ARTICLE
Journal of Cellular Biochemistry 116:1919–1931 (2015)

Journal of Cellular Biochemistry

Iron Regulatory Protein 1 Suppresses Hypoxia-Induced Iron Uptake Proteins Expression and Decreases Iron Levels in HepG2 Cells

Chun-Ming Cheng, Dan Wang, Xian Cao, Qian-Qian Luo, Ya-Peng Lu, and Li Zhu*

Department of Biochemistry, Institute for Nautical Medicine, Nantong University, Nantong, Jiangsu 226001, PR China

ABSTRACT

Transferrin receptor (TfR1) and divalent metal transporter 1 (DMT1) are important proteins for cellular iron uptake, and both are regulated transcriptionally through the binding of hypoxia-inducible factor 1 (HIF-1) to hypoxia-responsive elements (HREs) under hypoxic conditions. These proteins are also regulated post-transcriptionally through the binding of iron regulatory protein 1 (IRP1) to iron-responsive elements (IREs) located in the mRNA untranslated region (UTR) to control cellular iron homeostasis. In iron-deficient cells, IRP1–IRE interactions stabilize TfR1 and DMT1 mRNAs, enhancing iron uptake. However, little is known about the impact of IRP1 on the regulation of cellular iron homeostasis under hypoxia. Thus, to investigate the role of IRP1 in hypoxic condition, overexpression and knockdown assays were performed using HepG2 cells. The overexpression of IRP1 suppressed the hypoxia-induced increase in TfR1 and DMT1 (+IRE) expression and reduced the stability of TfR1 and DMT1 (+IRE) mRNAs under hypoxia, whereas IRP1 knockdown further increased the hypoxia-induced expression of both proteins, preventing the decrease in IRE-dependent luciferase activity induced by hypoxia. Under hypoxic conditions, ferrous iron uptake, the labile iron pool (LIP), and total intracellular iron reduced when IRP1 was overexpressed and further increased when IRP1 was knocked down. IRP1 expression declined and TfR1/DMT1 (+IRE) expression increased with the time of hypoxia prolonged, whereas the binding of IRP1 to the IRE of TfR1/DMT1 mRNA maintained. In summary, IRP1 suppressed TfR1/DMT1 (+IRE) expression, limited the cellular iron content and decreased lactate dehydrogenase (LDH) release induced by hypoxia. J. Cell. Biochem. 116: 1919–1931, 2015.

KEY WORDS: HIF-1; IRP1; TfR1; DMT1; IRON METABOLISM

Iron is an important nutrient for all mammalian cells, as it is an essential component of proteins involved in DNA synthesis, mitochondrial respiration, and oxygen transport [Romney et al., 2011]. However, excessive cellular iron catalyzes the generation of reactive oxygen species (ROS) that damage DNA and proteins, whereas cellular iron deficiency results in cell death [Altamura and Muckenthaler, 2009]. Thus, to avoid the consequences of iron depletion or excess, the cellular iron concentration must be regulated within a narrow range [Eckard et al., 2010]. Cellular iron homeostasis is regulated by iron regulatory proteins (IRPs, including IRP1 and IRP2), which sense cytosolic iron levels and post-transcriptionally regulate the expression of TfR1, one isoform of DMT1 (DMT1+IRE), ferritin-L chains (Ft-L), and several other iron metabolism proteins by interacting with conserved iron-responsive elements (IREs) present in the untranslated region (UTR) of target mRNAs [Sammarco et al., 2008; Styś et al., 2011; Wang et al., 2011]. IRP1 regulates gene expression by binding to IREs in either the 5′-UTR or 3′-UTR: the initiation of target gene translation is inhibited when IRP1 binds to the 5′-UTR IRE (e.g., Ft-L), whereas the mRNA is stabilized when IRP1 binds to the 3′-UTR IRE of a target gene (e.g.,

Abbreviations: DMT1, divalent metal transporter 1; FAS, ferric ammonium sulfate; Ft-L, ferritin L chain; HIF-1, hypoxia-inducible factor 1; HRE, hypoxia-responsive element; IRE, iron-responsive element; LIP, labile iron pool; TfR1, transferrin receptor 1; UTR, untranslated region.

Chun-Ming Cheng and Dan Wang contributed equally to this work.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 31171143, 31471141; Grant sponsor: 973 Program; Grant number: 2011CB510004; Grant sponsor: Priority Academic Program Development of Jiangsu Higher Education Institution (PAPD).

*Correspondence to: Li Zhu, PhD, Department of Biochemistry, Institute for Nautical Medicine, Nantong University, 19 QiXiu Road, Nantong, Jiangsu 226001, PR China. E-mail: zhulili65@126.com

Manuscript Received: 11 August 2014; Manuscript Accepted: 24 February 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 February 2015

DOI 10.1002/jcb.25147 • © 2015 Wiley Periodicals, Inc.

TfR1, DMT1+IRE,) [Gunshin et al., 2001; Malik et al., 2011; Sanchez et al., 2011].

Hypoxia-inducible factor 1α (HIF-1α) is a transcription factor that senses hypoxia [Semenza and Wang, 1992]. In the presence of oxygen, HIF-1α is degraded through the ubiquitin/proteasome pathway mediated by the tumor suppressor protein von Hippel-Lindau (VHL) [Doe et al., 2012]. Conversely, HIF-1α is stabilized by hypoxia and translocates to the nucleus, where it dimerizes with the constitutively expressed HIF-1β subunit [Song et al., 2008; Semenza and Wang, 1992]. The binding of the dimer to hypoxia-responsive elements (HRE) triggers the expression of a series of genes, including IRP1, TfR1, and DMT1 [Lok and Ponka., 1999; Luo et al., 2011; Qian et al., 2011].

Interestingly, on the one hand, there are HREs in the promoters of both TfR1 and DMT1, as well as IREs in 3′-UTR mRNA of TfR1 and DMT1, on the other hand, IRP1 has three functional HREs in its 5′-regulatory region [Casey et al., 1989; Gunshin et al., 2001; Wang et al., 2010]. Therefore, TfR1 and DMT1 (+IRE) are transcriptionally regulated by the HIF-1/HRE system and post-transcriptionally regulated by the IRP1/IRE system. Moreover, IRP1 is also regulated by the HIF-1/HRE system under hypoxia. Different studies have reported conflicting results about the effect of hypoxia on cellular iron metabolism; however, little is known about the role of IRP1 on iron metabolism under hypoxia [Toth et al., 1999; Meyron-Holtz et al., 2004]. In the present study, we found that IRP1 suppressed hypoxia-induced TfR1/DMT1 expression, restricted intracellular iron levels, including the labile iron pool and total cellular iron content, and reduced lactate dehydrogenase (LDH) release.

MATERIALS AND METHODS

CELL CULTURE AND HYPOXIA INDUCTION

Human HepG2 hepatoma cells were cultured in Dulbecco′s Modified Eagle′s Medium (DMEM, Hyclone, South Logan, UT) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air (Froma Series II, Thermo, Rockford, IL). For the hypoxia treatment, the cell culture plates were incubated in an incubator with an atmosphere of 5% CO_2 with 1% O_2 balanced with N_2 (Invivo2, Ruskinn, UK) for the indicated periods.

LACTATE DEHYDROGENASE ASSAY

To assess the cytotoxicity of hypoxia under our experimental conditions, LDH release was measured in treated cells grown in 96-well plates. Control cells not treated with hypoxia (0 h) were chosen to confirm the viability of the cells. The cell culture medium (20 μl) was collected, and then the absorbance at 450 nm was measured as described by the manufacturer (Jiancheng, China). The optical density was measured using an Elx800 reader (Bio-Tek, Winooski, VT).

TOTAL RNA EXTRACTION AND REAL-TIME PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer′s instructions and reverse transcribed with AMV reverse transcriptase (Promega, Madison, WI) and oligo-dT in a 20-μl reaction mixture. The primers for the real-time PCR analysis are listed in Table I. The transcript levels were assessed by quantitative real-time PCR (Rotor-Gene RG3000A, Qiagen, Hilden, Germany).

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in a buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO) according to the manufacturer′s instructions. The protein level was estimated using the bicinchoninic acid (BCA) assay. The proteins (20 μg) were separated on 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were incubated for 2 h in 5% skim milk to block non-specific binding. After washing three times with TBS, the membranes were incubated overnight at 4°C with primary antibodies against the following proteins: IRP1 (1:200, Santa Cruz, CA), TfR1 (1:1000, Zymed, South San Francisco, CA), DMT1 (1:500, Proteintech, Chicago, IL), Ft-L (1:2000, Abcam, Cambridge, MA), and β-actin (1:10000, Sigma). The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (Sigma) for 2 h at room temperature after washing three times with TBST. The protein bands were detected using the Super Signal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). Densitometry was conducted using Image-Pro Plus, and the relative band intensity for each protein was normalized to lane 1 after subtracting the respective β-actin values. Lane 4 was further normalized to lane 3 to eliminate the influence of IRP1 overexpression or knockdown under normoxia (Figs. 3 and 5).

PLASMID CONSTRUCTS

An IRP1 expression plasmid was constructed as previously described [Cheng et al., 2012]. The coding sequence (CDS) of the IRP1 sequence was obtained by subcloning a PCR-generated fragment (2,670 bp) into pcDNA3.1(+) (Promega) to generate pcDNA3.1-IRP1.

