Regular Article

RED CELLS, IRON, AND ERYTHROPOIESIS

The second transferrin receptor regulates red blood cell production in mice

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Key Points

- TFR2, a gene mutated in hemochromatosis and a partner of the EPO receptor, limits erythropoiesis expansion in mice.
- Iron deficiency mimics *TFR2* deletion in the erythroid compartment.

Transferrin receptor 2 (TFR2) contributes to hepcidin regulation in the liver and associates with erythropoietin receptor in erythroid cells. Nevertheless, *TFR2* mutations cause iron overload (hemochromatosis type 3) without overt erythroid abnormalities. To clarify TFR2 erythroid function, we generated a mouse lacking *Tfr2* exclusively in the bone marrow (*Tfr2^BMKO*). *Tfr2^BMKO* mice have normal iron parameters, reduced hepcidin levels, higher hemoglobin and red blood cell counts, and lower mean corpuscular volume than normal control mice, a phenotype that becomes more evident in iron deficiency. In *Tfr2^BMKO* mice, the proportion of nucleated erythroid cells in the bone marrow is higher and the apoptosis lower than in controls, irrespective of comparable erythropoietin levels. Induction of moderate iron deficiency increases erythroblasts number, reduces apoptosis, and enhances erythropoietin (Epo) levels in controls, but not in *Tfr2^BMKO* mice. Epo-target genes such as *Bcl-x_L* and *Epor* are highly expressed in the spleen and in isolated erythroblasts from *Tfr2^BMKO* mice. Low hepcidin expression in *Tfr2^BMKO* is accounted for by erythroid expansion and production of the erythroid regulator erythroferrone. We suggest that Tfr2 is a component of a novel iron-sensing mechanism that adjusts erythrocyte production according to iron availability, likely by modulating the erythroblast Epo sensitivity. (*Blood*. 2015;125(7):1170-1179)

Introduction

Transferrin receptor 2 (TFR2), the gene mutated in hemochromatosis type 3[1] is a transmembrane protein homologous to TFR1. Though not involved in iron transport, TFR2 binds the iron-loaded transferrin (holo-TF), even if with a lower affinity than TFR1,[2,3] a finding that suggests a potential regulatory role. TFR2 is expressed in the liver and, to a lower extent, in erythroid cells.[2,4] In iron-replete conditions, TFR2 protein is stabilized on the plasma membrane by binding to its ligand holo-TF. This induces a reduction of TFR2 lysosomal degradation[5] or a decreased shedding of the receptor from the plasma membrane (A.P., L.S., and C.C., unpublished manuscript). All of these properties make TFR2 a good candidate sensor for iron bound to circulating TF, measured as transferrin saturation (TS).

Humans with mutations of *TFR2* develop iron overload[1,6,7] with low hepcidin levels[8]; a similar phenotype occurs in mice with constitutive[9-12] or liver conditional[12,13] *Tfr2* deletion.

The hepatic form of TFR2 is proposed to cooperate with the hereditary hemochromatosis protein HFE, the atypical major histocompatibility complex class I protein, responsible for hemochromatosis type 1.[14] The TFR2/HFE complex is presumed to activate the transcription of hepcidin (*HAMP*), the master regulator of iron homeostasis,[15] in response to increased TS, although the molecular mechanisms remain poorly understood.

Although the involvement of TFR2 in the regulation of *HAMP* has been extensively studied, the erythroid function of the protein has not been investigated in depth. TFR2 and the erythropoietin receptor (EPOR) are activated synchronously and coexpressed during erythroid differentiation.[2,16,17] Moreover, in erythroid precursors, TFR2 associates with EPOR in the endoplasmic reticulum and is required for the efficient transport of the receptor to the cell surface. Finally, *TFR2* knockdown in vitro delays the terminal differentiation of human erythroid progenitors.[17] Thus, the erythroid TFR2 is a component of the EPOR complex and is required for efficient erythropoiesis.

We have recently demonstrated that the phenotype of *Tfr2* total (*Tfr2^−/−*) and liver-specific (*Tfr2^LCKO*) knockout (KO) mice lacking the hepcidin inhibitor *Tmprss6* switches from iron overload to iron deficiency, overlapping the phenotype of *Tmprss6^−/−* mice. An intriguing finding in the double KO mice that we generated was that only *Tmprss6^−/−Tfr2^−/−* mice developed erythrocytosis; this was not observed in *Tmprss6^−/−Tfr2^LCKO* mice.[18] We hypothesized that this abnormality was accounted for by the loss of the erythroid Tfr2 in *Tfr2^−/−*; thus, we proposed that TFR2 might be a limiting factor for erythropoiesis, particularly in conditions of iron restriction.[19] However, because *Tmprss6^−/−Tfr2^−/−* mice have lower hepcidin than *Tmprss6^−/−* and *Tmprss6^−/−* animals with liver-specific deletion of *Tfr2*, it remained

There is an Inside *Blood* Commentary on this article in this issue.

Figure 1. Iron parameters and hepatic genes expression of Tfr2^{BMKO} mice. Iron parameters and hepatic gene expression were determined in mice 4 months after transplantation with a WT (controls) or a Tfr2$^{-/-}$ (Tfr2^{BMKO}) BM. Three weeks before being sacrificed a group of mice was fed an ID or an IB diet. Graphed in the figure are: (A) TS; (B) splenic and (C) hepatic non-heme iron content (SIC and LIC); (D) liver messenger RNA (mRNA) expression of Tfr1, (E) bone morphogenetic protein 6 (Bmp6) and (F) Hepcidin (Hamp) relative to hypoxanthine phosphoribosyltransferase 1 (Hprt1). mRNA expression ratio was normalized to an IB control mean value of 1. Mean values of 3 to 8 animals for genotype (controls, Tfr2^{BMKO}) and treatment (IB, ID) are graphed. Error bars indicate standard error. Asterisks refer to a statistically significant difference. *$P <$ .05; ***$P <$ .005. C, controls.
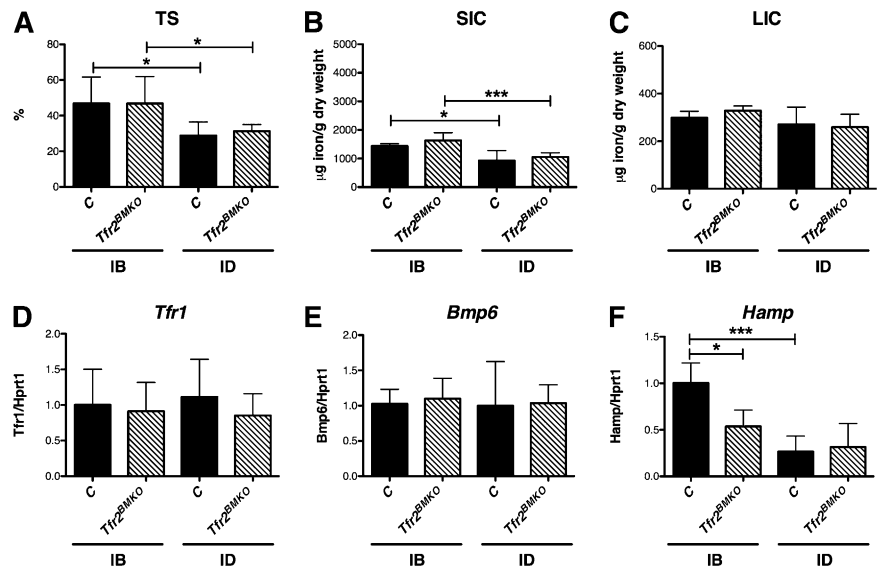
to be proven that Tfr2 deletion rather than iron deficiency or variable hepcidin levels explain the observed phenotype.

