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

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Supplementary Design and Methods

Progenitor cell assay

Light-density mononuclear cells (MNC) were isolated from heparinized peripheral blood and frozen before use in the *in vitro* colony-forming assay as previously described.[1] Briefly, $3x10^5$ MNC were plated in methylcellulose media (H4531; StemCell Technologies, Vancouver, Canada) with the addition of 0.12, 0.24, 0.5, or 1.0 U of recombinant human erythropoietin (rhEPO) (Janssen Pharmaceuticals) per milliliter of medium. In selected cultures, an iron chelate salicylaldehyde isocotinoyl hydrazone (SIH) saturated with iron (Fe-SIH) and a broad-spectrum caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-fmk) (Promega, Madison, WI, USA) were added at the beginning of the cultures. SIH, a gift from Dr. Prem Ponka (McGill University, Montreal, Canada), was saturated with iron (Fe), as previously described.[2] The final concentration of Fe-SIH used in the cultures was 10 μM.[3] Z-VAD-fmk was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Munich, Germany) to prepare a stock solution of 20 mM. A concentration of 30 μM of z-VAD-fmk was used in the hematopoietic colony assay on DMT1-mutant progenitors because it does not significantly alter the plating efficiency of healthy control burst-forming unit-erythroid (BFU-E) progenitors (*our unpublished observations*). BFU-E colonies were counted on day 14 under an inverted microscope and processed for cellularity determination and the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (see below).

Cellularity of erythroid colonies

Individual BFU-E colonies from cultures containing different concentrations of rhEPO (without Fe-SIH) were harvested into 1.5 mL of Iscove's modified Dulbecco's medium (Invitrogen) and pelleted by centrifugation (2000 rpm for 5 min). The cell pellets were resuspended in phosphate-buffered saline (Invitrogen) and the total cell number was determined using a Bürker's counting chamber (Meopta, Prerov, Czech Republic). Cellularity expresses the average number of cells per colony.

TUNEL assay

BFU-E colonies from cultures containing 1.0 U/mL of EPO were harvested and used for cytospin analysis. Archived May-Grünwald/Giemsa-stained bone marrow smears were destained overnight in Carnoy fixative.[4] The Fluorescein *In Situ* Cell Death Detection Kit (Roche Applied Science) was used to detect apoptosis according to the manufacturer's instructions. Apoptotic cells were identified as being TUNEL-positive ($TUNEL^+$). Cell nuclei were counterstained with 0.001% 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). Slides were analyzed using immunofluorescence microscopy.

Measurement of plasma iron parameters and tissue iron detection

The plasma iron levels and unsaturated iron binding capacity (UIBC) were determined using a colorimetric ferrozine-based assay and commercial kits from Roche (#1876996 and #2146398) (F. Hoffmann-La Roche Ltd.). The samples were processed according to the manufacturer's instructions and measured using Modular Analytics SWA Evo (Roche, Hitachi). Concentrations of non-heme iron in the liver and spleen were quantified as previously described.[5] Tissue non-heme iron was detected on deparaffinized tissue sections by the standard Perls' Prussian blue staining technique.

Quantification of erythropoietin

The plasma levels of EPO in mice were quantified according to the manufacturer's instructions for the Mouse/Rat Erythropoietin Quantikine ELISA Kit (R&D Systems).

Flow cytometry

Bone marrow and spleen cells were isolated and immunostained with fluorescein isothiocyanate (FITC)-conjugated CD71 antibody and/or phycoerythrin-conjugated Ter119 antibody (both from BD Biosciences, Franklin Lakes, NJ, USA) according to Socolovsky *et al.*[6] Apoptosis was detected using the Annexin V/FITC Kit (BD Biosciences), according to the manufacturer's instructions. Dead cells were excluded from the analysis by their co-staining with near infrared (IR)-fluorescent reactive dye (Invitrogen). The intensity of fluorescence was measured by FACSCalibur (BD Biosciences).

