Correlation between the expression of divalent metal transporter 1 and the content of hypoxia-inducible factor-1 in hypoxic HepG2 cells

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Abstract

Transferrin and transferrin receptor are two key proteins of iron metabolism that have been identified to be hypoxia-inducible genes. Divalent metal transporter 1 (DMT1) is also a key transporter of iron under physiological conditions. In addition, in the 5' regulatory region of human DMT1 (between –412 and –570), there are two motifs (CCAAAGTGCTGGG) that are similar to hypoxia-inducible factor-1 (HIF-1) binding sites. It was therefore speculated that DMT1 might also be a hypoxia-inducible gene. We investigated the effects of hypoxia and hypoxia/re-oxygenation on the expression of DMT1 and the content of HIF-1alpha in HepG2 cells. As we expected, a very similar tendency in the responses of the expression of HIF-1α, DMT1+IRE (iron response element) and DMT1–IRE proteins to chemical (CoCl2) or physical hypoxia was observed. A highly significant correlation was found between the expression of DMT1 proteins and the contents of HIF-1α in hypoxic cells. After the cells were exposed to hypoxia and subsequent normoxia, no HIF-1α could be detected and a significant decrease in DMT1+IRE expression (P<0.05), but not in DMT1–IRE protein (versus the hypoxia group), was observed. The findings implied that the HIF-1 pathway might have a role in the regulation of DMT1+IRE expression during hypoxia.

Keywords: hypoxia-inducible gene • human hepatoma HepG2 cells • divalent metal transporter 1 (DMT1) • hypoxia-inducible factor-1 α (HIF-1 α) • chemical and physical hypoxia • hypoxia/re-oxygenation

Introduction

Hypoxia-inducible factor-1 (HIF-1) was identified for the first time for its role in regulating the transcription of the erythropoietin (EPO) gene in 1992 [1]. This transcription factor is now recognized as a master regulator of cellular and systemic oxygen homeostasis. It consists of the HIF-1α and HIF-1β (or ARNT, aryl hydrocarbon nuclear receptor translocator) subunits [2, 3] and is expressed in many cell types [4]. HIF-1α is constitutively expressed and unaffected by hypoxia or normoxia [5, 6]. HIF-1β is continuously synthesized and destroyed in normoxia as a result of its ubiquitination and the subsequent degradation by the proteasomal system after hydroxylation [2, 4].

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Under hypoxic conditions, the degradation of HIF-1α is inhibited, thus allowing the protein to accumulate in cells. HIF-1α then heterodimerizes with HIF-1β [7] and relocates to the nucleus. The heterodimer can then bind to a specific DNA consensus sequence hypoxic-response elements (HRE), which is contained in the promoter regions of the target genes [2, 8, 9], to increase their expression. Genes regulated by HIF-1 broadly include those involved in oxygen homeostasis and glucose-energy metabolism and mediate adaptive physiological responses [4, 10, 11]. To date, nearly 100 genes operated in all cells have been identified as regulated by HIF-1 in response to hypoxia [12, 13].

Iron is a transition metal that is essential for oxygen transport, cell growth and cell survival. It has been known for a long time that transferrin (Tf) and transferrin receptor (TfR) are two key proteins which are involved in iron metabolism under physiological conditions [14]. The Tf-bound iron is the major form of iron transport and iron uptake by mammalian cells and is mainly mediated by TfR. In addition, recent studies have shown that divalent metal transporter 1 (DMT1, previously known as Nramp2 or DCT1) is an essential protein of iron homeostasis [15, 16]. DMT1 exists in at least four distinct isoforms, two of which are transcribed from alternative promoters encoding that differ in N-terminus (termed exon 1A and exon 2). Transcripts of both exon 1A and exon 2 can exist in two splice variants differing in the 3’ end which results in different C-terminal sequences [17, 18]. One of the two variants of the C-form possesses an IRE in the 3’ untranslated region of the message (termed +IRE) while the other does not (termed, –IRE). It has been well documented that DMT1 plays a physiological role in iron transport into the enterocyte [15, 16]. Also, this protein is required not only for the accumulation of non-Tf-bound iron (Fe^{2+}) by mammalian cells but also for the iron transport across endosome, and then its entrance into cytosol after the release of iron from Tf in the endosome [19].

