HEPCIDIN REGULATION IN A MOUSE MODEL OF ACUTE HYPOXIA

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Abstract

Objective. During hypoxia hepcidin expression is inhibited to allow iron mobilization to sustain erythropoietic expansion. We analysed molecular mechanisms underlying hypoxia-induced hepcidin inhibition in an *in vivo* model of acute hypoxia.

Methods. Mice were kept under normal or hypoxic conditions for 6h and 15h and treated with α-PDGF-BB antibody or PDGF-BB receptor inhibitor. Blood, liver, spleen, and bone marrow were collected to extract RNA and protein or to quantify EPO and PDGF-BB. mRNA and protein levels were assessed by RT-PCR and Western Blot.

Results. Hepcidin was strongly inhibited at 15 hours and this down-regulation followed erythropoiesis activation and up-regulation of several growth factors. PDGF-BB, Erythroferrone, GDF15 and TWSG1 were up-regulated by hypoxia in the bone marrow, but not in spleen or liver. Inactivation of PDGF-BB or its receptor suppressed the hypoxia-induced hepcidin inhibition.

Conclusion. Spleen and liver are not involved in the early stages of hypoxia-induced hepcidin down-regulation. Our data support the role of PDGF-BB and probably also of erythroferrone in the recruitment of iron for erythropoiesis in the hypoxia setting. The rapid normalization of all the erythroid factors against persistent hepcidin suppression suggests that other signals are involved that should be clarified in future studies.

Key words: Hypoxia, Hepcidin, Erythropoiesis, PDGF-BB, Erythroferrone

Introduction

Hepcidin is a small peptide hormone mainly produced by hepatocytes and codified by *HAMP* gene. It is the main negative regulator of systemic iron homeostasis, mediating internalization and degradation of ferroportin, the only known cellular iron exporter[1]. Hepcidin activity inhibits iron absorption from diet and iron release from the stores (liver and spleen macrophages), preventing excess iron absorption and maintaining normal iron levels. Hepcidin production is strictly controlled by different stimuli that up or down-regulates its transcription[2]. In particular, during hypoxia when the bone marrow needs iron to sustain the erythropoietic expansion, hepcidin expression is inhibited to allow iron mobilization[3]. Although much progress has been made in the last years, mechanisms of hypoxia-induced hepcidin regulation are still partially unknown. More evidences indicate that hypoxia acts indirectly stimulating the bone marrow to release soluble factors in response to the erythropoietin (EPO)-induced erythropoiesis[4-7]. Indeed, different studies in animal models showed that hepcidin down-regulation was blocked by inhibiting erythropoietic activity[8,9], and we previously showed that cell exposure to human hypoxic sera reduced hepcidin expression *in vitro* suggesting the existence of circulating factors[7]. Several erythrokines released by the expanded erythroid marrow have been proposed, such as Growth Differentiation Factor 15 (GDF15)[10] or Twisted Gastrulation Homolog 1 (TWSG1)[11] that could act through the BMP-SMAD pathway[11]. However, subsequent studies did not confirm these results in models of hepcidin down-regulation induced by acute blood loss[12]. Accordingly, we also confirmed that GDF15 cannot be a main hepcidin regulator in subjects exposed to hypobaric hypoxia[7,13]. Erythroferrone (ERFE) has been proposed as candidate erythroid inhibitor of hepcidin expression through a still undefined mechanism[14]. ERFE, codified by *FAM132B* gene, is a recent discovered TNFα-like protein released by the bone marrow in condition of enhanced erythropoiesis due to phlebotomy or EPO injection[14]. While ERFE is not directly regulated by hypoxia, Platelet-Derived Growth Factor-BB (PDGF-BB) is a target of HIF-1α[15]. Recently, Sonnweber et al[16] showed that it significantly increases in humans and mice exposed to hypobaric hypoxia, and inhibits *HAMP* transcription in hepatic cell lines. Overall, these findings suggest that hypoxia-induced hepcidin down-regulation might occur through an

74 interplay between factors directly activated by hypoxia and factors activated through the hypoxia-induced
75 erythropoiesis drive. Taking into account that both events may occur in the very first hours after hypoxia
76 exposure[13,14,16,17], we analysed the time course of hepatic hepcidin production and of different hepcidin
77 regulator candidates in a mouse model of acute hypoxia focusing our attention on PDGF-BB and ERFE.