To unambiguously elucidate the function of TFR2 in erythropoiesis, particularly when iron-restricted, we generated a mouse model lacking Tfr2 in the erythroid precursors by transplanting lethally irradiated wild-type (WT) mice with the bone marrow from Tfr2$^{-/-}$ donors and manipulated the dietary iron content of the transplanted animals.

This model straightforwardly indicates that erythroid Tfr2 is essential to balance the red cell number according to the available iron, a crucial mechanism of adaptation to iron deficiency.

Methods

Mouse strains and bone marrow transplantation

Tfr2$^{-/-}$ mice (129S2 strain) were as previously described.[12] Bone marrow (BM) cells were harvested from 12 weeks old female Tfr2$^{-/-}$ mice or control WT littermates. Five $\times 10^6$ cells/mouse were injected IV into lethally irradiated (950 cGy) 8-week-old C57BL/6-Ly-5.1 male mice (Charles River). The animals were maintained in the animal facility of San Raffaele Scientific Institute (Milano, Italy) in accordance with the European Union guidelines. The study was approved by the Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute.

Two months after BM transplantation (BMT), blood was collected by tail vein puncture into tubes containing 40 mg/mL EDTA for the evaluation of hematological parameters and donor/host chimerism. Mice were fed a standard diet (200 mg/kg carbonyl-iron, Scientific Animal Food and Engineering, SAFE, Augy, France) or an iron-deficient (ID) diet (iron content: 3 parts per million, SAFE), starting the last 3 weeks before being sacrificed and were sacrificed at 4 or 6 months after BMT. Blood was collected by tail vein puncture for hematological analysis, serum transferrin saturation, and serum erythropoietin (Epo) levels determination when mice were sacrificed. Livers and spleens were weighed, dissected, and immediately snap-frozen for RNA analysis or dried for tissue iron quantification. BM cells were harvested and processed for flow cytometry analysis and cell sorting.

Hematological analysis and tissue iron content

Hemoglobin (Hb) concentration, red blood cell (RBC) count, and erythrocyte indexes (mean corpuscular volume, mean corpuscular hemoglobin), transferrin saturation, and tissue iron content were determined by standard methods as described in detail in the supplemental Materials available on the *Blood* Web site.

Flow cytometry

For phenotypic analysis by flow cytometry, bone marrow cells were pretreated with rat-anti-mouse CD16/CD32 (BD Pharmingen) to block unspecific immunoglobulin binding, and subsequently stained with phycoerythrin rat anti-mouse Ter119 (BD Biosciences) and antigen-presenting cell rat anti-mouse CD44 (BD Biosciences) for 30 minutes in the dark at 4°C.

Donor/host chimerism was evaluated on mouse peripheral blood and BM cells from transplanted mice by using FITC-conjugated anti-mouse CD45.1 (BD Biosciences) and antigen-presenting cell–conjugated anti-mouse CD45.2 antibodies (BD Biosciences). Cells were analyzed at FACS Canto^{TM} II.

Apoptosis was evaluated by using fluorescein isothiocyanate (FITC)_Annexin V Apoptosis Kit I (BD Pharmingen 556547). Briefly, cells were washed twice with cold phosphate-buffered saline and then resuspend in 1X Binding Buffer at a concentration of 1×10^6 cells/mL. 1×10^5 cells were incubated with 5 µL of FITC Annexin V and propidium iodide (PI) for 15 minutes at room temperature in the dark and then analyzed by flow cytometry within 1 hour.

Cell sorting

BM cells were pretreated with a rat anti-mouse CD16/CD32 (BD Pharmingen) to prevent unspecific immunoglobulin binding, and subsequently stained with FITC-conjugated rat anti-mouse Ter119 (BD Biosciences) and phycoerythrin-conjugated rat anti-mouse CD71 (BD Biosciences) for 30 minutes in the dark at 4°C. Cells were sorted by using MoFlo XDP cell sorter.

qRT-PCR

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed on total liver and spleen and isolated BM cells as described in the supplemental Material.
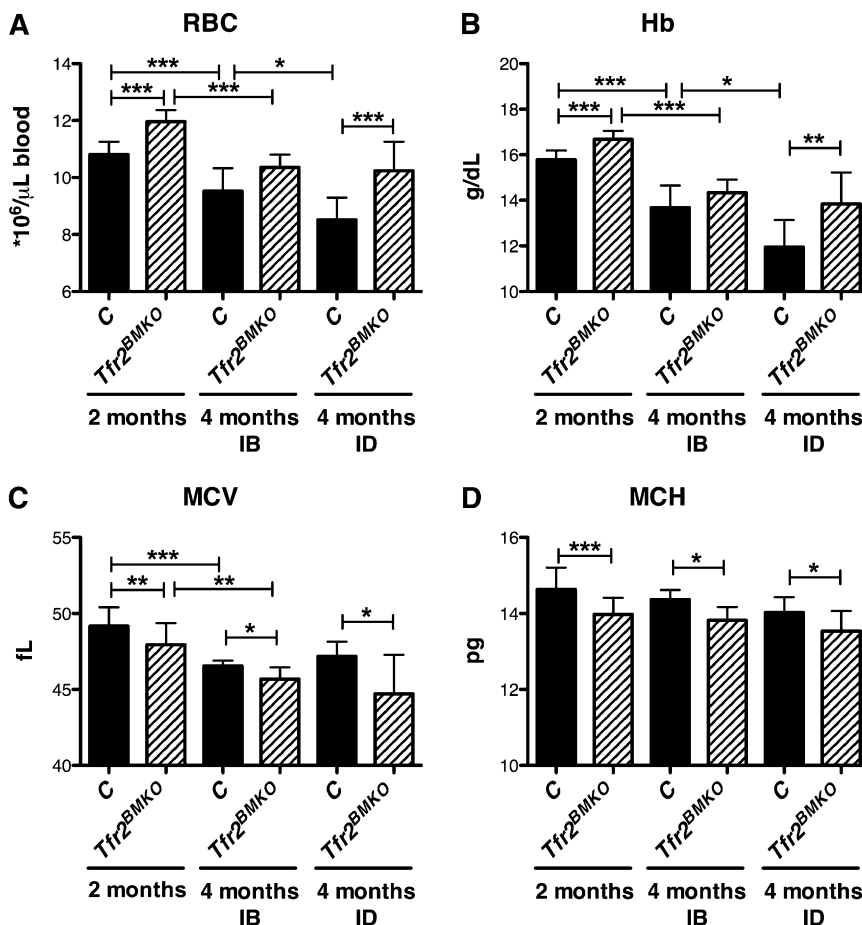
Statistics

Data are presented as mean ± standard deviation. Unpaired 2-tailed Student *t* test was performed using GraphPad Prism 5.0 (GraphPad). $P <$ .05 was considered statistically significant.

Results

Development of Tfr2^{BMKO} model

To determine the impact of Tfr2 deletion on erythropoiesis, we generated a mouse model lacking Tfr2 specifically in the erythroid

Figure 2. Hematological parameters of *Tfr2^BMKO* mice. The hematological parameters of mice were analyzed 2 and 4 months after transplantation with a WT (controls) or a *Tfr2^{-/-}* (*Tfr2^BMKO*) BM. Mice were fed as in legend to Figure 1 (see text for details). In the figure are graphed: (A) RBC; (B) Hb; (C) mean corpuscular volume (MCV) and (D) mean corpuscular hemoglobin (MCH). Mean values of 6 to 15 animals for genotype (controls, *Tfr2^BMKO*), time after BMT (2 or 4 months), and treatment (IB, ID) are graphed. Error bars indicate standard error. Asterisks refer to a statistically significant difference. $*P < .05$; $**P < .01$; $***P < .005$.

precursors by transplanting WT C57BL/6-Ly-5.1 mice with the BM cells obtained from *Tfr2^{-/-}* donors. Two different groups of BM transplantation chimeras were generated by transplanting 5×10^6 BM cells from *Tfr2^{-/-}* mice (*Tfr2^BMKO*) or from WT mice (controls) into lethally irradiated C57BL/6-Ly-5.1 mice (20 *Tfr2^BMKO* and 20 controls). Because BM donor and recipient mice were different in the alternative allelic form of the pan-leukocyte antigen CD45, it was possible to identify the donor (CD45.2) or recipient (CD45.1) origin of the hematopoietic cells by flow cytometry analysis using specific antibodies. Treated mice were analyzed at sacrifice for donor/host chimerism in the BM. Engraftment of donor cells, calculated as the proportion of CD45.2^+ to the total CD45^+ cells, was nearly complete (98% to 99%) at 4 months after transplantation (supplemental Figure 1) and was maintained in animals treated with an ID diet.