Previous studies have demonstrated that hypoxia can increase iron uptake by cells as well as the expression of Tf and TfR. Both Tf and TfR have been identified to be hypoxia-inducible genes [20, 21]. Like Tf and TfR, DMT1 is also a key transporter of iron under physiological conditions. In addition, it has been reported that there are two motifs (CCAAAGT-GCTGGG) that are similar to HIF-1 binding sites in the 5' regulatory region of human DMT1 (between –412 and –570) [17]. Therefore, it is reasonable to speculate that DMT1 might also be a hypoxia-inducible gene. In a recent study, Lis et al. [22] has demonstrated that hypoxia selectively increases expression of exon 1A, which contains species of DMT1, with lesser increases in either the +IRE or –IRE isoforms of this transporter in rat pheochromocytoma (PC12) cells. This provides important evidence for the hypothesis. However, more investigations are needed in order to reach a final conclusion on whether DMT1 is a hypoxia-inducible gene. In the present study, we therefore investigated the effects of chemical (CoCl₂) and physical (hypoxia chambers) hypoxia, as well as hypoxia/ re-oxygenation on the expression of DMT1 (+IRE and –IRE) and the content of HIF-1α in the human hepatoma HepG2 cells. Correlation analysis between the expression of DMT1 proteins and the content of HIF-1α was also conducted.

Materials and methods

Reagents

Cobalt chloride (CoCl₂), Poly-L-Lysine and anti-β-actin antibody were bought from Sigma Chemical Company, St. Louis, MO, USA. Anti-HIF-1α polyclonal antibody was purchased from Santa Cruz Biotechnology, CA, USA and anti-DMT1 antibodies (with and without IRE) were from ADI, San Antonio, TX, USA. The specificity of the anti-DMT1 antibodies was confirmed by a peptide competition test. The peptide was purchased from ADI. Secondary antibodies (HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG) were from Pierce, Rockford, IL, USA. Goat anti-rabbit conjugated to Alexa fluor 568 was purchased from Molecular Probes, Eugene, OR, USA.

Cell culture and treatment of hypoxia or hypoxia/re-oxygenation

Human hepatoma HepG2 cells were used in this study because they had been widely used to study the response of the liver gene expression to hypoxic stress [35]. HepG2 cells (HB 8065; American Type Culture Collection, Rockville, Maryland, USA) were cultured in a poly-L-Lysine coated 50 cm² flask (Corning, USA) in RPMI 1640 medium (Gibco, Grand Is., NY, USA) supplemented with 10% foetal...
bovine serum, 100 units/ml penicillin and 100 (mg/ml streptomycin in a humidified 95% air 5% CO₂ incubator (NAPCO 5400) at 37°C. The cells were suspended and forced ten times through a needle to form single-cell suspension. After the HepG2 cells had been grown to 80% confluence in the flask, the cells were treated by CoCl₂ (0, 0.05, 0.125, 0.5 or 1 mM) for 4 hrs, or exposed to hypoxia (1% O₂) for 0 (normoxia), 1, 3, 6, 12 or 24 hrs in an incubator (NAPCO 7101FC-1) humidified with 1%O₂, 94% N₂ and 5% CO₂ at 37°C. The cells were still viable after 24 hrs at 0.1% O₂ (98.3% viability). To investigate the effect of hypoxia/ re-oxygenation on the expression of DMT1 in HepG2 cells, the cells were exposed to hypoxia (1% O₂) for 6 hrs, then exposed to normoxia (21% O₂) for 24 hrs.

