Erythropoietin-driven signaling ameliorates the survival defect of DMT1-mutant erythroid progenitors and erythroblasts

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ABSTRACT

Background
Hypochromic microcytic anemia associated with ineffective erythropoiesis caused by recessive mutations in divalent metal transporter 1 (DMT1) can be improved with high-dose erythropoietin supplementation. The aim of this study was to characterize and compare erythropoiesis in samples from a DMT1-mutant patient before and after treatment with erythropoietin, as well as in a mouse model with a DMT1 mutation, the *mk/mk* mice.

Design and Methods
Colony assays were used to compare the *in vitro* growth of pre-treatment and post-treatment erythroid progenitors in a DMT1-mutant patient. To enable a comparison with human data, high doses of erythropoietin were administered to *mk/mk* mice. The apoptotic status of erythroblasts, the expression of anti-apoptotic proteins, and the key components of the bone marrow-hepcidin axis were evaluated.

Results
Erythropoietin therapy *in vivo* or the addition of a broad-spectrum caspase inhibitor *in vitro* significantly improved the growth of human DMT1-mutant erythroid progenitors. A decreased number of apoptotic erythroblasts was detected in the patient's bone marrow after erythropoietin treatment. In *mk/mk* mice, erythropoietin administration increased activation of signal transducer and activator of transcription 5 (STAT5) and reduced apoptosis in bone marrow and spleen erythroblasts. *mk/mk* mice propagated on the 129S6/SvEvTac background resembled DMT1-mutant patients in having increased plasma iron but differed by having functional iron deficiency after erythropoietin administration. Co-regulation of hepcidin and growth differentiation factor 15 (GDF15) levels was observed in *mk/mk* mice but not in the patient.

Conclusions
Erythropoietin inhibits apoptosis of DMT1-mutant erythroid progenitors and differentiating erythroblasts. Ineffective erythropoiesis associated with defective erythroid iron utilization due to DMT1 mutations has specific biological and clinical features.

Key words: DMT1, apoptosis, ineffective erythropoiesis, erythropoietin.

Citation: Horvathova M, Kapralova K, Zidova Z, Dolezal D, Pospisilova D, and Divoky V. Erythropoietin-driven signaling ameliorates the survival defect of DMT1-mutant erythroid progenitors and erythroblasts. Haematologica 2012;97(10):1480-1488. doi:10.3324/haematol.2011.059550

KK and ZZ contributed equally to this manuscript.

Funding: MH was supported by the Czech Grant Agency (grant P305/10/P210); MH, KK, ZZ, and VD were supported by an Internal Grant from Palacky University Olomouc, LF_2012_016. DP and VD were (in part) supported by the MSM 6198959205 program project (Ministry of Education, Youth, and Sports, Czech Republic).

Acknowledgments: we would like to thank P. Ponka for the SIH, M. Flemig for the mk/+ mice, J. Kucerova for assistance with the mk/mk mice genotyping and real-time polymerase chain reaction, and P. Luzna for assistance with immunohistochemistry.

Manuscript received on December 1, 2011. Revised version arrived on April 11, 2012. Manuscript accepted on April 19, 2012.

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The online version of this article has a Supplementary Appendix.

Introduction

Divalent metal transporter 1 (DMT1, also known as SLC11A2 and Nramp2) is a transmembrane protein important for intestinal iron (Fe^{2+}) absorption and erythroid iron utilization.[1-3] Microcytic anemia (*mk*) mice have systemic iron deficiency due to decreased intestinal iron absorption caused by the G185R mutation in DMT1.[4,5] Recessive mutations in DMT1 are associated with hypochromic microcytic anemia with ineffective erythropoiesis and with iron overload in the liver in the majority of patients.[6-10] Functional studies have shown that a reduction in the amount of DMT1 protein due to a partial loss-of-function mutation in *mk/mk* mice and human patients leads to iron-restricted erythropoiesis.[2,11,12] A comprehensive *in vitro* analysis using erythroid progenitors from the first DMT1-mutant (*1285G>C SCL11A2*) patient with severe anemia (hemoglobin of 7.4 g/dL)[6,13] revealed defective growth (i.e., decreased number, cellularity, and hemoglobinization) of the patient's erythroid colonies (burst-forming units-erythroid, BFU-E). This growth defect can be rescued by iron-saturated salicylaldehyde isonicotinoyl hydrazone (Fe-SIH),[13] a non-transferrin iron donor[14] that corrects heme synthesis defects in *mk/mk* reticulocytes.[2] Strikingly, high concentrations of erythropoietin (EPO; 1 U/mL or higher) are required for full recovery of BFU-E colonies in Fe-SIH-containing cultures. These data suggest that impaired DMT1 function in erythroid progenitors blunts certain aspects of EPO signaling and that activation of the EPO/EPO receptor (EPOR) pathway with high doses of EPO complements this defect. Based on these results, the DMT1-mutant patient was placed on recombinant human EPO (darbepoetin) therapy in 2005. Darbepoetin administration (2.4 μg/kg once a week) significantly increased this patient's hemoglobin level (9.1 g/dL).[15] This therapy-based experience led us to analyze the mechanistic link between EPO/EPOR signaling and the improvement of iron-deficiency anemia because the *in vivo* success following EPO treatment was also reproduced in two other DMT1-mutant patients.[16] Ineffective erythropoiesis in different erythroid disorders is thought to be associated with increased apoptosis in the erythroid compartment.[17,18] Because a detailed study of β-thalassemia mice revealed only a low level of apoptosis, demonstrating that prevention of erythroid differentiation was causing ineffective erythropoiesis,[19] we set out to study the erythroid parameters that are rescued by a recombinant EPO in DMT1-mutant patients and mice.

Design and Methods

The patient and sample processing

The present DMT1-mutant patient, originally described by Priwitzerova *et al.*,[13] has currently been treated with darbepoetin (6.76 μg/kg once every 2 weeks) for more than 60 months. Her hemoglobin levels oscillate around 9.5 g/dL. The level of fetal hemoglobin, determined as described elsewhere,[6] remained unchanged under darbepoetin administration (1.8±0.3% of total hemoglobin). Peripheral blood specimens of the patient and control subjects were processed for progenitor assays and for separation of plasma or serum. Samples collected prior to initiation of EPO therapy and at different time-points during therapy are referred to as pre-treatment and post-treatment samples, respectively. The patient's pre-treatment serum EPO concentrations were measured by a radioimmunoassay.[20] The plasma levels of growth differentiation factor 15 (GDF15) were quantified according to the manufacturer's instructions using the Human GDF-15 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA). Actual urinary hepcidin was measured on request by competitive ELISA by Intrinsic LifeSciences LLC (La Jolla, CA, USA). The patient's bone marrow smears were subjected to terminal deoxynucleotidyl transferase mediated dUTP nick-end-labeling (TUNEL) assay. For a detailed description of the methods, see the *Online Supplementary Design and Methods*. The study and informed consent request were approved by the Ethics Committee of Palacky University Hospital, Olomouc, Czech Republic.

