Transient decrease of serum iron after acute erythropoietin treatment contributes to hepcidin inhibition by ERFE in mice

Irene Artuso, Mariateresa Pettinato, Antonella Nai, Alessia Pagani, Ugo Sardo, Benjamin Billoré, Maria Rosa Lidonnici, Cavan Bennett, Giacomo Mandelli, Sant-Rayn Pasricha, et al.

Transient decrease of serum iron after acute erythropoietin treatment contributes to hepcidin inhibition by ERFE in mice

To coordinate iron supply with hemoglobin (Hb) synthesis, erythropoietin (EPO)-stimulated erythroid precursors release erythroferrone (ERFE), a soluble protein that suppresses the expression of hepcidin (*Hamp*).[1] Hepcidin is a short liver peptide that controls iron entry into the circulation by blocking the sole iron exporter ferroportin.[2] ERFE is essential in stress erythropoiesis since the *Erfe* knockout mice fail to suppress hepcidin after phlebotomy and show a delayed recovery from anemia.[1] Increased erythropoiesis and iron deficiency suppress hepcidin via ERFE-dependent and ERFE-independent mechanisms, respectively, culminating in chromatin remodeling due to reversible loss of the histone activation marks H3K4me3 and H3K9ac.[3]

Hepcidin expression in hepatocytes is mainly regulated by the Bone Morphogenetic Protein (BMP)-Son of Mother Against Decapentaplegic (SMAD) pathway. Increased plasma iron in the form of diferric transferrin (holoTF), measured *in vivo* as increased transferrin saturation, activates the signaling that is initiated by the ligand BMPs and requires the co-receptor hemojuvelin (HJV).

The mechanisms by which ERFE represses hepcidin are still unclear. Mounting evidence indicates that ERFE requires some basal BMP-SMAD activity,[4] but excessive activation of the BMP-SMAD pathway prevents its suppressive effects on hepcidin.[5] In *Tmprss6* KO mice, with an inappropriately active BMP-SMAD signaling, hepcidin levels are high, despite elevated EPO and ERFE levels.[6] The resistance of *Tmprss6* KO mice to ERFE is rescued by pharmacologic down-regulation of the pathway.[5] We thus hypothesized that EPO itself might attenuate the BMP-SMAD pathway to favor the ERFE regulatory function.

To investigate whether the liver BMP-SMAD pathway is downregulated by EPO, C57BL/6N male wild type (WT) mice were treated with a single 200 U/mouse intraperitoneal injection of EPO or saline and sacrificed at 3, 6, 9 and 15 hrs after treatment. Consistent with previous reports,[1] *Erfe* expression is upregulated both in bone marrow and spleen of EPO-treated mice (*Online Supplementary Figure S1A and S1B*) and *Hamp* expression is inhibited at 9 hrs post EPO (*Online Supplementary Figure*

S1C), paralleled by a downregulation of *Id1* (*Online Supplementary Figure S1D*) and *Smad7* (*Online Supplementary Figure S1E*). However, at 15 hours the pathway is active (increased *Id1* and *Smad7*) while hepcidin is inhibited. Although *Erfe* expression is upregulated at 3 and 6 hrs post-EPO, *Hamp* expression is not decreased until 9 hrs, when the pathway is attenuated. Altogether, these results demonstrate that the BMP-SMAD pathway is inhibited shortly after EPO injection.

To determine the contribution of ERFE at short time points after EPO, we next analyzed the response of *Erfe−/−* mice to EPO. As expected, *Hamp* was only slightly reduced in *Erfe−/−* animals 15 hrs post-EPO (Figure 1A). Interestingly, *Id1* (Figure 1B) and *Smad7* (Figure 1C) expression are also decreased in *Erfe*-deficient mice 9 hours after injection, suggesting an EPO-dependent but ERFE-independent inhibition of the pathway.

