Transferrin receptor 1 mRNA is downregulated in placenta of hepcidin transgenic embryos

Marie Elise Martin[a], Gaël Nicolas[a], Gilles Hetet[a], Sophie Vaulont[b], Bernard Grandchamp[a], Carole Beaumont[a,*]

[a] *INSERM U409, Faculté de Médecine X. Bichat, Institut Fédératif de Recherche 02, 16 rue Henri Huchard, 75018 Paris, France*
[b] *Département de Génétique, Développement et Pathologie Moléculaire, Institut Cochin, Faculté de Médecine Cochin-Port Royal, 24 rue du Faubourg S^T Jacques, 75014 Paris, France*

Received 8 June 2004; revised 12 July 2004; accepted 3 August 2004

Available online 18 August 2004

Edited by Lukas Huber

Abstract We have previously shown that hepcidin transgenic embryos are severely anemic and die around birth. Here, we report that embryonic hepcidin transgene expression decreases transferrin receptor 1 (TfR1) mRNA level in placenta, as shown by cDNA microarray analysis and quantitative RT-PCR, by a mechanism which is independent of placenta iron content and iron responsive element/iron regulatory protein (IRE/IRP) activity. On the contrary, iron injections into pregnant mothers result in increased placenta iron and ferritin content, and reduced IRE binding activity of IRP1 leading to decreased TfR1 mRNA level. Taken together, these results suggest that hepcidin action on placenta is mostly through transcriptional downregulation of the iron uptake machinery.

Keywords: Hepcidin; Placenta; Transferrin receptor; Transcriptional regulation; Iron; Iron regulatory protein

1. Introduction

Compelling evidence suggests that hepcidin, a small cysteine-rich peptide synthesized by the liver and secreted in serum, is a major regulator of iron homeostasis. Hepcidin deficient mice develop progressive iron overload of the parenchyma, with paradoxical iron depletion in tissue macrophages [1] and patients with homozygous hepcidin mutations develop a juvenile form of hemochromatosis [2]. Hepcidin is also a key mediator of the anemia of chronic disease [3]. Based on these observations, it has been proposed that hepcidin is the ''store regulator'', controlling intestinal iron absorption and iron recycling by macrophages [3].

Its role in the control of the materno-fetal iron transfer at the placenta level is not known but some of us have previously reported that forced expression of hepcidin during development in transgenic embryos leads to severe anemia and death around birth, whereas the control littermates are perfectly normal [4]. However, in these experiments, some transgenic embryos were viable without iron therapy and it was therefore possible to establish a transgenic mouse line (Thep27) constitutively expressing hepcidin [5].

These results suggest that hepcidin produced by the embryo can also block iron transfer from the mother to the foetus. Iron transfer across the placenta requires iron uptake at the brush border membranes by internalization of the maternal iron-transferrin complex by placenta transferrin receptors [6], release of iron in the endosomes and transfer to the cytoplasm, presumably by Nramp2/DMT1 [7], iron efflux at the basolateral membrane of the syncytiotrophoblast through the Fe(II) iron transporter ferroportin [8], also known as MTP1 [9] or IREG1 [10] and subsequent oxidation by a membrane-bound ceruloplasmin, which seems to be different from the duodenal hephaestin [11]. Placenta transferrin receptor 1 (TfR1) mRNA and protein have been shown to be both up-regulated by iron deficiency in the mother [12]. This compensatory mechanism probably results from a relative iron deficiency in the placenta, which stabilizes the TfR1 mRNA through the iron responsive element/iron regulatory protein (IRE/IRP) system. When cellular iron content is low, IRPs remain in their native form and bind with high affinity to IREs located in the 3′ untranslated region of the TfR1 mRNA, thereby protecting it from degradation by endonucleases (for review, see [13]).

The molecular targets of hepcidin remain mostly unknown. To better understand the mechanism underlying the onset of anemia in hepcidin transgenic embryos, we analyzed placenta mRNA by cDNA microarrays dedicated to genes of iron metabolism and anti-oxidant defenses and explored placenta iron metabolism. The effect of acute iron loading of the mother on placenta iron metabolism was also analyzed.