Tfr2^BMKO mice have low *Hamp* expression despite normal iron parameters

Four months after BMT, *Tfr2^BMKO* mice maintained with an iron balanced (IB) diet for 3 weeks had TS and spleen (SIC) and liver (LIC) iron content comparable to controls (Figure 1A-C). This finding also was confirmed at 6 months after BMT (supplemental Figure 2). The ID diet induced a similar decrease of TS (Figure 1A) and SIC (Figure 1B) in both groups, whereas LIC was unchanged (Figure 1C), indicating a mild degree of ID. Compatible with LIC, the hepatic expression of the *Tfr1* of WT and *Tfr2^BMKO* mice was comparable and was not modulated by ID (Figure 1D). This was also the case for bone morphogenetic protein 6, the iron-responsive *Hamp* activator (Figure 1E).

Conversely, *Hamp* expression (Figure 1F) was reduced in *Tfr2^BMKO* mice as compared with controls in mice kept an IB diet. Despite the normal hepatic iron content, ID induced a decrease of *Hamp* levels in control mice, but not in *Tfr2^BMKO* animals, which have already low basal levels.

Tfr2^BMKO mice have increased RBC and Hb levels

In normal iron (IB) conditions mice transplanted with *Tfr2^{-/-}* BM cells had higher red blood cell (RBC) (Figure 2A; supplemental Figure 3A) counts and Hb levels (Figure 2B) than controls, although the differences were statistically significant only at early time points after BMT. In the cohort of mice fed with the ID diet, RBC and Hb levels were unmodified in *Tfr2^BMKO* mice, whereas both parameters decreased in controls (Figure 2A-B), further strengthening the difference of hematological parameters between the 2 groups of mice. The increased RBC counts observed in *Tfr2^BMKO* were accompanied by reduction of mean corpuscular volume (Figure 2C; supplemental Figure 3C) and mean corpuscular hemoglobin (Figure 2D; supplemental Figure 3D), both in IB and in ID.

Terminal erythropoiesis is increased in *Tfr2^BMKO* mice

In adult organisms, mature erythrocytes are the terminally differentiated cellular product derived from hematopoietic stem and progenitor cells. These cells undergo a series of lineage choice fate decisions, ultimately committing to the erythroid lineage and beginning erythropoiesis. Traditionally, erythropoiesis has been divided into 3 stages: early erythropoiesis, terminal erythroid differentiation,
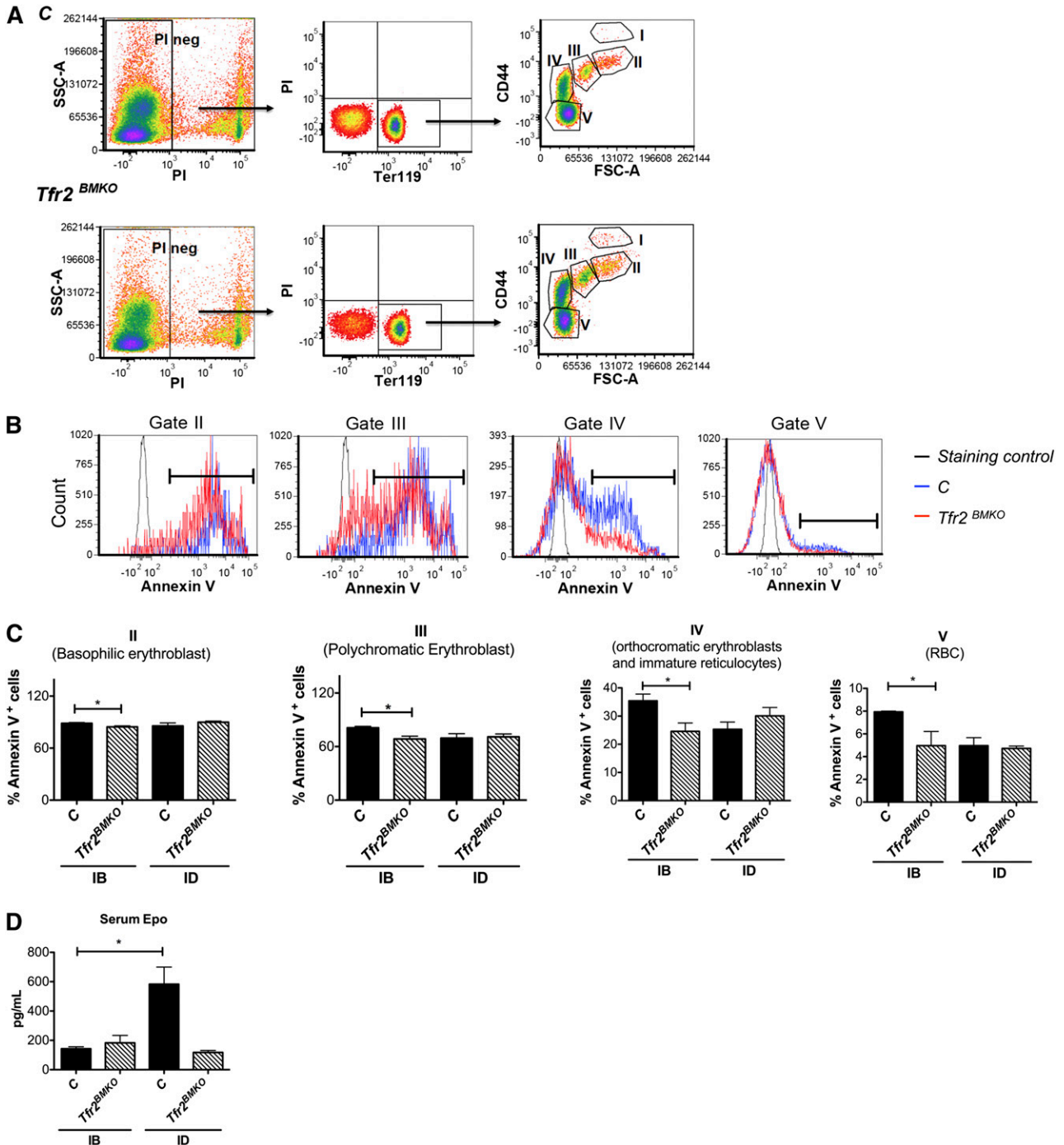
Figure 3. Analysis of erythropoiesis and serum Epo levels of Tfr2[BMKO] mice. Mice were analyzed 4 months after transplantation with a WT (controls) or a Tfr2^{−/−} (Tfr2[BMKO]) BM. Mice were fed as in legend to Figure 1 (see text for details). (A) Representative gating strategy for analysis of Ter119 subpopulations in controls and Tfr2[BMKO] mice. Viable cells (impermeable to PI) from BM were analyzed for Ter119/CD44 expression. Ter119 were gated and further analyzed with respect to FSC and CD44 surface expression for subpopulation composition (gated cluster I-V). (B) The survival of erythroblasts in the BM was examined for annexin V binding to assess apoptosis. Representative histograms of annexin V binding in basophilic (gate cluster II), polychromatic (gate cluster III), orthocromatic erythroblast (gate cluster IV), and RBC (gate cluster V). Staining control is a sample in which PI and annexin V were omitted. (C) Histogram represents percentage of apoptotic cells in basophilic (gate cluster II), polychromatic erythroblasts (gate cluster III), orthocromatic erythroblast (gate cluster IV), and RBC (gate cluster V). (D) Serum Epo levels. Mean values of 3 to 9 animals for genotype and treatment (control IB, Tfr2[BMKO] IB, control ID, Tfr2[BMKO] ID) are graphed. Bars indicate standard error. Asterisks refer to a statistically significant difference. *P < .05.

and reticulocyte maturation. Terminal erythroid differentiation begins with proerythroblasts differentiating into basophilic, polychromatic, and then orthochromatic erythroblasts that enucleate to become reticulocytes. In the final stage of erythropoiesis, reticulocytes mature into discoid erythrocytes, losing intracellular organelles.

