Duodenal mRNA expression of iron related genes in response to iron loading and iron deficiency in four strains of mice

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Gut 2002;51:648–653

Background: Although much progress has been made recently in characterising the proteins involved in duodenal iron trafficking, regulation of intestinal iron transport remains poorly understood. It is not known whether the level of mRNA expression of these recently described molecules is genetically regulated. This is of particular interest however as genetic factors are likely to determine differences in iron status among mouse strains and probably also contribute to the phenotypic variability seen with disruption of the haemochromatosis gene.

Aims: To investigate this issue, we examined concomitant variations in duodenal cytochrome b (Dcytb), divalent metal transporter 1 (DMT1), ferroportin 1 (FPN1), hephaestin, stimulator of Fe transport (SFT), HFE, and transferrin receptor 1 (TfR1) transcripts in response to different dietary iron contents in the four mouse strains C57BL/6, DBA/2, CBA, and 129/Sv.

Subjects: Six mice of each strain were fed normal levels of dietary iron, six were subjected to the same diet supplemented with 2% carbonyl iron, and six were fed an iron deficient diet.

Methods: Quantification of mRNAs isolated from the duodenum was performed using real time reverse transcription-polymerase chain reaction.

Results: There was a significant increase in mRNA expression of Dcytb, DMT1, FPN1, and TfR1 when mice were fed an iron deficient diet, and a significant decrease in mRNA expression of these molecules when mice were fed an iron supplemented diet. Strain to strain differences were observed not only in serum transferrin saturations, with C57BL/6 mice having the lowest values, but also in hepatic iron stores and in duodenal mRNA expression of Dcytb, DMT1, FPN1, hephaestin, HFE, and TfR1.

Conclusions: The results favour some degree of genetic control of mRNA levels of these molecules.

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Accepted for publication
28 March 2002

Iron is vital for all living organisms because it has essential roles in oxygen transport, electron transfer, and as a cofactor in many enzyme systems, including DNA synthesis. The biological importance of iron is underscored by evolution of very complex mechanisms for its acquisition, utilisation, and preservation. Although required for numerous cellular metabolic functions, iron is potentially toxic to the cell when present in excess, as seen in haemochromatosis.¹ The ability of mammals to excrete iron is limited and therefore iron acquisition by the absorptive epithelium of the small intestine must be carefully regulated.

Much progress has been made recently in characterising the proteins involved in duodenal iron trafficking. Non-haem dietary iron, mostly in the form of ferric iron complexes, is first converted to a transportable form by duodenal cytochrome b (Dcytb), a putative brush border surface ferric reductase.² Ferrous iron is then supplied to divalent metal transporter 1 (DMT1), formerly termed Nramp2 or DCT1, an apical transmembrane iron transporter that actively transports reduced dietary iron into intestinal enterocytes.^{3,4} Iron traverses the epithelial cell and is exported through the basolateral membrane by a process that involves a second transmembrane iron transporter, ferroportin 1 (or FPN1, also known as IREG1 or MTP1),⁵⁻⁷ and requires the transmembrane bound multicopper ferroxidase, hephaestin.⁸ Of note, another protein of iron metabolism, the stimulator of Fe transport (SFT), which facilitates both transferrin and non-transferrin bound iron uptake,⁹ is also expressed in the duodenum. Despite characterisation of these molecules, regulation of intestinal iron transport remains poorly understood. The protein mutated in haemochromatosis, HFE,¹⁰ which is

associated with transferrin receptor 1 (TfR1) in crypt enterocytes of the duodenum,¹¹ is thought to play a key role in this process.

To date, it is not known whether the level of mRNA expression of these recently described molecules is genetically regulated. This is of particular interest as inbred mouse strains exhibit considerable variability in their parameters of iron metabolism.¹²⁻¹⁴ Serum iron levels, serum transferrin saturations, and hepatic iron stores vary as much as twofold among inbred strains on a basal diet. Inbred strains also differ in the severity of iron loading when fed an iron supplemented diet¹² and on the impact of an iron deficient diet.¹³ Genetic factors are thus likely to determine differences in iron status among mouse strains and probably also contribute to the phenotypic variability seen with disruption of the *Hfe* gene.¹⁵⁻¹⁷

To investigate this issue, we examined, using real time reverse transcription-polymerase chain reaction (RT-PCR), concomitant variations in Dcytb, DMT1, FPN1, hephaestin, SFT, HFE, and TfR1 transcripts in response to different diets (iron deficient, iron balanced, or iron supplemented) in four strains of mice, C57BL/6, DBA/2, CBA, and 129/Sv.