2. Materials and methods

2.1. Animals

Thepc27 hepcidin transgenic males of mixed C57BL/6x129/SVxDBA/2 genetic background and three month old C57BL/6 female mice were maintained under normal housing conditions. To induce parenteral iron loading, three subcutaneous injections of 100 μl of an iron-saccharose preparation (Venofer®, Vifor International, Saint Gallen, Switzerland) containing 2 mg elemental iron were performed in the pregnant mothers on three consecutive days, 72, 48 and 24 h prior to sacrifice.

* Corresponding author. Fax: +33-1-42264624.

Abbreviations: TfR1, transferrin receptor 1; IRE, iron responsive element; IRP, iron regulatory protein; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; p.c., post-coïtum

0014-5793/$22.00
doi:10.1016/j.febslet.2004.08.010

2.2. RNA extraction

The same method was used to extract RNA for cDNA microarray analysis and for quantitative RT-PCR. Total RNA was isolated from pieces of liver or placenta using the RNA Plus Extraction Solution kit (Quantum Biotechnologies, Illkirch, France).

2.3. cDNA microarray analysis

We designed a custom oligonucleotide-based microarray for a selection of genes involved in iron metabolism and antioxidant-defenses. The corresponding list of genes and GenBank accession numbers, together with the conditions used for labeling, hybridization and scanning of the data are available on our web site http://www.bichat.inserm.fr/equipes/u409/suppl_art_MARTIN.htm.

2.4. Quantitative RT-PCR

Single-stranded cDNA was synthesized using SuperScript™ Rnase H⁻ Reverse Transcriptase (Invitrogen). Real-time quantification of transcripts was performed in 25 μl in ABI PRISM® 7700 Sequence Detector (PE Applied Biosystems, Courtaboeuf, France) using SYBR® Green PCR master mix (PE Applied Biosystems), 5 pmol of forward and reverse primers and 2.5 μl of reverse transcriptase reaction mixture. Sequences of the primers were as follows :

Tfr1 (69 bp), 5′-GAGGAACCAGACCGTTATGTTGT-3′ (forward) and

5′-CTTCGCCGCAACACCAG-3′ (reverse);

Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; 177 bp), 5′-TGCACCACCAACTGCTTAG-3′ (forward) and

5′-GAATGCAGGGATGATGTTC-3′ (reverse).

The result was normalized arbitrary on the sample with the lowest C_T value for Gapdh (named $C_{T\text{-Gapdh R}}$). For all others samples, the relative quantification was calculated using comparative C_T method with the following arithmetic formula: $(1+E_{\text{Gapdh}})^{(C_{T\text{-Gapdh R}}-C_{T\text{-Gapdh S}})}/(1+E_X)^{(C_{T\text{-X R}}-C_{T\text{-X S}})}$, where E_{Gapdh} is the efficiency of *Gapdh* target amplification and E_X the efficiency of amplification for the gene of interest. $C_{T\text{-Gapdh R}}$ and $C_{T\text{-X R}}$ are the respective threshold cycles for *Gapdh* and for the gene of interest of the reference sample; $C_{T\text{-Gapdh S}}$ and $C_{T\text{-X S}}$ are the respective threshold cycles for *Gapdh* and for the gene of interest of every sample but the reference sample. The amplification efficiency of each target was determined using serial 2-fold dilutions of cDNA.

2.5. Tissue homogenization

Mice tissues were collected, weighted, minced, and dissolved in 10 ml of lysis buffer (20 mM Tris–HCl, pH 7.4, 1 mM Na azide, 1 mM PMSF, 10 μM leupeptin, 1 μM pepstatin, and 1 mM benzamidine) per gram of wet tissue. Potter or sonication was used to disrupt the cells. Debris were precipitated by centrifugation at 10 000 rpm for 10 min. All the steps were performed at +4 °C. Supernatants were used to determine ferritin contents, or to analyze IRE/IRP interactions in some cases. Protein concentration was determined in triplicate using the BioRad protein assay and BSA as standard.

2.6. Quantification of tissue non-heme iron and L ferritin content

Quantification of iron levels was performed as described by Torrence and Bothwell [14] on tissue samples by using an IL test (Instrumentation Laboratory, Lexington, MA) on an Olympus AU400 automat. L ferritin in tissue extracts was measured on the same automat, using the Olympus human ferritin assay kit. Calibration curves obtained with the human ferritin standard included in the kit and with recombinant mouse L ferritin (kindly provided by Dr. Paolo Santambrogio, Milano, Italy) showed almost perfect overlap.