The IRE regions of human TfR1 and DMT1 were obtained by PCR using human cDNA as the template. The TfR1-Luc-IRE and DMT1-Luc-IRE primers were designed with Primer 5.0 and are shown in Table I. The PCR products were subcloned into the pGL3 vector and subcloned into the luciferase basic vector.

IRP1 siRNA

Prior to transfection, 5×10^4 cells were plated in each well of a 24-well plate; the transient transfection experiment was executed when the cells were grown to 50% confluence. For each transfection, HepG2 cells were transfected with a human IRP1-specific siRNA duplex (Dharmacon, Lafayette, CO) using the X-tremeGENE siRNA transfection reagent (Roche Indianapolis, IN) according to the manufacturer′s protocol.

TfR1 AND DMT1 mRNA STABILITY

HepG2 cells were transfected with pcDNA3.1(+)-IRP1 or pcDNA3.1(+)-con under normoxic conditions for 36 h and subsequently exposed to hypoxia for 6 h before actinomycin D (5 mg/ml) was

TABLE I. Primers Used for Real-Time PCR

β-Actin sense	CATGTACGTTGCTATCCAGGC
β-Actin antisense	CTCCTTAATGTCACGCACGAT
DMT1 sense	CTCAGCCACTCAGGTATCCAC
DMT1 antisense	CAGACTCCCCATGATCTCCA
IRP1 sense	TGCTTCCTCAGGTGATTGGCTACA
IRP1 antisense	TAGCTCGGTCAGCAATGGACAACT
TfR1 sense	AGTTGAACAAAGTGGCACGAGCAG
TfR1 antisense	AGCAGTTGGCTGTTGTACCTCTCA
TfR1-Luc-IRE sense	GGGGTACCCTTTTGGCACTGAGATAT
TfR1-Luc-IRE antisense	CCGCTCGAGTTATAGGAGTTGGGATACA
DMT1-Luc-IRE sense	GGGGTACCCCCATCCTCACATTTACG
DMT1-Luc-IRE antisense	CCGCTCGAGGCAACGGCACATACTTTT

added. Total RNA was isolated at 0, 1, 2, 4, and 6 h after the addition of actinomycin D, and real-time PCR was performed using primers for TfR1, DMT1, and β-actin, as described in Table I.

PLASMID TRANSFECTION AND LUCIFERASE ASSAY

To assess IRE function in HepG2 cells, 5×10^4 cells were seeded in each well of a 24-well plate and incubated overnight. On the following day, the TfR1-Luc-IRE plasmid and IRP1 interference fragments were co-transfected into the cells under normoxic conditions for 48 h (normoxia) or under normoxic conditions for 42 h followed by hypoxia for 6 h (hypoxia 6 h). pRL-TK (Promega) was co-transfected as an internal control for transfection efficiency. After 48 h, the luciferase activity was measured using a dual luciferase assay kit (Promega). All the plasmid transfections were performed using the FuGENE HD DNA Transfection Reagent (Roche Indianapolis) according to the manufacturer's instructions. The DMT1-Luc-IRE plasmid and IRP1 interference sequence were transfected in the same manner. The luciferase activity of the cells was normalized to the activity under normoxia, which was defined as 100.

CYTOSOLIC EXTRACT PREPARATION AND RNA SUPERSHIFT ANALYSIS

HepG2 cells were washed with phosphate-buffered saline (PBS) and lysed with 140 μl lysis buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid], 25 mM KCl, 0.5% nonidet P-40 [NP-40], and 1 mM dithiothreitol [DTT]). Cells were scraped and cell lysates cleared by centrifugation at 15,000*g* for 15 min at 4°C. The sequences of TfR1 IRE and DMT1 IRE probes (GenePharma, Shanghai, China) were shown in Table II. Biotins were labeled in 5′ end oligonucleotide. Twelve microgram of cytosolic extract protein was incubated for 20 min at room temperature in binding reaction system including: 1.5 μl 10×binding buffer, 1.5 μl poly(dI-dC, 1.0 μg/μl), and ddH_2O to meet a final volume of 15 μl. Then 1 μl Bio-TfR1/DMT1 IRE probe (500 fM) was added, and the reaction was incubated for 20 min at room temperature. Protein–RNA complexes were resolved by electrophoresis at 4°C on a 6.5% acrylamide gel and subjected to autoradiography. Electrophoresis was carried out on a 6.5% nondenaturing polyacrylamide gel at 175 V in 0.25 × TBE (1 × TBE is 89 mM Tris–HCl, 89 mM boric acid, and 5 mM EDTA, pH8.0) at 4°C for 1 h. Gels were transferred to the banding membrane at 394 mA in 0.5 × TBE at room temperature for 40 min. Then, crosslinked the membrane in UV crosslink apparatus for 10 min (immobilization), blocked, lableled with Streptavidin-HRP, washed, equilibrated, and obtained the pictures through the Imager apparatus (Alpha flurechemical).

TABLE II. RNA Supershift Analysis Probe For Each Probe, the Complementary Sequences are Shown. Biotins Were labeled in 5′ End Oligonucleotide

Probe	Sequence
TfR1 probe1	5′-UUUAUCAGUGACAGAGUUCACUAUAAA3′
TfR1 probe2	5′-AUUAUCGGAAGCAGUGCCUUCCAUAAU′3
TfR1 probe3	5′-AUUAUCGGGAGCAGUGUCUUCCAUAAU-3′
TfR1 probe4	5′-UGUAUCGGAGACAGUGAUCUCCAUAUG-3′
TfR1 probe5	5′-AUUAUCGGGAACAGUGUUUCCCAUAAU-3′
DMT1 probe1	5′-GCCAUCAGAGCCAGUGUGUUUCUAUGGU-3′

CALCEIN LOADING AND THE FERROUS UPTAKE ASSAY

The ferrous uptake assay was performed as described [Epsztejn et al., 1997]. Briefly, the cells were washed twice and incubated with 0.125 μM calcein-AM in a serum-free medium for 10 min at 37°C; excess calcein-AM on the cell surface was removed by three washes with Hank's balanced salt solution (HBSS, pH 7.4). Prior to the measurements, 100 μl of calcein-loaded cell suspension and 2 ml of HEPES were added to the cuvette. The fluorescence was measured using an ultraviolet spectrophotometer (Shimadzu RF-5301PC, Kyoto, Japan) at λ_{ex}=488 nm and λ_{em}=518 nm at 37°C. After the initial baseline of fluorescence intensity was collected, ferrous ammonium sulfate (FAS, 40 μM final concentration) was added to the cuvette, and calcein fluorescence quenching was recorded every 5 min for 30 min.

LABILE IRON POOL ASSAY

The labile iron pool (LIP) in the cells was measured using a modified protocol based on the fluorescent probe calcein [Epsztejn et al., 1997; Kakhlon and Cabantchik, 2002]. Briefly, the cells were washed three times with PBS and collected, and 1 ml of HEPES was added. Calcein-AM (0.125 μM final concentration) was added and incubated for 10 min at 37°C; any excess calcein-AM was removed by washing three times with HEPES. Immediately prior to the measurements, 3 ml of pre-warmed HBSS was added to the cells. The fluorescence was measured using an ultraviolet spectrophotometer (Shimadzu RF-5301PC) at λ_{ex}=488 nm and λ_{em}=518 nm for 200 s to establish a stable baseline. After adding 50 mM deferiprone (Sigma) in HEPES to each well, the fluorescence was measured every second for 1,000 s. Increases in fluorescence compared with the control during the plateau period were used for statistical analysis.

QUANTIFICATION OF THE TOTAL CELLULAR IRON CONTENT

An analysis of total cellular iron content was performed using atomic absorption spectroscopy (AAS) and fluorescence. HepG2 cells were grown in 6-well dishes at a density of 1×10^6 cells/well after incubation under normoxia or hypoxia for 2, 4, 6, 8, 12, and 24 h. After the treatment, the cells were washed twice with PBS, harvested, counted, and centrifuged; the cell pellets were lysed in 800 μl of 50 mM NaOH. Two methods were adopted to measure the cellular total iron content. First, aliquots of the lysate were incubated at 100°C, and the total amount of iron was quantified using AAS

[Hoepken et al., 2004]. Second, aliquots of the lysate were incubated overnight at 60°C, and hydrochloric acid (200 μl) and ultrapure water (800 μl) were added at 60°C for 4 h. This cellular solution (75 μl) and the detection reagent (0.08% $K_2S_2O_8$, 8% KSCN, and 3.6% HCl dissolved in water, 75 μl) were added to 96-well plates, and the absorbance was measured at an excitation wavelength of 490 nm after 10–15 min [Liu et al., 2011]. The iron content was determined by normalizing the obtained absorbance with a standard curve using $FeCl_3$ calibration standards.

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS 10.0. The data are presented as the means ± SD. The difference between the means was determined by a one-way ANOVA, followed by a Student-Newman–Keuls test for multiple comparisons. The data were considered statistically significant at $^{*}P < 0.05$.

RESULTS

LDH RELEASE AND CELLULAR IRON CONTENT INCREASED AFTER EXPOSURE TO 1% OXYGEN FOR MORE THAN 6 H

The release of LDH into the culture medium was measured to assess injury due to hypoxia in cultured cells. HepG2 cells were exposed to hypoxia (1% oxygen) for periods from 1 to 24 h. LDH release was found to increase progressively upon exposure to hypoxia for more than 6 h, indicating marked membrane damage caused by hypoxic exposure (Fig. 1A). Unless otherwise indicated, the HepG2 cells in the following experiments were treated with hypoxia for 6 h. To estimate the effect of hypoxia on the cellular iron level, the total iron content was quantified using AAS. The total iron content in the HepG2 cells increased with the time of hypoxia, from 6 to 16 h, reaching the highest levels at 8 h (Fig. 1B).