Viable murine erythroblasts at distinct stages of terminal differentiation were analyzed by flow cytometry using Ter119 and CD44 antibodies staining on BM cells and forward scatter (reflecting cell size), as described previously.[20] The expression levels of CD44 as a function of forward scatter (FSC) in all Ter119^{+} cells separate 5 distinct clusters: proerythroblasts (I), basophilic erythroblasts (II),

Table 1. Analysis of erythropoiesis of _Tfr2_^{BMKO} mice

Treatment	Population	_C_, %	_Tfr2_^{BMKO}, %	Statistics
IB	Ter119	16.08 ± 1.72	22.97 ± 1.61	*
	I (proerythroblasts)	0.16 ± 0.02	0.29 ± 0.07	NS
	II (basophilic erythroblasts)	1.14 ± 0.06	2.23 ± 0.21	**
	III (polychromatic erythroblasts)	2.36 ± 0.12	4.43 ± 0.61	*
	IV (orthochromatic erythroblasts)	12.43 ± 1.53	16.03 ± 0.8	NS
ID	Ter119	22.8 ± 1.47	18.41 ± 1.57%	NS
	I (proerythroblasts)	0.17 ± 0.02	0.17 ± 0.03	NS
	II (basophilic erythroblasts)	2.97 ± 0.36	2.37 ± 0.17	NS
	III (polychromatic erythroblasts)	5.34 ± 0.42	4.47 ± 0.28	NS
	IV (orthochromatic erythroblasts)	14.33 ± 1.14%	11.41 ± 1.48%	NS

Mice were analyzed 4 months after transplantation with a WT (controls) or a _Tfr2_^{−/−} (_Tfr2_^{BMKO}) BM. Mice were fed as in legend to Figure 1 (see text for details). Viable cells (impermeable to PI) from BM were analyzed for Ter119/CD44 expression. Ter119⁺ cells were gated and further analyzed with respect of FSC and CD44 surface expression as described in Figure 3A. Table represents percentage of Ter119⁺ cells on alive cells (RBC not included) and subpopulation composition (gated cluster I-IV). Values are shown as mean ± standard error of the mean (SEM). The number of the events corresponding to the % Ter119⁺ cells reported (mean ± SEM) is the following: in iron balance 8042 ± 859 for controls and 11 487 ± 806 for _Tfr2_^{BMKO}; in iron deficiency 11 402 ± 736 for controls and 9204 ± 786 for _Tfr2_^{BMKO} mice.

NS, not significant.

Asterisks refer to a statistically significant difference. *$P < .05$; **$P < .01$

polychromatic erythroblasts (III), orthochromatic erythroblasts and immature reticulocytes (IV), and mature red cells (V) (Figure 3A).

In the IB condition, the percentage of BM Ter119⁺ nucleated cells was higher in _Tfr2_^{BMKO} mice than in controls (22.97 ± 1.61% vs 16.08 ± 1.72%, respectively; $P < .05$). In mice fed the ID diet, the percentage of Ter119⁺ cells was comparable between the 2 groups (Table 1). Considering the differentiation stages, in IB we observed a higher proportion of stage II, III, and IV erythroblasts (Table 1) and reticulocytes in _Tfr2_^{BMKO} mice than in controls (data not shown). The ID diet increased the proportion of cells in stage II and III only in controls (Table 2).

Erythroid precursors of _Tfr2_^{BMKO} mice show reduced apoptosis in the presence of normal Epo levels

To evaluate the apoptosis state of the maturing erythroblasts, we examined the survival of BM erythroblasts on the basis of their annexin V binding. Freshly isolated BM cells from controls and _Tfr2_^{BMKO} mice were stained with antibodies against Ter119 and CD44, incubated with annexin and the viability dye PI, and examined by flow cytometry. Viable cells were analyzed for Ter119, CD44 expression and annexin V binding.

Four months after BMT, _Tfr2_^{BMKO} mice fed an IB diet showed reduced apoptosis of the erythroid precursors (basophilic, polychromatic, and orthochromatic erythroblasts) and mature RBC, as compared with control mice (Figure 3B-C). This difference was not observed at a later time point (6 months) (supplemental Figure 4A), when the RBC count remained elevated but Hb levels normalized. ID did not influence the rate of apoptosis of _Tfr2_^{BMKO} erythroblasts. On the contrary, the percentage of apoptotic erythroid precursors decreased in control mice, reaching the levels observed in _Tfr2_^{BMKO} animals (Figure 3C).

Tfr2^{BMKO} and control animals have comparable serum Epo levels both at 4 (Figure 3D) and 6 months after BMT (supplemental Figure 4B) when maintained in IB. In ID, serum Epo levels were not affected in _Tfr2_^{BMKO}, whereas they were strongly increased in the anemic controls (Figure 3D).

## _Tfr2_^{BMKO} mice show signs of splenic stress erythropoiesis in the presence of normal Epo levels

Four months after BMT, the splenic expression of erythroid specific genes, like glycophorin A (Figure 4A), _Tfr1_(Figure 4B) _Epor_ (Figure 4C), and erythroferrone[21] (_Erfe_; Figure 4D) was higher in _Tfr2_^{BMKO} than in control mice maintained in IB conditions, suggesting that stress erythropoiesis takes place in the spleen of _Tfr2_^{BMKO}. In ID, the level of expression was unchanged in _Tfr2_^{BMKO} mice and increased in control animals, reaching levels observed in mice lacking _Tfr2_. Accordingly other Epo-EpoR–dependent Janus kinase 2/signal transducer and activator of transcription 5 (Stat5) signaling pathway target genes as B-cell lymphoma extra large (_Bcl-xL_)[22-24] (supplemental Figure 5A) and serine peptidase inhibitor, clade A, member 3G (_Serpina3g_)[25] (supplemental Figure 5B) was upregulated, whereas cyclin G2 (_Ccng2_) (supplemental Figure 5C) was downregulated, as reported.[25]

The expression of these erythroid genes in the spleen of _Tfr2_^{BMKO} is compatible with stress erythropoiesis and in the absence of increased Epo levels is in agreement with an increased Epo sensitivity.