Previous studies have indicated that suppression of *Hamp* expression 3-4 days after EPO treatment leads to a reversible loss of the histone activation marks H3K4me3 and H3K9ac at the *Hamp* locus (*Online Supplementary Figure S2*). We sought to assess whether chromatin remodeling may contribute to hepcidin inhibition in WT mice at short time points after EPO. Consistent with the repression of hepcidin (*Online Supplementary Figure S1C*), chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR) assays demonstrated a reduction in H3K4me3 (Figure 1D) but not in H3K9ac (Figure 1E) at the *Hamp* locus 9 hrs, but not at 6 hrs after EPO. This indicates that loss of H3K4me3 at the hepcidin promoter occurs early after EPO administration to repress hepcidin even in the presence of H3K9ac.

At early time points (6 and 9 hrs) after EPO treatment, liver and spleen iron content are unchanged (*data not shown*), whereas, serum iron (*Online Supplementary Figure S1F*), TS (*Online Supplementary Figure S1F*), *Id1* and *Smad7* (*Online Supplementary Figures S1D and S1E*) are significantly reduced, in agreement with similar recently reported results.[7] Serum iron (Figure 2A) and TS (Figure 2B) are decreased also in the absence of ERFE. However, since hepcidin suppression is blunted, their levels remain low in *Erfe*-deficient mice at 15 hours (Figure 2A and 2B). In contrast, in WT mice, both parameters increase between 9-15 hrs, stimulating the expression of *Id1* and *Smad7* (Figure 1B and 1C, *Online Supplementary Figure S1D and S1E*). We thus surmise that the ERFE effect on

Table 1. Membrane TFR1 (CD71) expression in bone marrow and spleen-derived cells at different stages of maturation.

	Subpopulations	UT	3hr	6hr	3hr *vs.* UT	6hr *vs.* UT	3hr *vs.* 6hr
BM	I	835.2±170.9	2014±180.7	3682±355.9	*	***	**
	II	3401±538.4	8473±953.1	10895±683	**	**	ns
	III	3020±66.23	5245±484.7	7562±102.9	**	****	**
	IV	1950±525.4	2511±213.2	3862±144.6	ns	*	ns
	V	3013±558.5	2762±289.1	3312±354.7	ns	ns	ns
SP	II	3500±257.1	8220±929.5	10027±734.8	***	****	ns
	III	2997±199.4	5569±600	7977±841.4	ns	**	ns
	IV	3063±649.3	3064±338.9	3931±481.9	ns	ns	ns
	V	3467±693.6	2794±299.3	3660±329.3	ns	ns	ns