Western blot

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in a 100–200 µl cold lysis buffer [10 mmol/l Tris-HCl with pH 7.6, 1.5 mmol/l MgCl₂, 10 mmol/L KCl, 2 mmol/l DL-dithiothreitol (DTT), 0.4 mmol/l phenylmethylsulfonyl fluoride (PMSF)] (Sigma Chemical Company, St. Louis, MO, USA), 2 mg/L Aprotinin (Roche Diagnostica GmbH Mannheim, Germany), 2mg/l Leupeptin (Boehringer Mannheim GmbH, Germany), and 2 mg/l Pepstatin (Boehringer Mannheim Corp. USA). Lysates were kept in ice for 30 min, sonicated for 10 sec using Soniprep JY92-2D (Ningbo, China). After centrifugation at 12,500 × g for 10 min at 4°C, the supernatant was collected and the protein content was determined using a modified Lowry method. Equivalent amounts of protein (30 µg) were loaded in each well and separated by 10% SDS-polyacrylamide gels, then transferred to the polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) previously blocked with 5% non-fat milk in TBS-T (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20), and incubated overnight at 4°C with the indicated primary antibodies (DMT1+IRE, 1:1000; DMT1-IRE, 1:1000; HIF-1α, 1:200; β-actin; 1:10000), and then incubated with the secondary antibodies for 2 hrs at room temperature [38–40]. After washing, the complexes were visualized by the enhanced chemiluminescence (Pierce, Rockford, IL, USA) and exposed to X-ray film (Kodak, USA). The intensity of each band was scanned and quantified with the Shine-tech Image System (Shanghai, China).

Immunocytochemical experiment

HepG2 cells were seeded in 24-well plates pre-coated with Poly-L-Lysine at 1x10⁴ cells per well. Two days later, the cells were subsequently fixed with 100% methanol for 7 min at −20°C, permeabilized with fresh 4% paraformaldehyde for 20 min at room temperature, blocked with blocking buffer (10% goat serum in PBS including 0.3% Triton X-100 and 0.03% NaNO₃) for 2 hrs at room temperature, and incubated overnight at 4°C with the primary antibody diluted (DMT1+IRE, 1:1000; DMT1-IRE, 1:1000; HIF-1α, 1:200) in the blocking buffer. The cells were washed with PBS and incubated for 2 hrs with the secondary antibody (goat anti-rabbit conjugated to Alexa fluor 568) diluted (1:1000) in the blocking buffer at room temperature, and were then viewed under a confocal laser microscope (Leica, Germany).

Statistical analysis

Data are expressed as the means ± standard error of mean (SEM). All statistical evaluations were performed using a two-tailed Student’s t-test and the data were analyzed using the computer program SPSS (Version 11.0). A probability level of P<0.05 was considered to be significant.

Results

Effects of CoCl₂ on the expression of DMT1 proteins and the content of HIF-1α

CoCl₂ have been shown to mimic the hypoxic state in several hypoxia-inducible genes [23]. So we first investigated the effect of CoCl₂ on the expression of DMT1 and the content of HIF-1α. As we expected, a very similar tendency in the responses of the expression of DMT1+IRE and DMT1-IRE proteins and the level of HIF-1α to the CoCl₂ treatment was observed. The content of HIF-1α protein increased with the increase in the concentration of CoCl₂, so did the expression of the two isoforms of DMT1 proteins (Fig. 1). The expression of the two isoforms of DMT1 proteins reached the peak when the cells were treated with 0.5 mM CoCl₂ for 4 hrs, while the HIF-1α showed the highest level when they were treated with 1 mM CoCl₂ for 4 hrs. Although the expression of the two isoforms of DMT1 proteins was slightly lower in the cells treated with 1 mM than that with 0.5 mM CoCl₂ for 4 hrs, it was still significantly higher than that of the control. Also, no difference in the expression of the two isoforms of DMT1 proteins was found between the cells treated with 1 mM and those with 0.5 mM CoCl₂ for 4 hrs.
Effects of hypoxia on the expression of DMT1 proteins and the content of HIF-1α

We then examined the effects of physical hypoxia (1% O₂, 5% CO₂, 94% N₂) on the expression of DMT1 proteins and the content of HIF-1α. After the HepG2 cells were exposed to hypoxia for 0, 1, 3, 6, 12 or 24 hrs, western blot was performed to detect the expression of DMT1 proteins and the level of HIF-1α. The results demonstrated that both the level of HIF-1α and the expression of the two isoforms of DMT1 proteins were significantly affected by the hypoxia treatment (Fig. 2). The responses of the HIF-1α content to hypoxia were very similar to that of the two isoforms of DMT1 expression. All three proteins increased with the time of hypoxia, reached the highest at 3 hrs (HIF-1α) or 6 hrs (DMT1+IRE and DMT1-IRE), and then decreased gradually with the time of hypoxia (Fig. 2).