Material and methods

Animal care and in vivo mouse studies. Wild type C57BL/6 male mice 10 weeks old were purchased from Charles River Laboratories (Italy). They were housed at the animal facility of the University of Milano-Bicocca under pathogen-free conditions with fixed day and night cycles and free access to water and food. All animals received human care. All experiments were approved by the Italian ethics committee (N° 009/2013).

Hypoxia studies. Mice were housed under hypoxic conditions into a hypoxic chamber containing a fraction of 10% O_2 in nitrogen-saturated air for 6 and 15 hours. Normoxic control mice were kept under normal conditions (21% oxygen content of the air). Following hypoxic challenge, mice were anesthetized and blood was taken via intracardiac puncture. Normoxic and hypoxic mice were euthanized at the same time via cervical dislocation and liver and spleen were collected and snap-frozen in liquid nitrogen. Femur and tibia bones were collected to immediately perform the bone marrow flushing. Tissue samples were used to extract mRNA as described below; serum was used to quantify EPO and PDGF-BB levels by ELISA Quantikine Immunoassay kit (R&D Systems, Minneapolis, MN, USA). Mice received either an intraperitoneal injection of 500 μg AG1296 (Tyrphostin) (M-Medical S.r.l, Cornaredo, MI, Italy) or 100 μg of anti-PDGF-BB antibody (R&D Systems Inc., Minneapolis, MN, USA) each in 100 μL of PBS solvent. AG1296 and α-PDGF-BB antibody were delivered as a single dose immediately prior to 15 hours hypoxic challenge. Intraperitoneal injection of 100 μL of PBS was used as mock control.

Bone marrow flushing. For bone marrow cell suspension preparation, cells from mice's femur and tibia were flushed with PBS-2% FBS. The cell suspension was gently passed through a 21 G needle and then through a 40 μM cell strainer (BD Bioscience, San Jose, CA, U.S.A.). The suspension was then transferred to a 50-mL conical tube and centrifuged at 2000 rpm for 5 minutes. After centrifugation, the cell pellet was used for RNA extraction as described below.

RNA extraction and cDNA synthesis. RNA was extracted using ZR RNA miniprep (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's protocol. RNA was then quantified by

spectrophotometry and its integrity assessed by non-denaturing agarose gel. 2 µg of total RNA was used as a template for reverse transcription, performed using the High Capacity cDNA Archive kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacture's protocol.

Real-time quantitative-PCR. mRNA expression levels of candidate genes were evaluated by quantitative real time PCR (qRT-PCR); *Hprt* was chosen as housekeeping gene. The analysis were performed on an ABI 7900HT (Thermo Fisher Scientific, Waltham, MA, USA) using the Assays-on-Demand Gene Expression Products (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacture's protocol. Instrument was set up with default thermal cycler protocol provided by the producer: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min for 40 cycles. For each PCR reaction, 25 ng of cDNA were used as a template. All analyses were carried out in triplicate; results showing a discrepancy greater than 0.3 cycles between the samples were excluded. Relative quantities present in each sample were assessed using the $2^{-(\Delta Ct)}$ method[18]. Non-retrotranscribed RNAs were included in each amplification plate, and the analysis regarded as valid if the fluorescence intensity in the no-template control was zero.

Protein isolation and Western Blot analysis. Tissue samples were solubilized in NET buffer [150 mM NaCl, 5 mM EDTA, 10 mM Tris-Hcl pH 7.4, 1% Triton X-100 (pH 7.4), and 1X Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)] on a roller mixer for 10 min 4°C and cleared by centrifugation at 13500 rpm for 10 min 4°C. Protein concentrations were measured by using Bradford Assay (Sigma-Aldrich, St. Louis, MO, USA). Lysates were then analysed by Western Blot for CREB/H and L-Ferritin. 40 µg of protein extracts were diluted in reducing LDS sample buffer (Thermo Fisher Scientific, Waltham, MA, USA) and heated for 5 min at 97 °C. Proteins were separated electrophoretically on an SDS pre-cast 12% polyacrylamide gel (Thermo Fisher Scientific, Waltham, MA, USA), transferred to nitrocellulose (GE Healthcare, Amersham Biosciences Europe GmbH, Freiburg, Germany) and incubated for 1 hour 30 min in blocking buffer [5% non-fat dry milk in Tris-buffered saline with 0.1% Tween20 (TBST)]. Blots were incubated in blocking buffer containing rabbit anti-CREB/H (1:1000; Kerafast, Inc., Boston, MA, USA) or rabbit anti-L-Ferritin (1:2000; kindly provided by Prof. S. Levi). After wash with TBST, blots were incubated in TBST containing a donkey anti-