C57BL/6N wild type mice (3 mice for each time point) were treated with EPO (200 U/mouse) or saline and sacrificed at 3 and 6 hrs post-injection. BM and spleen cells were isolated and analyzed for Ter119/CD44 expression. Ter119 was gated and analyzed with respect to FSC and CD44 surface expression for subpopulation composition (gated cluster I-V) (see *Online Supplementary Figure S2A and S2B*). Membrane CD71/TFR1 expression in the BM and spleen derived erythroid populations was evaluated as mean fluorescent intensity (MFI). Unpaired two-tailed Student t-test was used for significance calculation. ns: not significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$. BM: bone marrow; SP: spleen.
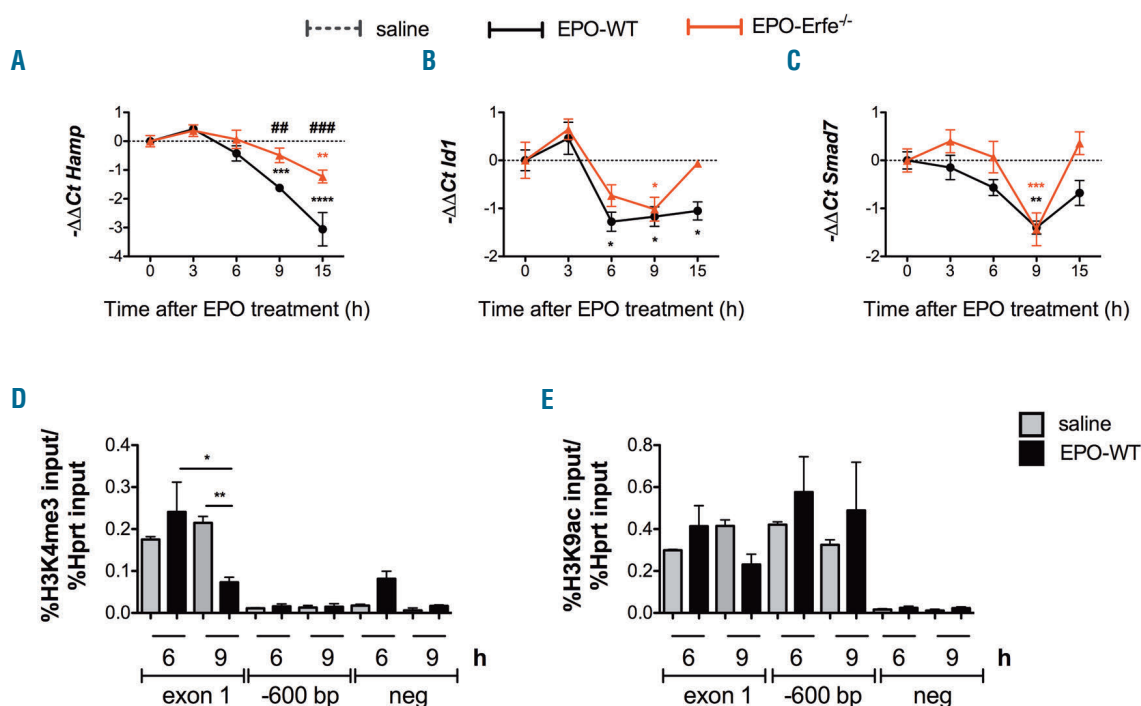
Figure 1. Time course analysis of the effect of a single EPO injection on hepcidin and BMP-SMAD target genes in WT and *Erfe*^-/- mice and analysis of chromatin modifications at the hepcidin promoter locus 6 and 9 hours after EPO. C57BL6/J wild type and *Erfe*-deficient male mice were treated with EPO (200 U/mouse) or saline (6-9 mice for each time point) and sacrificed at different time points. The liver expression of hepcidin (*Hamp*) (A) and the BMP-SMAD target genes *Id1* (B) and *Smad7* (C) was analyzed by qRT-PCR using *Hprt1* as housekeeping gene. Changes in the expression levels were represented as $-\Delta\Delta Ct$ (saline–EPO). Data shown are means ± s.e.m and were compared for each genotype at each time point to values at t = 0 (***$P<0.001$, **$P<0.01$) by one-way ANOVA and between WT and *Erfe*^-/- mice (###$P<0.001$, #$P<0.05$) by two-tailed Student *t*-test. ChIP-PCR assessing H3K4me3 (D) and H3K9ac (E) status at 2 regions around the *Hamp* promoter and a negative region was performed on liver tissue from C57BL6/N wild type mice taken 6 or 9 hours after EPO (200U/mouse) (black bars) or saline (grey bars) treatments (3 mice for each time point). Three-way ANOVA with Tukey's correction for multiple comparisons was used for significance calculation. *$P<0.05$; **$P<0.01$.

hepcidin is uncoupled from the iron effect on the BMP-SMAD pathway.

Circulating iron is uptaken mainly by maturing erythroid cells through binding of circulating holo-TF to transferrin receptor 1 (TFR1) and internalization of the complex. Mirciov and colleagues described an increase in *Tfr1* mRNA expression as a potential cause of reduction of circulating diferric TF.[7]