HYPOXIA DOWNREGULATED IRP1 EXPRESSION, UPREGULATED IRON UPTAKE PROTEINS EXPRESSION, AND KEPT THE ATTACHMENT OF IRP1 TO IRE ELEMENTS

To ascertain the effect of acute hypoxia on iron metabolism, the protein and mRNA expression of IRP1 and major iron uptake proteins (TfR1, DMT1) were examined by Western blotting and real-time PCR after exposure to hypoxia for 2, 4, 6, and 8 h. As shown in Figure 2A and B, hypoxia downregulated the protein and mRNA expression of IRP1. In contrast, hypoxia for 2, 4, 6, and 8 h up-regulated both the protein and mRNA expression of TfR1 and DMT1 (+IRE) in a time-dependent manner (Fig. 2A, C, and D).

As hypoxia downregulated IRP1 expression time dependently, we wondered whether TfR1 and DMT1 (+IRE) are post-transcriptionally regulated by IRP1 under hypoxia. We detected the IRP1/IRE interactions by using of RNA supershift analysis. The data showed that hypoxic exposure for 2–16 h maintained the binding of IRP1 to IRE of both TfR1 and DMT1 mRNA, which was similar to that in normoxia (Fig. 2E).

HYPOXIA-INDUCED IRON UPTAKE PROTEINS EXPRESSION WAS SUPPRESSED BY IRP1 OVEREXPRESSION

To determine the contribution of IRP1 to the regulation of iron metabolism in response to hypoxia, an IRP1 expression plasmid was constructed to assess the effect of IRP1 on TfR1 and DMT1 (+IRE) expression under hypoxia. Both IRP1 protein and mRNA increased after transfection with pcDNA3.1(+)-IRP1 for 48 h (Fig. 3A–C). Exposure to hypoxia for 6 h increased the protein expression of TfR1 and DMT1 (+IRE) in the pcDNA3.1(+)-con- and pcDNA3.1(+)-IRP1-trasnfected cells. However, under the hypoxic condition, the expression of TfR1 and DMT1 (+IRE) in the HepG2 cells transfected with pcDNA3.1(+)-IRP1 was less than that in the pcDNA3.1(+)-con-transfected cells, indicating that hypoxia-induced TfR1 and DMT1

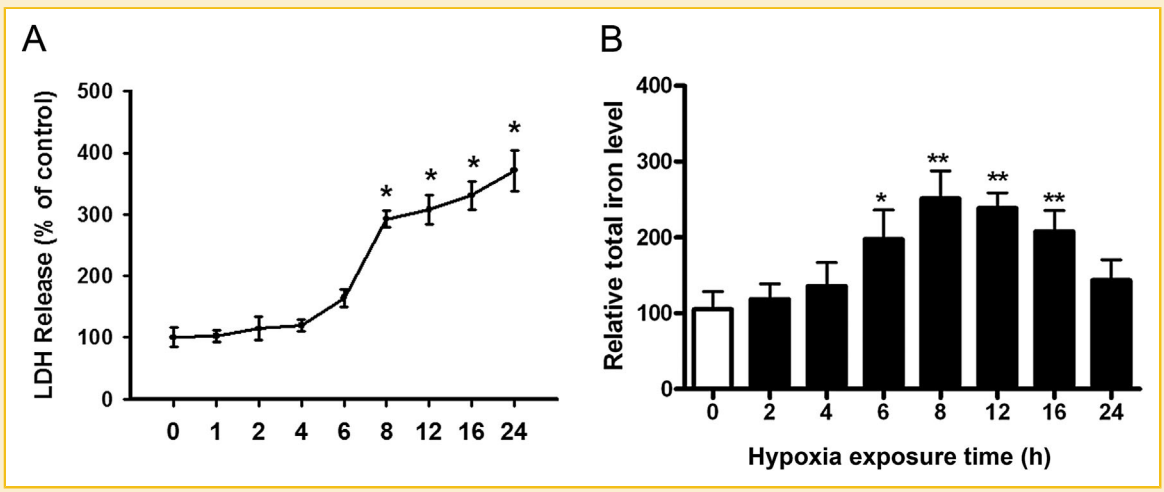

Fig. 1. Effect of hypoxia on LDH release and total cellular iron. HepG2 cells were exposed to hypoxia (1% O_2) from 0 h to 24 h and were subsequently harvested. A: After treatment for the indicated time, LDH release was measured in cells grown in 96-well plates. B: The total amount of iron was quantified using atomic absorption spectroscopy (AAS), as described in Materials and Methods. The data from three independent experiments were analyzed in duplicate and are represented as the means ± SD (n = 6). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the control (0 h).

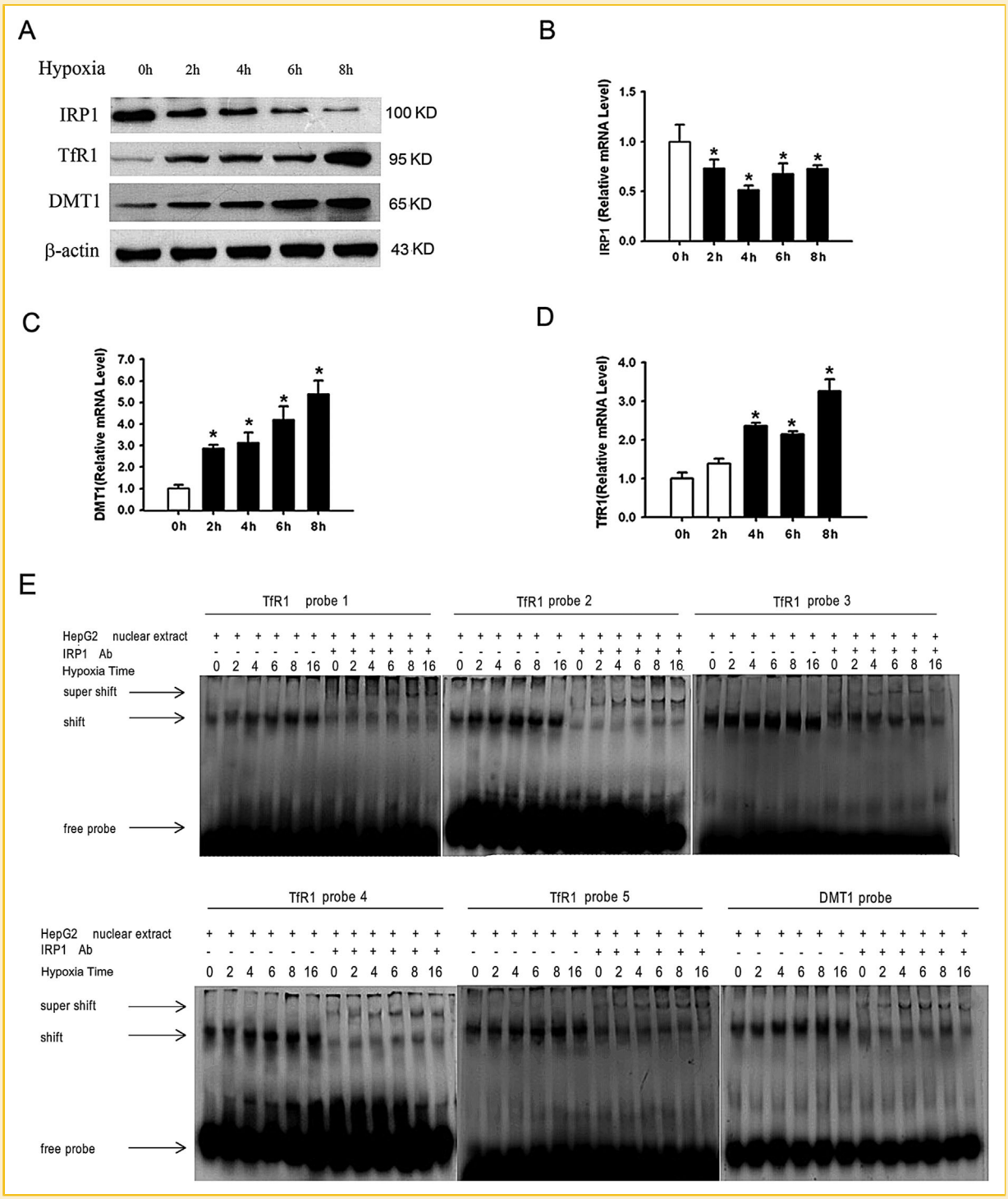

Fig. 2. The effect of hypoxia on the expression of IRP1, iron uptake proteins and IRP1/IRE binding. A–D: HepG2 cells were exposed to hypoxia for 0, 2, 4, 6, and 8 h, and total mRNA and protein were extracted. The protein and mRNA expression of IRP1, TfR1, and DMT1 (+IRE) was measured by Western blotting and real-time PCR. β-Actin was used as a control. The data are presented as the means ± SD. $^{*}P < 0.05$ compared to 0 h. E: HepG2 cells were exposed to hypoxia for 0, 2, 4, 6, 8, and 16 h. RNA supershift analysis of cytosolic cell extracts using biotin-labeled TfR1 IRE (prob1–5)/DMT1 probe anti-IRP1 antibodies.

(+IRE) expression was markedly suppressed by the overexpression of IRP1 (Fig. 3D–F).