rabbit secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). To confirm equivalent loading, samples were re-probed with rabbit anti-actin (1:5000; Sigma-Aldrich, St. Louis, MO, USA) followed by HRP-conjugated donkey anti-rabbit secondary antibody (1:10000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). After washes with TBST bands were visualized by using enhanced chemiluminescence (GE Healthcare, Amersham Biosciences Europe GmbH, Freiburg, Germany) and X-ray film. Densitometry analysis was performed using ImageJ calculating the intensities of L-Ferritin bands normalized to actin.

Fluorescence Activated Cell Sorting. Cells from bone marrow of normoxic and 6h-hypoxic mice were flushed as described above and centrifuged 10 min at 2000 rpm. To isolate erythroblasts at different stages of maturation, cells were stained in blocking-solution (PBS-5% FBS) for 15 minutes on ice in the dark with: APC/Cy7-conjugated anti-mouse CD45 (0.25 $\mu g/10^6$ cells), APC/Cy7-conjugated anti-mouse Ly-6G/Ly-6C (Gr-1) (0.25 $\mu g/10^6$ cells), Alexa Fluor® 700-conjugated anti-mouse/human-CD11b (0.25 $\mu g/10^6$ cells), APC-conjugated anti-mouse-TER-119 (1 $\mu g/10^6$ cells), Alexa Fluor® 488-conjugated anti-mouse CD117 (c-KIT) (0.25 $\mu g/10^6$ cells) and PE-conjugated anti-mouse/human CD44 (2 $\mu g/10^6$ cells) (all from Biolegend, San Diego, CA, USA). Cell sorting of the different erythroid precursors was performed following a gating strategy previously described[19] on MoFLO Astrios cell sorter equipped with Summit 6.3 software (both from Beckman Coulter, Miami, FL, USA). Briefly, the different erythroid precursors (pro-erythroblasts, basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts) were identified on a plot of CD44 versus FSC of $CD45^-$/$c\text{-}KIT^-$/ $CD11b^-$/$Gr\text{-}1^-$/$TER\text{-}119^+$ single cells and sorted. mRNA from different population was extracted, retro-transcribed and expression levels of target genes were evaluated by qRT-PCR.

Statistical analysis. One-way Anova and Tukey or Dunn test post hoc analysis were used for paired comparison. All tests were two sided and with a significance level of α equal to 0.05. Analyses were carried out by the GRAPHPAD PRISM statistical analysis software (version 3.02) (GraphPad Software, Inc., La Jolla, CA, USA).

Results

To assess the erythropoietic activity of the hypoxic mice we first examined serum EPO levels. EPO levels doubled at 6 hours of hypoxia exposure, reaching their highest values after 15 hours of treatment [median (1^{st}-3^{rd} quartiles): 627.9 (355.4-699.2) pg/ml in normoxic vs 1202.8 (881.2-1716.6) pg/ml in HYP-6h vs 1351.8 (374.1-4875.8) pg/ml in HYP-15h]. We then evaluated the modulation of hepatic hepcidin production measuring *Hamp1* mRNA levels. As reported in Figure 1, *Hamp1* mRNA expression increased after 6 hours of hypoxic challenge compared to controls and then significantly decreased after 15 hours.

