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ROS mediate the hypoxic repression of the *hepcidin* gene by inhibiting C/EBPα and STAT-3

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Abstract

Hepcidin, a liver peptide, systemically inhibits iron utilization and is downregulated under hypoxic conditions. However, little is known about the mechanism underlying the hypoxic suppression of hepcidin. Here, we tested the possibility that HIF-1 and ROS are involved in hepcidin regulation. Hepcidin mRNA, pre-mRNA, and protein levels were reduced in mouse livers and in HepG2 cells after hypoxic incubation, and HIF-1 overexpression and knock-down studies showed that hepcidin regulation is independent of HIF-1. On the other hand, ROS levels were significantly elevated in hypoxic HepG2 cells, and anti-oxidants prevented the hypoxic down-regulation of hepcidin. Conversely, a prooxidant, H_2O_2, suppressed hepcidin expression in these cells even in normoxia. Of the various transcription factors examined, C/EBPα and STAT-3 were found to dissociate from hepcidin promoter under hypoxia, but to become fully engaged after anti-oxidant treatment. These results suggest that ROS repress the *hepcidin* gene by preventing C/EBPα and STAT-3 binding to hepcidin promoter during hypoxia.

Keywords: Hepcidin; Reactive oxygen species; C/EBPα; STAT-3; HIF-1

Iron deficiency causes anemia or growth retardation, and conversely, an iron overload induces oxidative toxicities by causing the production of reactive oxygen species (ROS). Therefore, iron homeostasis is tightly regulated by modulating gut iron absorption and by regulating iron release from storage sites, such as, from macrophages, and hepatocytes [1,2]. Moreover, iron absorption and release are known to be systemically regulated by hemoglobin, oxygen tension, serum iron, and proinflammatory cytokines [3]. The above prompt the question as what controls systemic iron absorption and release?

Hepcidin is mainly produced by hepatocytes, and the *hepcidin* gene encodes an 84-aa prohepcidin, which is subsequently cleaved to 25-aa hepcidin and secreted into the circulation. Initially, hepcidin was considered to be an anti-microbial peptide, because it contains a cysteine-rich, defensin-like motif [4]. However, animal studies later identified that it has a more extensive physiological role. In one study, hepcidin production was found to be increased in mice overloaded with iron, and that this overproduction of hepcidin subsequently reduced serum iron levels [5]. In another supportive study, hepcidin transgenic mice showed severe iron-deficient anemia [6]. Mechanistically, hepcidin binds to the iron exporter ferroportin (FPN) on the surfaces of enterocytes, macrophages and hepatocytes, and these bindings lead to FPN internalization and degradation [7]. Moreover, since FPN is the main iron exporter [8], its inhibition by hepcidin effectively reduces both iron absorption by enterocytes and iron release by macrophages and hepatocytes. Therefore, hepcidin is regarded as a key player in the systemic regulation of iron homeostasis.

Hepcidin expression in liver is actively regulated by the body's need for iron, and during anemia, hepcidin expression is suppressed to increase the iron supply required for erythropoiesis. Then, perhaps the relevant question that should be asked is: How is hepcidin expression regulated?

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In the case of anemia, low serum iron might serve as the primary stimulant of hepcidin down-regulation. However, when anemia was induced by phlebotomy or phenylhydrazine treatment, hepcidin expression in mouse liver reduced without significant changes in liver and plasma iron levels [9], which suggests that some factor other than iron is responsible for hepcidin regulation. Since tissue hypoxia is the main consequence of anemia, hypoxia could signal hepatocytes to suppress hepcidin. Indeed, it has been demonstrated that hypoxia alone is enough to repress the *hepcidin* gene [9]. Moreover, hypoxia, rather than iron deficiency, was proposed to directly stimulate hepcidin down-regulation in anemia [10]. However, little is known about the mechanism whereby hepcidin expression is controlled by oxygen tension. Since the main features of hypoxia are HIF-1 activation and a change in redox state, we examined whether these factors are involved in the hypoxia-induced suppression of hepcidin.