TfR1 and DMT1 (+IRE) mRNAs are typically stabilized due to the interactions of IRPs (IRP1 and IRP2) with the IREs present in the 3′-UTRs of the transcripts, which protect the mRNA molecule. To understand why IRP-1 suppressed TfR-1 and DMT1 (+IRE) expression in response to hypoxia, we measured the stability of both mRNAs. The overexpression of IRP1 resulted in an obvious

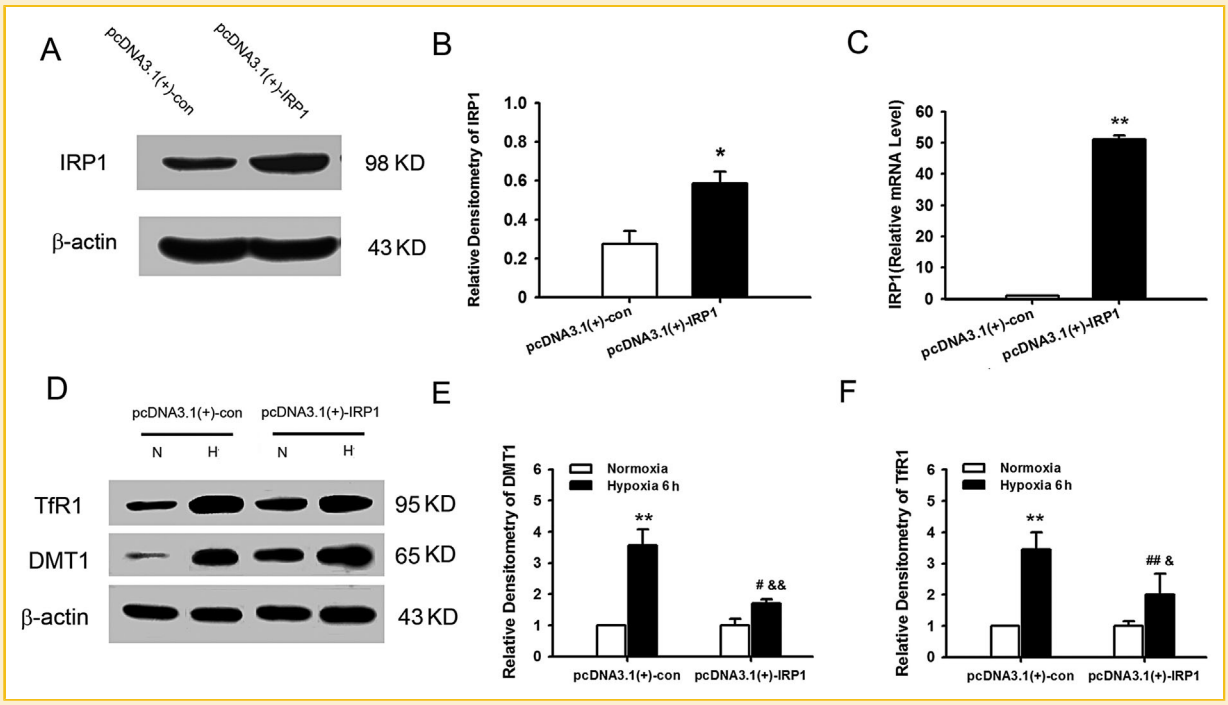

Fig. 3. Hypoxia-induced iron uptake proteins expression was suppressed by IRP1 overexpression. A–C: HepG2 cells were transfected with the pcDNA3.1(+)-IRP1 or pcDNA3.1(+)-con plasmid under normoxia (N). After 48 h, IRP1 protein (A and B) and mRNA (C) expression were measured by Western blotting or real-time PCR. D: HepG2 cells were transfected with the pcDNA3.1(+)-IRP1 or pcDNA3.1(+)-con plasmid under normoxia (N) or hypoxia for 6 h (H). After 48 h, TfR1 and DMT1 expression was measured by Western blotting. E,F: The bands were quantified by densitometry and normalized as described in Materials and Methods. β-Actin was used as a control. The results are expressed as the means ± SD (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to pcDNA3.1(+)-con under normoxia. $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ compared to pcDNA3.1(+)-IRP1 under normoxia. $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ compared to pcDNA3.1(+)-con under hypoxia.

increase of TfR1 and DMT1 (+IRE) mRNA stability versus the control under normoxia (Fig. 4 A, B, E, and F). As the overexpression of IRP1 enhanced the stability of TfR1 and DMT1 (+IRE) mRNA, it appeared to induce TfR1 and DMT1 expression in normoxic cells (Fig. 3D). However, the mRNA stability was not notably altered when IRP1 was overexpressed under hypoxia, suggesting that the capacity of IRP1 to stabilize the TfR1 and DMT1 mRNAs was restricted under hypoxia (Fig. 4C, D, G, and H).

HYPOXIA-INDUCED IRON UPTAKE PROTEINS EXPRESSION WAS ENHANCED BY IRP1 KNOCKDOWN

Using IRP1 interference, we further assessed the effect of IRP1 on TfR1 and DMT1 (+IRE) expression under hypoxia. Both the protein and mRNA expression of IRP1 were reduced after the cells were transfected with the IRP1 interference sequence (Fig. 5A–C), and exposure to hypoxia for 6 h increased the expression of TfR1 and DMT1 (+IRE) in the control and IRP1-knockdown cells. However, the expression of TfR1 and DMT1 (+IRE) in the IRP1-knockdown cells was higher than that of the control under hypoxia, suggesting that IRP1 knockdown facilitated hypoxia-induced TfR1 and DMT1 (+IRE) expression (Fig. 5D–F). Similarly, TfR1 and DMT1 mRNA levels in the IRP1-knockdown cells were also higher than that of the control under hypoxia (supplemental Fig. S1).

In addition, we assessed the function of the IRE in iron uptake proteins by measuring IRE-dependent luciferase reporter activity. The luciferase activity decreased under hypoxia in the siRNA-con cells; however, this decrease was prevented by treatment with siRNA-IRP1 (Fig. 5G–H). These results explain why the expression of iron-uptake proteins further increased when IRP1 was knocked down and indirectly suggest that the suppression of iron uptake protein expression by IRP1 was partially due to the inhibition of IRE function under hypoxia.

IRP1 RESTRICTED HYPOXIA-INDUCED FERROUS IRON UPTAKE AND REDUCED THE LABILE IRON POOL AS WELL AS THE TOTAL CELLULAR IRON CONTENT

To explore the effect of IRP1 on cellular iron homeostasis under hypoxia, we first measured ferrous iron uptake when IRP1 was overexpressed or knocked down. Without the addition of FAS, the fluorescence remained steady during the 30-min recording period, whereas the fluorescence dramatically decreased after FAS was added, indicating that a large amount of ferrous iron was taken up by the cells (Fig. 6A and C). Exposure to hypoxia for 6 h significantly increased ferrous iron uptake, which was inhibited when IRP1 was overexpressed (Fig. 6B); however, the increase in ferrous iron uptake caused by hypoxia was further enhanced when IRP1 was knocked down (Fig. 6D). Overall, these data revealed that ferrous uptake in HepG2 cells was restricted by IRP1 under hypoxia.

We then determined the levels of the labile iron pool (LIP), which represents the amount of free iron in cells. The fluorescence was