In the liver, mRNA levels of *Gdf15* did not significantly change, *Twsg1* significantly decreased after 15 hours of treatment and *Pdgf-b* levels slightly increased at 6 hours of hypoxic challenge compared to control and then decreased after 15 hours (Table 1). *Fam132b* was not expressed in the liver. Since previous study suggested that Hepatocyte Growth Factor (*Hgf*) and Epithelial Growth Factor (*Egf*) could both inhibit hepcidin expression in chronic liver failure[20], we evaluated their mRNA levels to assess whether they could have a role in hepcidin inhibition during hypoxia. However, we did not observe changes between hypoxic and control mice at 6 hours, while at 15 hours they even decreased compared to controls or 6-hours hypoxic mice, respectively (Table 1). At 6 hours of hypoxia exposure, *Bmp6* and *Tmprss6* showed mild but not significant changes and returned at basal levels at 15 hours, suggesting that BMP-SMADs pathway was not primarily involved in hypoxia-induced hepcidin modulation. In mice exposed to hypoxia, L-ferritin markedly increased at 6 hours and then decreased at 15 hours, close to the level observed in normoxia (Supplementary Figure 1), suggesting a relationship with the observed transient up-regulation of hepcidin. The expression of ceruloplasmin, *Tnf-*α and *Saa1* (Serum amyloid A1) mRNAs as well as P-STAT3 protein levels, as indices of inflammatory activation, did not significantly change at 6 and 15 hours (data not shown). Liver (LIC) and spleen (SIC) iron concentrations did not change at 6 and 15 hours of hypoxia exposure [Median LIC (1st-3rd) mg Fe/g dry in normoxic: 0.33 (0.31-0.43); HYP-6h: 0.35 (0.22-0.39); HYP-15h: 0.30 (0.22-0.43); Median SIC (1st-3rd) mg Fe/g dry in normoxic: 1.45 (1.42-1.91); HYP-6h: 1.81 (1.47-2.50); HYP-15h: 1.19 (0.85-1.49). p value: ns].

In the bone marrow, mRNA expression of *Gdf15, Twsg1, Pdgf-B* and *Fam132b* (ERFE) were significantly up-regulated after 6 hours of hypoxia exposure and then returned to basal level after 15 hours of treatment with the exception of *Fam132b* whose mRNA levels remained still higher than controls at 15 hours, but not enough to reach the statistical significance (Figure 2). Transferrin receptor 1 (TfR1) progressively increased from normoxic to 15 hours hypoxia exposure [Median (1st-3rd) AU in normoxic: 2.13 (1.69-2.83); HYP-6h: 3.32 (2.88-3.37); HYP-15h: 4.9 (3.93-5.2). ANOVA p value: 0.08]. Conversely, in the spleen we did not observe significant changes of the mRNAs expression after hypoxic challenge, compared to controls.

After sorting erythroid precursors (pro-erythroblasts, basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts), we observed that *Fam132b* was significantly up-regulated by hypoxia in polychromatic and orthochromatic erythroblasts (Figure 3A). As shown in Figure 3B, *Pdgf-b* expression was very low in erythroid and monocyte cells while it was significantly higher in the non-erythroid TER119^{neg} than in the other populations. However, differently to what observed in the whole bone marrow, we could not find significant differences of *Pdgf-b* mRNA levels in the sorted erythroid and non-erythroid TER119^{neg} populations according to normoxic or hypoxic status.

To explore if PDGF-BB was directly involved in hypoxia-mediated hepcidin down-regulation, we injected mice with the specific α-PDGF-BB antibody or with the specific PDGF receptor (PDGF-R) kinase antagonist AG1296 (tyrphostin) and exposed them to hypoxia for 15h. We confirmed that treatment with α-PDGF-BB antibody resets to zero serum PDGF-BB levels while treatment with AG1296 did not change them compared to untreated mice (data not shown). As shown in Figure 4A and B, pre-treatment with α-PDGF-BB or AG1296 blocked the effect of hypoxia on liver hepcidin expression, while it induced no significant changes in normoxic mice. To demonstrate that EPO was not involved in these processes, we showed that non-erythroid TER119^{neg} cells did not express EPO receptor (data not shown), and that serum EPO levels did not significantly change in α-PDGF-BB or AG1296 treated or untreated in normoxic and hypoxic status. Serum EPO [Median (1st-3rd quartile): normoxic untreated mice: 627.9 (355.4-699.2) pg/ml; normoxic mice + α-PDGF-BB: 269.8 (260.6-404.1) pg/ml; normoxic mice + AG1296: 529.6 (441.5-583.9) pg/ml; hypoxic

untreated mice: 1351.8 (374.1-4875.8) pg/ml; hypoxic mice + α-PDGF-BB: 1642.1 (1141.1-2189.9) pg/ml; hypoxic mice + AG1296: 1246.9 (1144.3-1518.1) pg/ml].