Tfr2^{BMKO} erythroid precursors have increased expression of Epo target genes

To study the expression of _Epor_ and Epo target genes in specific erythroid subpopulations, BM cells from WT and _Tfr2_^{BMKO} mice were sorted based on the relative expression levels of Ter119 and CD71 (Tfr1). As described previously,[24,26] by using this sorting strategy, basophilic and most of polychromatic erythroblasts CD71^{high}/Ter119^{high} (corresponding to gate II and gate III using CD44/Ter119), which are both increased in _Tfr2_^{BMKO} mice, can be isolated and further analyzed as a single population. Four different populations of nucleated cells were sorted: CD71^{high}/Ter119^{low}, corresponding to proerythroblasts (R3); CD71^{high}/Ter119^{high} containing a mixture of basophilic and polychromatic erythroblasts (R4), CD71^{intermediate}/Ter119^{high} polychromatic erythroblasts (R5),

Table 2. Analysis of erythropoiesis according to iron status

Population	_C_, IB, %	_C_, ID, %	Statistics	_Tfr2_^{BMKO} IB, %	_Tfr2_^{BMKO} ID, %	Statistics
Ter119	16.08 ± 1.72	22.8 ± 1.47	*	22.97 ± 1.61	18.41 ± 1.57	NS
I (proerythroblasts)	0.16 ± 0.02	0.17 ± 0.02	NS	0.29 ± 0.07	0.17 ± 0.03	NS
II (basophilic erythroblasts)	1.14 ± 0.06	2.97 ± 0.36	*	2.23 ± 0.21	2.37 ± 0.17	NS
III (polychromatic erythroblasts)	2.36 ± 0.12	5.34 ± 0.42	*	4.43 ± 0.61	4.47 ± 0.28	NS
IV (orthochromatic erythroblasts)	12.43 ± 1.53	14.33 ± 1.14	NS	16.03 ± 0.81	11.41 ± 1.48	NS

Mice were treated as indicated in Table 1. Gating strategy is reported in the legend of Table 1. Values are shown as mean ± SEM.

Asterisks refer to a statistically significant difference. *$P < .05$.

Figure 4. Erythroid genes are expressed in the spleen of Tfr2BMKO mice. The expression of erythroid genes was analyzed in the spleen of mice 4 months after transplantation with a WT (controls) or a Tfr2$^{-/-}$ (*Tfr2BMKO*) BM. Mice were fed as in legend to Figure 1 (see text for details). In the figure are graphed: (A) splenic mRNA expression of glycophorin A (*Gpa*), (B) *Tfr1*, (C) *Epor*, and (D) splenic mRNA expression of *Erfe* relative to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). mRNA expression ratio was normalized to an IB control mean value of 1. Mean values of 3 to 8 animals for genotype (controls, *Tfr2BMKO*) and treatment (IB, ID) are graphed. Error bars indicate standard error. Asterisks refer to a statistically significant difference. *P < .05; **P < .01; ***P < .005.
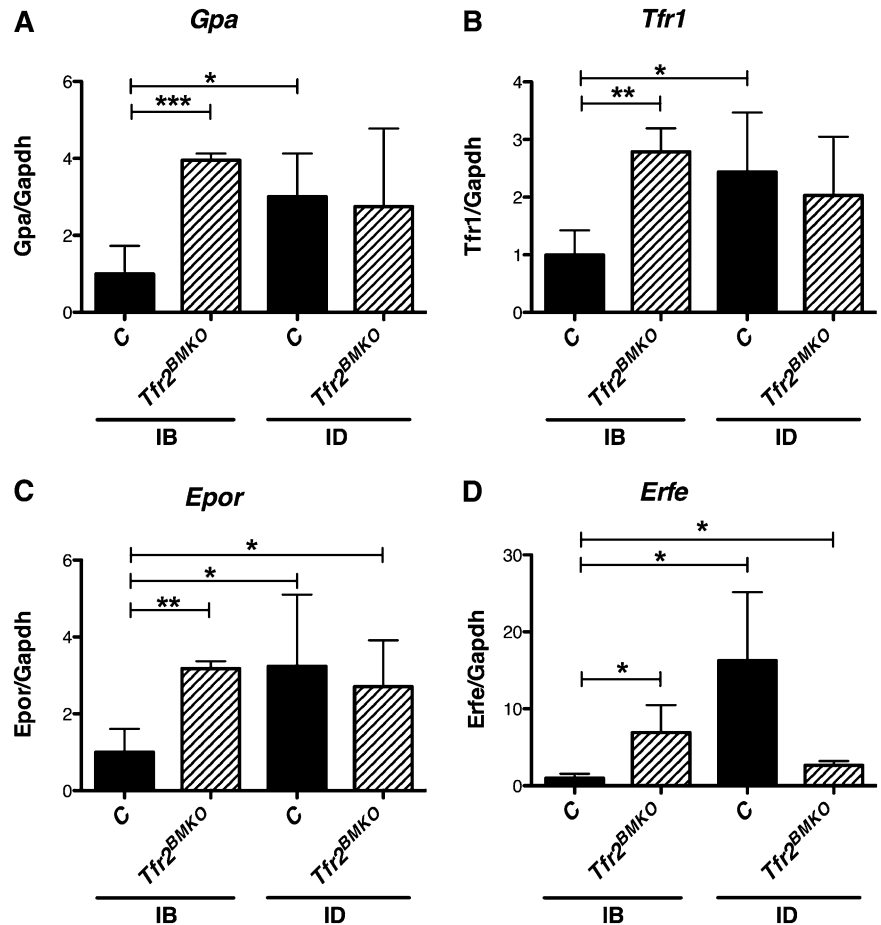
and CD71^{low}/Ter119^{high} corresponding to orthochromatic erythroblasts (R6) (Figure 5A). The percentage of cells in stage R4 was higher in *Tfr2BMKO* mice than in control mice (Figure 5B), further proving the increased proportion of erythroid precursors observed by Ter119 and CD44 expression analysis (see the previous section).

By evaluating the expression of *Tfr2* (Figure 5C) in R3, R4, and R5 stage cells, we confirmed that erythroid cells derived from *Tfr2BMKO* mice do not express *Tfr2*. In addition, we showed that in controls, both *Tfr2* (Figure 5C) and *Epor* genes (Figure 5D) are highly expressed in R3 and then downregulated during maturation. *TFR2* and *EPOR* show a similar pattern of expression, as observed[17]; interestingly, the downregulation of *Epor* expression was delayed in the absence of *Tfr2,* occurring only at R5 stage (Figure 5D). As for the expression profile of the Epo-Epor signaling target genes, R4 erythroblasts from *Tfr2BMKO* mice, compared with cells derived from mice transplanted with WT BM, expressed higher levels of the Epo-Stat5 target genes *Bcl-x$_L$* (Figure 6A) and *Serpina3g* (Figure 6B), lower levels of the apoptotic protein inhibited by the phosphatidylinositol 3-kinase/Akt pathway[26,27] Fas ligand (*Fasl*; Figure 6C) and similar levels of Baculoviral IAP repeat-containing protein 2 (*Birc2* or *cIap2*)[28] (data not shown).

Erythroblasts from *Tfr2BMKO* mice produce high levels of the erythroid regulator *Erfe*

Because expanded erythropoiesis signals iron needs through the erythroid regulator leading to hepcidin suppression, considering that hepcidin is strongly reduced in *Tfr2BMKO* mice, and because of the

presence of spleen erythropoiesis, we analyzed the splenic and erythroblast expression of the candidate erythroid regulator erythroferrone.

Erfe is a protein belonging to the family of tumor necrosis factor-α produced by maturing erythroblasts, recently proposed as the early suppressor of hepcidin after the physiological expansion of erythropoiesis that follows bleeding or Epo treatment.[21] *Erfe* is a target of the Epo-induced Janus kinase 2-Stat5 signaling pathway and is produced during erythroblast terminal differentiation. We observed that IB *Tfr2BMKO* mice express higher levels of *Erfe* than control mice in the spleen (Figure 4D), whereas, in ID, *Erfe* levels are unmodified in *Tfr2BMKO* mice but increase in controls, reaching the levels observed in mice lacking *Tfr2*. We confirm that also R4 erythroid precursors from *Tfr2BMKO* mice produce higher levels of *Erfe* than controls (Figure 6D).