Correlation analysis

The relationship between the expression of DMT1 proteins and the content of HIF-1α in the HepG2 cells treated with chemical (different concentrations of CoCl₂) and physical hypoxia (different durations) was determined by plotting the values for these two indicators against one another (Table 1). Highly significant correlations were found in the cells treated with chemical hypoxia (DMT1+IRE: R² = 0.6266, P<0.01; DMT1-IRE: R² = 0.8621, P<0.001) as well as physical hypoxia (0–6 hrs period: DMT1+IRE, R² = 0.764, P<0.005; DMT1-IRE, R² = 0.6978, P<0.05, and 3-24 hrs period: DMT1+IRE: R² = 0.7056, P<0.05).

Fig. 1 Expression of DMT1+IRE and DMT1–IRE proteins and the content of HIF-1α in HepG2 cells treated with or without CoCl₂. HepG2 cells were cultured as described in Material and methods. After being grown to 80% confluence in a flask, the cells were treated with CoCl₂ (0, 0.05, 0.125, 0.5 or 1 mM) for 4 hrs, and then Western blot analysis was performed to determine the levels of HIF-1α (A, B), DMT1+IRE (C, D) and DMT1–IRE (E, F) in HepG2 cells. (A, C and E): A representative experiment of Western blot of HIF-1α (A), DMT1+IRE (C) and DMT1–IRE (E); (B, D and F): Quantification of expression of HIF-1α (B), DMT1+IRE (D) and DMT1–IRE (F) protein in HepG2 cells. Expression values were normalized for β-actin and the data were presented as mean ± SEM (n = 3). *P<0.05, **P<0.01 versus the control (0 mM CoCl₂).
Effects of Hypoxia/re-oxygenation on the expression of DMT1 proteins and the content of HIF-1α

We also investigated the effect of hypoxia/re-oxygenation on the expression of DMT1 proteins and the content of HIF-1α in the HepG2 cells. We exposed the HepG2 cells to hypoxia (1% O₂, 5% CO₂, 94% N₂) for 6 hrs, then exposed them to normoxia (21% O₂) for 24 hrs. Our data showed that no HIF-1α could be detected after the HepG2 cells were treated with hypoxia/re-oxygenation (Fig. 3). The treatment of hypoxia/re-oxygenation induced a significant decrease in the expression of DMT1+IRE (P<0.05 versus hypoxia group). However, no significant difference was found in the expression of DMT1–IRE protein between the hypoxia/ re-oxygenation and the hypoxia groups (P>0.05).

Effect of hypoxia on the distribution of HIF-1α and DMT1 proteins

Our data showed that hypoxia could significantly affect the expression of the two isoforms of DMT1 proteins. However, it was unknown whether hypoxia could affect the distribution of the DMT1 in the HepG2 cells. Therefore, we also investigated the effects of hypoxia on DMT1 distribution in cells using immunofluorescent staining. The data (Fig. 4) revealed that the HepG2 cells were spindle-shaped. There was almost no expression of HIF-1a. The two isoforms of DMT1 were located in both the nucleus and the cytoplasm as found in the astrocyte and the astrocytomas [24] under normoxia. After being exposed to hypoxia for 6 hr, the shape of the HepG2 cells changed to an ellipse. Moreover, HIF-1α to 80% confluence in a flask, the cells were exposed to hypoxia (1% O₂) for 0, 1, 3, 6, 12 or 24 hrs in stainless steel hypoxia chambers. Western blot analysis was then performed to determine the levels of HIF-1α (A, B), DMT1+IRE (C, D) and DMT1–IRE (E) in HepG2 cells. (A, C and E): A representative experiment of Western blot of HIF-1α (A), DMT1+IRE (C) and DMT1–IRE (E) in HepG2 cells. (B, D and F): Quantification of expression of HIF-1α (B), DMT1+IRE (D) and DMT1–IRE (F) protein in HepG2 cells. Expression values were normalized for β-actin and the data were presented as mean ± SEM (n = 3). *P<0.05, **P<0.01 versus the control (0h hypoxia).
became distinctly expressed in the nucleus while DMT1+IRE became concentrated in the cytoplasm. Hypoxia resulted in the majority of DMT1–IRE clustering closer to the outer cellular membrane with little changes in the nucleus.

