% sucrose in HB-EDTA and HB without EDTA, and early endosomes were recovered at the interface between 35% sucrose in HB-EDTA and 25% sucrose in HB-EDTA.

Flame atomic iron absorption

Iron content of the endosomes was determined by atomic absorption flame emission spectroscopy (Perkin Elmer Mod Analyst 800 spectrometer) according to the procedure of Kreeftenberg *et al.*[4,5] Briefly, endosomes extracted from the same number of cells (8×10^6 cells) were washed with phosphate-buffered saline, resuspended in 200 μL aliquots, and digested with 0.5 mL destruction solution (perchloric acid/nitric acid, 4:1) overnight at 60°C. The final aliquot samples were diluted to 4 mL with distilled water and iron content was quantified by atomic absorption analysis. The iron concentrations of the samples were calculated by interpolation from a standard curve (of known iron reference standards).

Reticulocyte, granulocyte and lymphocyte separation

Reticulocytes were extracted according to a previously described protocol.[6] Briefly, whole blood samples were centrifuged at 1,700x g for 10 min at 4°C and the plasma removed. The mature red blood cells were lysed in a cold pre-mixed solution of five volumes of 0.144 M NH_4Cl and 0.5 volume 0.01 M NH4HCO3. The resulting suspension was chilled in ice for 30 min before centrifugation at 1,700x g for 10 min at 4°C to separate lymphocytes and granulocytes. The supernatant was transferred to another tube and 0.1 volume of 1.5 M sucrose and 0.15 M KCl solution was added to the supernatant; the cell suspension was then centrifuged at 4,800x g for 20 min at 4°C. The supernatant was then transferred to a clean tube and 700 μL 10% acetic acid was added. Finally, the reticulocytes were pelleted by centrifugation at 4,800x g for 20 min at 4°C.

The PITA tool

The PITA tool is an algorithm that is used to predict miRNA targets. It is based on a parameter-free model for microRNA-target interactions that computes the differences between the free energy gained from the formation of the microRNA-target duplex and the energetic cost of unpairing the target to make it accessible to the microRNA. The site accessibility is as important as the sequence match in the seed for determining the efficacy of microRNA-mediated translational repression, and the effective microRNA binding also requires the local region flanking the target to be unpaired. The energy-based scores for microRNA-target interactions, $\Delta\Delta G$, predicted by the PITA tool are equal to the difference between the free energy gained by the binding of the microRNA to the target, ΔG_{duplex}, and the free energy lost by unpairing the target-site nucleotides, ΔG_{open}.

Vector cloning

Pre-miR-Let-7d was cloned into a pcDNA3.1 vector (Promega, Madison, WI, USA) using the EcoRI–XhoI restriction sites. The full-length DMT1 3'-UTRs of both of the mRNA isoforms were cloned into the pRL-TK vector (Promega) downstream of the coding region of renilla luciferase in the XbaI site. The 3'-UTR of DMT1-IRE and DMT1+IRE were amplified from genomic DNA. The primer sequences are available upon request.

Site-directed mutagenesis

The site-directed mutagenesis of the miR-Let-7d binding site of DMT1-IRE and +IRE mRNA 3'-UTR and of the seed region of miR-Let-7d were generated according to the manufacturer's protocol of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The following primers were used for the mutagenesis (only the sense sequences are given, with the mutated nucleotides within the consensus sequence underlined): 3'-UTR DMT1-IRE-Mut: CACTGTAACCGCAAACACCCCGGTTTAATCGATTC; and miR-Let-7d-Mut: CCTAGGAACACGAATTAGATTGCATAGTTTTAG.