Bone marrow (BM) and spleen erythroblasts of EPO or saline treated WT mice were analyzed by flow cytometry using Ter119 and CD44 antibodies staining and forward scatter to separate cells at distinct stages of terminal differentiation: proerythroblasts (I), basophilic erythroblasts (II), polychromatic erythroblasts (III), orthochromatic erythroblasts and immature reticulocytes (IV), and mature red cells (V) (*data not shown*). We show that despite unchanged proliferation and differentiation of bone marrow (BM) (*Online Supplementary Figure S3A*) and spleen derived erythroid cells (*Online Supplementary Figure S3B*) surface TFR1 protein is increased after EPO treatment in immature erythroid cells both in the BM and spleen of WT mice (Table 1). The same approach was used to isolate erythroid cell population in *Erfe*^-/- mice and WT animals (*Online Supplementary Figure S4A*). Irrespective of unchanged proliferation and differentiation of erythroid cells (*Online Supplemental Figure S4B*), TFR1 increase was observed in the BM of *Erfe*^-/- and WT at 6 hrs after EPO (*Online Supplementary Figure S4C*).

To confirm that the early downregulation of hepcidin is driven by EPO-induced decrease of holoTF, WT mice were treated with EPO and 1 hr later received intravenous human holo transferrin (holoTF) (0.3 g/kg) to augment circulating iron[8] and sacrificed at 6, 9 and 15 hrs after EPO. Human holoTF injection does not interfere with *Erfe* expression in BM (Figure 2C) and spleen (Figure 2D) erythroid cells. Since *Erfe* is an EPO target gene, we also assume that the erythroid response to EPO is comparable in the two groups of mice. Liver iron content is unchanged after holoTF injection (data not shown). However, downregulation of *Hamp* (Figure 2E) and *Id1* (Figure 2F) mRNA expression is delayed after EPO-holoTF combinatory treatment compared with mice treated with EPO only. This confirms that decreased circulating iron mitigates the BMP-SMAD pathway.