Discussion

It has been demonstrated that Tf and TfR are hypoxia-inducible genes [20, 21]. Hypoxia can significantly affect the expression of these two proteins as well as some other proteins which are involved in iron homeostasis, including ferritin, ceruloplasmin, EPO, duodenal cytochrome B (Dcytb) and ferroportin 1 (Ireg1) [20, 21, 25–28]. The hypoxia-induced changes in the expression of these proteins are compensatory to the low oxygen environment and presumably restore metabolism towards normal or functionally acceptable homeostatic conditions which are required for cell survival [4, 13].

DMT1, like Tf and TfR, is a key transporter of iron. In addition, it has been reported that there are two putative HIF-1 recognition sequences in the 5’ regulatory region of human DMT1 [17]. Therefore, DMT1 might also be a hypoxia-inducible gene. In the present study, we first investigated the effects of chemical (CoCl₂) and physical (hypoxia chambers) hypoxia on the expression of DMT1 as well as the content of HIF-1α in the human hepatoma HepG2 cells. These cells were used in this investigation because they had been widely used to study the response of the liver gene expression to hypoxic stress [5]. We demonstrated that hypoxia was able to increase the expression of DMT1 as well as to accumulate the contents of HIF-1α in the HepG2 cells. The tendency in the hypoxia- and CoCl₂-induced changes in the content of HIF-1α was very similar to that in the expression of DMT1 protein. Our correlation analysis provided evidence for the first time for the existence of a highly significant correlation between the level of HIF-1α and the expression of DMT1 protein in cells during the process of physical as well as chemical hypoxia. These data suggested that the increased expression of DMT1 might be partly due to the accumulation of HIF-1α. In other words, the HIF-1 pathway might play a role in the regulation of DMT1 expression during hypoxia.

We also investigated the effects of hypoxia/re-oxygenation on the expression of DMT1 as well as the content of HIF-1α in the cells. We found that treatment of the HepG2 cells with hypoxia for 6 hrs and subsequently normoxia for 24 hrs (hypoxia/re-oxygenation) induced a significant decrease in the expression of DMT1+IRE, but not in the expression

| Table 1  | Correlation analysis of the relationship between expression of HIF-1α and DMT1 proteins in HepG2 cells treated with CoCl₂ (different concentrations) and hypoxia (Different durations of times) |
|----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| y        | R²                                                                  | P                      |                                                                 |
| CoCl₂ (0-0.5mM) |                                                                                           |                         |                                                                 |
| DMT1+IRE | 0.5177x + 1.0266                                                      | 0.6266                  | <0.01 (=0.708)                                                  |
| DMT1–IRE | 0.6362x + 0.86                                                       | 0.8621                  | <0.001 (=0.823)                                                   |
| Hypoxia (0-6 h) |                                                                                           |                         |                                                                 |
| DMT1+IRE | 0.5649x + 1.4167                                                      | 0.764                   | <0.005 (=0.750)                                                  |
| DMT1–IRE | 0.5484x + 1.452                                                      | 0.6978                  | <0.005 (=0.576)                                                  |
| Hypoxia (3-24 h) |                                                                                           |                         |                                                                 |
| DMT1+IRE | 0.7814x + 1.1127                                                      | 0.7056                  | <0.005 (=0.576)                                                  |
| DMT1–IRE | 0.229x + 1.8766                                                     | 0.1495                  | >0.05                                                             |

n=12-2=10
of DMT1–IRE protein (versus hypoxia group). No HIF-1α could be detected after the HepG2 cells were exposed to hypoxia/re-oxygenation. Similar tendencies in the hypoxia/reoxygenation-induced changes in the content of HIF-1α and the expression of DMT1+IRE protein suggested that HIF-1α might have a role to regulate the expression of DMT1+IRE instead of DMT1–IRE. Further studies are needed to precisely define the interaction between HIF-1α and the DMT1+IRE DNA.