Immunohistochemistry

All samples were fixed in neutral-buffered formalin for 24 h and embedded in paraffin. Immunohistochemical staining was performed on 3 μm to 5 μm-thick slides. Unconjugated AffiniPure Fab Fragment Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Labs, West Grove, PA, USA) was used to block endogenous mouse IgG. The following primary antibodies were used: rat anti-mouse Ter119 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-mouse phospho-STAT5 (Tyr 694/Tyr 699) (recognizing Tyr 694 phosphorylated Stat5a and Tyr 699 phosphorylated Stat5b; 1:100; Santa Cruz Biotechnology). Antigen revitalization was performed in 10 mM citrate buffer (Sigma-Aldrich, pH 6.0 in the histoprocessor: high pressure, 120°C, 15 min). The secondary antibodies and detection system used were as follows: biotinylated goat anti-rat Ig specific polyclonal antibody (1:200; BD Pharmingen), biotinylated donkey anti-goat IgG-B antibody (1:200; Santa Cruz Biotechnology) and streptavidin peroxidase (1:40; Biogenex, San Ramon, CA, USA). DAB+ (DAKO, Glostrup, Denmark) was used as a visualization chromogen. During the staining process, slides were washed twice for 5 min in 0.5 M Tris-HCl and once for 5 min in 0.5 M Tris-HCl with 0.5% Tween 20 (both Sigma-Aldrich). Ter119 staining was followed by analysis of apoptosis using the Alkaline Phosphatase *In Situ* Cell Death Detection Kit (Roche Applied Science), according to the manufacturer's instructions. The slides were analyzed by light microscopy.

Immunoblotting

Western blots of bone marrow and spleen protein lysates and detection of specific proteins were performed as previously described.[1] The primary antibodies used were as follows: anti-BC_L-X_L (BCL2L1, 1:400), anti-MCL-1 (1:500) (both from Abcam, Cambridge, United Kingdom), anti-tubulin (1:500, Sigma-Aldrich), anti-STAT5 (1:500; Cell Signaling Technologies, Danvers, MA, USA), and anti-phospho-STAT5 (recognizing Tyr 694 phosphorylated Stat5a and Stat5b; 1:250; Cell Signaling Technologies).

Real-time polymerase chain reaction

Total RNA was isolated from mouse bone marrow and livers using Trizol extraction (Invitrogen, Carlsbad, CA, USA) and the RNeasy Mini Kit (Qiagen Inc., Venlo, the Netherlands), respectively. A total of 1 μg of DNAse-treated RNA was subjected to cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany). Real-time polymerase chain reaction was performed in triplicate on a LightCycler 480 (Roche Applied Science) using the LightCycler 480 Probes Master (for GDF15, TWSG1, and all housekeeping genes) and the LightCycler 480 SYBR Green I Master (for hepcidin) under the following conditions: 95°C for 5 min followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. Gene-specific primers were designed by the Assay Design Center for UPL Systems (Roche Applied Science) with the exception of the hepcidin primers, which were designed using the Primer3 application (*http://frodo.wi.mit.edu/*). Expression of specific genes was normalized against two housekeeping genes; beta-actin and GAPDH for bone-marrow samples and UBC and GAPDH for liver and spleen samples. The primer sequences and corresponding UPL probes were as follows:

GDF15: Forward: GAGCTACGGGGTCGCTTC;
Reverse: GGGACCCCAATCTCACCT;
Probe #62 (cat. no. 04688619001);

TWSG1: Forward: CTGTTCCCAGCAACAATGTC;
Reverse: ACTGTGCACATGCGCTCTT;
Probe #60 (cat. no. 04688589001);

Beta-actin: Forward: AAGGCCAACCGTGAAAAGAT;
Reverse: GTGGTACGACCAGAGGCATAC;
Probe #56 (cat. no. 04688538001);

mGAPDH: Forward: AGCTTGTCATCAACGGGAAG;
Reverse: TTTGATGTTAGTGGGGTCTCG;
Probe #9 (cat. no. 04685075001);

mUBC: Forward: TCTTCGTGAAGACCCTGACC;
Reverse: CGTTCTCGATGGTGTCACTG
Probe #10 (cat. no. 04685091001);

Hepcidin: Forward: CCTGAGCAGCACCACCTATCT;
Reverse: TCAGGATGTGGCTCTAGGCTATGT

References

1. 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.
2. Ponka P, Schulman HM. Acquisition of iron from transferrin regulates reticulocyte heme synthesis. J Biol Chem. 1985;260(27):14717-21.
3. 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.
4. Nersesyan A, Kundi M, Atefie K, Schulte-Hermann R, Knasmüller S. Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. Cancer Epidemiol Biomarkers Prev. 2006;15 (10):1835-40.
5. Torrance JD, Bothwell TH. Tissue iron stores. In: Methods in Hematology, Vol. 1. Cook JD (ed), Churchill Livingstone, New York, 1980, pp 90-115.
6. Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF. Ineffective erythropoiesis in Stat5a−/−5b−/− mice due to decreased survival of early erythroblasts. Blood. 2001;98(12):3261-73.

Online Supplementary Table S1. EPO decreases apoptosis of erythroid cells in *mk/mk* bone marrow.