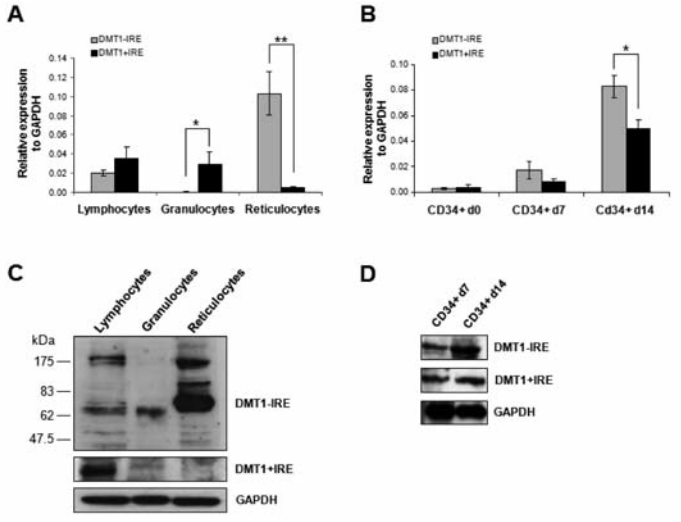
Cell transfections and luciferase assays

To evaluate the activity of miR-Let-7d on the repression of the full-length luciferase 3'-UTR constructs, the pcDNA3.1 miR-Let-7d construct (0.5 μg) and the pRL-TK 3'-UTR target construct (0.1 μg) were co-transfected into K562 and HEL cells, using the TransIT-LT1 transfecting reagent (Mirus, Bio Corporation, Madison, USA) with a pGL3 CMV firefly luciferase vector for normalization. Luciferase activities were analyzed using a Dual Luciferase Reporter Assay system (Promega, Madison, WI, USA).

Creation of the stable clones in K562 cells and their erythroid differentiation

K562 cells (1×10^6) were transfected using the TransIT-LT1 transfecting reagent (Mirus, Bio Corporation, Madison, USA) according to the manufacturer's recommendations. For both transfection methods, four groups of cells were transfected: K562 cells without the pcDNA 3.1 vector (control cells), K562 cells transfected with the negative control (empty vector), K562 cells transfected with miR-Let-7d (Mir Let-7d WT cells), and K562 cells with miR-Let-7d-Mut (Mir Let-7d mut cells). Following transfections, 600 mg/mL neomycin (Invitrogen) were added to the culture medium of the K562 cells to select the populations of cells that stably expressed the negative control or miR-Let-7d. After the death of the control K562 cells (approximately 14 days after neomycin addition), as evaluated by Trypan Blue dye exclusion (0.4% w/v; Invitrogen), 50 μM hemin (Sigma, St Louis, MO, USA) was added to the culture medium of the wild-type, negative control and miR-Let-7d K562 cells (2×10^5/mL). During the culture of these K562 cell groups, samples were collected at specific time points: before transfection, before hemin addition, and at days 4 and 6 after hemin addition. Erythroid differentiation was assessed by FACS analysis for transferrin receptor 1 (CD71) and glycophorin A (CD235A), and benzidine staining for the more mature erythroid cells. Gamma-globin (HBG) mRNA was also detected by RT-PCR, and its mRNA levels served as an indicator of undifferentiated and more mature erythroid cells.

The total samples evaluated in the present study were obtained from three independent experiments, each performed in duplicate, as described above for each transfection method.



Supplementary Figure S1. DMT1 expression in blood cells and during erythroid differentiation. (A) Relative mRNA expression of DMT1+IRE and DMT1-IRE in lymphocytes (n = 7), granulocytes (n = 7) and reticulocytes (n = 10) from healthy volunteers. *P=0.02, **P=0.0003. (B) Relative expression of DMT1+IRE and DMT1-IRE 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=0.002. Data are representative of four independent experiments. (C) Representative Western blot of protein expression of DMT1+IRE and DMT1-IRE in lymphocytes, granulocytes and reticulocytes from pooled cells from healthy volunteers. Data are representative of three independent experiments. (D) Representative Western blot of protein expression of DMT1+IRE and DMT1-IRE on days 7 (d7) and 14 (d14) of erythroid differentiation of CD34$^+$ cells from two cell pools, each from 4 healthy volunteers. Data are representative of three independent experiments. P value >0.05 (not significant) are not shown.