Animals

129S6/SvEvTac-*mk*/+ mice (with the G185R DMT1 mutation)[3] were provided by M. Fleming (Children's Hospital, Boston, USA). Homozygous *mk/mk* mice were identified among the offspring of *mk*/+ x *mk*/+ breeding by genotyping.[4] Mice were maintained on a standard diet. Eight- to 12-week old *mk/mk* mice and their wild-type (wt) littermates were included in the study. The basic hematologic parameters were measured using tail blood and a VET ABC animal blood counter (Scil Diagnostics Sdn. Bhd., Viernheim, Germany). Recombinant human EPO (Janssen Pharmaceuticals, Inc., Beerse, Belgium) was injected intravenously (50 IU/mouse) on 4 consecutive days.[2,21] A longer, 8-day EPO treatment regime was also tested. The tail blood sample, femora, tibiae, spleen, and liver were collected 16 h after the last injection. The samples were subjected to real-time polymerase chain reaction analysis, flow cytometry, immunohistochemistry, and immunoblotting. The EPO level and iron status parameters in the plasma were also determined. A detailed description of these assays is given in the *Online Supplementary Design and Methods*. Three to 12 mice per genotype were analyzed in individual experiments. Experiments were performed in accordance with the regulations of Palacky University Institutional Animal Care and Use Committee, Olomouc, Czech Republic.

Statistical analyses

Student's t-test was used to determine the statistical significance of the results. *P* values less than 0.05 were considered statistically significant. All analyses were conducted using Origin 6.1 software (OriginLab Corporation, Northampton, MA, USA).

Results

Fe-SIH rescue of the defective growth of DMT1-mutant erythroid progenitors requires high doses of erythropoietin

In vitro colony-forming assays reflect the *in vivo* potential of hematopoietic progenitors to survive and differentiate. Erythroid progenitors in cultures respond to increasing concentrations of EPO, the principal hormone indispensable for erythropoietic growth, by gradual increases in the plating efficiency and cellularity of erythroid colonies (Figure 1A).[22] We have previously reported that a partial loss-of-function DMT1 mutation leads to suppression of the colony-forming capacity of erythroid progenitors and that this defect can be corrected by the addition of Fe-SIH to cultures containing 1 or 2 U/mL of EPO.[11,13]

Interestingly, the EPO dose of 0.5 U/mL only partially rescued the growth of DMT1-mutant BFU-E colonies, while no effect was observed with concentrations of 0.24 U/mL or lower (previously unpublished observations, Figure 1B). These data demonstrate that DMT1-mutant progenitors require high doses of EPO to stimulate their survival, proliferation, and/or differentiation.

Erythropoietin therapy improved the patient's anemia and defective growth of DMT1-mutant burst-forming units-erythroid

In accordance with the *in vitro* data, the administration of high doses of darbepoetin to our DMT1-mutant patient led to a marked increase in her hemoglobin.[15] The comparison of the patient's pre-treatment and post-treatment hematologic and iron status parameters is shown in Table 1. During EPO therapy, the majority of these parameters, apart from hemoglobin, did not change significantly, including urinary hepcidin, which remained below the normal range[15] (Table 1). This finding indicates that there were no profound changes in iron metabolism following EPO treatment. Serum soluble transferrin receptor levels remained high (Table 1). Two short-term discontinuations of EPO treatment led to immediate drops in the patient's hemoglobin (to 7.4±0.3 g/dL) within 4 weeks of the discontinuations. A similar observation was also documented in an Italian DMT1-mutant patient.[7] Together, these clinical observations show that DMT1-mutant erythropoiesis requires increased EPO stimulation and indicate the need for continuous EPO administration. In fact, the pre-treatment serum EPO level of our patient was already increased up to twice the normal level (70.2 mU/mL; normal range: 24-42 mU/mL).

We next aimed to determine the underlying cause of the success of EPO therapy in DMT1-deficient erythropoiesis. *In vitro* cultures of our patient's DMT1-mutant cells on methylcellulose without Fe-SIH revealed a significant increase ($P<0.001$) in the plating efficiency of post-treatment BFU-E progenitors when compared with that of the pre-treatment sample at all EPO concentrations tested (Figure 1C). This improved capacity of post-treatment BFU-E progenitors to form colonies *in vitro* was accompanied by a significant increase ($P<0.05$) in their cellularity (Figure 1C). These two characteristics of *in vitro* colony growth reflect the improved survival and *in vitro* differentiation of post-treatment DMT1-mutant BFU-E progenitors.

Defective growth of pre-treatment DMT1-mutant burst-forming unit-erythroid progenitors involves caspase-dependent apoptosis

Because EPO is the main survival/anti-apoptotic factor of erythroid cells,[23] we assessed whether apoptosis is involved in the observed decreased survival of pre-treatment DMT1-mutant BFU-E progenitors and in the defective growth of corresponding colonies. Given that apoptosis of erythroid progenitors and their progeny is mediated by caspases,[24,25] we decided to test whether the broad-spectrum caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-fmk) improves the plating efficiency of DMT1-mutant BFU-E progenitors. We first tested an optimal z-VAD-fmk dose in EPO-containing cultures of three normal controls because caspases are also activated during physiological differentiation of erythroid progenitors[17,25] and the effects of caspase inhibition could be complex. We performed a series of erythroid progenitor assays using 1 U/mL of EPO and various concentrations of z-VAD-fmk and determined that 30 μM z-VAD-fmk did not significantly alter BFU-E plating efficiency in healthy controls at this EPO dose (*data not shown*). Importantly, a significant increase in pre-treatment DMT1-mutant BFU-E colony number (from 12±2 to 23±4 per 3×10^5 plated mononuclear cells) ($P<0.01$) was achieved by the addition of 30 μM z-VAD-fmk to media containing 1 U/mL of EPO. These results show that caspase-dependent apoptosis contributes to the defective growth of pre-treatment BFU-E progenitors.

Erythropoietin therapy reduces apoptosis of DMT1-mutant erythroblasts in vitro and in vivo

To analyze the aforementioned defect in the colony-forming capacity of DMT1-mutant BFU-E progenitors further, we assessed the apoptotic status within *in vitro*-differentiated erythroblasts using cytospin analysis of the pre-treatment and post-treatment BFU-E colonies and the TUNEL assay. The number of TUNEL$^+$ erythroblasts in the post-treatment culture (7.5±2.1%) was significantly lower than the number in the pre-treatment culture (23±4.2%) ($P=0.043$, n=2) (Figure 1D). The extent to which apoptosis occurred within erythroblasts in the post-treatment BFU-E colonies was only slightly higher than that of normal BFU-E colonies (4.0±2.8%), indicating improved survival of erythroid cells in the post-treatment culture.

The analysis of pre-treatment bone marrow smears revealed erythroid hyperplasia (39.2%) with a predominant presence of polychromatophilic erythroblasts (*Online Supplementary Figure S1*). The erythroid compartment further increased (to 60%) following EPO therapy; disappearance of early immature erythroblasts and additional expansion of polychromatophilic erythroblasts were observed in the post-treatment bone marrow smear (*Online Supplementary Figure S1*). Consistent with the *in vitro* data, the TUNEL assay on the bone marrow smears showed a reduction in the number of TUNEL$^+$ erythroblasts after EPO supplementation (from 4% to 1.5%) (Figure 1E).

Table 1. Selected hematologic values and iron status parameters in the DMT1-mutant patient.