To figure out the molecular pathway involved in PDGF-BB-mediated hepcidin inhibition, we evaluated CREB/H and BMP/SMAD pathways. Differently to Sonnweber *et al.*[16] who reported a down-regulation of CREB/H at 48 hours of hypoxic challenge, in our experiments CREB/H levels did not change after 6 and 15 hours of hypoxia exposure suggesting that it was not involved in the early response of hypoxia-mediated hepcidin down-regulation (Figure 5). We then measured *Id1* mRNA levels as a marker of activation of the BMP-SMADs pathway[21] and we found that *Id1* was slightly but not significantly down-regulated at 15 hours of hypoxia exposure (data not shown).

Discussion

In the present paper we showed that: *i.* After a very early and transient increase of hepatic hepcidin expression, hepcidin mRNA markedly decreased at 15 hours of hypoxia exposure; *ii.* At 6 hours of hypoxia exposure, *Gdf15*, *Twsg1, Pdgf-B* and *Fam132b* mRNA levels significantly increased in the bone marrow, but rapidly normalized, while no change was observed in the spleen; *iii.* In the liver, *Pdgf-b*, *Gdf15, Twgs1, Hgf* and *Egf* expression were unchanged or decreased; *iv.* The inhibition of PDGF-BB protein activity by neutralization of circulating PDGF-BB or the specific PDGF receptor (PDGF-R) abolished hepdicin mRNA levels down-regulation in hypoxic mice. These findings allow us to conclude that: a. Hepcidin down-regulation had a latency period from hypoxia exposure and was preceded by erythropoiesis activation and up-regulation of the expression of several growth factors including *Gdf15*, *Twsg1, Pdgf-B* and *Fam132b* in the bone marrow*;* b. Spleen and liver seemed not involved in the early stages of hypoxia-induced hepcidin down-regulation as none of the studied growth factors inhibiting hepcidin synthesis were up-regulated in those tissues; c. PDGF-BB and ERFE might be major factors involved in the early stages of hepcidin down-regulation by hypoxia; d. Although hepcidin down-regulation persists beyond 15 hours of hypoxia exposure, as shown in animal and human studies[7,13,16,17,22,23], all the growth factors analysed rapidly returned to normal levels in the bone marrow, suggesting that other actors might be involved in maintaining hepcidin down-regulation in hypoxia.

Despite some controversies, there is evidence that hypoxia inhibits hepcidin expression indirectly, stimulating the bone marrow to release soluble factors in response to EPO-induced erythropoiesis[4-9]. However, hypoxia induces inhibition or activation of several pathways, some of them directly involved in iron metabolism[24-26], suggesting that hypoxia-induced hepcidin down-regulation *in vivo* might depend to more complex events involving both erythroid and non-erythroid related factors. Different erythrokines have been proposed to be the hepcidin erythroid regulators, such as GDF15[10], TWSG1[11] and, more recently, PDGF-BB[16] and Erythroferrone[14]. In our model, both *Gdf15* and *Twsg1* mRNAs were up-regulated in the bone marrow after hypoxia exposure confirming that their transcription is induced under erythropoietic

activation. However, previous studies did not support their role as hepcidin inhibitors[7,12]. ERFE has been proposed as the erythroid regulator of hepcidin during stressed or enhanced erythropoiesis induced by phlebotomies or EPO injections in animal models[14]. Our findings suggest that ERFE might be one of the erythroid-derived inhibitors of hepcidin expression also in the hypoxia setting and confirm that it was mainly produced by polychromatic and orthochromatic erythroblasts[14]. However, Talbot et al[27] were unable to find a clear role for ERFE in linking erythropoietic drive to hepcidin suppression in high altitude residents that underwent generous phlebotomies. Thus, further studies are needed to clarify the role of ERFE in the hypoxia setting and the mechanism of ERFE-induced hepcidin inhibition. Sonnweber et al[16] recently investigated the effects of hypoxia on iron metabolism in mice at 48 hours of exposure indicating PDGF-BB as a new "iron-molecule" able to inhibit hepcidin during hypoxia acting through the CREB/H pathway. We showed that the hypoxia challenge at 6 hours associated with the increase of PDGF-BB mRNA in the whole bone marrow and that the neutralization of PDGF-BB activity prevented hepcidin down-regulation providing evidence of a role of PDGF-BB in hepcidin inhibition in the early stage of hypoxia. It was previously demonstrated *in vitro* that EPO induces PDGF-BB expression in HUVEC cells (endothelial cell line) during inflammatory condition (TNFα treatment)[28], but our findings did not confirm this hypothesis in our model. Accordingly, Sonnweber et al[16] found only a non-significant trend towards higher PDGF-BB serum levels after EPO injection, suggesting that erythropoietic activity does not play a major effect on PDGF-BB serum concentrations.