It is generally accepted that cobalt exposure can induce hypoxia-like stress by the activation of HIF-1α and hypoxia-inducible genes [29, 30], although the relevant mechanism is not fully understood [31]. The results obtained from the present study showed that CoCl₂ treatment induced a significant increase in the contents of HIF-1α as well as the expression of DMT1 protein. Although it is possible that the increased expression of the DMT1 protein is due to the increased contents of HIF-1α by CoCl₂ as we proposed, it is also highly likely that it is the result of the direct role of CoCl₂. In addition, it has been well determined that DMT1 is a protein which transports a number of divalent metals including Fe²⁺, Mn²⁺, Zn²⁺, Co²⁺, Cd²⁺, Cu²⁺, Ni²⁺ and Pb²⁺ [15, 16] and that the 5’ regulatory region of human DMT1 contains five potential metal response elements (MRE’s) (17). The increased concentration of Co²⁺ outside the cells itself might have a direct effect to increase responsively the expression of the DMT1 protein. A decreased Fe²⁺ uptake induced by the increased extracellular Co²⁺ (a competitive inhibition) might also be one of the causes for the increased expression of the DMT1 protein.

In addition to the potential role of HIF-1α, hepcidin, a newly identified peptide and a key player in iron metabolism [32–34], might also be responsible for the increased expression of the DMT1 protein.

Fig. 3 Effect of hypoxia/re-oxygenation on the expression of DMT1 proteins and the content of HIF-1α in HepG2. HepG2 cells were cultured as described in Material and methods. After being grown to 80% confluence in a flask, the cells were exposed to hypoxia (1% O₂) for 6 hrs (H: Hypoxia only) or hypoxia (1% O₂) for 6 hrs and then exposed to normoxia (21% O₂) for 24 hrs (H/R: hypoxia/re-oxygenation). Western blot analysis of HIF-1α, DMT1+IRE and DMT1–IRE was then conducted. (A and C): A representative experiment of Western blot of HIF-1α, DMT1+IRE and DMT1–IRE; (B and D): Quantification of expression of HIF-1α, DMT1+IRE and DMT1–IRE proteins in HepG2 cells. Expression values were normalized for β-actin and the data were presented as mean ± S.E.M. (n = 3). *P<0.05 versus Hypoxia only.
during the process of hypoxia. It was recently demonstrated that hypoxia was able to reduce the level of hepcidin transcripts in vitro in the human HepG2 and Hep3B hepatoma cells and in vivo in mice housed in hypobaric hypoxia chambers [35]. It was also reported [36] that normal mice which were exposed to hypoxia for 3 days exhibited a two-to threefold increase in iron absorption. It had been hypothesized that the increase in intestinal absorption seen in hypoxia [36, 37] was due to the decreased expression of hepcidin [33]. At the same time, the decrease in hepcidin expression induced by hypoxia might also result in an increase in the DMT1 expression in the HepG2 cells.

Our immunocytochemical experiments clearly demonstrated that under normoxia, the two isoforms of DMT1 were located in both the cytoplasm and the nucleus in the HepG2 cells. Under hypoxia, almost all of the DMT1+IRE were concentrated in the cytoplasm, while DMT1–IRE was not remarkably altered by hypoxia. Hypoxia only resulted in the majority of DMT1–IRE in the cytoplasm clustering closer to the outer cellular membrane with little changes in the
nucleus. These results might imply that the two isoforms of DMT1 have different functions.

In summary, our studies demonstrated that exposure to physical and chemical hypoxia could significantly increase the level of HIF-1α and the expression of the two isoforms of DMT1 in the HepG2 cells. Correlation analysis provided evidence for the existence of a highly significant correlation between the level of HIF-1α and the expression of DMT1 proteins in the cells during hypoxia. Hypoxia/re-oxygenation experiments showed that the tendency in the changes of HIF-1α content is similar to that of the expression of DMT1+IRE protein. The findings are in favour of the hypothesis that HIF-1α might play a direct role in the regulation of DMT1+IRE expression during hypoxia. However, it should be pointed out that the increased expression of DMT1 in the hypoxic cells might be due to multiple factors, and the potential involvement of HIF-1α as discussed in the preceding paragraph is not the only one. Hypothesized mechanisms for the regulation of DMT1 expression in the hypoxic HepG2 cells are proposed in Figure 5.

To conclude whether DMT1 is a hypoxia-inducible gene, transfection analysis is absolutely needed to determine the putative HRE in the regulatory regions of DMT1 and direct evidence for the binding of HIF-1 to the DNA of DMT1 using EMSA (electrophoretic mobility shift assay) is also absolutely necessary.

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