	Patient		
	Pre-treatment	Post-treatment	Normal values
Red blood cell count ($x10^{12}$/L)	5.0±0.4	6.1±0.6	4.0-5.4
Hemoglobin level (g/dL)	7.5±0.5	9.5±0.5	12.0-15.6
Hematocrit (%)	29.0±1.4	33.5±0.7	36-45
Mean corpuscular volume (fL)	56.1±1.0	57.0±0.8	80-90
Mean corpuscular hemoglobin (pg)	15.2±0.2	15.4±0.2	27-34
Serum iron (μmol/L)	44.0±1.4	43.5±4.2	14.5-26.0
Total iron binding capacity (μmol/L)	50.7±0.6	50.7±1.2	44.8-71.6
Ferritin (ng/mL)	179±26	175±27	20-150
Serum transferrin receptor (mg/L)	24.1±10.8	24.5±3.1	1.9-4.4
Urinary hepcidin (ng/mg creatinine)	See Mims *et al.*[6]	55.3	126-986

The average values for individual post-treatment parameters were calculated from blood samples analyzed at different time-points during therapy. Values are means ± standard deviations.

Erythropoietin improves anemia in mk/mk *mice*

To confirm and extend the data obtained from the patient's samples, DMT1-mutant *mk/mk* mice were treated with EPO. Due to the reduced viability of *mk/mk* mice on the original MK/ReJ background,[4,5] we used the *mk/mk* mouse strain 129S6/SvEvTac, created by Gunshin *et al.*[3] Analysis of hematologic parameters confirmed severe microcytic anemia in 129S6/SvEvTac-*mk/mk* mice (Table 2); peripheral blood smears showed marked anisocytosis and poikilocytosis, which are characteristic of severe iron deficiency. In contrast to the hyposideremia documented in MK/ReJ-*mk/mk* mice,[5] the 129S6/SvEvTac-*mk/mk* showed significantly increased plasma iron (48.6±11.7 μmol/L) compared to wt littermates (33.7±3.7 μmol/L) (P=0.0140) (Table 2). In accordance with these results the saturation of transferrin in 129S6/SvEvTac-*mk/mk* mice reached 90-100%. However, the tissue non-heme iron concentrations in the 129S6/SvEvTac-*mk/mk* were reduced compared to those in age-matched, control wt mice (*mk/mk* liver: 20.7±4.1 μg/g wet tissue; wt liver 130.1±33.1 μg/g, $P=4.7\times10^{-5}$; *mk/mk* spleen: 73.5±16.6 μg/g; wt spleen: 1172.6±141.9 μg/g, $P=1.03\times10^{-5}$; n=5 for each group). Diminished iron stores in 129S6/SvEvTac-*mk/mk* mice in comparison to wt mice were also confirmed by Perls' Prussian blue staining of spleen and liver sections (*Online Supplementary Figure S2*).

The pre-treatment plasma EPO level in 129S6/SvEvTac-*mk/mk* mice was higher than that in their wt littermates (331.5±137.7 pg/mL, n=5, and 153.8±33.5 pg/mL, n=8, respectively). This corresponded to a 2-fold elevation in pre-treatment EPO in the patient. Four days of EPO administration to the *mk/mk* mice led to a significant increase in hemoglobin (7.5±0.6 to 9.5±0.4 g/dL) ($P=2.5\times10^{-4}$), red blood cell count (5.9±1.4 to 8.3±1.5×10^6/μL) (P=0.036), and reticulocytes (13.0±3.1 to 30.8±7.1) ($P=5.3\times10^{-5}$) (Table 2). In contrast to the EPO-treated DMT1-mutant patient, slight, non-significant decreases in mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were observed in EPO-treated *mk/mk* mice (Table 2). None of the hematologic parameters changed further with the 8-day application of EPO (*data not shown*). EPO supplementation in *mk/mk* mice resulted in a significant 2.6-fold reduction in plasma iron (P=0.0025) and 3.8-fold reduction in transferrin saturation ($P=6.4\times10^{-6}$), with a concomitant 1.3-fold increase in total iron-binding capacity (P=0.0011) (Table 2). These changes in iron metabolism parameters likely reflect increased iron demand during stimulated erythropoiesis in EPO-treated *mk/mk* mice.

Table 2. EPO increases red blood cell count and hemoglobin level and alters iron indices in *mk/mk* mice.

	mk/mk	mk/mk EPO	wt
Red blood cell count (x10^{12}/L)	5.9±1.4	8.3±1.5	8.3±1.2
Hemoglobin level (g/dL)	7.5±0.6	9.5±0.4	13.9±2.2
Hematocrit (%)	22.4±6.2	30.2±5.6	44.9±6.5
Mean corpuscular volume (fL)	37.2±1.8	36.4±1.1	54.3±0.8
Mean corpuscular hemoglobin (pg)	13.1±2.5	11.7±1.7	16.8±0.6
Reticulocytes (%)	13.0 ±3.1	30.8±7.1	3.8±1.2
Plasma iron (μmol/L)	48.6±11.7	18.4±2.2	33.7±3.7
Total iron binding capacity (μmol/L)	56.5±5.2	76.4±7.7	48.0±5.8
Transferrin saturation (%)	90.8±10.8	24.0±2.2	67.0±2.0

Values are means ± standard deviations of the indicated genotypes; n = 6 each.

Erythropoietin ameliorates apoptosis in mk/mk *erythroblasts*

We subsequently analyzed the *in vivo* apoptosis rate in *mk/mk* erythroblasts using annexin V binding. The bone marrow and spleen cells were co-stained with Ter119 antibody and annexin V. Dead cells were excluded from the analysis based on positive staining with IR-fluorescent reactive dye. Flow cytometry analysis revealed that 4 days of EPO treatment significantly reduced the numbers of erythroid Ter119$^+$ precursors undergoing apoptosis (annexin V$^+$) in *mk/mk* bone marrow (12.4±2.3% to 5.4±0.9%) ($P=2.7\times10^{-4}$) and spleens (7.3±0.7% to 3.1±0.9%) ($P=1.97\times10^{-4}$) (Figure 2A). The degrees of amelioration of apoptosis were comparable between the 4- and 8-day EPO treatment regimens (*Online Supplementary Tables S1 and S2*). Nevertheless, the number of Ter119$^+$/annexin V$^+$ cells still remained 2- to 3-fold higher than that in the wt littermates (*Online Supplementary Tables S1 and S2*).

To confirm the reduction in the number of erythroblasts undergoing apoptosis following 4 days of EPO supplementation, immunohistochemical staining of bone marrow and spleen histological sections was performed with Ter119 antibody and then followed by TUNEL assays. A decreased number of Ter119$^+$/TUNEL$^+$ apoptotic cells was detected in the bone marrow and spleen of the EPO-supplemented *mk/mk* mice compared to pre-treatment (Figure 2B). The exact values were as follows: non-treated *mk/mk* mice, 4.6±1.1% for bone marrow and 7.4±0.9% for spleen; EPO-treated *mk/mk* mice, 1.7±0.8% for bone marrow and 1.4±0.6% for spleen. The number of Ter119$^+$/TUNEL$^+$ cells in the bone marrow and spleen of wt mice was approximately 2% (*data not shown*).