We were not able to confirm the involvement of the CREB/H pathway in the first 15 hours after hypoxia challenge. Also, our results did not support the involvement of the BMP-SMADs pathway in the same setting. So, further studies are needed to clarify the signalling pathways involved in the early stages of PDGF-BB-mediated hepcidin down-regulation in hypoxia.

By sorting bone marrow cells, we showed that PDGF-BB was mainly produced by a non-erythroid Ter^{119neg} population. However, hypoxia did not seem to modulate PDGF-BB mRNAs in any of the sorted bone marrow populations. We had no clear explanation of these findings and we can only speculate that they

could be related to the sorting procedures. At our knowledge, there is only one study available showing increased expression of PDGF-BB in bone marrow-derived mesenchymal stem cells[29] exposed to hypoxia. If these cells are involved in the production of PDGF-BB in hypoxia-induced erythropoietic drive *in vivo* remains a mere speculation that we could not confirm using FACS technique.

Although our findings indicated that both ERFE and PDGF-BB might act as hepcidin down-regulators in hypoxia, they also showed that up-regulation has short duration. This confirms what previously found by Kautz et al[14] in another model of erythropoietic activation and might suggest that hypoxia itself or hypoxia-induced erythropoiesis activate other pathways in the bone marrow or in other tissues to maintain hepcidin down-regulation. Indeed, we previously demonstrated that hepcidin inhibition persisted after 72 hours of hypoxia in mice[22] and even after one month in human volunteers[13]. The high iron requirement by the bone marrow, as also confirmed by the progressive increase of TfR1 expression observed in our model, leading to an iron deficient erythropoiesis, might be one of these additional factors. Transferrin saturation, as we previously suggested[13,30], and Transferrin receptor (TfR)-2 and its soluble form, as recently proposed by Camaschella et al[31], could be the connectors between erythropoiesis and hepcidin regulation through the BMP-SMADs pathway. We did not observe changes in LIC and SIC in the earliest stages of hypoxia exposure, but the decrease of hepatic iron stores and serum ferritin that generally occurs after 48-72 hours of hypoxia exposure[7,13,17,32-34], might contribute in maintaining hepcidin down-regulation and iron supply to the erythroid bone marrow.

Our results, taken together, demonstrated that the hypoxia-mediated hepcidin regulation occurs quickly and follows the release of circulating factors from the activated erythropoietic bone marrow. This study also confirmed that the bone marrow plays a key role in the inhibition of hepcidin during hypoxia and that both PDGF-BB and, possibly ERFE, could be good candidates as hepcidin inhibitors. However, future studies are needed to better understand the complex and coordinated mechanisms occurring between the bone marrow and the liver during hypoxia exposure.

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Conflict of interest

The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in this manuscript. The authors declare no conflict of interest.

Author's contribution

GR: Performed the experiments; analysis and interpretation of data; drafting of the manuscript; approval of the final version of the manuscript; SP: Performed the experiments, analysis and interpretation of data; revision of the manuscript; approval of the final version of the manuscript; GBC: Performed the experiments; approval of the final version of the manuscript; FG: Performed the experiments; approval of the final version of the manuscript; RM: Revision of the manuscript; approval of the final version of the manuscript; IP: Revision of the manuscript; approval of the final version of the manuscript; SB: Performed FACS experiments; approval of the final version of the manuscript; RP: Revision of the manuscript; approval of the final version of the manuscript; DB: Revision of the manuscript; approval of the final version of the manuscript; AP: Conception and design of the study; analysis and interpretation of data; drafting and revision of the manuscript; approval of the final version of the manuscript.

References

1. 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.

2. Piperno A, Mariani R, Trombini P, Girelli D. Hepcidin modulation in human diseases: from research to clinic. *World J Gastroenterol*. 2009;15(5):538-551.

3. Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest*. 2002;110(7):1037-1044.