2.7. RNA gel shift assays of IRP activity

Mouse tissue extracts were prepared as described above and diluted at 1 μg/μl in lysis buffer before use. IRE/IRP interactions were measured as previously described [15], by incubating a [^{32}P]-labeled IRE-HFt mRNA probe (3×10^4 cpm at 10^4 cpm/ng) transcribed in vitro from pIL2CAT with 5 μg of cytoplasmic extracts. After 15 min. incubation at room temperature, 5 mg/ml heparin was added for another 10 min. IRE-protein complexes were run on a 6% non-denaturing polyacrylamide gel. In parallel experiments, extracts were treated with 2% β-mercaptoethanol for 10 min. prior to the addition of the IRE probe to allow full expression of IRE binding activity. The radioactivity associated with the IRE/IRP complexes was quantified with an Instant Imager (Packard Instruments, Rungis, France).

2.8. Statistical analysis

Statistical significance was evaluated using the non-parametric Mann Whitney test (unpaired, two-tailed) for comparison between two medians. Correlation was performed by linear regression. GraphPad Prism software (GraphPad Software, San Diego, CA) was used for statistical evaluation.

3. Results

3.1. Hepcidin induces TfR1 mRNA downregulation in placenta

To analyze the effect of embryonic hepcidin expression on placenta iron metabolism, we crossed transgenic Thepc27 males constitutively expressing hepcidin with C57BL/6 wild type females. Hepcidin transgenic and non-transgenic embryos from the same littermate and their respective placenta were isolated at day 16.5 post coïtum, at a stage where endogenous embryonic hepcidin is not normally expressed. As previously reported, the transgenic embryos were very pale as compared to their non-transgenic littermates [4]. To identify changes in gene expression that might be induced in the placenta by the hepcidin produced by the embryonic liver, we developed 50-mer oligonucleotide-based cDNA microarrays dedicated to mouse iron genes. Pooled RNAs from two placenta of transgenic and two placenta of non-transgenic embryos were labeled with Cy3 and Cy5, respectively, and co-hybridized in duplicate on the mouse dedicated cDNA microarrays. A second hybridization experiment was performed with the reverse labeling. Most of the studied genes including both ferritin subunits, Nramp2, ferroportin, and Dcytb had a clearly detectable expression in placenta (>5 times the background signal) but did not show any change in their expression. Only transferrin receptor mRNA displayed a reduced expression (2.5-fold) in placenta from transgenic embryos. These results were confirmed by quantitative RT-PCR. At 16.5, 17.5 and 18.5 days p.c., there was a 3- to 5-fold reduction in TfR1 mRNA in placenta of transgenic embryos as compared to those from non-transgenic embryos (Fig. 1). These results were highly significant ($P < 0.005$). TfR1 mRNA stability is known to be regulated by intracellular iron through the IRE/IRP system. Our results suggest that sustained hepcidin expression by the embryo induces iron accumulation in the placenta, inactivation of IRE binding activity of the IRPs and destabilization of TfR1 mRNA. To test this hypothesis, we measured iron content in the placenta of transgenic and non-transgenic embryo but found no difference between the two conditions (Fig. 2A), although there was a moderate but statistically significant increase in placenta L ferritin content (Fig. 2B). Evaluation of the IRE binding activity of the IRPs by gel retardation assay (Fig. 2C) showed that on an average, 60–70% of IRP1 is in the apo form possessing an IRE binding activity, both in control and in hepcidin transgenic placenta (Fig. 2D). No IRP2 binding activity was detected, as previously observed in mouse tissues [15] or human placenta [16]. Since there is no change in placenta iron content and no modification in the IRE binding activity of the IRP, it is likely that the strong reduction in TfR1 mRNA results from transcriptional repression of the TfR1 gene, rather than from degradation of TfR1 mRNA.