Consistently, increased expression of two anti-apoptotic proteins, BCL-X$_L$ and MCL-1,[26,27] was observed in the bone marrow and spleen cell lysates prepared from *mk/mk* mice treated for 4 days with EPO when compared to untreated *mk/mk* mice (Figure 2C). These data further confirm a positive effect of EPO on the survival of DMT1-mutant erythroid precursors. Subsequent immunoblotting revealed increased phosphorylation of signal transducer and activator of transcription 5 (STAT5) in the bone marrow and spleen of EPO-treated *mk/mk* mice in comparison to untreated *mk/mk* mice (Figure 2D). Immunohistochemical staining with anti-phospho-STAT5 antibody confirmed this result and showed nuclear localization of activated STAT5 (Figure 2E). These data suggest that high EPOR stimulation results in STAT5 hyperactivation which is essential to maintain survival signaling in iron-deficient erythropoiesis.

Additionally, we tested whether and how increased differentiation of DMT1-mutant erythroid cells contributes to the amelioration of anemia in EPO-treated *mk/mk* mice. Flow cytometry analysis of viable Ter119 and CD71 double-stained cells showed abnormal differentiation patterns in *mk/mk* bone marrow (*Online Supplementary Figure S3* and *Online Supplementary Table S3*) and spleen (*Online Supplementary Figure S3* and *Online Supplementary Table S4*) when compared to the patterns in wt littermates. Immature Ter119^{high}CD71^{high} (Region II) erythroblasts pre-

dominated in *mk/mk* mice; the difference was more profound in the spleen, reflecting dramatic expansion of splenic erythropoiesis in *mk/mk* mice. EPO administration for 4 days significantly increased the total number of Ter119$^+$ cells in the bone marrow, although the distribution of erythroblasts at different stages of maturation in the *mk/mk* bone marrow and spleen did not change (*Online Supplementary Tables S3 and S4*). Eight days of EPO treatment led to additional induction of erythropoiesis in the bone marrow (more Ter119$^+$ cells), with an increased percentage of mature erythroid cells (Region IV) in comparison to immature erythroblasts (Regions I through III) (*Online Supplementary Figure S3* and *Online Supplementary Table S3*). No significant expansion of erythropoiesis or changes in erythroid differentiation were observed in the spleen, with the exception of an increased number of proerythroblasts (Region I) (*Online Supplementary Figure S3* and *Online Supplementary Table S4*).

Analysis of key components of the bone marrow-hepcidin axis

To address the interconnection of deregulated erythropoiesis and iron homeostasis, we assessed three known or putative factors involved in these processes: hepcidin,[28] GDF15,[29] and twisted gastrulation protein homolog 1 (TWSG1).[30] Real-time polymerase chain reaction analysis of *mk/mk* mice showed very low expression of hepcidin mRNA in the liver (Figure 3A), which became completely undetectable following EPO treatment. The expression of GDF15 was higher in *mk/mk* mice than in wt mice, although the difference was more significant in the bone marrow (19.1-fold difference) than in the spleen (2-fold