METHODS

Animals and treatments

C57BL/6, DBA/2, and CBA mice were purchased from the Centre d'Elevage Robert Janvier (Le Genest St Isle, France).

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; Dcytb, duodenal cytochrome b; DMT1, divalent metal transporter 1; FPN1, ferroportin 1; SFT, stimulator of Fe transport; TfR1, transferrin receptor 1.

Table 1 Primer pairs used to quantify iron related transcripts expressed in the duodenum

Gene	Sense primer	Antisense primer	GenBank accession No (position)
Dcytb	5'-GCAGCGGGCTCGAGTTA-3'	5'-TTCCAGGTCCATGGCAGTCT-3'	AF354666 (137–235)
DMT1	5'-GGCTTTCTTATGACATTGCCTA-3'	5'-GGAGCACCCAGAGCAGCTTA-3'	L33415 (289–385)
SFT	5'-CTGTGCTCATGAGAGGACCTT-3'	5'-TCTGGTGTCTTCTCAGTCACG-3'	AA178012 (232–329)
FPN1	5'-TTGAGGAGTCACTTGCTGCTA-3'	5'-TGGAGTCTGCACACCATGAT-3'	AF226613 (1670–1789)
Hephaestin	5'-TTGTCTCATGAAGAACATTACAGCAC-3'	5'-CATATGGCAATCAAAGCAGAAGA-3'	AF082567 (3803–3963)
HFE	5'-CTGAAAGGGTGGGACTACATGTC-3'	5'-GGACACCACTCCCACTTCGT-3'	U66849 (362–454)
TfR1	5'-TCATGAGGGAAATCAATGATCGTA-3'	5'-GCCCCAGAAAGATATGTCGGAA-3'	X57349 (2018–2118)
β-Actin	5'-GACGGCCAAGTCATCACTATTG-3'	5'-CCACAGGATTCATACCCAAGA-3'	M12481 (652–740)

Dcytb, duodenal cytochrome b; DMT1, divalent metal transporter 1; SFT, stimulator of Fe transport; FPN1, ferroportin 1; TfR1, transferrin receptor 1.

129/Sv mice, originally obtained from Iffa Credo (L'Arbresle, France), were maintained at the IRF30 animal facility. After weaning, mice were fed a standard diet (R03; UAR, Epinay-sur-Orge, France) with 280 mg Fe/kg over two weeks. Dietary iron deficiency was induced by placing six five week old mice of each strain on a diet with virtually no iron content (212 Fe; UAR) and demineralised water for a period of two weeks. Dietary iron loading was obtained by placing six five week old animals of each strain on the R03 diet supplemented with 2% (wt/wt) carbonyl iron (Sigma Immunochemicals, Saint-Quentin Fallavier, France) for two weeks. Six control animals of each strain received the iron balanced diet of the same composition (R03). All mice were analysed at seven weeks and fasted for 14 hours before blood sampling. After blood was obtained, mice were sacrificed and the duodenum (the 2 cm length of small intestine distal to the pylorus) was dissected for RNA isolation. All animal experiments were performed in accordance with institutional and governmental guidelines.

Measurement of serum transferrin saturation

Blood was obtained by inferior vena cava puncture. Serum iron and total iron binding capacity were measured on a Roche/Hitachi 717 Automatic Analyser (Roche Diagnostics, Meylan, France). Transferrin saturation was calculated as (serum iron/total iron binding capacity)×100%.

Assessment of liver iron content

Hepatic iron content was evaluated as described previously.¹⁸ Briefly, liver specimens (0.5–4 mg) were first desiccated for 24 hours at 120°C in a ventilated oven. Thereafter, the dried samples were weighed and mineralised by strong acid digestion and heating. Iron was then complexed to the bathophenanthroline sulphonate chromogen and absorbance measured at 535 nm.