	Bone marrow			
	wt	mk/mk	mk/mk EPO 4 days	mk/mk EPO 8 days
% Ter119+/ annexin V+ cells	2.8±1.0	12.4±2.3	5.4±0.9***	5.0±1.1***

*Values indicate means ± standard deviations of the indicated genotypes; n = 5 each. 4d and 8d represent 4-day and 8-day EPO application, respectively. ***P< 0.001 when compared to non-treated* mk/mk *mice. The difference between* mk/mk *EPO 4d and* mk/mk *EPO 8d is not statistically significant.*

Online Supplementary Table S2. EPO decreases apoptosis of erythroid cells in the *mk/mk* spleen.

	Spleen			
	wt	mk/mk	mk/mk EPO 4 days	mk/mk EPO 8 days
% Ter119+/ annexin V+ cells	1.1±0.5	7.3±0.7	3.1±0.9***	2.8±0.9***

*Values are means ± standard deviations of the indicated genotypes; n = 5 each. ***P< 0.001 when compared to non-treated* mk/mk *mice. The difference between* mk/mk *EPO 4 days and* mk/mk *EPO 8 days is not statistically significant.*

Online Supplementary Table S3. Distribution of maturation stages of *mk/mk* erythroid precursors in the bone marrow (see also *Online Supplementary Figure S3*).

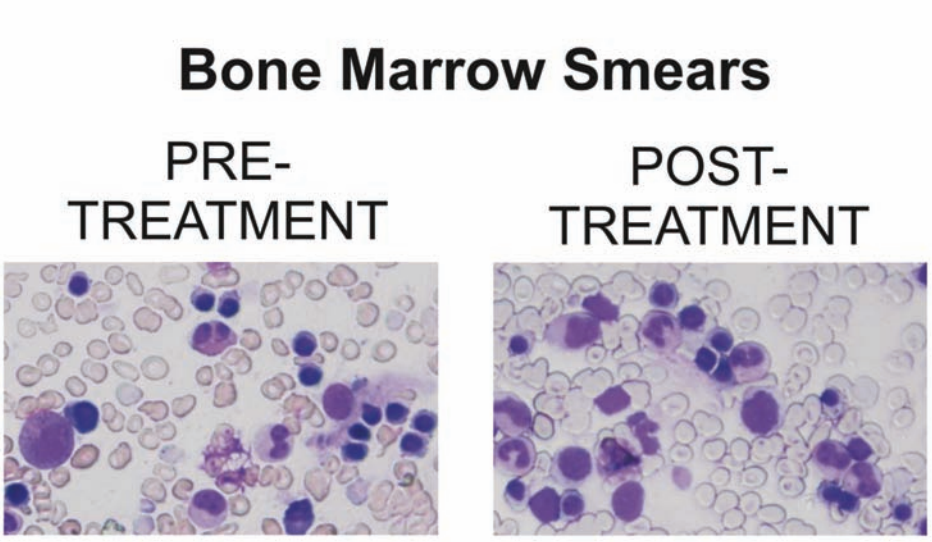
	wt	mk/mk	mk/mk EPO 4 days	mk/mk EPO 8 days
Total Ter119+ cells (%)	43.6±5.1	41.5±4.5	58.1±8.9*	64.8±9.8**
Region I (%)	2.0±0.5	3.2±0.3	3.8±0.4	6.2±1.0***
Region II (%)	63.8±7.1	86.3±1.5	84.6±2.5	72.3±7.7*
Region III (%)	1.8±0.3	1.0±0.2	2.1±0.8	1.7±0.5*
Region IV (%)	32.9±7.1	9.6±1.6	9.5±1.8	19.8±7.5*

Values are means ± standard deviations of the indicated genotypes; n = 8 for wt, mk/mk, *and* mk/mk *EPO 4 days and n = 4 for* mk/mk *EPO 8 days. Cells were divided into the regions based on the level of Ter119 and CD71 expression according to Socolovsky* et al.[6] *The relative number of cells in regions I to IV is expressed as a percentage of all viable erythroid cells. Significant differences between* mk/mk *and* mk/mk *treated with EPO for either 4 days or 8 days are indicated by asterisks; *P<0.05, **P<0.01, ***P<0.001.*