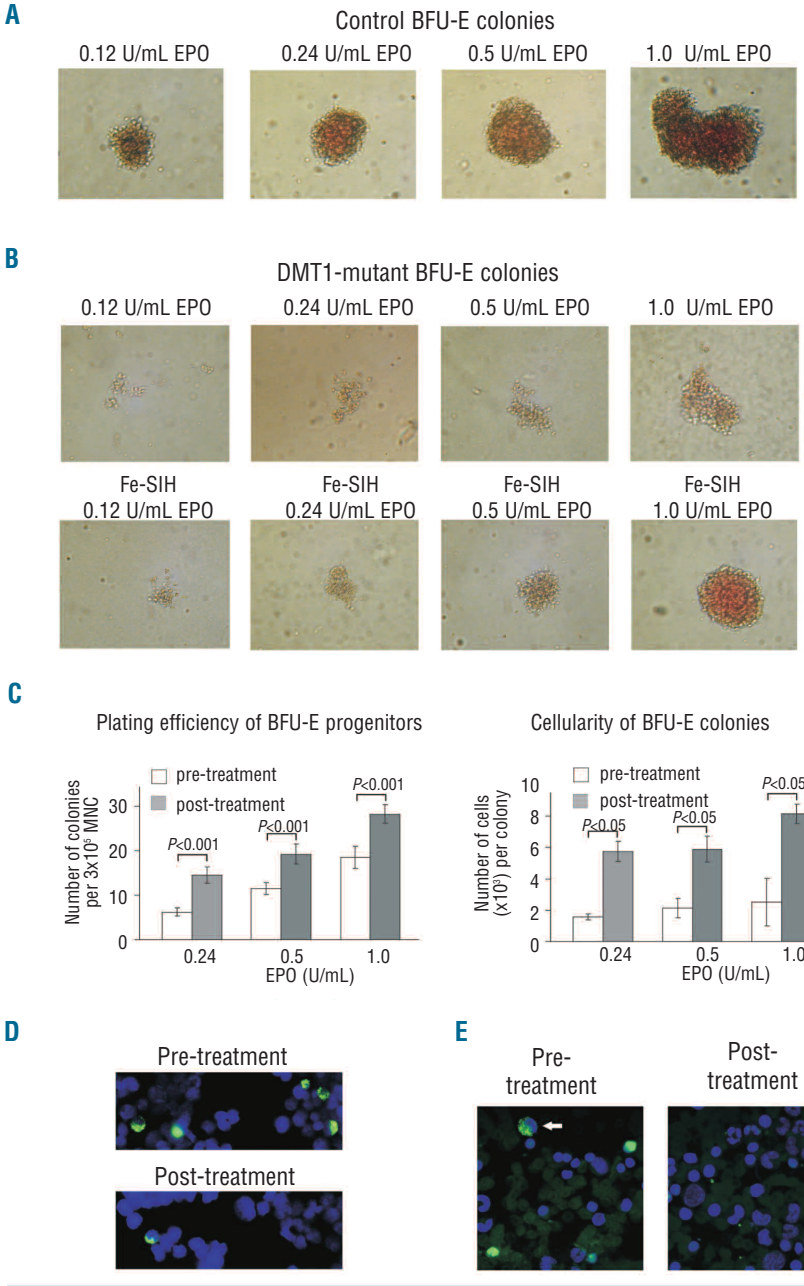

Figure 1. Hematopoietic colony assay and TUNEL detection of apoptosis. (A) The colony formation of normal control BFU-E progenitors was dependent on the EPO dose, i.e., the plating efficiency (not shown), cellularity, and hemoglobinization gradually increased with increasing concentrations of EPO. (B) Fe-SIH rescue of the defective growth of pre-treatment DMT1-mutant BFU-Es required high EPO doses (≥ 1 U/mL). An EPO concentration of 0.5 U/mL had only a partial effect, and EPO doses lower than 0.24 U/mL did not rescue the growth of DMT1-mutant BFU-E colonies, although these concentrations are sufficient to support the growth of relatively large, normal control BFU-E colonies (see A). (C) EPO therapy had a positive effect on the plating efficiency and cellularity of our patient's DMT1-mutant BFU-E colonies. The plating efficiency of BFU-E progenitors in the post-treatment sample was significantly higher than in the pre-treatment sample ($P<0.001$) for all tested concentrations of EPO. Values indicate means ± standard deviations (SD); (n=4). A significant increase in BFU-E cellularity ($P<0.05$) was also documented for all tested EPO concentrations. Values are means ± SD; (n=2). MNC, mononuclear cells. No Fe-SIH was used in these cultures. (D) A fluorescein *in situ* cell death detection kit revealed significantly fewer TUNEL$^+$ cells (green fluorescence) in the post-treatment BFU-E colonies relative to the pre-treatment BFU-E colonies. (E) Fewer TUNEL$^+$ polychromatophilic normoblasts (green fluorescence) were detected in the DMT1-mutant patient's post-treatment bone marrow smear in comparison to the pre-treatment bone marrow smear. Polychromatophilic normoblasts, the predominant erythroid cells in the bone marrow compartment, were identified based on their typical morphology. The arrow indicates non-specific staining of cytoplasmic granules in a white blood cell precursor. The nuclei in panels (D) and (E) were counterstained with DAPI (blue fluorescence). BFU-E colonies (A and B) were visualized with an Olympus IX 71 light microscope (Olympus, Hamburg, Germany), magnification 100x. Immunostained cells [panels (D) and (E)] were analyzed with an Olympus BX 51 fluorescence microscope (Olympus), magnification 400x. Digital images were acquired with an Olympus DP 50 camera driven by DP controller software (provided by Olympus). Images were cropped, assembled, and labeled using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

difference) (Figure 3B). EPO treatment increased the expression of GDF15 in *mk/mk* mice, which correlated with completely suppressed expression of hepcidin. The expression of TWSG1 was similar in both genotypes and remained unchanged with EPO supplementation (Figure 3C), indicating that TWSG1 is not involved in hepcidin suppression under conditions of DMT1-mutant erythropoiesis. No significant differences in the expression of the analyzed genes were found between the groups of *mk/mk* mice that underwent the two EPO treatments of different durations (*data not shown*).

Corroborating these mouse data, the patient's actual urinary hepcidin was decreased to 55.3 ng/mg creatinine (normal range, 126-986). Although this value cannot be directly compared to the pre-treatment urinary hepcidin published by Mims *et al.*[6] (4 ng/mg creatinine; normal range, 10-200) because of the different measurement techniques, in both cases the hepcidin level fell below half the lower limit of normal. These hepcidin levels are inconsistent with the patients' iron overload and confirm the influence of a bone marrow-derived regulator of hepcidin expression that overrides hepcidin regulation by iron.[28] Surprisingly, the actual post-treatment plasma GDF15 level in the DMT1-mutant patient was only 3.4-fold higher (973.7±593.5 pg/mL) than that of gender- and age-matched controls (288.4±56.9 pg/mL). The relatively mild elevation of GDF15 in our patient in comparison to the dramatically increased GDF15 levels in β-thalassemia patients[31] suggests that there may be an additional bone marrow-derived regulator of hepcidin production.

Discussion

The aim of this study was to reveal the molecular bases for the clinically demonstrated success of high-dose EPO supplementation in a patient with a rare form of anemia caused by a mutation in DMT1. It has been suggested that the EPO-triggered therapeutic improvement of iron-deficiency anemia could be due to the anti-apoptotic effect of EPO.[7,32] The present study confirms this hypothesis. Colony-forming assays showed significantly improved *in vitro* growth of our patient's DMT1-mutant post-treatment BFU-E progenitors compared to pre-treatment BFU-E pro-