3.2. Acute iron overload in the mother does not induce iron overload of the embryo

To determine to what extent placenta iron loading was capable of regulating TfR1 mRNA expression and IRP activity,

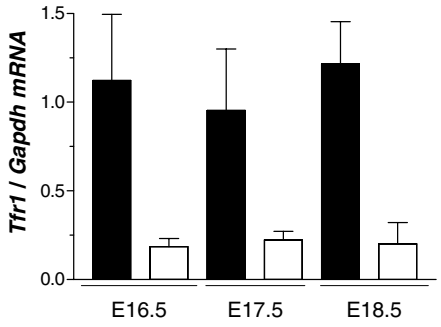

Fig. 1. Hepcidin transgene expression in embryonic liver downregulates TfR1 mRNA in the placenta. Quantitative RT-PCR experiments were performed for placenta TfR1 mRNAs and normalized to Gapdh mRNA. Placentas from non-transgenic (black bars) and from transgenic (open bars) embryos were analyzed. At each stage of development, results are means ± S.D. of data obtained for between 5 and 7 embryos of the same genotype resulting from two different matings between a wild type C57BL/6 mother with a Thepc27 hepcidin transgenic male. Differences between controls and transgenic TfR1 mRNA levels were highly significant ($P < 0.005$).

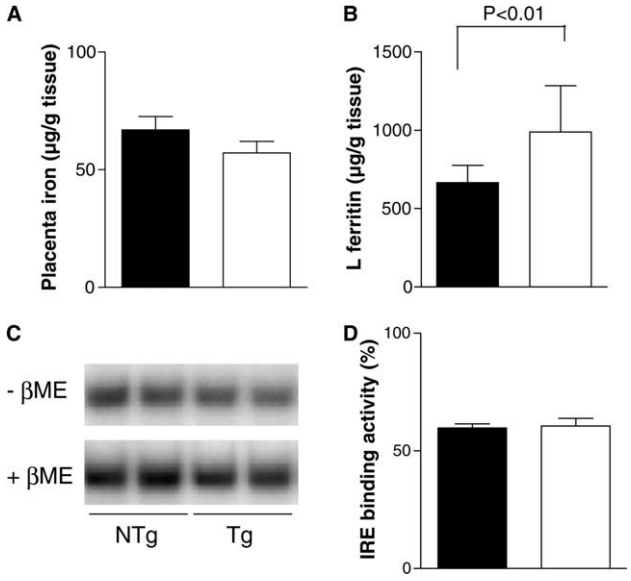

Fig. 2. Hepcidin transgene expression in embryonic liver does not change IRP activity in the placenta. One litter from a mating with a Thepc27 male was analyzed for each of the three stages of development. Each litter consisted in three to five non-transgenic (black bars) or three to five transgenic (open bars) embryos. Results are means ± S.D. of data obtained with all the placenta from the same genotype. (A) Placenta non-heme iron. (B) Placenta L ferritin content, determined with an Olympus automat. (C) IRE binding activity of the IRP was evaluated by gel retardation assay using an IRE probe. An autoradiogram of a typical gel shift experiments is shown, with the IRE/IRP complex found in two non-transgenic (NTg) and two transgenic (Tg) embryos at 18.5 days of development, in the presence or absence of 2% βME. (D) Quantifications of the bands by Instant Imager, expressed as percent of the total IRE binding activity in the placenta from non-transgenic and transgenic embryos.

we induced acute iron overload in pregnant mothers mated to wild type males and the consequences on placenta iron metabolism were followed. Iron was injected to pregnant mothers on three consecutive days prior to sacrifice at 17.5 day p.c. Iron injections in pregnant mothers induced roughly a 10-fold iron overload in their liver, although the total iron in the embryo was not modified. In the placenta, there was a 5-fold increase in iron load (Fig. 3A), a moderate but statistically significant ($P < 0.05$) reduction in TfR1 mRNA expression (Fig. 3B), and a 2-fold increase in L ferritin content (Fig. 3C). Iron accumulation in the placenta reduced the IRE-binding activity of IRP1, with only 30–35% of the IRP remaining in the apo form in placenta from iron-overloaded mother (Fig. 3D and E). These results suggest that transfer of iron from the placenta to the embryo is a rate-limiting step and that iron accumulation in the placenta subsequently downregulates TfR1 mRNA expression through inactivation of the IRP. Correlation between IRP binding activity and TfR1 mRNA in the placenta of embryos from both control and iron overloaded mothers suggests that placenta iron content regulates TfR1 mRNA levels through the IRE/IRP system (Fig. 3F).