Materials and methods

Animals and cells. six-week-old Balb/c mice purchased from Orient (Seoul, Korea) were used to measure hepatic hepcidin levels. To induce whole-body hypoxia, mice were placed in normobaric chamber continuously flushed with the 12% O_2/88% N_2 gas mixture. All animal procedures were performed according to the established procedures of the Seoul National University Laboratory Animal Maintenance Manual. HepG2 human hepatoma cells were cultured in Minimal Essential Medium (Gibco, Auckland, New Zealand) with 10% heat inactivated fetal bovine serum and 1% penicillin–streptomycin. To give hypoxic environment, cells were either placed in the hypoxic chamber (1% O_2) or in the GasPak pouch (BD science, Becton Drive Franklin Lakes, NJ). *N*-Acetyl-L-cysteine (NAC), dithiothreitol (DTT), or H_2O_2 were purchased from Sigma–Aldrich (St. Louis, MO), and pretreated an hour before hypoxia.

Plasmids and siRNAs. HIF-1α and ARNT expression plasmids were generous gifts from Dr. Eric Huang (University of Utah School of Medicine, Salt Lake City, UT). To knock-down HIF-1α, a synthesized siRNA duplex was obtained from Invitrogen (Carlsbad, CA). The sequence targeting HIF-1α (GenBank No. NM_001530) was 5′-AGUUAGU UCAAACUGAGUUAAUCCC-3′, which corresponds to nucleotides 360–384 of its coding region. The sequence of the control siRNA used was 5′-CAAGACCCGCGCCGAGGUGAAUU-3′. For transient transfection, about 40% confluent cells in 60-mm cell culture dishes were transfected with siRNA using Lipofectamine (Invitrogen).

RT-PCR analysis. To quantify mRNA expressions, highly sensitive semi-quantitative RT-PCR was performed [11]. RNA was isolated with TRIzole Reagent (Invitrogen), and quantified by measuring absorbance at 260 nm. One microgram of RNA was used for reverse transcription using Superscript One-step kit (Invitrogen) and the cDNA was amplified over 25 PCR cycles with [α-^{32}P]dCTP. Five microliters of the PCR products were electrophoresed on a 4% polyacrylamide gel at 100 V in a TAE buffer, and the dried gels were autoradiographed. The sequences of primers were 5′-CACCACCTATCTCCATCAAC-3′ and 5′-GGAGGGCAGGAATAA ATAAT-3′ for mouse hepcidin mRNA, 5′-CTGTTTTCCCACAACAG AC-3′ and 5′-GCAGGAATAAATAAGGAAGG-3′ for human hepcidin mRNA, 5′-GGTAAAACCCCGTCTCTACT-3′ and 5′-ACGGTCACTC TACCACTGTC-3′ for human hepcidin pre-mRNA, and 5′-ACACCTTC TACAATGAGCTG-3′ and 5′-CATGATGGAGTTGAAGGTAG-3′ for human β-actin mRNA.

Western blot analysis. Cell was lysed with in SDS buffer containing 10% β-mercaptoethanol, and the lysate was separated on 8% or 10% SDS/polyacrylamide gel, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in Tris–buffered saline with 0.1% Tween-20 (TTBS) and then incubated overnight with primary antibodies; anti-HIF-1α [12], anti-ARNT (Santa-Cruz Biotechnology Inc., SantaCruz, CA), anti-β-tubulin (SantaCruz), anti-C/EBPα (SantaCruz), anti-STAT-3 (Cell Signaling Technology, Danvers, MA), anti-phosphoSTAT-3 (Cell Signaling), USF-1 (SantaCruz), or USF-2 (SantaCruz). Horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat (Zymed Laboratories Inc., South San Francisco, CA) were used as a secondary antibody. Antigen–antibody complexes were visualized by using Enhanced Chemiluminescence Plus Kit (Amersham Bioscience, Piscataway, NJ).

Prohepcidin assay. Prohepcidin assay was performed according to the manufacturer's protocol (DRG Instruments, Marburg, Germany). Cells were lysed with a buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, and 0.1% NP-40, and centrifuged at 1200*g* for 30 min. The supernatants were collected for the assay. Determinations were performed in duplicates using 96 well coated with prohepcidin antibody. In brief, 50 μl of each standards and samples were added into the wells with 100 μl N-terminally biotinylated hepcidin-(28–47) and incubated for 2 h at room temperature. The biotinylated antigen–antibody complexes were detected by streptavidin peroxidase enzyme with the substrate tetramethylbenzidine; the color reaction was stopped with 0.5 M H_2SO_4 was read at 450 nm wavelength.