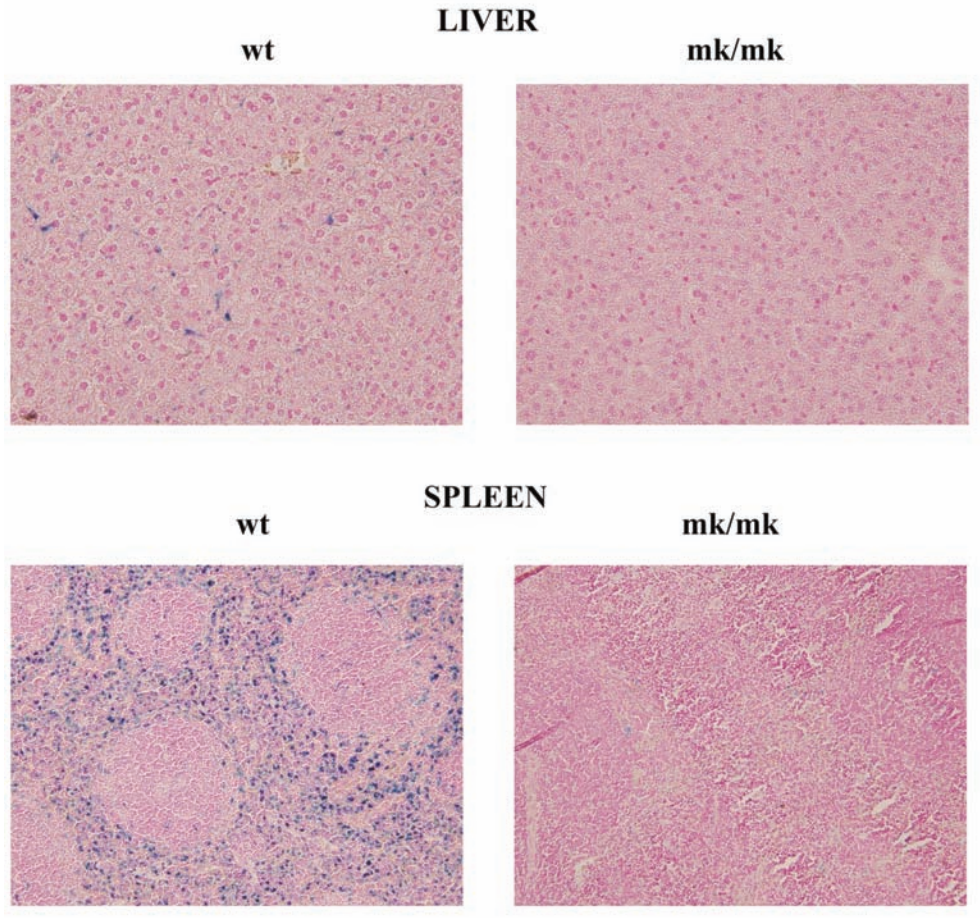
Online Supplementary Table S4. Distribution of maturation stages of *mk/mk* erythroid precursors in the spleen (see also *Online Supplementary Figure S3*).

	wt	mk/mk	mk/mk EPO 4 days	mk/mk EPO 8 days
Total Ter119+ cells (%)	61.5±6.8	77.3±3.9	77.6±0.6	74.6±3.8
Region I (%)	0.2±0.1	1.5±0.3	2.4±1.0	2.5±0.5*
Region II (%)	15.7±1.5	82.9±5.0	84.3±5.8	80.9±3.2
Region III (%)	1.1±0.2	1.4±0.4	2.3±1.3	1.6±0.1
Region IV (%)	83.1±1.4	14.2±4.9	11.1±5.5	15.1±2.7