4. Discussion

In this paper, we demonstrate that hepcidin constitutively produced by embryonic liver during development reduces transferrin receptor mRNA in the placenta. This is the first description of a molecular effect that can only be ascribed to changes in native hepcidin expression. So far, all the evidence we have that hepcidin is a major regulator of iron homeostasis relies mostly on two lines of evidence. One is the observation that hepcidin gene expression is upregulated by iron and LPS [17,18] and downregulated by iron deficiency [19], phlebotomy, or hypoxia [20]. The other one is brought about by the phenotype of animal models with modified hepcidin expression [1] or by the identification of hepcidin mutations in patients with juvenile hemochromatosis [2]. Hepcidin is thought to downregulate intestinal iron absorption by duodenal enterocytes and iron recycling by macrophages. The current view is that hepcidin interacts with some components of the iron export machinery [21], although the precise mechanism of these effects remains completely unknown. Our results suggest that long term adaptation of the placenta to sustained embryonic hepcidin expression is through reduction of the iron uptake protein at the maternal side, although we cannot rule out the possibility that hepcidin also downregulates iron efflux from the placenta. This is in keeping with a recent publication showing that intraperitoneal injection of synthetic hepcidin in mice decreases duodenal iron uptake, as shown by tied off loop experiments [22].

It is likely that this TfR1 mRNA reduction results from transcriptional downregulation of the gene rather than reduced stability of the mRNA, since placenta iron was not increased by embryonic expression of hepcidin, and the IRE binding activity of the IRP1 was not modified. On the contrary, experimental placenta iron overload inactivated the IRE binding activity of the IRP1, increased ferritin synthesis and reduced TfR1 mRNA accumulation, as expected. The strong correlation between TfR1 mRNA and IRP1 activity in placenta from both control and iron loaded mothers highlights the importance of the iron-mediated post-transcriptional regulations in the control of the iron exchanges between the mother and the foetus. The results obtained following acute iron loading of the mother extend previous observations that in rats, iron deficiency in the mother results in activation of TfR1 mRNA expression [17] and that in humans, placentas from diabetic