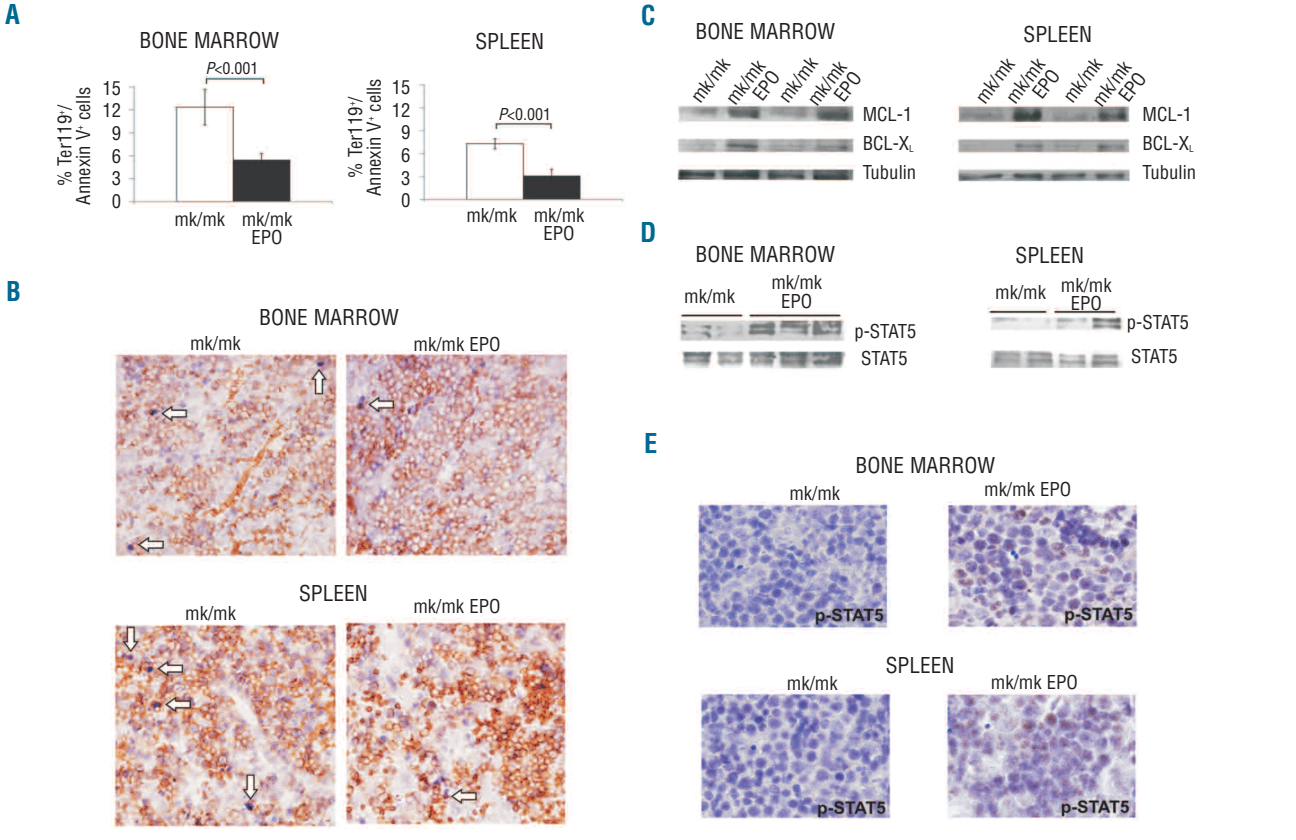

Figure 2. Effects of EPO supplementation on *mk/mk* erythroid cells. (A) The number of early-apoptotic (annexin V$^+$) erythroid cells (Ter119$^+$) was significantly lower in the bone marrow and spleen of EPO-treated *mk/mk* mice than in non-treated *mk/mk* mice. (B) TUNEL assays confirmed significant reductions in the numbers of Ter119$^+$ cells undergoing apoptosis in the bone marrow (P=0.03986, n = 3) and spleen (P=6.2519x10^{-4}, n=3) of EPO-treated *mk/mk* mice compared to non-treated *mk/mk* mice. The arrows indicate Ter119$^+$/TUNEL$^+$ cells. (C) Immunoblotting of bone marrow and spleen lysates showed increased expression of anti-apoptotic proteins BCL-X$_L$ and MCL-1 in EPO-treated *mk/mk* mice compared with non-treated *mk/mk* mice. (D) and (E) EPO treatment led to augmented phosphorylation of STAT5 in *mk/mk* mice. (E) Immunohistochemical staining of bone marrow and spleen sections confirmed nuclear localization of activated STAT5 (brown nuclear staining). Immunohistochemical slides (B and E) were analyzed with an Olympus BX 51 light microscope (Olympus), magnification 200x (B) and magnification 1000x (E). Digital images were acquired with an Olympus DP 50 camera driven by DP controller software (provided by Olympus). Images were cropped, assembled, and labeled using Adobe Photoshop software (Adobe Systems).

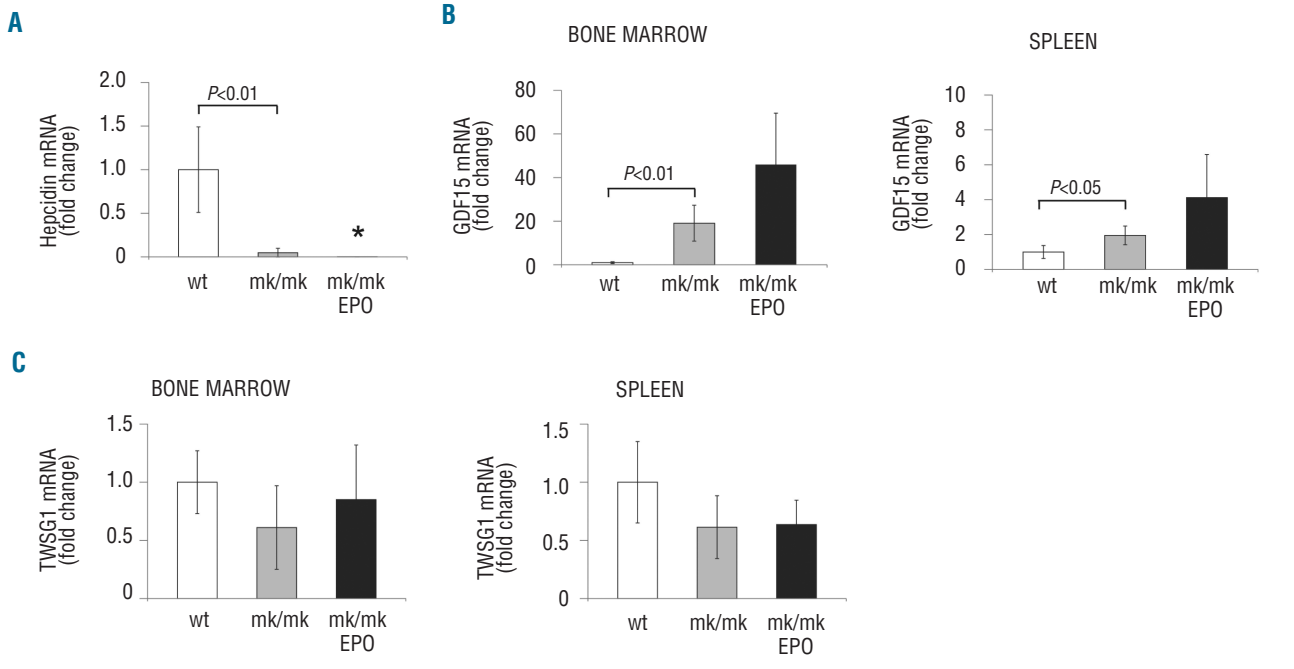

Figure 3. Analysis of the known and putative components of the bone marrow-hepcidin axis that regulate iron homeostasis in *mk/mk* mice. (A) Hepcidin mRNA expression was dramatically reduced in *mk/mk* livers (*P*=0.00352) compared to in the livers of wt littermates and became completely repressed (marked with asterisk) upon EPO treatment. (B) The expression of GDF15 was higher in *mk/mk* mice than in wt littermates (19.1-fold difference in bone marrow, *P*=0.00344; 2-fold difference in the spleen, *P*=0.046) and further increased upon EPO treatment. (C) No differences in TWSG1 mRNA expression were observed among wt mice, non-treated *mk/mk* mice, and EPO-treated *mk/mk* mice. Expression is in arbitrary units normalized against housekeeping genes. The results are represented as means ± standard deviations, n = 8 for wt mice, n = 5 for *mk/mk* mice. mk/mk EPO: *mk/mk* mice supplemented for 4 days with EPO.

genitors. Because growth (plating efficiency) of the pre-treatment BFU-E progenitors can also be corrected *in vitro* by the addition of the broad-spectrum caspase inhibitor z-VAD-fmk, we propose that caspase-dependent apoptosis is activated and dominates over survival signaling in DMT1-mutant BFU-E progenitors. The decreased rates of apoptosis detected in the post-treatment bone marrow erythroblasts and within the post-treatment *in vitro*-differentiated BFU-E colonies confirmed that EPO-driven signaling also complements the survival defect of DMT1-mutant erythroblasts.

High doses of EPO were also tested in *mk/mk* mice. The effectiveness of 4 days of administration of EPO was verified by the induction of splenic erythropoiesis and the reticulocyte count in wt mice (*data not shown*). All subsequent analyses in *mk/mk* mice supplemented with EPO were performed at the time of their best experimental response (i.e. increase in hemoglobin level). EPO administration to *mk/mk* mice led to increased expression of BCL-X_L and MCL-1 and to increased activation of STAT5 in the bone marrow and spleen. These data suggest that the improved survival of EPO-treated erythroblasts was likely mediated by STAT5 signaling.[33] In this regard; the condition of *mk/mk* mice treated with EPO resembles that of β-thalassemia mice with dramatically elevated endogenous EPO and increased BCL-X_L expression, which is responsible for the prevention of apoptosis of thalassemic erythroid cells in this model.[19]

The possible involvement of any substantial increase in iron utilization by individual erythroid cells as a contributing factor to the positive effects of EPO therapy can be ruled out by the fact that the patients' erythrocyte characteristics (MCV and MCH) remained unchanged with EPO supplementation.[7,32] Our observation of decreased MCV and MCH in the *mk/mk* mice following EPO treatment further supports this conclusion.

We also suggest that inhibition of apoptosis rather than increased differentiation of DMT1-mutant erythroid cells predominantly accounts for the partial improvement of anemia in EPO-treated *mk/mk* mice. Despite significant expansion of the erythroid component in the bone marrow, no profound changes in erythroblast maturation were noted at the time of induction of the experimental response to EPO supplementation (4-day period). Although EPO treatment for 8 days influenced erythroid differentiation, its contribution to the amelioration of anemia seems to be minor based on the fact that the hematologic parameters of these mice did not improve further compared to those of mice treated with EPO for 4 days. In the spleen, it seems likely that the high erythropoietic rate generated by the endogenous conditions of *mk/mk* mice cannot be further stimulated by EPO.