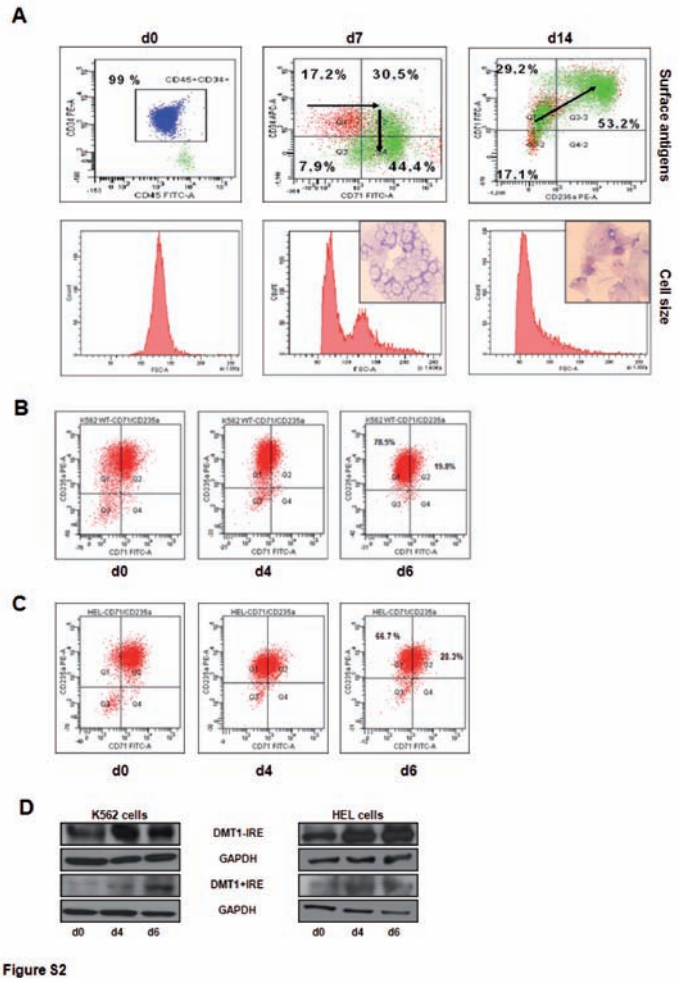


Figure S2

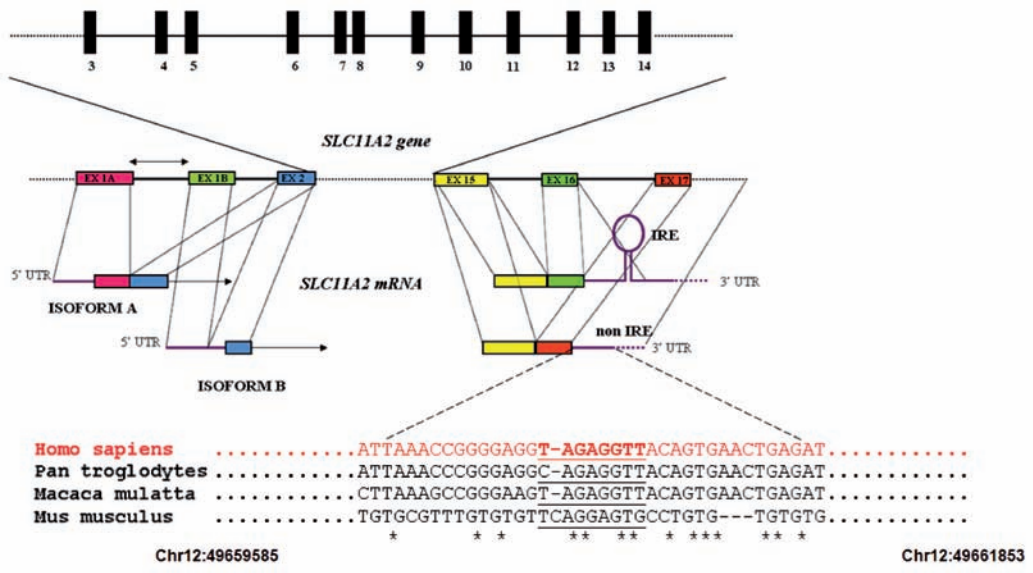
Supplementary Figure S2. Erythroid differentiation analysis in CD34$^+$ cells, K562 and HEL cell lines. (A) FACS analysis of CD34$^+$ cells during erythroid differentiation induced by erythropoietin, on days 0 (d0), 7 (d7) and 14 (d14), with the relative percentages of positive cells as indicated, according to the CD71 and CD235 markers. Monoparametric forward scatter (FSC) analysis showed the progressive reduction in CD34$^+$ cell size at days 0, 7, 14 of culture with the relative May–Grunwald–Giemsa staining at 7 and 14 days in the boxes. (B) FACS analysis as in (A), for K562 cells during erythroid differentiation induced by hemin, on days 0 (d0), 4 (d4) and 6 (d6). (C) FACS analysis as in (B), for HEL cells during erythroid differentiation. (D) Representative Western blot of DMT1+IRE and DMT1-IRE during erythroid differentiation of the K562 cells (left panel) and the HEL cells (right panel), on days 0 (d0), 4 (d4) and 6 (d6) after hemin treatment. Data are representative of three independent experiments.