Quantification of duodenal transcripts through real time PCR

Expression of duodenum specific transcripts was analysed by RT-PCR. Total RNA from mouse duodenum was isolated using the SV Total RNA Isolation System (Promega, Charbonnières, France). RNA was then reverse transcribed into cDNA with the M-MLV reverse transcriptase RNase H- (Promega). Quantification of different mRNAs was performed by real time PCR on a 5700 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). The PCR reaction mix contained the cDNA quantity converted from 75 ng RNA, 0.3 μM of each primer (sequences in table 1), 4×10⁻⁵× SYBR Green I (Sigma-Aldrich, Saint-Quentin Fallavier, France), 1× ROX (Life Technologies, Cergy Pontoise, France) to normalise for non-PCR related fluctuations in fluorescence signal, and 1× Platinum Quantitative PCR SuperMix-UDG (Life Technologies). PCR amplification began with one cycle of 50°C for two minutes (UDG PCR carry over decontamination) and 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for

15 seconds and annealing/extension at 60°C for 60 seconds. In real time PCR, each reaction is characterised by the point during cycling when amplification of the PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. Direct detection of PCR products is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to double stranded DNA. The higher the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed. A threshold is defined as the fractional cycle number at which the fluorescence passes a fixed threshold above baseline. Quantification was obtained by comparing the threshold cycles of unknown samples against calibration curves with known copy numbers. All experiments were performed in duplicate. Primers were designed using Primer Express software (Applied Biosystems). Primer specificity was checked by systematic sequencing of PCR products. In addition, dissociation curves were used as a quality control tool to check the absence of primer dimers and other non-specific products in the amplification reactions.

Data analyses

Raw values obtained for Dcytb, DMT1, FPN1, hephaestin, SFT, TfR1, and HFE were first standardised to the β-actin endogenous control. Briefly, for each experimental sample, the target amount was divided by the endogenous reference amount and multiplied by 10⁶ to obtain the standardised target value—that is, the number of target copies per 10⁶ β-actin copies. The amounts of the different transcripts were not normally distributed. Therefore, for statistical analyses of the effect of iron diet and strain on mRNA levels, mRNA values were transformed by taking their natural logarithm, and the relative contributions of diet and strain on mean levels of the measured parameters were determined by two way analysis of variance (ANOVA). All main effects and interaction terms were considered significant when p values were less than 0.05. Where a significant effect was seen, individual comparisons between groups were made by Duncan's multiple range tests. Associations between parameters were assessed by the non-parametric Spearman's rank order correlation test. Geometric means were used to estimate levels of upregulation or downregulation of the duodenal transcripts under different dietary conditions. Geometric means, which were obtained by taking the antilogarithms of the means of the log transformed values, have the advantage of recovering the original units. Statistical analyses were performed using the Statistical Analysis System (SAS Institute Inc., Cary, North Carolina, USA).

RESULTS

Serum transferrin saturation and liver iron content

Two way ANOVA showed that strain (p<0.0001), diet (p<0.0001), and the interaction between strain and diet (p<0.0001) each contributed to the variance in transferrin

Figure 1 Transferrin saturation (A) and hepatic iron content (B) (mean (SD)) in the four different strains of mice (C57BL/6, DBA/2, CBA, and 129/Sv) fed each of the three diets.

Figure 2 Duodenal expression of duodenal cytochrome b (Dcytb) (A) and divalent metal transporter 1 (DMT1) (B) mRNAs in the four different strains of mice (C57BL/6, DBA/2, CBA, and 129/Sv) fed each of the three diets. Transcript levels were log transformed to induce normality of the distributions and results are expressed as means (SD).

saturation across the different strains and diet combinations. The significant interaction term in this variance analysis suggests that the effect of dietary iron content varies according to strain. As shown on fig 1, on a basal diet, C57BL/6 mice had a significantly lower transferrin saturation (mean 34.6%) than CBA (mean 48.3%), DBA/2 (mean 52.2 %), or 129/Sv (mean 60.8%) mice. Mean transferrin saturations also differed across strains when mice were fed an iron deficient diet, with the 129/Sv strain having significantly higher transferrin saturations than the other strains. Of note, DBA/2 and CBA strains appeared more susceptible to iron deficiency than the other strains as they had significantly lower transferrin saturations than mice of the same genetic backgrounds fed an iron balanced diet. No strain differences were seen when mice were fed an iron supplemented diet.