Unexpectedly, the phenotype of iron-restricted erythropoiesis with concomitant hypersideremia and increased saturation of transferrin observed in 129S6/SvEvTac-*mk/mk* mice is reminiscent of the clinical picture of DMT1-mutant patients but differs from that the originally described MK/ReJ-*mk/mk* mice.[5] The striking phenotypic difference between these two mouse strains with the same DMT1 mutation is likely caused by genetic factors

that modify iron homeostasis and which differ between the MK/ReJ and 129S6/SvEvTac strains. In this regard, 129S6/SvEvTac-*mk/mk* mice seem to be a more accurate model for comparison with human samples, although they still lack liver iron overload. Comparable iron status characteristics, i.e., elevated serum iron but a lack of tissue iron deposits, have also been reported for Belgrade (*b*) rats with the same DMT1 mutation as the *mk/mk* mice.[34] The liver iron overload in DMT1-mutant patients likely results from the combined effect of increased intestinal iron (heme or non-heme) absorption and redistribution of iron from erythroid tissues to the liver, as proposed by Priwitzerova *et al.*[13] and Gunshin *et al.*,[35] respectively. In agreement with this proposal, *b/b* rats fed a high-iron diet eventually develop liver iron overload.[36] On the other hand, enhanced erythropoiesis after EPO treatment in 129S6/SvEvTac-*mk/mk* mice results in significantly decreased plasma iron and confirms a persistent functional iron deficiency.[37] This imbalance between the iron needs of the erythroid compartment after EPO therapy and the inadequate iron supply from iron stores distinguishes the murine model from the patient.

Abnormal iron metabolism in association with ineffective erythropoiesis underlying some common erythroid disorders (such as β-thalassemia) is only starting to be understood.[38-40] The regulation of iron homeostasis as well as the direct regulation of hepcidin production is complex, involving iron status, inflammation, erythropoietic activity, and hypoxia.[28,41,42] It is accepted that accelerated erythropoiesis overrides hepcidin regulation by iron, which is also the case for DMT1-mutant patients and mice with reduced hepcidin levels in spite of increased serum iron. The hepcidin suppression is likely driven by ineffective erythropoiesis and by increased EPO levels due to tissue hypoxia. A direct effect of hypoxia-inducible factor (HIF) signaling to down-regulate hepcidin should also be taken into account because Gordeuk *et al*. showed that hypoxia suppresses hepcidin independently of erythropoietin or hemoglobin concentration.[43] On the other hand, the *in vivo* suppression of hepcidin by EPO appears to be predominantly indirect because EPO-treated mice with erythropoiesis disrupted by bone marrow irradiation are unable to repress hepcidin.[44] The specific negative erythroid regulators of hepcidin production need to be characterized.

In this study we demonstrated that almost undetectable expression of hepcidin mRNA in *mk/mk* liver correlated with increased expression of GDF15. Based on the fact that the expression of GDF15 increased after EPO supplementation in *mk/mk* mice and also in EPO-treated wt mice (*data not shown*), we suggest that GDF15 up-regulation might reflect stimulated erythropoiesis rather than increased apoptosis, which would be in agreement with the accelerated need for iron supply and thus for reduced hepcidin. This result, however, disagrees with the suggestion that GDF15 is produced by apoptotic erythroblasts,[31] as decreased apoptosis was observed with EPO treatment in *mk/mk* mice.

In contrast to the apparent co-regulation of hepcidin and GDF15 in *mk/mk* mice, the correlation between hepcidin and GDF15 levels in our DMT1-mutant patient seems to be weak. The maximal level of GDF15 measured in this patient's plasma was 1393.4 pg/mL. Tanno *et al.*[29] demonstrated that significant suppression of hepcidin required a GDF15 level higher than 5000 pg/mL. This discrepancy between human and mouse data might reflect differences in homology and the tissue expression of GDF15 in these two organisms.[31] Our results favor the existence of an additional bone marrow-derived regulator of hepcidin expression and are consistent with a recent study demonstrating no correlation between GDF15 and hepcidin levels in healthy individuals after EPO administration.[45]

In conclusion, we propose that inhibition of apoptosis of erythroid progenitors and differentiating erythroblasts plays a crucial role in the success of EPO therapy under conditions of DMT1-deficient erythropoiesis. Our data also suggest specific biological and clinical features of ineffective erythropoiesis associated with defective erythroid iron utilization due to the DMT1 mutation. In contrast to ineffective erythropoiesis associated with β-thalassemia,[29,30,38] iron overload of varying severities in DMT1-mutant patients is restricted to the liver;[16] they show no reticulocytosis, and hepcidin reduction seems to be regulated differently. At the cellular level, DMT1 deficiency affects not only developing erythroblasts but also early erythroid progenitors because iron is required for other vital functions in addition to hemoglobin synthesis. We conclude that ineffective erythropoiesis associated with iron-deficient anemia due to a partial-loss-of-function DMT1 mutation is primarily caused by a survival defect of the erythroid progenitors and differentiating erythroblasts.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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References

1. Canonne-Hergaux F, Gruenheid S, Ponka P, Gros P. Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. Blood. 1999;93(12):4406-17.
2. Canonne-Hergaux F, Zhang AS, Ponka P, Gros P. Characterization of the iron transporter DMT1 (NRMAP2/DCT1) in red blood cells of normal and anemic mk/mk mice. Blood. 2001;98(13):3823-30.
3. Gunshin H, Fujiwara Y, Custodio AO, Direnzo C, Robine S, Andrews NC. Slc11a2 is required for intestinal iron absorption and erythropoiesis but dispensable in placenta and liver. J Clin Invest. 2005;115(5):1258–66.
4. Fleming MD, Trenor CC 3rd, Su MA, Foernzler D, Beier DR, Dietrich WF, et al. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. Nat Genet. 1997;16(4):383-6.
5. Bannerman RM, Edwards JA, Kreimer-Birnbaum M, McFarland E, Russell ES. Hereditary microcytic anaemia in the mouse; studies in iron distribution and metabolism. Br J Haematol. 1972;23(2):235-45.
6. Mims MP, Guan Y, Pospisilova D, Priwitzerova M, Indrak K, Ponka P, et al. Identification of a human mutation of DMT1 in a patient with microcytic anemia and iron overload. Blood. 2005;105(3):1337-42.
7. Iolascon A, d'Apolito M, Servedio V, Cimmino F, Piga A, Camaschella C. Microcytic anemia and hepatic iron overload in a child with compound heterozygous mutations in DMT1 (SCL11A2). Blood. 2006;107(1):349-54.
8. Beaumont C, Delaunay J, Hetet G, Grandchamp B, de Montalembert M, Tchernia G. Two new human DMT1 gene