Discussion

TFR2 mutations in humans lead to iron overload with low hepcidin in the absence of erythroid abnormalities,[1,8] highlighting only the receptor hepatic function. Consequently, most efforts have been concentrated into unraveling the liver role of TFR2 in hepcidin control and the significance of the TFR2 erythroid expression has remained unexplained. Although molecular mechanisms remain incompletely clarified, defective TFR2 impairs hepcidin upregulation in response to increased holo-TF both in humans[29] and in mice.[30] This is clearly related to the TFR2 ability to bind iron-loaded TF and emphasizes its function as a liver sensor of circulating iron excess.
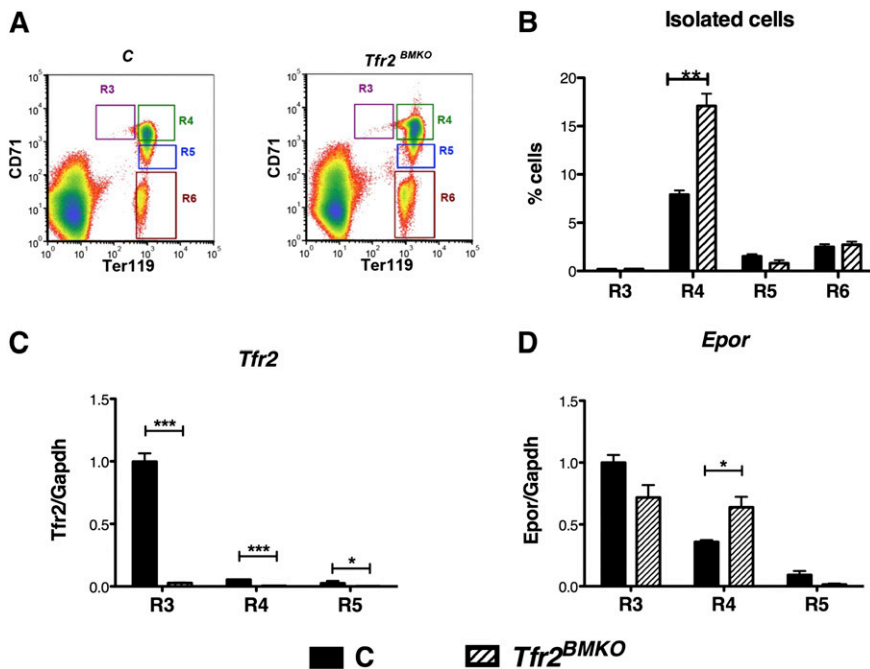
Figure 5. Analysis of *Tfr2* and *Epor* in isolated erythroblasts. BM cells from control and *Tfr2^BMKO^* mice maintained with an IB diet were sorted as described in the text. (A) Gating strategy for freshly isolated bone marrow erythroid subsets: R3, R4, R5, and R6. Nucleated cells were selected and subsets gated based on Ter119 and CD71. (B) Flow cytometry analysis of BM cells stained with antibodies against Ter119 and CD71. Histograms show the percentage of cells in stage R3, R4, R5, and R6. The number of events is reported in supplemental Table 3. (C-D) mRNA expression of *Tfr2* (C) and *Epor* (D) relative to *Gapdh* in the R3, R4, and R5 stages. mRNA expression ratio was normalized to the R3 control mean value of 1. mRNA expression ratio was normalized to the control mean value of 1. Mean values of 3 samples for genotype (controls, *Tfr2^BMKO^*) and stage (R3, R4, R5, R6) are graphed. Error bars indicate standard error. Asterisks refer to a statistically significant difference. $*P < .05$; $***P < .005$.

A major advance in deciphering the extrahepatic role of TFR2 was the serendipitous identification of its partnership with EPOR.[17] Foretnikova et al suggested that the TFR2/EPOR association stabilizes EPOR on the cell surface, although TFR2 does not directly bind EPO and does not seem to interfere with the EPO binding to EPOR. They also showed that silencing *TFR2* in vitro delays erythroid differentiation. However, these results did not provide clear insights into its function in erythropoiesis, and the consequences of *TFR2* ablation in vivo remained uncertain. Increased Hb levels were found in *Tfr2^−/−^* mice as compared with WT littermates, but this finding was ascribed to the concomitant iron overload.[12,18] An in-depth analysis revealed that only mice with ubiquitous *Tfr2* deletion are characterized by increased Hb levels, whereas *Tfr2* liver-specific KO[12,18] are not, suggesting that high Hb levels might result from the absence of Tfr2 in the erythroid compartment, rather than from the increased iron supply.

In this article, we directly analyzed the erythroid function of TFR2, showing that it has a crucial role in limiting erythropoiesis and avoiding erythrocytosis, a function disguised in iron overload but evident in iron deficiency. At the same time, we discovered that iron deficiency mimics TFR2 deletion in the erythroid compartment.

We started with the observation that erythrocytosis develops in the *Tfr2-Tmprss6* double KO mice, but not in the liver-specific *Tfr2-Tmprss6* double KO[18] animals, although both models showed the same degree of iron deficiency. This led us to propose that TFR2 might limit the erythrocyte numbers especially in iron deficiency.[19]

Our observation agrees with the results of genomewide association studies, which have identified association of *TFR2* genetic variants with RBC number[31] and hematocrit and Hb levels,[32] suggesting that TFR2 may be involved in the control of erythropoiesis.

To unravel the modalities of this control, we generated a straightforward murine model characterized by specific deletion of *Tfr2* in the BM (*Tfr2^BMKO^*), where its expression is mostly restricted to the erythroid lineage.[4,16] Loss of Tfr2 in the erythroid compartment remarkably increased the erythroid precursor frequency and their terminal differentiation, especially the proportion of maturing erythroblasts and reticulocytes. This was accompanied by decreased rate of apoptosis of cells during the phases of maturation and by signs of stress erythropoiesis (increased Ter119^+^ cells in the spleen). We

cannot exclude a role for Tfr2 in early erythropoiesis because that was not investigated, but we consider it unlikely, because the first stage of terminal differentiation (proerythroblasts) was not expanded in *Tfr2^BMKO^*. The changes observed suggest the effect of EPO stimulation[33-37] but, surprisingly, serum Epo levels of *Tfr2^BMKO^* were not increased. Considering that the survival of the erythroblasts depends on EPO[38] and that TFR2 is an EPOR partner,[17] we speculate that the genetic loss of *Tfr2* increases the EPO sensitivity of erythroid precursor cells. We found an increased expression of several Epo target genes (as *Bcl-x_L*, *Fasl*, *Serpina3g*,
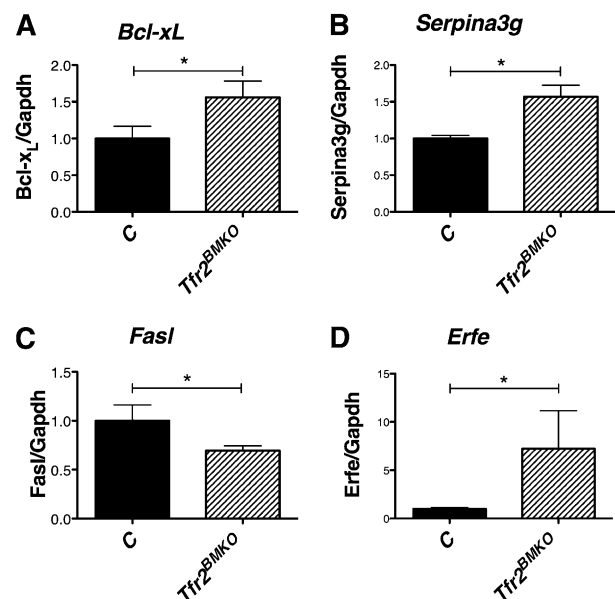
Figure 6. Analysis of Epo target genes in basophilic and polychromatic erythroblasts (R4). (A) mRNA expression of *Bcl-x_L*, (B) *Serpina3G*, (C) *Fasl*, and (D) *Erfe* relative to *Gapdh* in R4 erythroblasts. mRNA expression ratio was normalized to the control mean value of 1. Mean values of 3 samples for genotype (controls, *Tfr2^BMKO^*) are graphed. Error bars indicate standard error. Asterisks refer to a statistically significant difference. $*P < .05$; $***P < .005$.