4. Mastrogiannaki M, Matak P, Mathieu JR, et al. Hepatic hypoxia-inducible factor-2 down-regulates hepcidin expression in mice through an erythropoietin-mediated increase in erythropoiesis. *Haematologica*. 2012;97(6):827-834.

5. Volke M, Gale DP, Maegdefrau U, et al. Evidence for a lack of a direct transcriptional suppression of the iron regulatory peptide hepcidin by hypoxia-inducible factors. *PLoS One*. 2009;4(11):e7875.

6. Liu Q, Davidoff O, Niss K, Haase VH. Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis. *J Clin Invest*. 2012;122(12):4635-4644.

7. Ravasi G, Pelucchi S, Greni F, et al. Circulating factors are involved in hypoxia-induced hepcidin suppression. *Blood Cells Mol Dis*. 2014;53(4):204-210.

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

9. Vokurka M, Krijt J, Sulc K, Necas E. Hepcidin mRNA levels in mouse liver respond to inhibition of erythropoiesis. *Physiol Res*. 2006;55(6):667-674.

10. Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nat Med*. 2007;13(9):1096-1101.

11. Tanno T, Porayette P, Sripichai O, et al. Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. *Blood*. 2009;114(1):181-186.

12. Casanovas G, Vujić Spasic M, Casu C, et al. The murine growth differentiation factor 15 is not essential for systemic iron homeostasis in phlebotomized mice. *Haematologica*. 2013;98(3):444-447.

13. Piperno A, Galimberti S, Mariani R, et al. Modulation of hepcidin production during hypoxia-induced erythropoiesis in humans in vivo: data from the HIGHCARE project. *Blood*. 2011;117(10):2953-2959.

14. 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.

15. Schito L, Rey S, Tafani M, et al. Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells. *Proc Natl Acad Sci U S A*. 2012;109(40):E2707-2716.

16. Sonnweber T, Nachbaur D, Schroll A, et al. Hypoxia induced downregulation of hepcidin is mediated by platelet derived growth factor BB. *Gut*. 2014;63(12):1951-1959.

17. Talbot NP, Lakhal S, Smith TG, et al. Regulation of hepcidin expression at high altitude. *Blood*. 2012;119(3):857-860.

18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.

19. Liu J, Zhang J, Ginzburg Y, et al. Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis. *Blood*. 2013;121(8):e43-49.

20. Goodnough JB, Ramos E, Nemeth E, Ganz T. Inhibition of hepcidin transcription by growth factors. *Hepatology*. 2012;56(1):291-299.

21. Meynard D, Vaja V, Sun CC, et al. Regulation of TMPRSS6 by BMP6 and iron in human cells and mice. *Blood*. 2011;118(3):747-756.

22. Barisani D, Buoli-Comani G, Rusconi M, et al. BMP/SMAD pathway: regulation by hypoxia. European Iron Club. Verona; 2014.

23. Goetze O, Schmitt J, Spliethoff K, et al. Adaptation of iron transport and metabolism to acute high-altitude hypoxia in mountaineers. *Hepatology*. 2013;58(6):2153-2162.

24. Silvestri L, Pagani A, Camaschella C. Furin-mediated release of soluble hemojuvelin: a new link between hypoxia and iron homeostasis. *Blood*. 2008;111(2):924-931.

25. Lakhal S, Schödel J, Townsend AR, Pugh CW, Ratcliffe PJ, Mole DR. Regulation of type II transmembrane serine proteinase TMPRSS6 by hypoxia-inducible factors: new link between hypoxia signaling and iron homeostasis. *J Biol Chem*. 2011;286(6):4090-4097.

26. Patel N, Varghese J, Masaratana P, et al. The transcription factor ATOH8 is regulated by erythropoietic activity and regulates HAMP transcription and cellular pSMAD1,5,8 levels. *Br J Haematol*. 2014;164(4):586-596.

27. Talbot NP, Smith TG, Lakhal-Littleton S, et al. Suppression of plasma hepcidin by venesection during steady-state hypoxia. *Blood*. 2016;127(9):1206-1207.

28. Janmaat ML, Heerkens JL, de Bruin AM, Klous A, de Waard V, de Vries CJ. Erythropoietin accelerates smooth muscle cell-rich vascular lesion formation in mice through endothelial cell activation involving enhanced PDGF-BB release. *Blood*. 2010;115(7):1453-1460.