ROS assay. To measure intracellular ROS level, 30 μM 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) was used as a fluorescent probe. This probe was loaded onto the cell with Hanks solution (Gibco) immediately after cell was displaced from the hypoxic chamber. After 20 min incubation in the dark, cells were trypsinized for 2 min and the reaction was stopped with MEM with 10% FBS. Cells were pelleted by centrifugation and washed twice with PBS. The cell pellet was resuspended with 350 μl of PBS and was used for FACS analysis. The oxidized form of DCFDA, fluorescent dichlorofluorescein was excited at 488 nm and detected at 530 nm.

Chromatin immunoprecipitation assay. After displacing cells from hypoxia chamber, we crosslinked chromatin and protein complex by treating with 1% formaldehyde for 15 min. After cross linking, chromatin was sonicated and then incubated with salmon sperm DNA/protein A agarose-50% slurry for 6 h. After incubation, sample was washed sequentially with low salt, high salt, LiCl, and TE buffers. The complexes were then eluted with 0.5% SDS, 300 mM NaCl, 5 mM EDTA, and 10 mM Tris–Cl by incubating overnight at 65 °C. RNA and proteins were digested by adding RNase A at 37 °C for 30 min, and by adding protease K at 55 °C for 60 min. DNA was purified by phenol/chloroform extraction and was precipitated with ethanol. DNA isolated from immunoprecipitated material was amplified by semi-quantitative PCR with [α-^{32}P]dCTP. The PCR primer sequences used were 5′-GTGAGAGC TTAAAGCAATGGAT-3′ and 5′-TCACAGACACACACTGCTCAC-3′, which produced 250 bp fragments including −432 to −183 bp of the *hepcidin* gene.

Results and discussion

Hepcidin gene transcription is repressed under hypoxic conditions

The *hepcidin* gene consists of three exons and two introns. To differentiate mature mRNA from pre-mRNA, primers for hepcidin mRNA were designed to bind exon 1 or 3 site and primers for pre-mRNA were designed to bind an intron 1 site. To examine the effects of hypoxia on hepcidin mRNA expression *in vivo*, five mice were subjected to 24 h of whole-body hypoxia in an air-tight chamber containing a 12% O_2 atmosphere. Hepcidin mRNA levels in the livers of these mice were found to be significantly downregulated vs. normoxic controls (Fig. 1A). Moreover, in HepG2 human hepatoma cells, hepcidin mRNA levels were found to be markedly downregulated

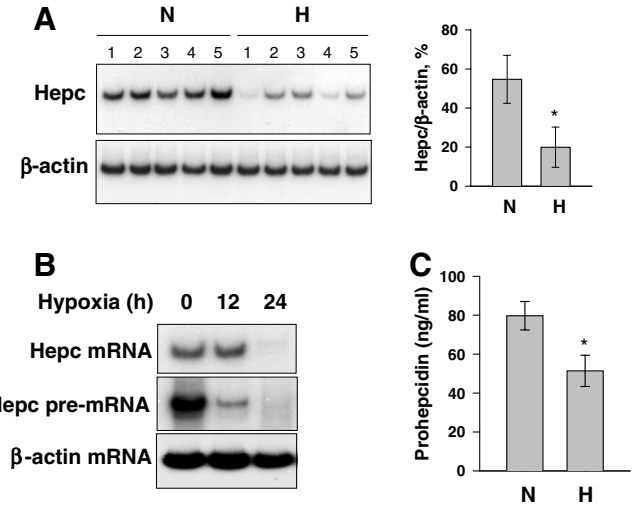

Fig. 1. Hypoxia downregulates *hepcidin* gene expression. (A) Mice were exposed to 24 h normoxia (N, $n = 5$) or hypoxia (H, $n = 5$), and RNAs were extracted from excised livers. Hepcidin mRNA levels were determined by semi-quantitative RT-PCR and autoradiography. β-Actin mRNA was analyzed as a loading control. Band intensities were quantified using ImageJ 1.36b image analysis software (NIH, USA). The percentages of hepcidin to β-actin are presented in the right panel. $^{*}p<0.01$ vs. the normoxic control. (B) Hepcidin mRNA and pre-mRNA were determined in HepG2 cells incubated under hypoxic conditions for indicated time. (C) HepG2 cells were incubated under normoxic or hypoxic conditions for 24 h, and lysed. The supernatants were used for prohepcidin assay using an ELISA kit provided from DRG Instruments. $^{*}p<0.05$ vs. the normoxic value.