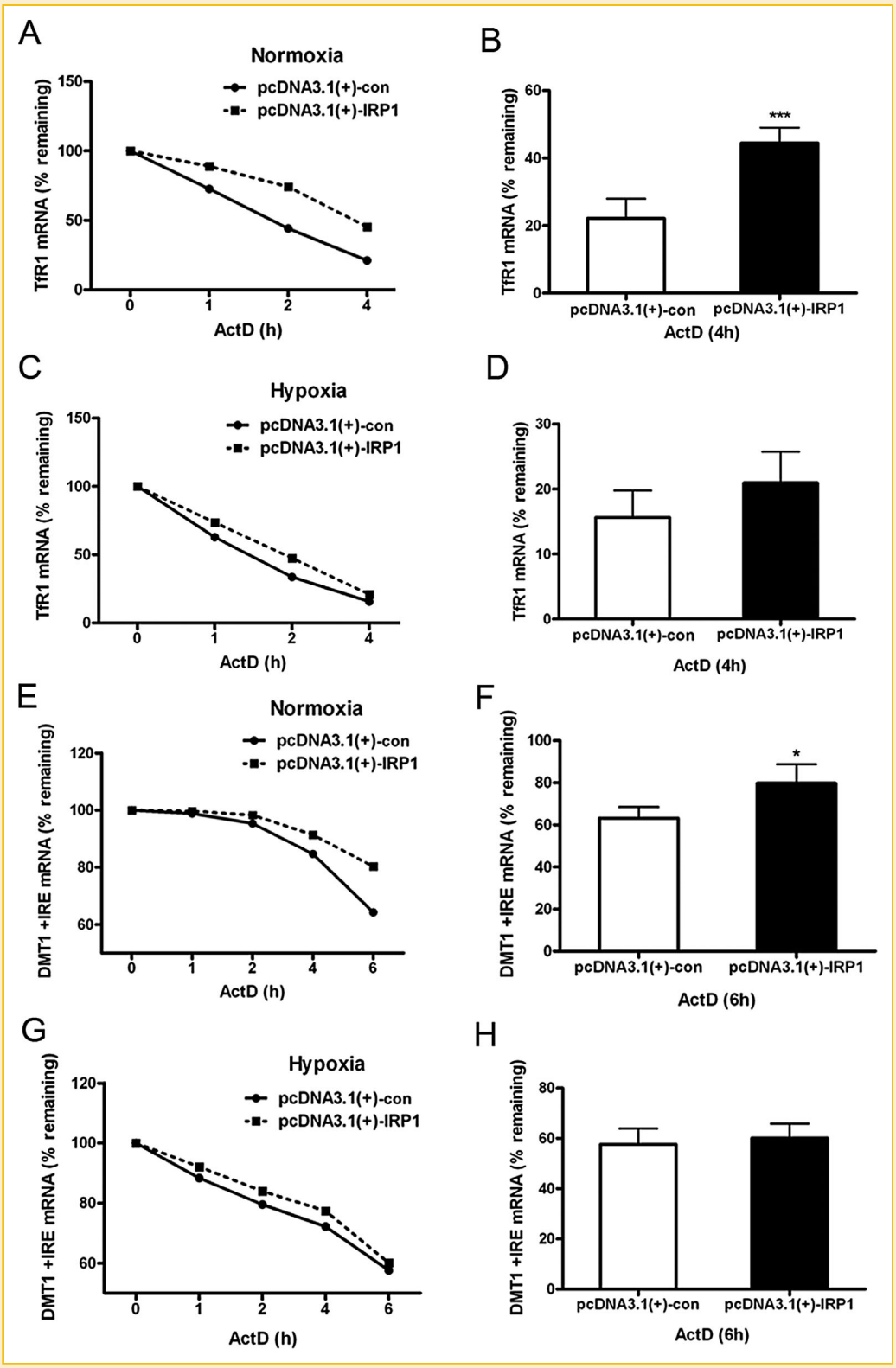

Fig. 4. The mRNA stability of TfR1 and DMT1 (+IRE) under normoxia or hypoxia. HepG2 cells were transfected with the IRP1 expression plasmid (pcDNA3.1(+)-IRP1) under normoxia (42 h) or hypoxia (36 h of normoxia + 6 h of hypoxia), and actinomycin D (5 mg/ml) was subsequently added to the cells for 0–6 h. Total RNA was isolated, and qRT-PCR was performed with specific primers for human TfR1, DMT1, and β-actin. A,C: The stability of TfR1 mRNA was assessed under normoxia or hypoxia. B,D: The mRNA stability of TfR1 was normalized at the 4-h time point. E,F: The mRNA stability of DMT1 (+IRE) was assessed under normoxia or hypoxia. G,H: The mRNA stability of DMT1 (+IRE) was normalized at the 6-h time point. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to the cells transfected with pcDNA3.1(+)-con.

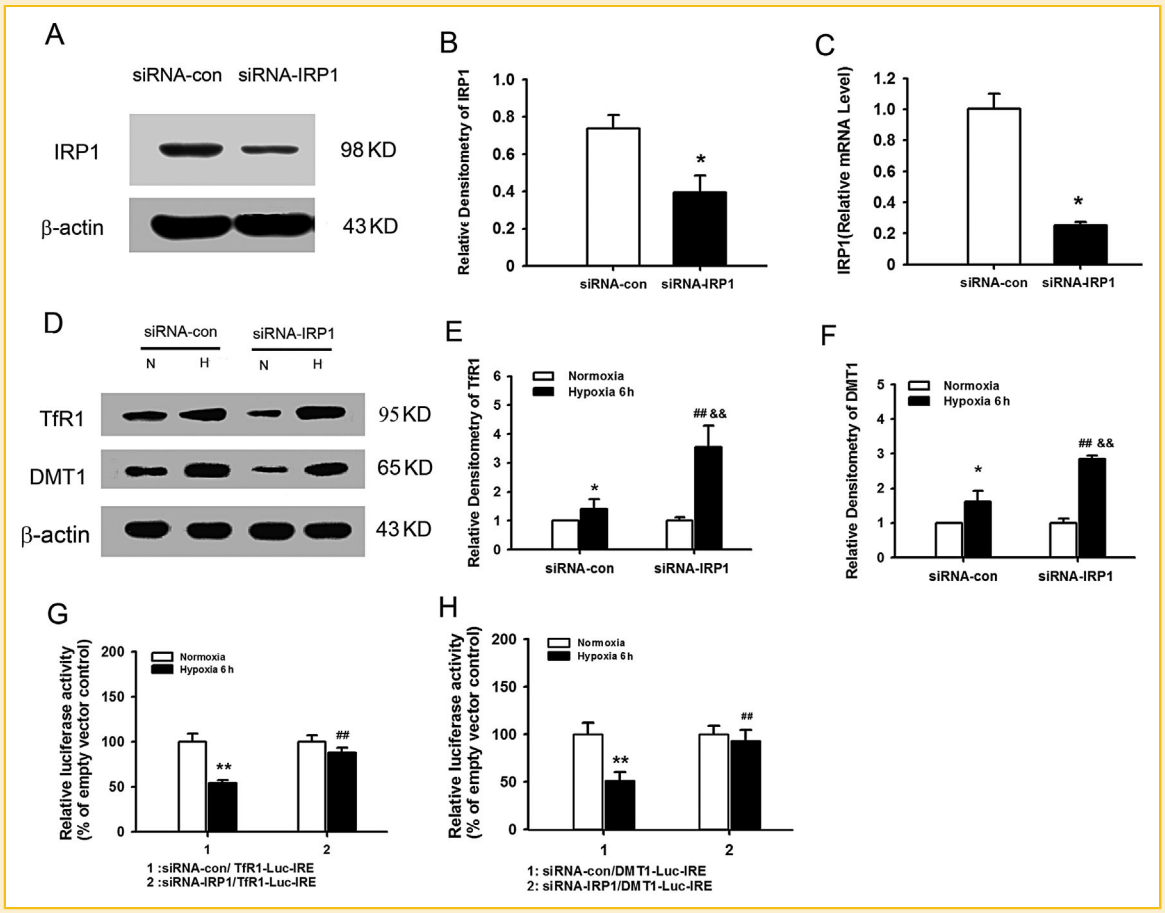

Fig. 5. Hypoxia-induced iron uptake proteins expression was enhanced by IRP1 knockdown. A–C: HepG2 cells were transfected with the IRP1 interference fragments under normoxia. After 48 h, the protein (A and B) and mRNA (C) expression of IRP1 was measured by Western blotting and real-time PCR. D: HepG2 cells were transfected with the IRP1 interference sequence (siRNA-IRP1) or control sequence (siRNA-con) under normoxia or hypoxia for 6 h. After 48 h, the expression of TfR1 and DMT1 was measured by Western blotting. E,F: The bands were quantified by densitometry and normalized as described in Materials and Methods. β-Actin was used as a control. G,H: TfR1 and DMT1 luciferase plasmids were co-transfected into HepG2 cells with the IRP1 interference sequence under normoxia or hypoxia, and the luciferase activity was determined by a dual luciferase assay; the luciferase activity of cells under normoxia was defined as 100. The results are expressed as the means ± SD (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to siRNA-con under normoxia. $^{\&\&}P < 0.01$ compared to siRNA-con under hypoxia. $^{\&\&}P < 0.01$ compared to siRNA-IRP under normoxia.

dramatically increased after deferiprone was added, which indicated that the levels of LIP increased (Fig. 7A and C). Exposure to hypoxia for 6 h caused an increase in LIP, which was inhibited when IRP1 was overexpressed (Fig. 7B); in contrast, the levels of LIP further increased under hypoxia when IRP1 was knocked down (Fig. 7D). These results showed that IRP1 decreased the cellular LIP under hypoxia.

Lastly, a similar effect of IRP1 on the total cellular iron content was observed under hypoxia; the overexpression of IRP1 decreased the total iron content under hypoxia (Fig. 7E), whereas IRP1 knockdown further increased the total iron content (Fig. 7F).

Taken together, these results indicate that IRP1 not only suppressed the expression of iron uptake proteins but also restricted the increases in the cellular iron levels, thereby maintaining cellular iron homeostasis under hypoxia.

IRP1 AMELIORATED HYPOXIA INDUCED CELL INJURY

As IRP1 inhibited iron uptake proteins expression and restricted the cellular iron level in response to hypoxia, we speculated that IRP1 might protect cells against hypoxic stimuli. IRP1 knockdown significantly increased LDH release under hypoxia for 6–10 h, suggesting that IRP1 reduced hypoxia-induced cell injury (Fig. 8A). Conversely, the overexpression of IRP1 did not affect LDH release under hypoxia(Fig. 8B).