We have shown that BMP-SMAD pathway attenuation is required for hepcidin inhibition.[5] While we were preparing this manuscript, it was reported that serum diferric transferrin is decreased early after EPO with a consequent reduction of SMAD1/5/8 phosphorylation in the liver.[7] The reduction of serum iron and TS was ascribed to increased *Tfr1* expression by EPO in BM and spleen.[7] We confirm this mechanism showing that TFR1 protein is highly expressed on the surface of erythroid precursors before any increase in *Tfr1* mRNA levels (*data not shown*). The consequent iron uptake of the acutely expanded erythropoiesis overcomes the capacity of iron
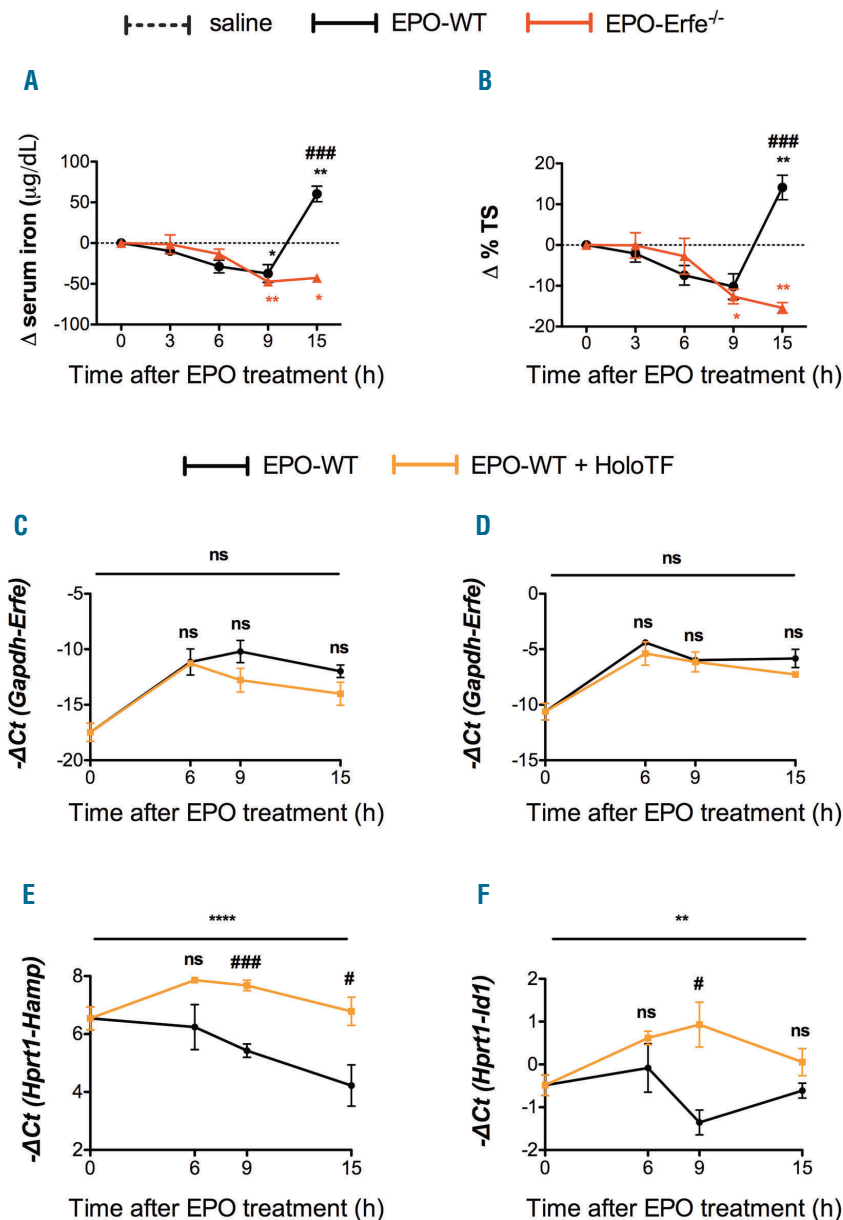
Figure 2. Circulating iron is reduced after EPO treatment both in WT and *Erfe*⁻/⁻ mice, and human holo-transferrin injection prevents the downregulation of the BMP-SMAD pathway by EPO and attenuates the hepcidin suppressive effect of ERFE. Serum iron (A) and transferrin saturation (B) were analyzed in C57BL/6J wild type and *Erfe*-deficient mice (6-9 mice for each time point) treated with a single EPO injection (200 U/mouse) and expressed as a difference between the EPO-treated and the saline-treated values. Data shown are means ± s.e.m and were compared for each genotype at each time point to values at t=0 (*P<0.05; **P<0.01) by one-way ANOVA and between WT and *Erfe*⁻/⁻ mice (###P<0.001) by two-tailed Student *t*-test. EPO-treated C57BL/6N wild type mice (3-4 mice for each time point) were administered intravenously with human holo-transferrin (HoloTF) and sacrificed at 6, 9 and 15 hrs post-EPO. *Erfe* expression was evaluated in the bone marrow (C) and spleen (D) by qRT-PCR and normalized on *Gapdh* expression. Liver hepcidin (*Hamp*; E) and *Id1* (F) mRNA levels were analyzed by qRT-PCR. *Hprt1* was used as housekeeping gene. Data are shown as means ± s.e.m. Two way-ANOVA was used to compare EPO and EPO-hHoloTF treated mice (ns: not significant; **P<0.01; ****P<0.0001). Two-tailed *t*-test was used to calculate statistically significant differences of EPO treatment at different time points compared to t=0; ns: not significant; #P<0.05; ###P<0.001.

release from absorption and store sites, causing a transient reduction of serum iron and TS levels until hepcidin is suppressed.