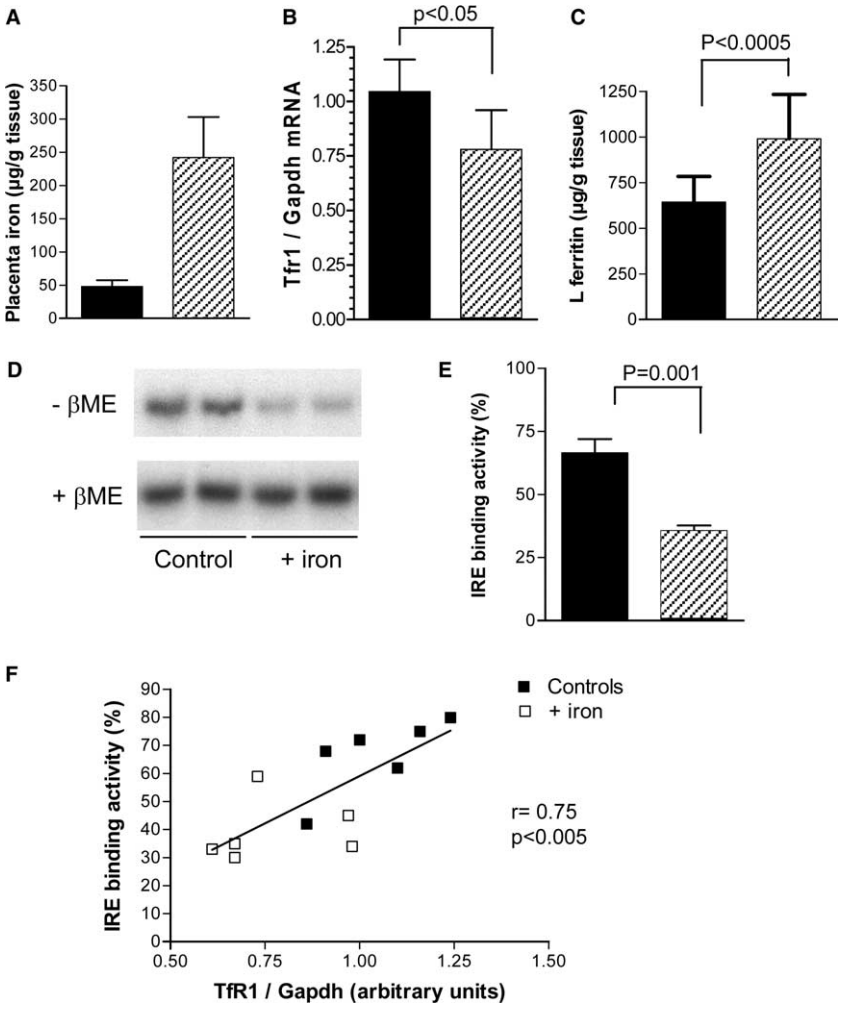

Fig. 3. Placenta iron overload reduces IRP binding activity. Placentas from control mothers (black bars) or from two iron overloaded mothers (hatched bars) were analyzed. (A) Non-heme iron was measured on six placentas for each condition. (B) TfR1 mRNA was measured by quantitative RT-PCR and normalized to Gapdh mRNA, on six placentas for each condition. (C) Placenta L ferritin contents were determined with an Olympus automat. Results are means ± S.D. of placentas ferritin measurement from 12 placentas from two litters for each condition. (D) IRE binding activity of the IRP was evaluated by gel retardation assay using an IRE probe. An autoradiogram of a typical gel shift experiment is shown, with the IRE/IRP complex found in two control and two iron-loaded placentas. (E) Quantification of the bands by Instant Imager, expressed as percent of the total IRE binding activity evaluated following exposure to 2% βME, is shown for six placentas from one control and six placentas from one overloaded mother. (F) Correlation between IRE binding activity and TfR1 mRNA level for each placenta was made by linear regression.

mothers also show increased TfR1 mRNA expression and increased IRE binding activity of IRP1 [16]. The moderate increase in L ferritin synthesis in transgenic placenta is difficult to explain, considering that the RNA-binding activity of IRP1 is not modified. However, decreased stability of IRP2, which could not be evaluated in our experimental conditions, could account for this effect. IRP2 has been shown to dominate iron-mediated post-transcriptional regulations in certain tissues [23].

Transcriptional regulation of TfR1 gene expression has not been studied in great details but it is known to be upregulated when resting cells are induced to proliferate [24] or during erythroid differentiation [25]. In addition, a functional HIF-1α binding site located about 90 nucleotides upstream of the transcription start site can mediate transcriptional activation of the TfR1 gene in hypoxic conditions [26]. Interestingly, iron is a key factor in the regulation of HIF-1α stability [27] and cellular iron depletion can stabilize HIF-1α, even in normoxic conditions [28]. Therefore, in some conditions, transcriptional regulations of TfR1 gene expression can override the regulation of mRNA stability by the IRP/IRE complexes, as it is probably the case in response to embryonic hepcidin.

Finally, it will be important to establish whether hepcidin action on one of its natural target organ such as the duodenum also involves downregulation of proteins involved in iron uptake like the iron reductase Dcytb or the iron transporter Nramp2/DMT1.

Acknowledgements: This work was supported by a grant "Action Concertée Incitative" from the French Ministry of Education and Research. The authors are very grateful to Laurent Gouya and Jacqueline Bauchet for their help with the ferritin assay.

References

[1] Nicolas, G., Bennoun, M., Devaux, I., Beaumont, C., Grandchamp, B., Kahn, A. and Vaulont, S. (2001) Proc. Natl. Acad. Sci. USA 98, 8780–8785.