Strain ($p < 0.0001$), diet ($p < 0.0001$), and the interaction between strain and diet ($p < 0.0001$) also contributed to the variance in liver iron content across the different strain and diet combinations. As shown in fig 1, on an iron balanced diet, no significant differences were seen between strains. However, when mice were fed an iron deficient diet, mean hepatic iron content was significantly lower in C57BL/6 mice than in the three other strains. Conversely, when mice were fed an iron supplemented diet, 129/Sv mice appeared significantly more resistant to hepatic iron loading (mean hepatic iron concentration 18.4 μmol/g dry liver) than DBA/2 (mean concentration 34.2), C57BL/6 (mean concentration 38.0), or CBA (mean concentration 43.8) strains. These results show that strain differences strongly influence hepatic iron accumulation resulting from an iron supplemented diet.

There was no correlation between serum transferrin saturation and liver iron concentration among animals within

each strain and diet category. These observations suggest that different genetic factors determine serum transferrin saturation and hepatic iron concentration. Leboeuf and colleagues¹² reached a similar conclusion from studies of strain differences in sensitivity to dietary iron loading.

Messenger levels of molecules known to be expressed at the apical membrane of villus enterocytes

Transcripts of molecules involved in reducing dietary iron to a transportable form (Dcytb) and transporting reduced iron from the intestinal lumen into enterocytes (DMT1) were examined first.

Both diet ($p < 0.0001$), strain ($p < 0.006$) and, to a lesser degree, the interaction between diet and strain ($p = 0.02$), significantly influenced expression of Dcytb mRNA. Indeed, as shown in fig 2, for each strain mean values of (log) Dcytb mRNA differed significantly across diets, with an increase in expression of Dcytb caused by dietary iron deficiency and a decrease in this expression caused by dietary iron loading, except for the 129/Sv strain. For the latter strain, there was no significant decrease in expression of Dcytb induced by an iron supplemented diet, an observation that contrasts with the fact that this strain accumulates less hepatic iron than the three other strains as a consequence of dietary iron loading. Of interest, expression of Dcytb mRNA following dietary iron deficiency in the C57BL/6 strain was significantly lower than in the DBA/2, 129/Sv, and CBA strains. This correlates with the observation that liver iron stores were significantly lower in the C57BL/6 strain than in the other strains.

Similarly, both diet ($p < 0.0001$), strain ($p = 0.005$) and, to a lesser extent, the interaction between diet and strain ($p = 0.01$), significantly influenced expression of DMT1

Figure 3 Duodenal expression of ferroportin 1 (FPN1) (A) and hephaestin (B) mRNAs in the four different strains of mice (C57BL/6, DBA/2, CBA, and 129/Sv) fed each of the three diets. Transcript levels were log transformed to induce normality of the distributions and results are expressed as means (SD).

Figure 4 Duodenal expression of HFE (A) and transferrin receptor 1 (TfR1) (B) mRNAs in the four different strains of mice (C57BL/6, DBA/2, CBA, and 129/Sv) fed each of the three diets. Transcript levels were log transformed to induce normality of the distributions and results are expressed as means (SD).

mRNA. As shown on fig 2, within each strain mean values of (log) DMT1 mRNA differed significantly across diets, with upregulation following dietary iron deficiency and downregulation following dietary iron loading, except for the C57BL/6 and CBA strains. For these two strains, there was no significant decrease in expression of DMT1 induced by an iron supplemented diet. Interestingly, these two strains also have the highest liver iron contents when fed an iron supplemented diet. As observed for Dcytb, DMT1 mRNA expression following dietary iron deficiency was significantly lower in the C57BL/6 strain than in the DBA/2, 129/Sv, and CBA strains.

Interestingly, in each strain, Dcytb and DMT1 transcript levels were significantly correlated (Spearman rank correlation coefficient $r=0.63$, $p=0.005$ for C57BL/6; $r=0.88$, $p<0.0001$ for CBA; and $r=0.94$, $p<0.0001$ for both DBA/2 and 129/Sv strains), indicating coordinated regulation of Dcytb and DMT1 at the apical membrane over the range of dietary iron contents used in this study. Of note, neither diet nor strain significantly influenced transcript levels of SFT, previously shown to be localised in the subapical part of villus cells.¹⁹

Messenger levels of molecules known to be expressed at the basolateral membrane of villus enterocytes

Transcripts of the molecules involved in the export of iron through the basolateral membrane, FPN1 and hephaestin, were examined next.