after 24 h of hypoxia (Fig. 1B). To examine this mRNA suppression at the transcription level, we also analyzed hepcidin pre-mRNA levels, and found that they were downregulated as early as 12 h after hypoxia, indicating that hepcidin suppression by hypoxia is mainly due to reduced transcription (Fig. 1B). Moreover, the time gap of 12 h found between the down-regulations of pre-mRNA and mRNA appears reasonable in terms of the time required to produce mature mRNA from pre-mRNA. In addition, prohepcidin, which is the translation product of hepcidin mRNA, was also found to be downregulated under hypoxic conditions (Fig. 1C).

The hypoxic down-regulation of hepcidin does not involve HIF-1

HIF-1 was examined initially as a potential inducer of hepcidin down-regulation under hypoxic conditions. HIF-1 is composed of HIF-1α and ARNT subunits, and HIF-1α acts as the primary transcription factor and is normally degraded by proteasomes [13]. During hypoxia, HIF-1α stabilizes, dimerizes with ARNT, and the complex formed acts as a transcription factor for those genes required for adaptation to a hypoxic environment [14]. To date, about 60 hypoxia-inducible genes have been found to be regulated by HIF-1 transcription factor [15]. Thus, to determine whether hepcidin transcription depends on HIF-1, HIF-1 was overexpressed using HIF-1α and ARNT plasmids or knocked-down using HIF-1α siRNA in HepG2 cells; HIF-1 levels were monitored to check transfection efficiency (Fig. 2, lower panel). However, despite successfully induced changes in HIF-1 expression, hepcidin mRNA levels were unaffected (Fig. 2, upper panel). Moreover, hepcidin mRNA levels during normoxia and hypoxia remained the same as in respective controls. These findings indicate that the hypoxic down-regulation of hepcidin mRNA is not mediated by HIF-1.

ROS production increases during hypoxia

We next examined the possibility that ROS mediates the hypoxic suppression of hepcidin. Since molecular oxygen is required for ROS generation, ROS production is stimulated under hyperoxic conditions. Thus, ROS production would be expected to be reduced under hypoxic conditions. But, evidence demonstrates that ROS levels are paradoxically elevated during hypoxia [16,17]. Mechanistically, this hypoxic stimulation of ROS production has been proposed to be due to hypoxia-induced changes in the lipid–protein structures of mitochondrial inner membranes, and a resultant increase in the transfer of electrons from semi-ubiquinone to O_2 [18]. In the present study, ROS levels were measured in hypoxic HepG2 cells, using DCFH-DA fluorescent dye. During hypoxia, the fluorescence peak was found to shift to the right, demonstrating that hypoxia increased intracellular ROS levels (Fig. 3A). Moreover, according to mean measured fluorescence levels, ROS levels were enhanced ~4-fold during hypoxia vs. normoxia (Fig. 3A).

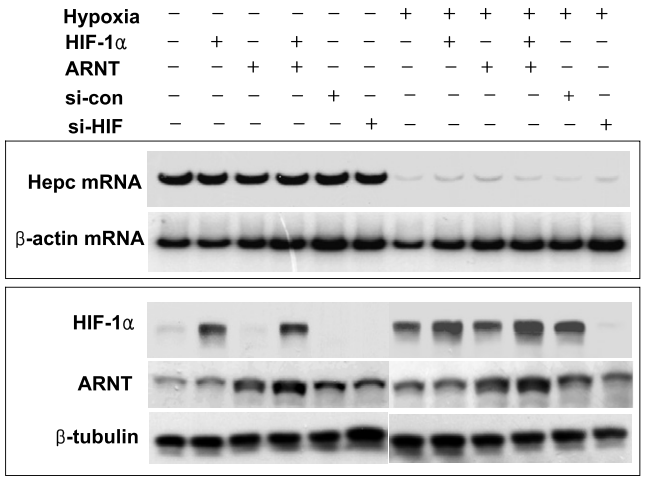

Fig. 2. Hypoxic suppression of hepcidin is independent of HIF-1. Hepcidin and β-actin mRNA levels during normoxia (lanes 1–6) and hypoxia (lanes 7–12) were determined by RT-PCR and autoradiography. HepG2 cells were transfected with 2 μg of plasmid (pcDNA, pHIF-1α or pARNT) or with 40 nM of siRNA (HIF-1α or control RNA), and then incubated under normoxic or hypoxic conditions for 24 h. To identify expressed proteins and knock-down of HIF-1α, HIF-1α, ARNT and β-tubulin proteins were analyzed by Western blotting.