DISCUSSION

Hypoxia is a severe cellular stress and an important component of several physiological and pathophysiological processes, including the development of tumors and ischemia [Wong et al., 2012]. It has been proposed that hypoxia can regulate iron uptake, storage, and utilization via the HIF-1/HRE regulatory system [Li et al., 2011]. Iron is indispensable for the function of many proteins, whereas excessive free iron is an intrinsic producer of ROS when combined with oxygen. Because ROS, including the superoxide radical and hydroxyl free radical, oxidize and damage cellular proteins, nucleic

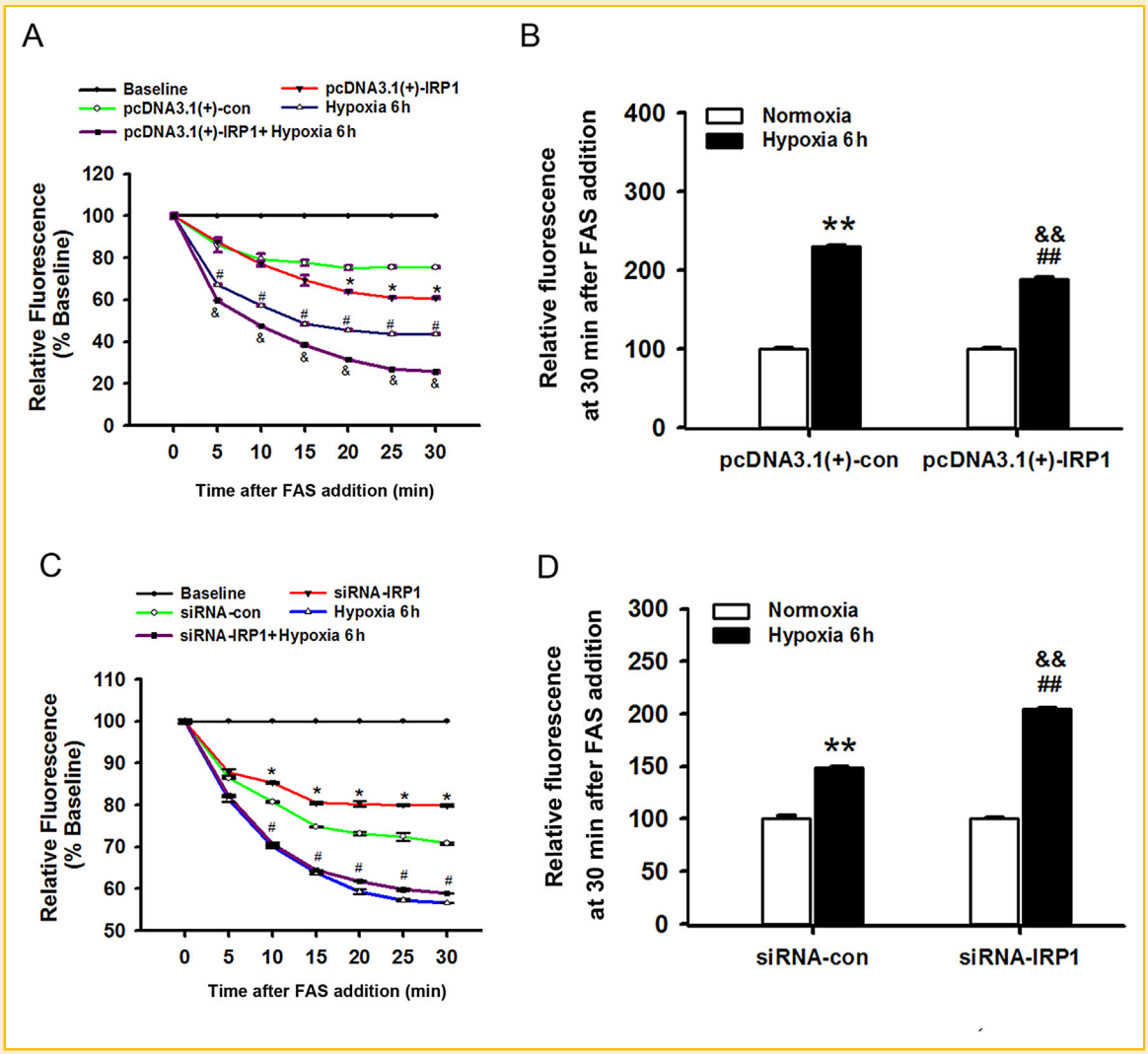

Fig. 6. IRP1 restricted the ferrous iron uptake induced by hypoxia. HepG2 cells were transfected with the IRP1 expression plasmid (pcDNA3.1(+)-IRP1) or the IRP1 interference fragments (siRNA-IRP1) under normoxia or hypoxia. After 48 h, the fluorescence was evaluated as described in Materials and Methods. A decrease in the fluorescence between the baseline and the other groups indicates that a large amount of ferrous iron was taken up by the cells. A,C: Relative fluorescence of the cells transfected with pcDNA3.1 (+)-IRP1 or siRNA-IRP1. B,D: The relative fluorescence at 30 min after FAS was added to the pcDNA3.1(+)-IRP1 or siRNA-IRP1 cells and was normalized as described in Materials and Methods. The results are expressed as the means ± SD (n = 3). $^{**}P < 0.01$ compared to the control under normoxia. $^{\#\#}P < 0.01$ compared to pcDNA3.1(+)-IRP1 or siRNA-IRP1 under normoxia. $^{\&\&}P < 0.01$ compared to the control under hypoxia.

acids, and lipids [Rouault and Klausner, 1996; Altamura and Muckenthaler, 2009], mammalian cells have evolved complex mechanisms to control the concentrations of cellular iron. IRP1 and IRP2 are two key mediators of cellular iron homeostasis that control iron transport and storage [Garrick and Garrick, 2009]. These IRPs respond to both iron-dependent and iron-independent signals, such as hypoxia, H_2O_2, and NO [Pantopoulos, 2003]. However, little is known about the function of IRPs in cellular iron metabolism under hypoxia. This study is the first to reveal that IRP1 suppresses hypoxia-induced TfR1 and DMT1 expression to affect cellular iron metabolism.

First, we measured the expression of IRP1, TfR1, and DMT1 (+IRE) for 2, 4, 6, and 8 h under hypoxia and found that hypoxia increased the expression of TfR1 and DMT1 (+IRE) but progressively reduced IRP1. The results suggest that these genes are regulated by the HIF-1/HRE system under hypoxia, in agreement with the results of previous research [Lok and Ponka, 1999; Wang et al., 2010; Luo et al., 2011]. It is well known that TfR1 and DMT1 (+IRE) are post-transcriptionally regulated by the binding of IRP1 to the 3′-UTR IRE, we detected the binding of IRP1 to IRE for 2–16 h of hypoxia. We found that although IRP1 expression decreased with the time of hypoxia, the binding of IRP1 to the IRE of target mRNAs maintained, suggesting the role of IRP1 on TfR1 and DMT1 expression under hypoxia. In previous studies, hypoxia was showed to enhance IRE/IRP-1 binding in Hep3B cell lines [Toth et al., 1999] or inhibit the RNA-binding activity of IRP1 by protecting its 4Fe-4S cluster and maintaining the protein in the cytosolic aconitase form [Meyron-Holtz et al., 2004]. In the present study, we found that the elevated expression of TfR1 and DMT1 (+IRE)