Only diet significantly influenced expression of FPN1 mRNA ($p<0.0001$) and, to a minor extent, strain ($p=0.03$), with no significant interaction between diet and strain. As

shown in fig 3, for each strain mean values of (log) FPN1 mRNA were significantly increased as a consequence of dietary iron deficiency, and significantly decreased, except for the CBA strain, following dietary iron loading. The CBA strain also had no significant decrease in expression of DMT1 when fed an iron supplemented diet.

Strain ($p=0.006$), diet ($p=0.002$), and the interaction between diet and strain ($p=0.001$) each contributed to the variance in the level of expression of the hephaestin transcripts across the different strain and diet combinations. As shown in fig 3, mean values of (log) hephaestin mRNA were significantly increased by dietary iron deficiency in the CBA and 129/Sv strains, and significantly decreased by dietary iron loading in the 129/Sv strain. Of note, expression of hephaestin in C57BL/6 and DBA/2 mice was not significantly influenced by diet.

Interestingly, a significant correlation between FPN1 and hephaestin transcript levels was also observed both in CBA ($r=0.85$; $p<0.0001$) and in 129/Sv mice ($r=0.82$; $p<0.0001$), indicating coordinated regulation of the two mRNAs expressed at the basolateral membrane in these two strains.

Messenger levels of molecules known to be expressed in crypt enterocytes

For the purpose of completeness, expression of the transcripts of the HFE and TfR1 molecules that associate in the duodenal crypt cells were also examined.

Only strain significantly influenced expression of HFE mRNA ($p<0.0001$), with no effect of diet and no significant interaction between diet and strain (fig 4). Mean separation

tests suggested that the mean value of (log) HFE mRNA was significantly lower in the C57BL/6 strain than in the three other strains.

Finally, strain ($p < 0.0001$), diet ($p = 0.0003$), and the interaction between diet and strain ($p = 0.002$) each contributed to the variance in expression of the TfR1 transcript across the different strain and diet combinations. As shown in fig 4, for each strain, mean values of (log) TfR1 mRNA were significantly increased by dietary iron deficiency and significantly decreased by dietary iron loading. In addition, compared with the three other strains, 129/Sv mice fed an iron supplemented diet had significantly lower levels of duodenal TfR1 mRNA expression.

DISCUSSION

In this study, we used real time RT-PCR to quantify the transcripts of all of the molecules involved in duodenal iron absorption described so far in 18 mice of each of the C57BL/6, DBA/2, CBA, and 129/Sv strains, six fed an iron balanced diet, six subjected to the same diet supplemented with 2% carbonyl iron, and six fed an iron deficient diet. It is known that tight linkage of dietary iron absorption with body iron stores occurs in the proximal intestine where duodenal crypt cells are thought to sense the iron needs of the body and to be consequently "programmed" as they mature into absorptive enterocytes to express appropriate levels of the molecules involved in iron absorption.²⁰ Our results demonstrate that manipulating body iron stores leads to significant changes in expression of Dcytb, DMT1, FPN1, and TfR1 mRNAs. These transcripts were globally upregulated by feeding mice a diet with virtually no iron for two weeks (11.7-fold increase in geometric means for Dcytb, 14.2-fold for DMT1, 2.8-fold for FPN1, and 2.2-fold for TfR1) and downregulated by feeding mice the iron balanced diet supplemented with 2% carbonyl iron (5.1-fold decrease between the geometric means for Dcytb, 2.1-fold for DMT1, 1.9-fold for FPN1, and 2.6-fold for TfR1). Of interest, transcripts of the molecules involved in the reduction of ferric iron to the ferrous state and in iron uptake steps at the apical membrane of the villus cells, Dcytb and DMT1, were regulated in a coordinated way, as can be deduced from the significant correlation between the two transcript levels over the range of dietary iron contents used in this study. Although FPN1 and TfR1 transcripts were also upregulated as a consequence of the decrease in body iron stores, the amplitudes of the variations were less remarkable. In contrast, transcripts levels of SFT, HFE and, in two strains, hephaestin, were not significantly modified by manipulating iron stores, which suggests that variations in their expression levels are not necessary for duodenal enterocytes to adjust iron absorption to the body's needs. Although it is possible that increased mRNA production does not result in increased protein production, this hypothesis is unlikely, at least for Dcytb, DMT1, and FPN1. Indeed, McKie and colleagues² observed that iron deficiency caused by feeding mice an iron deficient diet strongly induced both Dcytb mRNA levels and protein expression in duodenal extracts, and that ferric reductase activity was significantly higher in membranes prepared from CaCo-2 cells transfected with Dcytb than in membranes prepared from untransfected cells. Furthermore, Zoller and colleagues²¹ recently showed that, in humans, differences in DMT1 and FPN1 mRNA expression reflected corresponding changes in expression of the respective proteins in the duodenum.