RNA isolation, cDNA preparation and quantitative real-time PCR

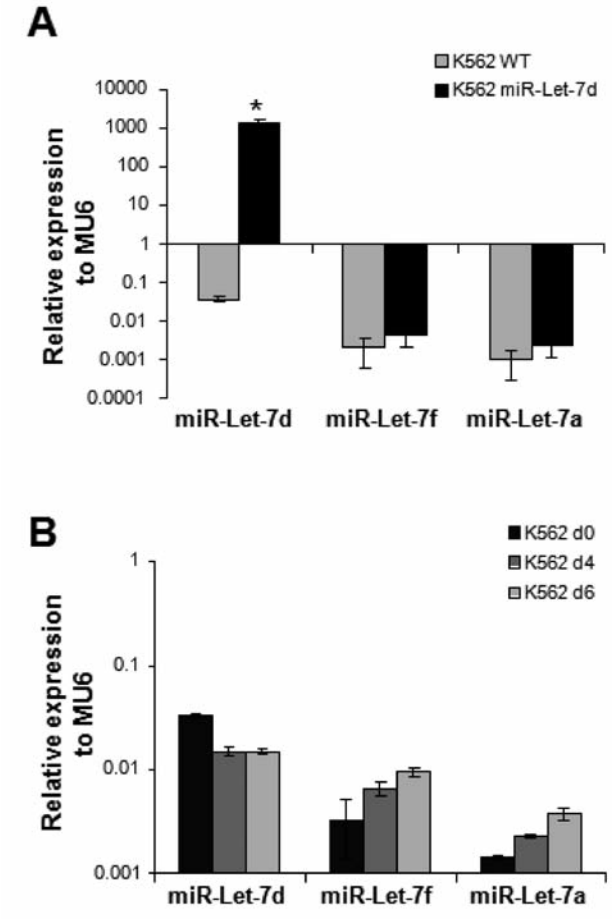
Total RNA was extracted from cell lines, lymphocytes, granulocytes and reticulocytes using Trizol reagent (Invitrogen). Super Script II First Strand kits (Invitrogen) was used for synthesis of cDNA from total RNA (2 μg). Quantitative RT-PCR (qRT-PCR) was performed using the SYBR-green method following standard protocols with an Applied Biosystems ABI PRISM 7900HT Sequence Detection system. Relative gene expression was calculated using the $2^{(-\Delta Ct)}$ method, where Δt indicates the differences in the mean Ct between selected genes and the internal control. The GAPDH or β-actin genes were the internal controls for each gene (Figure 4 and *Online Supplementary Figure S1*). Mean fold-change was calculated using the $2^{-(\text{average }\Delta\Delta Ct)}$ method,[7] where ΔΔCt is the mean difference in the ∅Ct between the genes and the internal control. QRT-PCR primers for each gene were designed using Primer Express software version 2.0 (Applied Biosystems). Primer sequences are available upon request. The significance of the gene expression differences were determined using the Student's t-test; statistical significance was established at $P<0.05$. All statistical analysis was performed by Excel included in Microsoft Office 2007.