[2] Roetto, A., Papanikolaou, G., Politou, M., Alberti, F., Girelli, D., Christakis, J., Loukopoulos, D. and Camaschella, C. (2003) Nat. Genet. 33, 21–22.
[3] Ganz, T. (2003) Blood 102, 783–788.
[4] Nicolas, G., Bennoun, M., Porteu, A., Mativet, S., Beaumont, C., Grandchamp, B., Sirito, M., Sawadogo, M., Kahn, A. and Vaulont, S. (2002) Proc. Natl. Acad. Sci. USA 99, 4596–4601.
[5] Nicolas, G., Viatte, L., Lou, D.Q., Bennoun, M., Beaumont, C., Kahn, A., Andrews, N.C. and Vaulont, S. (2003) Nat. Genet. 34, 97–101.
[6] McArdle, H.J., Danzeisen, R., Fosset, C. and Gambling, L. (2003) Biometals 16, 161–167.
[7] Georgieff, M.K., Wobken, J.K., Welle, J., Burdo, J.R. and Connor, J.R. (2000) Placenta 21, 799–804.
[8] Donovan, A., Brownlie, A., Zhou, Y., Shepard, J., Pratt, S.J., Moynihan, J., Paw, B.H., Drejer, A., Barut, B., Zapata, A., Law, T.C., Brugnara, C., Lux, S.E., Pinkus, G.S., Pinkus, J.L., Kingsley, P.D., Palis, J., Fleming, M.D., Andrews, N.C. and Zon, L.I. (2000) Nature 403, 776–781.
[9] Abboud, S. and Haile, D.J. (2000) J. Biol. Chem. 275, 19906–19912.
[10] McKie, A.T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S., Bomford, A., Peters, T.J., Farzaneh, F., Hediger, M.A., Hentze, M.W. and Simpson, R.J. (2000) Mol. Cell 5, 299–309.
[11] Danzeisen, R. and McArdle, H.J. (2000) Trace Elements in Man and Animals (Roussel et al, Ed.), pp. 947–950.
[12] Gambling, L., Danzeisen, R., Gair, S., Lea, R.G., Charania, Z., Solanky, N., Joory, K.D., Srai, S.K. and McArdle, H.J. (2001) Biochem. J. 356, 883–889.
[13] Hentze, M.W. and Kuhn, L.C. (1996) Proc. Natl. Acad. Sci. USA 93, 8175–8182.
[14] Torrance, J.D. and Bothwell, T.H. (1968) S. Afr. J. Med. Sci. 33, 9–11.
[15] Ferreira, C., Santambrogio, P., Martin, M.E., Andrieu, V., Feldman, G., Henin, D. and Beaumont, C. (2001) Blood 98, 525–532.
[16] Georgieff, M.K., Berry, S.A., Wobken, J.D. and Leibold, E.A. (1999) Placenta 20, 87–93.
[17] Pigeon, C., Ilyin, G., Courselaud, B., Leroyer, P., Turlin, B., Brissot, P. and Loreal, O. (2001) J. Biol. Chem. 276, 7811–7819.
[18] Nemeth, E., Valore, E.V., Territo, M., Schiller, G., Lichtenstein, A. and Ganz, T. (2003) Blood 101, 2461–2463.
[19] Frazer, D.M., Wilkins, S.J., Becker, E.M., Vulpe, C.D., McKie, A.T., Trinder, D. and Anderson, G.J. (2002) Gastroenterology 123, 835–844.
[20] Nicolas, G., Chauvet, C., Viatte, L., Danan, J.L., Bigard, X., Devaux, I., Beaumont, C., Kahn, A. and Vaulont, S. (2002) J. Clin. Invest. 110, 1037–1044.
[21] Hentze, M., Muckenthaler, M.U. and Andrews, N. (2004) Cell 117, 285–297.
[22] Laftah, A.H., Ramesh, B., Simpson, R.J., Solanky, N., Bahram, S., Schumann, K., Debnam, E.S. and Srai, S.K. (2004) Blood 103, 3940–3944.
[23] Meyron-Holtz, E.G., Ghosh, M.C., Iwai, K., LaVaute, T., Brazzolotto, X., Berger, U.V., Land, W., Ollivierre-Wilson, H., Grinberg, A., Love, P. and Rouault, T. (2004) EMBO J. 23, 386–395.
[24] Enns, C.A. (2002) in: Molecular and Cellular Iron Transport (Templeton, D.M., Ed.), pp. 71–94, Marcel Dekker, New York.
[25] Marziali, G., Perrotti, E., Ilari, R., Lulli, V., Coccia, E.M., Moret, R., Kuhn, L.C., Testa, U. and Battistini, A. (2002) Oncogene 21, 7933–7944.
[26] Lok, C.N. and Ponka, P. (1999) J. Biol. Chem. 274, 24147–24152.
[27] Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) Science 292, 468–472.
[28] Wang, G.L. and Semenza, G.L. (1993) Blood 82, 3610–3615.