mutations in a patient with microcytic anemia, low ferritinemia, and liver iron overload. Blood. 2006;107(10):4168-70.
9. Blanco E, Kannengiesser C, Grandchamp B, Tasso M, Beaumont C. Not all DMT1 mutations lead to iron overload. Blood Cells Mol Dis. 2009;43(2):199-201.
10. Bardou-Jacquet E, Island ML, Jouanolle AM, Détivaud L, Fatih N, Ropert M, et al. A novel N491S mutation in the human SLC11A2 gene impairs protein trafficking and in association with the G212V mutation leads to microcytic anemia and liver iron overload. Blood Cells Mol Dis. 2011;47(4):243-8.
11. Priwitzerova M, Nie G, Sheftel AD, Pospisilova D, Divoky V, Ponka P. Functional consequences of the human DMT1 (SLC11A2) mutation on protein expression and iron uptake. Blood. 2005;106(12):3985-7.
12. Lam-Yuk-Tseung S, Mathieu M, Gros P. Functional characterization of the E399D DMT1/NRAMP2/SLC11A2 protein produced by an exon 12 mutation in a patient with microcytic anemia and iron overload. Blood Cells Mol Dis. 2005;35(2):212-6.
13. Priwitzerova M, Pospisilova D, Prchal JT, Indrak K, Hlobilkova A, Mihal V, et al. Severe hypochromic microcytic anemia caused by a congenital defect of the iron transport pathway in erythroid cells. Blood. 2004;103(10):3991-2.
14. Ponka P, Schulman HM. Acquisition of iron from transferrin regulates reticulocyte heme synthesis. J Biol Chem. 1985;260(27):14717-21.
15. Pospisilova D, Mims MP, Nemeth E, Ganz T, Prchal JT. DMT1 mutation: response of anemia to darbepoetin administration and implications for iron homeostasis. Blood. 2006;108(1):404-5.
16. Iolascon A, Camaschella C, Pospisilova D, Piscopo C, Tchernia G, Beaumont C. Natural history of recessive inheritance of DMT1 mutations. J Pediatr. 2008;152(1):136-9.
17. Testa U. Apoptotic mechanisms in the control of erythropoiesis. Leukemia. 2004;18(7):1176-99.
18. Mathias LA, Fisher TC, Zeng L, et al. Ineffective erythropoiesis in beta-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. Exp Hematol. 2000;28(12):1343-53.
19. Libani IV, Guy EC, Melchiori L, Schiro R, Ramos P, Breda L, et al. Decreased differentiation of erythroid cells exacerbates ineffective erythropoiesis in beta-thalassemia. Blood. 2008;112(3):875-85.
20. Zadrazil J, Horak P, Horcicka V, Zahalkova J, Strebl P, Hruby M. Endogenous erythropoietin levels and anemia in long-term renal transplant recipients. Kidney Blood Press Res. 2007;30(2):108-16.
21. Peng H, Truksa J, Lee P. EPO-mediated reduction in Hamp expression in vivo corrects iron deficiency anaemia in TMPRSS6 deficiency. Br J Haematol. 2010;151(1):106-9.
22. Prchal JF, Adamson JW, Murphy S, Steinmann L, Fialkow PJ. Polycythemia vera. The in vitro response of normal and abnormal stem cell lines to erythropoietin. J Clin Invest. 1978;61(4):1044-7.
23. Constantinescu SN, Ghaffari S, Lodish HF. The Erythropoietin Receptor: Structure, Activation and Intracellular Signal Transduction. Trends Endocrinol Metab. 1999;10(1):18-23.
24. Gregoli PA, Bondurant MC. Function of caspases in regulating apoptosis caused by erythropoietin deprivation in erythroid progenitors. J Cell Physiol. 1998;178(2):133-43.
25. Ribeil JA, Zermati Y, Vandekerckhove J, Cathelin S, Kersual J, Dussiot M, et al. Hsp70 regulates erythropoiesis by preventing caspase-3-mediated cleavage of GATA-1. Nature. 2007;445(7123):102-5.
26. Rhodes MM, Kopsombut P, Bondurant MC, Price JO, Koury MJ. Bcl-x(L) prevents apoptosis of late-stage erythroblasts but does not mediate the antiapoptotic effect of erythropoietin. Blood. 2005;106(5):1857-63.
27. Gregoli PA, Bondurant MC. The roles of Bcl-X(L) and apopain in the control of erythropoiesis by erythropoietin. Blood. 1997;90(2):630-40.
28. Ganz T. Hepcidin in iron metabolism. Curr Opin Hematol. 2004;11(4):251-4.
29. Tanno T, Bhanu NV, Oneal PA, Goh SH, Staker P, Lee YT, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med. 2007;13(9):1096-101.
30. Tanno T, Porayette P, Sripichai O, Noh SJ, Byrnes C, Bhupatiraju A, et al. Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. Blood. 2009;114(1):181-6.
31. Tanno T, Noel P, Miller JL. Growth differentiation factor 15 in erythroid health and disease. Curr Opin Hematol. 2010;17(3):184-90.
32. Iolascon A, de Falco L, Beaumont C. Molecular basis of inherited microcytic anemia due to defects in iron acquisition or heme synthesis. Haematologica. 2009;94(3):395-408.
33. Sathyanarayana P, Dev A, Fang J, Houde E, Bogacheva O, Bogachev O, et al. EPO receptor circuits for primary erythroblast survival. Blood. 2008;111(11):5390-9.
34. Sladic-Simic D, Zivkovic N, Pavic D, Marinkovic D, Martinovic J, Martinovitch PN. Hereditary hypochromic microcytic anemia in the laboratory rat. Genetics. 1966;53(6):1079-89.
35. Gunshin H, Jin J, Fujiwara Y, Andrews NC, Mims M, Prchal J. Analysis of the E399D mutation in SLC11A2. Blood. 2005;106(6):2221.
36. Thompson K, Molina RM, Brain JD, Wessling-Resnick M. Belgrade rats display liver iron loading. J Nutr. 2006;136(12):3010-4.
37. M Loo, Y Beguin. The effect of recombinant human erythropoietin on platelet counts is strongly modulated by the adequacy of iron supply. Blood. 1999;93(10):3286-93.
38. Ginzburg Y, Rivella S. {beta}-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism. Blood. 2011;118(16):4321-30.
39. Tanno T, Miller JL. Iron loading and overloading due to ineffective erythropoiesis. Adv Hematol. 2010;2010:358283.
40. Schrier SL. Pathophysiology of thalassemia. Curr Opin Hematol. 2002;9(2):123-6.
41. Ganz T. Hepcidin and iron regulation, 10 years later. Blood. 2011;117(17):4425-33.
42. Yoon D, Pastore YD, Divoky V, Liu E, Mlodnicka AE, Rainey K, et al. Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. J Biol Chem. 2006;281(35):25703-11.
43. Gordeuk VR, Miasnikova GY, Sergueeva AI, Niu X, Nouraie M, Okhotin DJ, et al. Chuvash polycythemia VHLR200W mutation is associated with down-regulation of hepcidin expression. Blood. 2011;118(19):5278-82.
44. Vokurka M, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. Physiol Res. 2006;55(6):667-74.
45. Ashby DR, Gale DP, Busbridge M, Murphy KG, Duncan ND, Cairns TD, et al. Erythropoietin administration in humans causes a marked and prolonged reduction in circulating hepcidin. Haematologica. 2010;95(3):505-8.