29. Tong C, Hao H, Xia L, et al. Hypoxia pretreatment of bone marrow-derived mesenchymal stem cells seeded in a collagen-chitosan sponge scaffold promotes skin wound healing in diabetic rats with hindlimb ischemia. *Wound Repair Regen*. 2016;24(1):45-56.

30. Trombini P, Coliva T, Nemeth E, et al. Effects of plasma transfusion on hepcidin production in human congenital hypotransferrinemia. *Haematologica*. 2007;92(10):1407-1410.

31. Camaschella C, Pagani A, Nai A, Silvestri L. The mutual control of iron and erythropoiesis. *Int J Lab Hematol*. 2016;38 Suppl 1:20-26.

32. Robach P, Fulla Y, Westerterp KR, Richalet JP. Comparative response of EPO and soluble transferrin receptor at high altitude. *Med Sci Sports Exerc*. 2004;36(9):1493-1498; discussion 1492.

380 33. Robach P, Cairo G, Gelfi C, et al. Strong iron demand during hypoxia-induced erythropoiesis is
381 associated with down-regulation of iron-related proteins and myoglobin in human skeletal muscle. *Blood*.
382 2007;109(11):4724-4731.
383 34. Richalet JP, Souberbielle JC, Antezana AM, et al. Control of erythropoiesis in humans during
384 prolonged exposure to the altitude of 6,542 m. *Am J Physiol*. 1994;266(3 Pt 2):R756-764.

Figures legend

Figure 1. *Hamp1* mRNA expression in the liver of normoxic (CTRL, N=12) and hypoxic mice (HYP) after 6h (N=6) and 15h (N=11) of hypoxia exposure. Boxes denote lower quartile, mean and upper quartile, and whiskers show maximum and minimum ranges.

Figure 2. A) *Gdf15*, **B)** *Twsg1*, **C)** *Pdgf-b* and **D)** *Erfe (Fam132b)* mRNA expression in the bone marrow of normoxic (CTRL, N=12) and hypoxic mice (HYP) after 6h (N=6) and 15h (N=11) of hypoxia exposure. Boxes denote lower quartile, mean and upper quartile, and whiskers show maximum and minimum ranges.

Figure 3. mRNA levels of *Erfe (Fam132b)* (Fig **3A**) and *Pdgf-b* (Fig **3B**) evaluated *in* pro-erythroblasts (I), basophilic erythroblasts (II), polychromatic erythroblasts (III), orthochromatic erythroblasts (IV), Monocyte and $Ter119^{neg}$ (TER-) population of normoxic and 6-hours hypoxic mice (N=5). Results are expressed as mean and SEM (Standard Error of the Mean).

Figure 4. *Hamp1* mRNA expression of liver of normoxic untreated mice (CTRL); normoxic mice treated with PBS (CTRL+PBS), α-PDGF-BB (CTRL+aPDGF) (Fig **4A**) or AG1296 (CTRL+AG) (Fig **4B**); hypoxic mice after 15h of hypoxia challenge untreated (HYP) and treated with PBS (HYP+PBS), α-PDGF-BB (HYP+aPDGF) (Fig **4A**) or AG1296 (HYP+AG) (Fig **4B**). Values of normoxic mice untreated and treated with PBS were considered as a sole group in statistical analysis. Results are expressed as mean and standard deviation.

Figure 5. Western Blot analysis of total lysate protein in liver of normoxic mice (CTRL; N=3) and mice subjected to hypoxia challenge for 6 (HYPOXIA 6h; N=3) and 15 (HYPOXIA 15h; N=3) hours. After protein extraction, Western Blot analysis of CREB/H (70 KDa) was performed. Results were then normalized with actin (42 KDa) expression.

Supplementary figure 1. A) Western Blot analysis of total lysate protein in liver of normoxic mice (CTRL; N=2) and mice subjected to hypoxia challenge for 6 and 15 hours. After protein extraction, Western Blot analysis of L-Ferritin (19 KDa) was performed. Results were then normalized with actin (42 KDa) expression. **B)** Densitometry analyses performed on three independent experiments. The intensities of L-ferritin bands

409 were normalized with actin. Boxes denote lower quartile, mean and upper quartile, and whiskers show
410 maximum and minimum ranges.