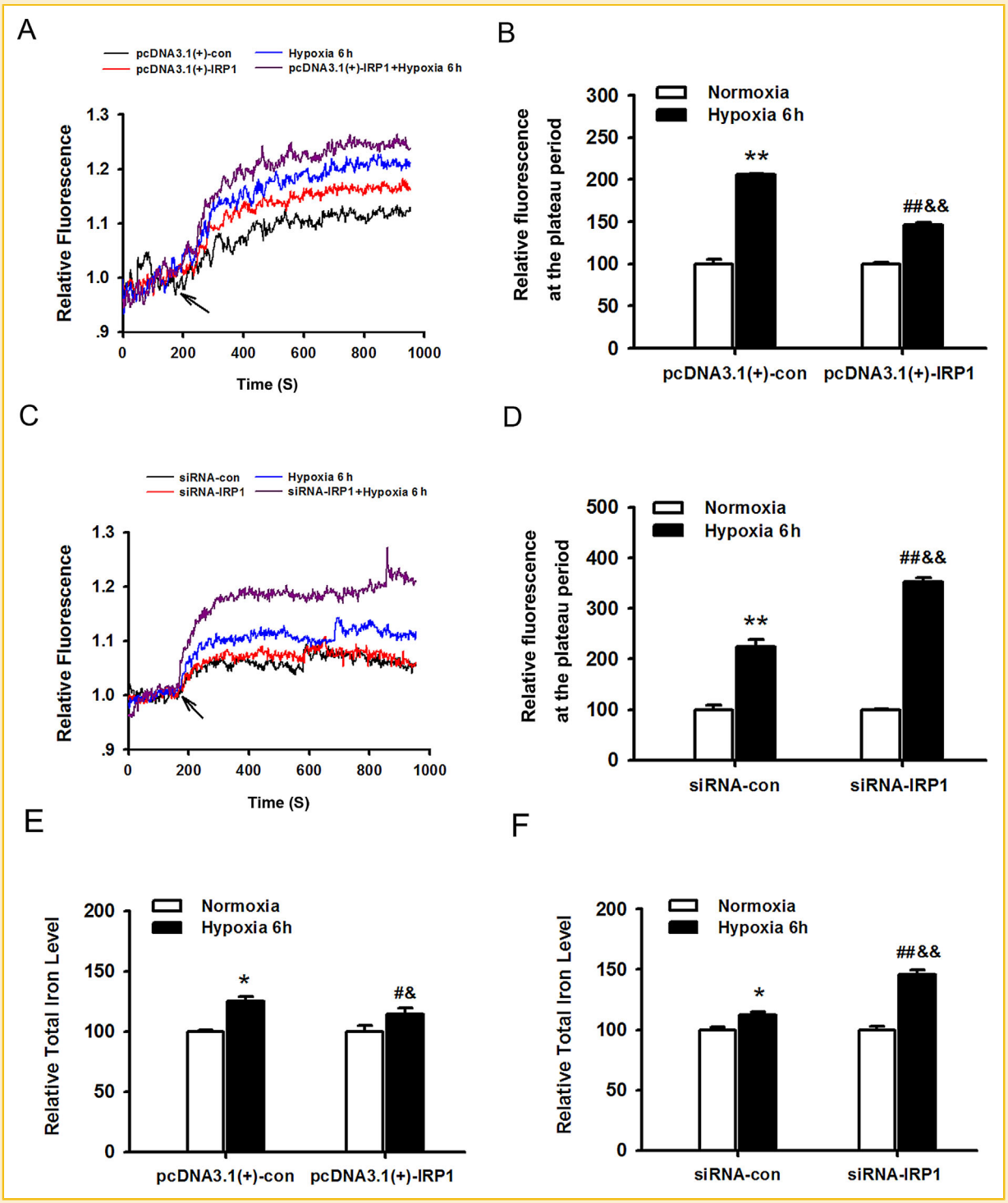

Fig. 7. IRP1 reduced the labile iron pool and total cellular iron induced by hypoxia. HepG2 cells were transfected with the IRP1 expression plasmid (pcDNA3.1(+)-IRP1) or IRP1 interference sequence (siRNA-IRP1) under normoxia or hypoxia. After 48 h, the relative fluorescence value was evaluated as described in Materials and Methods. An increase in the fluorescence indicates an increase in the LIP. A,C: Relative fluorescence of the cells transfected with pcDNA3.1(+)-IRP1 or siRNA-IRP1. B,D: The increase in fluorescence during the plateau period was assessed and normalized as described in Materials and Methods after the addition of deferiprone at 200 s (arrow). E: The change in total cellular iron when IRP1 was overexpressed. F: The change in total cellular iron when IRP1 was knocked down. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the control under normoxia. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to pcDNA3.1(+)-IRP1 or siRNA-IRP1 under normoxia. $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ compared to control under hypoxia.

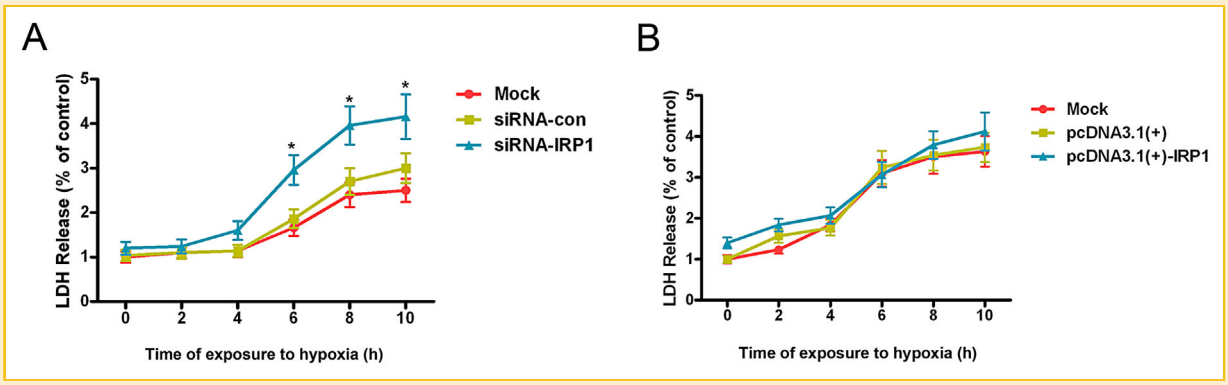

Fig. 8. IRP1 ameliorated hypoxia-induced cell injury. HepG2 cells were transfected with an IRP1 expression plasmid or interference fragments under normoxia. The cells were harvested after exposure to hypoxia from 0 to 10 h. The release of LDH was measured and normalized in the treated cells. $^{*}P < 0.05$ compared to siRNA-con.

induced by hypoxia was significantly inhibited when IRP1 was overexpressed under hypoxia yet further increased when IRP1 was knocked down. These results indicate that IRP1 inhibited the expression of iron uptake proteins under hypoxic conditions. To elucidate the mechanism by which IRP1 suppresses iron uptake proteins under hypoxia, we investigated the stability of TfR1 and DMT1 (+IRE) mRNAs and examined IRE function. The results showed that the ability of IRP1 to stabilize the DMT1 and TfR1 mRNAs was limited under hypoxia, which suggested that the negative regulation of IRP1 on the expression of TfR1 and DMT1 (+IRE) under hypoxia is differed from what was observed under normoxia. A dual luciferase assay revealed that the IRE-dependent reduction in luciferase activity was rescued when IRP1 was knocked down under hypoxia, suggesting that IRP1 inhibits the function of TfR1 and DMT1 (+IRE) IREs under hypoxia. Because exogenous IRP1 only containing the coding sequence (CDS) would not be regulated by hypoxia, we did not perform the luciferase assay in the pcDNA3.1(+)-IRP1 cells [Cheng et al., 2012].

Although we showed that IRP1 suppresses the expression of iron uptake proteins (TfR1 and DMT1+IRE) by reducing mRNA stability and inhibiting IRE function under hypoxic conditions, the effect of IRP1 on cellular iron homeostasis under hypoxia remains unclear. Therefore, we assessed the ferrous iron influx, and we found that the observed increase in ferrous influx was consistent with TfR1 and DMT1 (+IRE) expression upon exposure to hypoxia. However, increases in iron uptake might damage cells via increased ROS [Wang et al., 2010]. LIP is defined as the pool of accessible iron ions and has been reported to be the main determinant of the cellular response to oxidative stress [Greenberg, 1964; Kruszewski, 2003]. Moreover, an increase in ferrous uptake might also affect the total cellular iron content [Yang et al., 2012]. Accordingly, IRP1 inhibited the increases in both LIP and the total iron content induced by hypoxia, suggesting that IPR1 regulates cellular iron homeostasis under hypoxia in a manner that differs from that under normoxia. By overexpressing IRP1 and knocking down its expression, we were able to explore the effects of IRP1 under hypoxia. We found that ferrous iron uptake, LIP, and total cellular iron content increased under hypoxia for 6 h; however, all of these increases were partially reversed by IRP1. Our findings provide novel insight into the role of IRP1 in cellular iron homeostasis via the suppression of TfR1 and DMT1 (+IRE) under hypoxia. Adaptation to hypoxia is an essential cellular response controlled by the oxygen-sensitive master transcription factor HIF-1: after exposure to hypoxia, HIF-1 activates the expression of downstream genes, including TfR1 and DMT1, enhancing iron uptake in response to hypoxia [Du et al., 2012; Yang et al., 2012]. However, excessive ferrous iron influx not only oxidizes and damages cellular proteins, nucleic acids, and lipids by generating highly reactive hydroxyl radicals [Jomova and Volko, 2011], but also influences the total cellular iron content [Yang et al., 2012]. IRP1 might play a critical role in the suppression of increased cellular iron levels caused by hypoxia.

We also observed that LDH release from cultured HepG2 cells rapidly increased after exposure to hypoxia for more than 6 h, which indicated that the HepG2 cells responded to hypoxia in a compensatory manner and were not markedly damaged within 6 h. Therefore, the suppressive effect of IRP1 on iron uptake proteins and the cellular iron level under hypoxia was a compensatory response. It can be speculated that IRP1 benefits cells by reducing injury upon exposure to hypoxia. Our results showed that the knockdown of IRP1 significantly increased LDH release under hypoxia for 6–10 h, suggesting that IRP1 ameliorated hypoxia-induced cell injury. However, the expression of IRP1 itself decreased with the time of hypoxia exposure (0–8 h), and the effect of IRP1 on cellular iron homeostasis were not very strong.

It has been reported that the effect of hypoxia on iron uptake due to the RNA-binding activity of IRP is a biphasic response [Schneider and Leibold, 2003]. In the present study, the expression of iron uptake proteins and the cellular iron level increased progressively under hypoxia within 8 h, whereas the expression of IRP1 decreased. We observed that IRP1 inhibited iron uptake proteins expression and decreased the cellular iron level when HepG2 cells were exposed to hypoxia for 6 h. Further experiments are needed to determine the effect of IRP1 on iron homeostasis under long-term exposure to hypoxia. In fact, the total cellular iron content increased with the time of hypoxia, reaching a maximum at 8 h, and was maintained at a higher lever than the control, suggesting that the influence of IRP1 on iron homeostasis is complicated and that other factors are likely to be involved.

As noted above, IRP1 alleviated the effect of hypoxia by preventing excess accumulation of cellular iron through the inhibition of hypoxia-induced TfR1 and DMT1 (+IRE) expression. IRP2, the homolog of IRP1, is reported to have a key role in the maintenance of iron homeostasis [Moroishi et al., 2011]. The IRE binding activities of the two IRPs are regulated by distinct mechanisms. In addition to being regulated by bioavailable iron levels, IRP2 is regulated in an O_2-dependent manner. IRP2 has a half-life of ~6 hr in normoxia which increases to greater than 12 hr in hypoxic cells [Hanson et al., 2003]. Thus, the contribution of IRP2 on iron homeostasis under hypoxia should be further analyzed.

HIF2 is a close homolog of HIF1 and plays an important role in the expression of iron uptake genes in response to hypoxic stimulation [Yeh et al., 2011]. Interestingly, a conserved, functional IRE has been identified within the 5′-UTR of the HIF-2α mRNA [Sanchez et al., 2007], increasing the complexity of the effect of hypoxia on iron metabolism. It has been recently reported that IRP1 regulates HIF-2α synthesis, which is critical for coordinating the oxygen- and iron-dependent regulation of cell differentiation and iron metabolism [Anderson et al., 2013]. Furthermore, a novel in vivo function of IRP1 as oxygen/iron sensor and regulator of HIF2α-dependent pathophysiological responses has been described [Wilkinson and Pantopoulos, 2013]. Taken together, these findings emphasize the importance of IRP1 in iron metabolism under hypoxia.