Western blotting

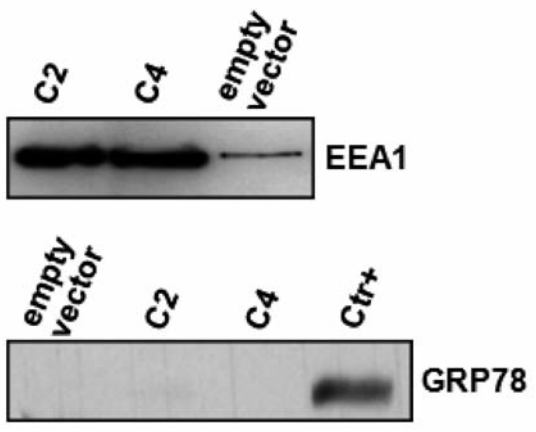
Thirty μg total lysates were loaded onto 12% polyacrylamide gels, and blotted onto polyvinylidene difluoride membranes (BioRad, Milan, Italy). The membranes were then incubated with a polyclonal rabbit anti-DMT1+IRE antibody (Alpha Diagnostics) (1:500) and with a polyclonal rabbit anti-DMT1 -IRE antibody (Alpha Diagnostic) (1:500). Anti-β-actin and anti-GAPDH antibodies (1:1,000) (Sigma) were used as controls for equal loading of total lysates. An anti-EEA1 antibody (AbCam) (1:1,000) was used as a marker of endosome purity and an anti-GRP78 antibody (Santa Cruz) (1,500). The bands were quantified by densitometry to obtain optical densities (ODs) which were then normalized with respect to the normalized protein OD. We used the GS-800 Calibrated Densitometer and the Quantity One 4.6.3 program (BioRad, Milan, Italy) for densitometric analyses. The significance of protein expression differences were determined using the Student's t-test. Statistical significance was established at $P<0.05$.



Online Supplementary Figure S3. Phylogenetic conservation of miR-Let-7d binding site in the DMT1-IRE 3'UTR. Schematic representation that shows the location of the DMT1+IRE and DMT1-IRE sequence, the let7d binding site with the phylogenetic conservation of the binding site among four species analyzed. Evolutionary conservation of miR-Let-7d binding site across four species analyzed. Multiple sequence alignment was performed using ClustalW2, an on-line freeware tool (*http://www.ebi.ac.uk/Tools/clustalw2/index.html*); *nucleotides identical in all sequences in the alignment.



Online Supplementary Figure S4. Gene expression analysis of the Let-7 cluster in the K562 cells. (A) Relative expression of the miRNA cluster: miRNAs Let-7d, Let-7f and Let-7a, in wild-type K562 cells and with their overexpression of miR-Let-7d. (B) Relative expression of the miRNA cluster as for (A), during erythroid differentiation of the K562 cells induced by hemin, on days 0 (d0), 4 (d4) and 6 (d6).



Online Supplementary Figure S5. EEA1 and GRP78 expression in endosomes from K562 C2 and C4. Representative Western blot showing EEA1 (endosomes marker) expression in the endosomes from the K562 C2 and C4 stable clones over-expressing miR-Let-7d, and the empty-vector clone and GRP78 (endoplasmatic reticulum marker) in the endosomes from the K562 C2 and C4 stable clones over-expressing miR-Let-7d, in the empty-vector clone and in a positive control (CTR+ K562 WT whole lysate).

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