Taken together, our data demonstrate an iron-dependent and ERFE-independent mechanism of hepcidin regulation by EPO, which is crucial for efficient inhibition of hepcidin by ERFE. EPO treatment rapidly increases the expression of TFR1 on plasma membrane of erythroid precursors. We hypothesize that upregulation of membrane TFR1 by EPO results in a strong and acute increase in iron uptake by maturing erythroblasts, as observed in EPO-treated human (K562) and murine erythroleukemic (MEL) cells,[9] thus reducing the levels of circulating iron and TS. The (acute) expansion of erythropoiesis overcomes the capacity of iron release from absorption and recycling until hepcidin is suppressed. The mechanism is ERFE-independent since the same decrease of circulating iron is observed shortly after EPO injection in *Erfe*⁻/⁻ mice, followed by a downregulation of BMP-SMAD tar-

get genes, including hepcidin. We also show that concomitant to the early (9 hrs) hepcidin decrease, Hamp locus undergoes epigenetic modification with loss of H3K4me3, involved in facilitating gene transcription,[10] but not of H3K9ac. Taken together with previous data,[3] these findings indicate the time course of loss of chromatin activation marks, which closely mirror reductions in gene expression.

The holoTF injection counteracts the early decrease of serum iron, delays the downregulation of hepcidin, further supporting that the transient drop of iron is essential for ERFE function on hepcidin. In summary, the EPO effect on hepcidin expression is biphasic. The immediate and transient effect on serum iron that allows the early repression of BMP-SMAD pathway and hepcidin repression is Erfe independent; the late, ERFE-mediated effect, persists until the increased iron needs are met. Our findings are a further example of the tight crosstalk between erythroid cells and iron homeostasis.

Irene Artuso,[1,#] Mariateresa Pettinato,[1,2] Antonella Nai,[1,2] Alessia Pagani,[1] Ugo Sardo,[3] Benjamin Billoré,[3] Maria Rosa Lidonnici,[4] Cavan Bennett,[5] Giacomo Mandelli,[4] Sant-Rayn Pasricha,[5,6] Giuliana Ferrari,[4] Clara Camaschella,[1] Léon Kautz[3] and Laura Silvestri[1,2]

[1]Regulation of Iron Metabolism Unit, Division of Genetics and Cell Biology, IRCCS San Raffaele Scientific Institute, Milan, Italy; [2]Vita-Salute San Raffaele University, Milan, Italy; [3]IRSD, Université de Toulouse, INSERM U1220, INRA U1416, ENVT, UPS, France; [4]SR-Tiget Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy; [5]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia and [6]Department of Medical Biology, The University of Melbourne, VC, Australia

IA and MP contributed equally to this work

[#]Present address: Cellular and Molecular Immunology, Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milano, Italy

Correspondence: LAURA SILVESTRI - silvestri.laura@hsr.it

doi:10.3324/haematol.2018.199810

References

1. 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.
2. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004;306(5704):2090-2093.
3. Pasricha SR, Lim PJ, Duarte TL, et al. Hepcidin is regulated by promoter-associated histone acetylation and HDAC3. Nat Comm. 2017;8(1):403.
4. Wang CY, Core AB, Canali S, et al. Smad1/5 is required for erythropoietin-mediated suppression of hepcidin in mice. Blood. 2017;130(1):73-83.
5. Nai A, Rubio A, Campanella A, et al. Limiting hepatic Bmp-Smad signaling by matriptase-2 is required for erythropoietin-mediated hepcidin suppression in mice. Blood. 2016;127(19):2327-2336.
6. Finberg KE, Whittlesey RL, Fleming MD, Andrews NC. Down-regulation of Bmp/Smad signaling by Tmprss6 is required for maintenance of systemic iron homeostasis. Blood. 2010;115(18):3817-3826.
7. Mirciov CSG, Wilkins SJ, Hung GCC, Helman SL, Anderson GJ, Frazer DM. Circulating iron levels influence the regulation of hepcidin following stimulated erythropoiesis. Haematologica. 2018 Jun 14. [Epub ahead of print]
8. Li H, Rybicki AC, Suzuka SM, et al. Transferrin therapy ameliorates disease in beta-thalassemic mice. Nat Med. 2010;16(2):177-182.
9. Weiss G, Houston T, Kastner S, Johrer K, Grunewald K, Brock JH. Regulation of cellular iron metabolism by erythropoietin: activation of iron-regulatory protein and upregulation of transferrin receptor expression in erythroid cells. Blood. 1997;89(2):680-687.
10. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. Cell Res. 2011;21(3):381-395.