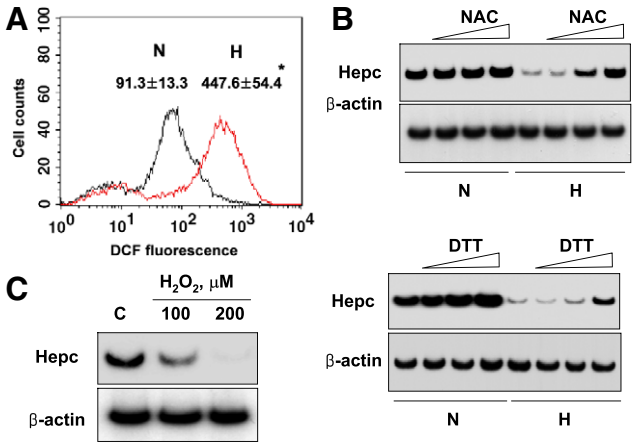

Fig. 3. ROS mediate hypoxic suppression of hepcidin. (A) HepG2 cells were incubated under normoxic or hypoxic conditions for 24 h. Cells were treated with DCFH-DA for 20 min, and harvested to determine ROS level by using FACS. Intracellular ROS levels were determined by measuring the fluorescence intensity at Ex = 485 nm and Em = 530 nm. $^{*}p<0.001$ vs. the normoxic value. (B) HepG2 cells were pretreated with anti-oxidants NAC (0.5, 2, and 5 mM) or DTT (40, 100, and 200 μM) for 1 h, and then incubated under normoxic or hypoxic conditions for 24 h. Hepcidin and β-actin mRNA levels were determined by RT-PCR and autoradiography. (C) HepG2 cells were treated with hydrogen peroxide (100 and 200 μM) in a serum-free medium for 24 h, and prepared for RT-PCR of hepcidin and β-actin mRNA.

ROS mediated the hypoxic down-regulation of hepcidin

In terms of their biological roles, ROS are often considered harmful because they are responsible for oxidizing and cross-linking macromolecules. However, organisms have learned to exploit this reactivity in many intracellular signal pathways. ROS are known to activate AP-1, NF-κB, and Ref-1 transcription factors by stimulating protein expression, nuclear translocation, or transcriptional activity, and in the context of transcriptional activation, ROS are known to enhance the DNA-binding affinities of transcription factors by altering DNA core structures and facilitating interactions. It should also be added that some transcription factors are deactivated by ROS [19]. To test the hypothesis that ROS regulate hepcidin expression, we examined the effects of the anti-oxidants *N*-acetyl cysteine (NAC) or dithiothreitol (DTT) on hepcidin expression in hypoxic HepG2 cells. It was found that both prevented the hypoxic down-regulation of hepcidin in a dose-dependent manner (Fig. 3B). On the other hand, H_2O_2 noticeably suppressed hepcidin expression even during normoxia (Fig. 3C). These results suggest that *hepcidin* gene transcription is inhibited under hypoxic conditions via an ROS-mediated pathway.

C/EBPα and pSTAT-3 are involved in the hypoxic down-regulation of hepcidin

Relatively few transcription factors are known to participate in hepcidin expression. C/EBPα was the first transcription activator of the *hepcidin* gene identified. In addition, the liver-specific deletion of the *C/EBPα* gene in mice prevented hepcidin expression and induced a severe iron overload [20]. Upstream stimulatory factor (USF) is another transcription factor of hepcidin expression via *cis*- and *trans*-regulation. USF−/− mice were found to be hepcidin deficient and to progressively develop an iron overload [21]. In addition, hepcidin promoter and DNA-binding studies showed that USF-1 or USF-2 directly bind to and transactivate hepcidin promoter [22]. In addition, STAT-3 has been suggested to transactivate hepcidin promoter in the context of hepcidin up-regulation during inflammation [23]. To clarify the mechanism of hypoxic hepcidin repression, we monitored the expressions of these four transcription factors, and HIF-1α expression (a marker of hypoxic status). C/EBPα and phospho-STAT-3 (pSTAT-3) levels were found to reduce under hypoxic conditions in a time-dependent manner, but USF-1 and USF-2 levels were not (Fig. 4A). To investigate the DNA bindings of C/EBPα and pSTAT-3, we performed chromatin immunoprecipitation assays. Both C/EBPα and pSTAT-3 bindings to hepcidin promoter were found to be reduced by hypoxia, and to recover fully after anti-oxidant treatment (Fig. 4B). However, C/EBPα and pSTAT-3 protein levels only slightly recovered after anti-oxidant treatment