Figure 7. Model of regulation of erythropoiesis and hepcidin by Tfr2. Regulation of erythropoiesis and hepcidin in WT mice in normal iron status (upper left panel) and in ID (upper right panel). Regulation of erythropoiesis and hepcidin in the absence of Tfr2 in normal conditions (lower left panel) and in ID (lower right panel). Arrow thickness indicates activity or binding. Bold character indicates increase concentration. Dotted lines indicate lack of binding. Dotted Tfr2 represents membrane destabilization from a lack of the ligand (2Fe-Tf). Tf, apotransferrin; 2Fe-Tf, diferric transferrin.
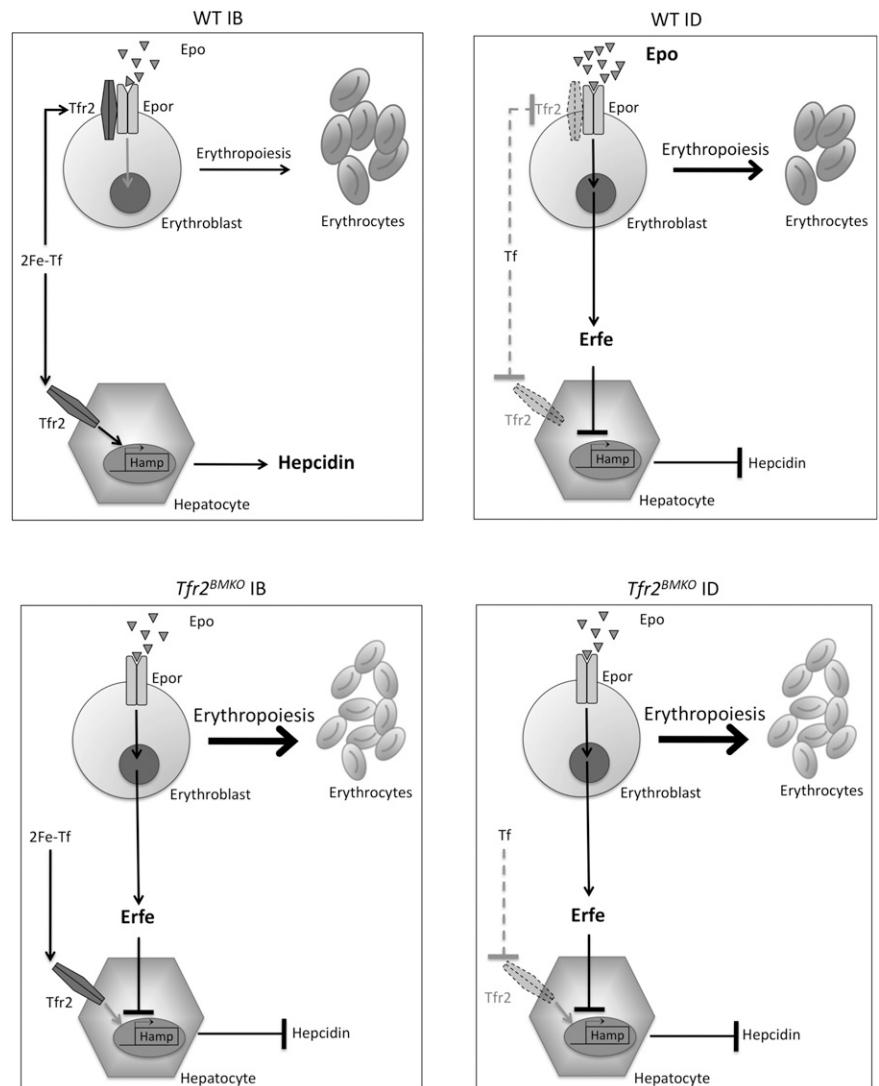
Ccng2, Epor, and *Erfe*) in *Tfr2$^{-/-}$* erythroblasts in the presence of normal Epo levels that is in agreement with this interpretation. We also considered that *Epor* expression—that in the absence of reliable anti-Epor antibodies we analyzed only at transcriptional level—was maintained longer in *Tfr2$^{-/-}$* erythroblasts during maturation, thus favoring a prolonged Epo responsiveness.

The second surprising and unexpected finding of our model is the observation that the erythroid maturation of control mice (transplanted with a WT BM) fed an ID diet becomes similar to that observed in iron-replete *Tfr2^{BMKO}* mice. The degree of ID reached with 3 weeks of an iron-poor diet was mild. However, control animals develop anemia with high levels of Epo, whereas the ID diet has no effect on the erythropoiesis of *Tfr2^{BMKO}* mice, whose Hb levels, RBC number, and percentage of erythroid precursors remain unmodified.

The finding that the erythropoiesis of ID mice with high Epo mimics that of *Tfr2^{BMKO}* mice with normal Epo and that Tfr2-deficient erythropoiesis is unmodified in ID is another point favoring the suggested increased Epo sensitivity of *Tfr2^{BMKO}* erythroid precursors.

All of these data may be compatible with our recent finding that TFR2, as TFR1, is shed from plasma membrane when not stabilized by the ligand holo-TF, whose levels are especially low in ID. This has been documented both in hepatoma-derived cells, in erythroid cell lines, and in cultures of primary erythroid cells (Pagani et al, in revision). Findings

of the present article suggest that loss of Tfr2 mimics ID. We speculate that in IB, Tfr2 might sequester EpoR on the plasma membrane to avoid excessive erythropoiesis expansion (Figure 7).

A high number of RBCs in relation with Hb levels is a feature of ID that explains the reduced Hb content per cell (mean corpuscular hemoglobin). This occurs to optimize Hb production, to ensure oxygen transport, and to provide the best metal usage in conditions of iron restriction. Small red cells may have a normal or only slightly reduced lifespan, as demonstrated by ^{51}Cr labeling red cells in humans.[39] Thus, TFR2 acts to produce efficient red cells but at the same time to save iron when its supply is limited as a means of maintaining a global body iron economy. This function is difficult to disclose when TFR2 is mutated in hemochromatosis type 3, because high transferrin saturation stabilizes TFR2 on plasma membrane and excess iron modifies erythropoiesis. The TFR2 hepatic function appears dominant over its erythroid function.

If TFR2 senses the degree of iron-loaded transferrin simultaneously in BM and hepatocytes, an important implication is that TFR2 adapts hepcidin and erythropoiesis mutual needs (Figure 7). Sensing the degree of available circulating iron (expressed as transferrin saturation), TFR2 would represent a natural link between the 2 distant tissues, although it does not play the role of erythroid regulator.[40-42] Indeed, *Tfr2^{BMKO}* mice have reduced *Hamp* levels secondary to erythropoiesis

expansion and in the absence of alterations of systemic iron homeostasis. It is likely that the increased iron release, resulting from low hepcidin levels, is used by the expanded erythropoiesis, leaving iron parameters unmodified. Among the proposed erythroid regulators able to suppress hepcidin production, we found that the transcription of the recently discovered erythroid regulator $Erfe$[21] was upregulated both in total spleen and in erythroid precursors isolated from $Tfr2^{BMKO}$ mice. This finding is in agreement with the proposed increased Epo sensitivity of $Tfr2^{-/-}$ erythroblasts, because $Erfe$ is an Epo-target gene.[21] $Erfe$ transcription is also enhanced in control animals kept in ID, again strengthening the concept that Tfr2-deficient erythropoiesis mimics the adaptation that occurs in ID, because both conditions are characterized by low hepcidin levels.