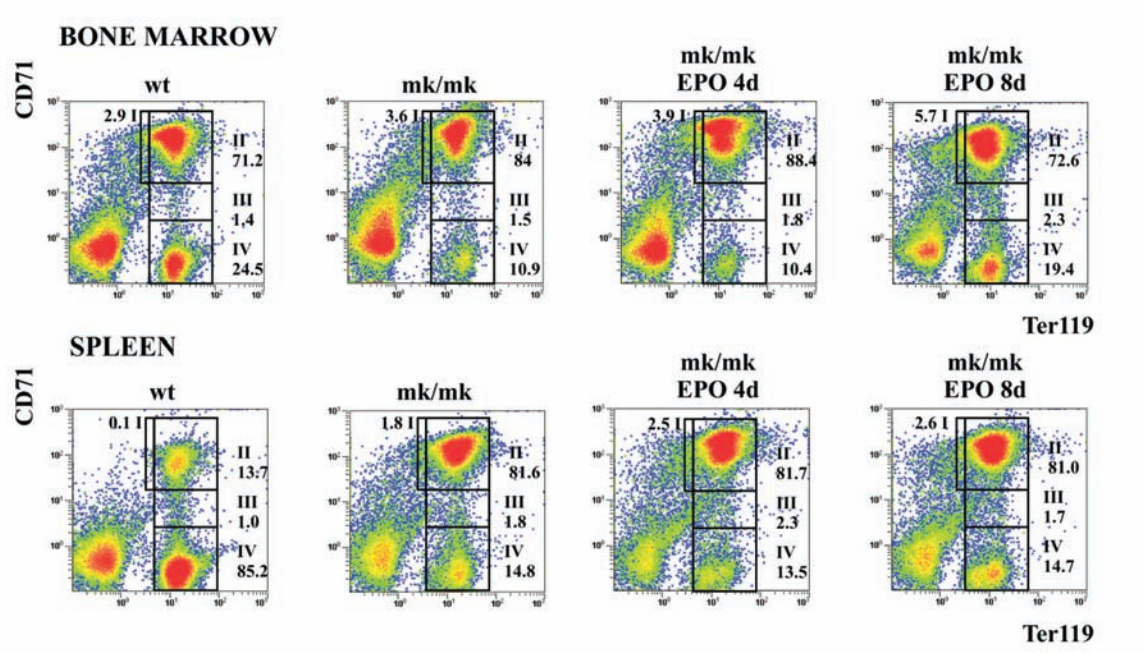
Values are means ± standard deviations of the indicated genotypes; n = 8 for wt, mk/mk, *and* mk/mk *EPO 4 days and n = 4 for* mk/mk *EPO 8 days. Cells were divided into the regions based on the level of Ter119 and CD71 expression according to Socolovsky* et al.[6] *The relative number of cells in regions I to IV is expressed as a percentage of all viable erythroid cells. A significant difference between* mk/mk *and* mk/mk *treated with EPO for either 4 days or 8 days is indicated by an asterisk; *P<0.05.*



Online Supplementary Figure S1. Comparison of pre-treatment and post-treatment bone marrow smears of the DMT1-mutant patient. May-Grünwald/Giemsa staining of bone marrow smears revealed erythroid hyperplasia with signs of dyserythropoiesis and a predominant presence of polychromatophilic normoblasts. Pre-treatment bone marrow smear: erythroid compartment represents 39.2%, proerythroblasts: 0.4% (normal range, 0-1%), basophilic normoblasts: 5.2% (normal range, 0-5%), polychromatophilic normoblasts: 17.6% (normal range, 6-16%), and orthochromatic normoblasts: 16% (normal range, 4-12%). Post-treatment bone marrow smear: erythroid compartment represents 60%, proerythroblasts: 0.0%, basophilic normoblasts: 4%, polychromatophilic normoblasts: 50.4%, and orthochromatic normoblasts: 6%. Erythrocytes are hypochromic and microcytic. Granulopoiesis is reduced, and it represents 39.6% and 32% of the pre-treatment and post-treatment bone marrow, respectively. Monocytes, lymphocytes, megakaryocytes, and plasmacytes are normal. Bone marrow smears were examined with an Olympus BX 51 microscope (Olympus, Hamburg, Germany), magnification 200x. Digital images were acquired with an Olympus DP 50 camera driven by DP controller software (both provided by Olympus). Images were cropped, assembled, and labeled using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).



Online Supplementary Figure S2. Histological detection of iron. Perls' Prussian blue staining (blue stain for iron) showed reduced iron content in the liver and spleen of *mk/mk* mice when compared to wild-type (wt) littermates. The slides were analyzed with an Olympus BX 51 light microscope (Olympus), magnification 200x for liver and 100x for spleen. Digital images were acquired with an Olympus DP 50 camera driven by DP controller software (Olympus). Images were cropped, assembled, and labeled using Adobe Photoshop software (Adobe Systems).



Online Supplementary Figure S3. The distribution of maturation stages of *mk/mk* erythroid precursors. Flow cytometry density plots of the bone marrow (top) and spleen (bottom) from a representative wild-type (wt) mouse, *mk/mk* mouse, and *mk/mk* mice treated for 4 days (4d) and 8 days (8d) with EPO. Cells were divided into the regions based on the level of Ter119 and CD71 expression according to Socolovsky *et al.*[6] The relative number of cells in regions I to IV as a percentage of all viable erythroid cells is indicated on each plot. The average values for each genotype are presented in *Online Supplementary Tables S3 and S4*. Distribution of erythroblasts of different maturation stages in *mk/mk* bone marrow and spleens differs when compared to wt mice. Immature Ter119^{high}CD71^{high} erythroblasts predominate in *mk/mk* mice; the difference is more profound in the spleen, reflecting dramatic expansion of splenic erythropoiesis in *mk/mk* mice.