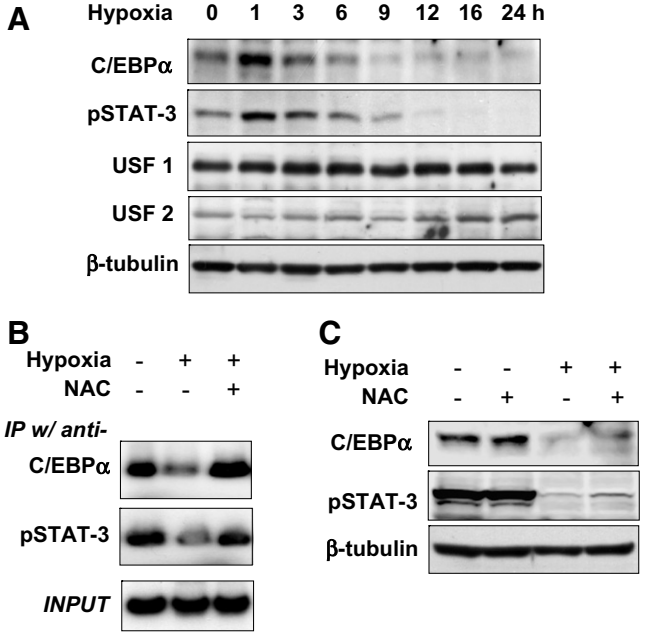

Fig. 4. C/EBPα and STAT-3 are involved in hypoxic suppression of hepcidin. (A) After HepG2 cells were exposed to hypoxia for various incubation times, protein levels of transcription factors were determined using Western blotting. (B) C/EBPα and pSTAT-3 binding to hepcidin promoter were analyzed using chromatin immunoprecipitation (CHIP) with anti-C/EBPα or anti-pSTAT-3. Proximal promoter DNAs of the *hepcidin* gene were amplified by 30 cycles of PCR, electrophoresed, and autoradiographed. Input shows amplification of hepcidin promoter using cell lysate without immunoprecipitation. (C) C/EBPα and pSTAT-3 protein levels were determined after 24 h hypoxic incubation with or without 5 mM NAC.

(Fig. 4C). Although both the protein levels and DNA bindings of C/EBPα and pSTAT-3 decreased during hypoxia, it appears that DNA-binding inhibition, rather than protein reduction, is the underlying cause of the ROS-dependent, hypoxic repression of the *hepcidin* gene. Our hypothesis regarding the mechanism of the hypoxic down-regulation of hepcidin is summarized in the Supplementary figure.

Implications and speculations

Since hepcidin is a major regulator of iron homeostasis, abnormal hepcidin levels should cause iron-related disorders. And, in chronic inflammatory diseases, hepcidin is induced by IL-6 (an inflammatory cytokine) and causes iron-deficient anemia [24]. Moreover, in hereditary hemochromatosis (the most commonly encountered iron-overload condition), HFE, TfR2, or HJV genes are mutated, and interestingly, these gene mutations have been associated with *hepcidin* gene suppression and iron overload [25–27]. Thus, because hepcidin appears to be involved in diseases involving an iron deficiency or excess, the molecular entities responsible for hepcidin regulation become attractive therapeutic targets for the treatment of iron-related diseases. In the present study, we found that ROS repress the *hepcidin* gene by preventing C/EBPα and pSTAT-3 binding to hepcidin promoter during hypoxia. If this is in fact the case, prooxidants or inhibitors of C/EBPα or STAT-3 might be useful therapeutic tools for the treatment of iron-deficiency anemia or hypoproliferative anemia in combination with erythropoietin supplementation. Conversely, anti-oxidants or C/EBPα or STAT-3 activators might be therapeutically useful for preventing iron overload in hereditary hemochromatosis or thalassemia patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.137.

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