Strain to strain variations in serum iron levels, transferrin saturation, and hepatic iron stores have been reported in the literature,^{12–14} suggesting that genetic factors control iron metabolism. As previously reported, our analysis of iron parameters in C57BL/6, DBA/2, CBA, and 129/Sv mice revealed marked differences, with serum transferrin saturations and liver iron stores varying about twofold among the different

inbred strains. As it was not known whether strain to strain variations could also be observed in the level of mRNA expression of the duodenal iron transporters, DMT1 and FPN1, as well as in the level of mRNA expression of the other molecules involved in iron absorption and expressed in the duodenum (Dcytb, hephaestin, SFT, HFE, and TfR1), we quantified the transcripts of these molecules in the four strains. Interestingly, the 129/Sv strain appears able to better maintain iron homeostasis than the other strains. Indeed, these mice are more resistant to dietary iron loading; they have lower hepatic iron stores although no significant decrease in expression of Dcytb and DMT1 mRNAs. They also have higher transferrin saturations when fed an iron deficient diet than other strains. In contrast, the CBA strain appears less prone to maintain iron homeostasis on dietary iron loading. Although CBA mice have the highest hepatic iron stores, they have no significant decrease in expression of DMT1 and FPN1 mRNAs. Finally, C57BL/6 mice, which have the lowest transferrin saturations among the four strains analysed, were not able to upregulate Dcytb and DMT1 transcripts in response to dietary iron deficiency as much as the other strains (3.9-fold upregulation *v* 16.9-fold in the other strains for Dcytb; 4.9-fold upregulation *v* 20.7-fold in the other strains for DMT1), despite having lower hepatic iron stores than the other strains. Interestingly, C57BL/6 mice did not significantly downregulate expression of DMT1 when fed an iron supplemented diet, despite having very high liver iron stores. The mechanisms by which expression of Dcytb, DMT1, and FPN1 are regulated in response to the needs of the body for iron and the reasons why their expression does not appear to be correlated to iron stores in C57BL/6 and CBA mice remain to be determined.

In conclusion, our results are in favour of some degree of genetic control of mRNA levels of these molecules. These genetic factors may well contribute to the phenotypic variability seen with disruption of the *Hfe* gene.^{15–17} There are indeed marked differences in levels of serum transferrin saturation and iron deposition in hepatic parenchymal cells in *Hfe* knockout mice, with C57BL/6 *Hfe*^{−/−} mice having the lowest propensity to accumulate iron of all of the *Hfe*^{−/−} strains studied to date. In addition, there appear to be differences in duodenal expression of DMT1 mRNA in *Hfe*^{−/−} mice of different genetic backgrounds.^{17,22} A recent study of a large community sample of mono- and dizygotic twins²³ demonstrated that genetic factors also influence iron metabolism in humans. Identification of genetic factors that regulate iron absorption in the mouse could thus provide new insights into the pathways that mediate the phenotypic variation in iron stores observed in humans.

ACKNOWLEDGEMENTS

This work was supported by EU (QLK6-CT-1999-02237). The authors thank Christine Mazeres for assessment of liver iron content, Michèle Stebenet (Service de Biochimie, Hôpital Purpan) for iron analyses, Claudie Offer (Service Commun de Séquençage, IFR30) for PCR product sequencing, and Maryline Calise (Service de Zootechnie, IFR30) for assistance in the mouse breeding.

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