In summary, with the aid of overexpression and knockdown assays, we disclosed that the regulate model of IRP1 on TfR1 and DMT1 expression under hypoxia was different from that in normoxia; IRP1 suppressed the hypoxia-induced expression of iron uptake proteins by reducing the stability of the TfR1 and DMT1 mRNAs and inhibiting IRE function under hypoxia in HepG2 cells. Moreover, IRP1 limited the intracellular iron level and decreased LDH release to prevent the potential toxicity of iron ions under hypoxia.

ACKNOWLEDGMENTS

This study was financially supported by the National Natural Science Foundation of China (grant nos. 31171143 and 31471141), 973 Program (2011CB510004), and the Priority Academic Program Development of Jiangsu Higher Education Institution (PAPD).

REFERENCES

Altamura S,Muckenthaler U. 2009. Iron toxicity in diseases of aging: Alzheimer′s disease, Parkinson′s disease and atherosclerosis. J Alzheimers Dis 16:879–895.

Anderson SA, Nizzi CP, Chang YI, Deck KM, Schmidt PJ, Galy B, Damnernsawad A, Broman AT, Kendziorski C, Hentze MZ, Fleming MD, Zhang J, Eisenstein RS. 2013. The IRP1-HIF-2alpha axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption. Cell Metab . 17: 282–290.

Casey JL, Koeller DM, Ramin VC, Klausner RD, Harford JB. 1989. Levels requires iron-responsive elements and a rapid turnover determinant in the 3′ untranslated region of the mRNA. EMBO J 8:3693–3699.

Cheng CM, Wang D, Zhu L. 2012. Construction and identification of eukaryotic expression plasmid of human Iron regulation protein 1 and function in HepG2 cells. Med J Commun 26,103–107.

Doe MR, Ascano JM, Kaur M, Cole MD. 2012. Myc posttranscriptionally induces HIF1 protein and target gene expression in normal and cancer cells. Cancer Res 72:949–957.

Du F, Fan M, Gong Q, Zhu LL, Zhu ZJ, Lu L, Ke Y. 2012. Effects of hypoxic preconditioning on the expression of iron influx and efflux proteins in primary neuron culture. Neurochem Int 60:335–343.

Eckard J, Dai J, Wu J, Jian J, Yang Q, Chen H, Costa M, Frenkel K, Huang. X. 2010. Effects of cellular iron deficiency on the formation of vascular endothelial growth factor and angiogenesis. Cancer Cell Int 10:1–11.

Epsztejn S, Kakhlon O, Glixkstein H, Breuer W, Cabantchik H. 1997. Fluorescence analysis of the labile iron pool of mammalian cells. Anal Biochem 248:31–40.

Garrick MD, Garrick LM. 2009. Cellular iron transport. Biochim Biophys Acta 1790:309–325.

Greenberg GR, Wintrobe MM. 1964. A labile iron pool. J Biol Chem 165:397–398.

Gunshin H, Allerson CR, Polycarpou-Schwarz M, Rofts A, Rogers JT, Kishi F, Hentze MW, Rouault TA, Andrews NC, Hediger. MA. 2001. Iron-dependent regulation of the divalent metal ion transporter. FEBS Lett 509:309–316.

Hanson ES, Rawlins ML, Leibold EA. 2003. Oxygen and iron regulation of iron regulatory protein 2. J Biol Chem 278:40337–40342.

Hoepken HH, Korten T, Robinson SR, Dringen. R. 2004. Iron accumulation, iron-mediated toxicity and altered levels of ferritin and transferrin receptor in cultured astrocytes during incubation with ferric ammonium citrate. J Neurochem 88:1194–1202.

Jomova K, Volko M. 2011. Advances in metal-induced oxidative stress and human disease. Toxicology 283:65–87.

Kakhlon O, Cabantchik IZ. 2002. The labile iron pool: Characterization, measurement, and participation in cellular processes(1). Free Radic Biol Med 33:1037–1046.

Kruszewski M. 2003. Labile iron pool: The main determinant of cellular response to oxidative stress. Mut Res 531:81–92.

Li HZ, Liu YQ, Wang HT, Liu SX, Zhao B, Yang J. 2011. Effects of hypoxia on iron metabolism of rat L6 skeletal muscle cells. Acta Physiologica Sinica 63:347–352.

Liu YX, Chen ZP, Wang JK. 2011. Systematic evaluation of biocompatibility of magnetic Fe3O4 nanoparticles with six different mammalian cell lines. J Nanoparticle Res 13:199–212.

Lok CN, Ponka. P. 1999. Identification of a hypoxia response element in the transferrin receptor gene. J Biol Chem 274:24147–24152.

Luo QQ, Wang D, Yu MY, Zhu L. 2011. Effect of hypoxia on the expression of iron regulatory proteins 1 and the mechanisms involved. LUBMB Life 63:120–128.

Malik IA, Naz N, Sheikh N, Khan S, Moriconi F, Blaschke M, Ramadori G. 2011. Comparison of changes in gene expression of transferrin receptor-1 and other iron-regulatory proteins in rat liver and brain during acute-phase response. Cell Tissue Res 344:299–312.

Meyron-Holtz EG, Ghosh MC, Rouault TA. 2004. Mammalian tissue oxygen levels modulate ironregulatory protein activities in vivo. Science 5704: 2087–2090.

Moroishi T, Nishiyama M, Takeda Y, Nakayama KI. 2011. The FBXL5-IRP2 axis is integral to control of iron metabolism in vivo. Cell Metabol 14:339–351.

Pantopoulos K. 2003. Iron regulatory proteins and oxidative stress (Meeting abstract). Proceedings of the meeting of the society for free radical research, European section, 55–60.

Qian ZM, Wu XM, Fan M, Yang L, Du F, Yung WH, Ke Y. 2011. Divalent metal transporter 1 is a hypoxia-inducible gene. J Cell Physiol 226:1596–1603.

Romney SJ, Newman BS, Thacker C, Leibold. EA. 2011. HIF-1 regulates iron homeostasis in caenorhabditis elegans by activation and inhibition of genes involved in iron uptake and storage. PLoS Genet 7:1–12.

Rouault TA, Klausner. DR. 1996. Post-transcriptional regulation of genes of iron metabolis in mammalian cells. J Biol Inorganic Chem 1:494–499.

Sammarco MC, Ditch S, Banerjee A, Grabczy E. 2008. Ferritin L and H subunits are differentially regulated on a post-transcriptional level. J Biol Chem 283:4578–4587.

Sanchez M, Galy B, Muckenthaler MU, Hentze. MW. 2007. Iron-regulatory proteins limit hypoxia-inducible factor-2α expression in iron deficiency. Nat Struct Mol Biol 14:420–426.

Sanchez M, Galy B, Schwanhaeusser B, Blake J, Bähr-Ivacevic T, Benes V, Selbach M, Muckenthaler MU, Hentze MW. 2011. Iron regulatory protein-1 and -2: Transcriptome-wide definition of binding mRNAs and shaping of the cellular proteome by iron regulatory proteins. Blood 118:168–179.

Schneider BD, Leibold EA. 2003. Effects of iron regulatory protein regulation on iron homeostasis during hypoxia. Blood 102:3404–3411.

Semenza GL, Wang GL. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 12: 5447–5454.

Song G, Kim J, Bazer FW, Spenser TE. 2008. Progesterone and interferon tau regulate hypoxia-inducible factors in the endometrium of the ovine uterus. Endocrinology 149:1926–1934.

Styś A, Galy B, Starzyński1 RR, Smuda E, Drapier J-C, Lipiński P, Bouton C. 2011. Iron regulatory protein 1 outcompetes iron regulatory protein 2 in regulating cellular iron homeostasis in response to nitric oxide. J Biol Chem 286:22846–22854.

Toth I, Yuan L, Rogers JT, Boyce H, Bridges KR. 1999. Hypoxia alters iron-regulatory protein-1 binding capacity and modulates cellular iron homeostasis in human hepatoma and erythroleukemia cells. J Biol Chem 7:4467–4473.

Wang Q, Luo W, Zhang W, Liu M, Song H, Chen. J. 2011. Involvement of DMT1+IRE in the transport of lead in an in vitro BBB model. Toxicol In Vitro 25:991–998.

Wang D, Wang L-H, Zhao Y, Lu Y-P, Zhu. L. 2010. Hypoxia regulates the ferrous iron uptake and reactive oxygen species level via divalent metal transporter 1 (DMT1) Exon1B by hypoxia-inducible factor-1. LUMB Life 62:629–636.

Wilkinson N, Pantopoulos K. 2013. IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2α mRNA translation. Blood 9:1658–1668.

Wong CC, Zhang H, Gilkes DM, Chen J, Wei H, Chaturvedi P, Hubbi ME, Semenza. GL. 2012. Inhibitors of hypoxia-inducible factor 1 block breast cancer metastatic niche formation and lung metastasis. J Mol Med 90:803–815.

Yang L, Fan M, Du F, Gong Q, Bi ZG, Zhu ZJ, LLZhu, Ke Y. 2012. Hypoxic preconditioning increases iron transport rate in astrocytes. Biochim Biophys Acta 1822:500–508.

Yeh K-Y, Yeh M, Polk P, Glass J. 2011. Hypoxia-inducible factor-2 alpha and iron absorptive gene expression in Belgrade rat intestine. Am J Physiol Gastrointest Liver Physiol 301:G82–G90.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.