In conclusion, we have identified a new mechanism for regulating erythropoiesis in an ID manner. TFR2 is a modulator of erythropoiesis, in keeping with its role of EPOR partner. We speculate that, by modulating the EPO sensitivity of the erythroid precursors, TFR2 may act as a control system of RBC number to maintain a correct balance between their production and the available iron.

Acknowledgments

Authorship

Contribution: A.N. designed the experimental work, performed research, analyzed data, and wrote the paper; M.R.L. performed research, analyzed data, and cowrote the paper; M.R., G.M., and A.P. performed research and analyzed data; L.S. contributed to the experimental design and to analyzing the data; G.F. contributed to the experimental design, to analyzing the data, and to writing the manuscript; C.C. designed research and critically reviewed the paper; and all authors approved the final version of the manuscript.

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References

1. Camaschella C, Roetto A, Calì A, et al. The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22. Nat Genet. 2000;25(1):14-15.

2. Kawabata H, Yang R, Hirama T, et al. Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. J Biol Chem. 1999;274(30):20826-20832.

3. Fleming RE, Migas MC, Holden CC, et al. Transferrin receptor 2: continued expression in mouse liver in the face of iron overload and in hereditary hemochromatosis. Proc Natl Acad Sci USA. 2000;97(5):2214-2219.

4. Kawabata H, Germain RS, Ikezoe T, et al. Regulation of expression of murine transferrin receptor 2. Blood. 2001;98(6):1949-1954.

5. Johnson MB, Chen J, Murchison N, Green FA, Enns CA. Transferrin receptor 2: evidence for ligand-induced stabilization and redirection to a recycling pathway. Mol Biol Cell. 2007;18(3):743-754.

6. Roetto A, Totaro A, Piperno A, et al. New mutations inactivating transferrin receptor 2 in hemochromatosis type 3. Blood. 2001;97(9):2555-2560.

7. Roetto A, Daraio F, Alberti F, et al. Hemochromatosis due to mutations in transferrin receptor 2. Blood Cells Mol Dis. 2002;29(3):465-470.

8. Nemeth E, Roetto A, Garozzo G, Ganz T, Camaschella C. Hepcidin is decreased in TFR2 hemochromatosis. Blood. 2005;105(4):1803-1806.

9. Fleming RE, Ahmann JR, Migas MC, et al. Targeted mutagenesis of the murine transferrin receptor-2 gene produces hemochromatosis. Proc Natl Acad Sci USA. 2002;99(16):10653-10658.

10. Kawabata H, Fleming RE, Gui D, et al. Expression of hepcidin is down-regulated in TfR2 mutant mice manifesting a phenotype of hereditary hemochromatosis. Blood. 2005;105(1):376-381.

11. Wallace DF, Summerville L, Lusby PE, Subramaniam VN. First phenotypic description of transferrin receptor 2 knockout mouse, and the role of hepcidin. Gut. 2005;54(7):980-986.

12. Roetto A, Di Cunto F, Pellegrino RM, et al. Comparison of 3 Tfr2-deficient murine models suggests distinct functions for Tfr2-alpha and Tfr2-beta isoforms in different tissues. Blood. 2010;115(16):3382-3389.

13. Wallace DF, Summerville L, Subramaniam VN. Targeted disruption of the hepatic transferrin receptor 2 gene in mice leads to iron overload. Gastroenterology. 2007;132(1):301-310.

14. Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet. 1996;13(4):399-408.

15. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. Cell. 2010;142(1):24-38.

16. Kawabata H, Nakamaki T, Ikonomi P, Smith RD, Germain RS, Koeffler HP. Expression of transferrin receptor 2 in normal and neoplastic hematopoietic cells. Blood. 2001;98(9):2714-2719.

17. Forejtnikovà H, Vieillevoye M, Zermati Y, et al. Transferrin receptor 2 is a component of the erythropoietin receptor complex and is required for efficient erythropoiesis. Blood. 2010;116(24):5357-5367.

18. Nai A, Pellegrino RM, Rausa M, et al. The erythroid function of transferrin receptor 2 revealed by Tmprss6 inactivation in different models of transferrin receptor 2 knockout mice. Haematologica. 2014;99(6):1016-1021.

19. Silvestri L, Nai A, Pagani A, Camaschella C. The extrahepatic role of TFR2 in iron homeostasis. Front Pharmacol. 2014;5:93.

20. Chen K, Liu J, Heck S, Chasis JA, An X, Mohandas N. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci USA. 2009;106(41):17413-17418.

21. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet. 2014;46(7):678-684.

22. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell. 1999;98(2):181-191.

23. Silva M, Benito A, Sanz C, et al. Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent cell lines. J Biol Chem. 1999;274(32):22165-22169.

24. 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):3261-3273.

25. Singh S, Dev A, Verma R, et al. Defining an EPOR- regulated transcriptome for primary progenitors, including Tnfr-sf13c as a novel mediator of EPO- dependent erythroblast formation. PLoS ONE. 2012;7(7):e38530.

26. Liu Y, Pop R, Sadegh C, Brugnara C, Haase VH, Socolovsky M. Suppression of Fas-FasL coexpression by erythropoietin mediates erythroblast expansion during the erythropoietic stress response in vivo. Blood. 2006;108(1):123-133.

27. Kashii Y, Uchida M, Kirito K, et al. A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. Blood. 2000;96(3):941-949.

28. Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature. 2001;412(6847):641-647.

29. Girelli D, Trombini P, Busti F, et al. A time course of hepcidin response to iron challenge in patients with HFE and TFR2 hemochromatosis. *Haematologica.* 2011;96(4):500-506.

30. Ramos E, Kautz L, Rodriguez R, et al. Evidence for distinct pathways of hepcidin regulation by acute and chronic iron loading in mice. *Hepatology.* 2011;53(4):1333-1341.

31. Soranzo N, Spector TD, Mangino M, et al. A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. *Nat Genet.* 2009; 41(11):1182-1190.

32. Auer PL, Teumer A, Schick U, et al. Rare and low-frequency coding variants in CXCR2 and other genes are associated with hematological traits. *Nat Genet.* 2014;46(6): 629-634.

33. Goldwasser E. Erythropoietin and its mode of action. *Blood Cells.* 1984;10(2-3):147-162.

34. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science.* 1990; 248(4953):378-381.

35. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell.* 1995;83(1):59-67.

36. Dolznig H, Habermann B, Stangl K, et al. Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts. *Curr Biol.* 2002;12(13): 1076-1085.

37. Elliott S, Pham E, Macdougall IC. Erythropoietins: a common mechanism of action. *Exp Hematol.* 2008;36(12):1573-1584.

38. Kelley LL, Koury MJ, Bondurant MC, Koury ST, Sawyer ST, Wickrema A. Survival or death of individual proerythroblasts results from differing erythropoietin sensitivities: a mechanism for controlled rates of erythrocyte production. *Blood.* 1993;82(8):2340-2352.

39. Temperley IJ, Sharp AA. The life span of erythrocytes in iron-deficiency anaemia. *J Clin Pathol.* 1962;15:346-349.

40. Finch CA, Cook JD, Labbe RF, Culala M. Effect of blood donation on iron stores as evaluated by serum ferritin. *Blood.* 1977;50(3):441-447.

41. Finch C. Regulators of iron balance in humans. *Blood.* 1994;84(6):1697-1702.

42. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. *Blood.* 2006;108(12): 3730-3735.