Early Embryonic Lethality of H Ferritin Gene Deletion in Mice*  
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Ferritin molecules play an important role in the control of intracellular iron distribution and in the constitution of long term iron stores. In vitro studies on recombinant ferritin subunits have shown that the ferroxidase activity associated with the H subunit is necessary for iron uptake by the ferritin molecule, whereas the L subunit facilitates iron core formation inside the protein shell. However, plant and bacterial ferritins have only a single type of subunit which probably fulfills both functions. To assess the biological significance of the ferroxidase activity associated with the H subunit, we disrupted the H ferritin gene (Fth) in mice by homologous recombination. Fth+/− mice are healthy, fertile, and do not differ significantly from their control littersmates. However, Fth−/− embryos die between 3.5 and 9.5 days of development, suggesting that there is no functional redundancy between the two ferritin subunits and that, in the absence of H subunits, L ferritin homopolymers are not able to maintain iron in a bioavailable and nontoxic form. The pattern of expression of the wild type Fth gene in 9.5-day embryos is suggestive of an important function of the H ferritin gene in the heart.  

Iron is essential to all living organisms, but to prevent its toxicity it must be associated to specialized molecules. Of those, ferritins (Fts) play special roles because of their ubiquitous distribution in all tissues, to the tight iron-dependent gene expression, and to their capacity to store large amounts of iron (up to 4,000 Fe atoms per molecule) inside a large protein shell, in a nontoxic and bioavailable form (reviewed in Ref.1). Mammalian ferritins are heteropolymers made of two different subunit types named H and L. The H chain carries a ferroxidase center which appears to be essential for iron incorporation (2), whereas the L chain facilitates iron mineralization inside the cavity (3). In prokaryotes and plants, ferritins are made of 24 identical subunits which all carry the ferroxidase activity. In mammals, multiple transcriptional regulations operate which modify H ferritin mRNA levels in response to cytokines (4), heme (5, 6), oncogenes (7), or to cell proliferation or differentiation (reviewed in Ref 8). In addition, ferritin mRNAs have unique features which allow efficient (9) and tissue-specific (10) translational regulation according to the iron status of the cell. Therefore, the conservation of the ferritin ferroxidase activity throughout evolution as well as the very complex genetic regulation of ferritin expression suggest that this catalytic activity is essential for ferritin biological function.  

We disrupted the H ferritin (Fth) gene in mice and found that H subunit-associated ferroxidase is necessary for early embryonic development because no Fth−/− embryos were found after 3.5 days post coitus. Our data also demonstrate that L ferritin gene product cannot substitute for the H subunit. In contrast, heterozygous Fth+/− were healthy and indistinguishable from their control littersmates.  

EXPERIMENTAL PROCEDURES  

Disruption of the Fth Gene—A 128/Sv genomic library was screened with a radiolabeled probe corresponding to a 483-bp HindIII fragment from intron 1 of the murine Fth gene, and a phage clone containing a 13-kb insert was identified. To construct the targeting vector, a 1.5-kb 5′-homology PCR fragment from the transcription start site to the first 137 nucleotides of exon 2 was subcloned into a PGEM1Z with a modified polylinker upstream of a 4.8-kb IRES β-geo-poly(A) signal cassette (11). A HindIII 6.5-kb long-arm 3′-homology fragment starting from intron 2 was then subcloned downstream of the IRES β-geo. The resulting promoterless targeting vector (see Fig. 1A) was linearized at the 5′-end by ScaI, and 20 μg of plasmid DNA was introduced into CK35 ES cells (gift from C. Babinet, Institut Pasteur, Paris, France) by electroporation. After 10 days of G418 selection, surviving colonies were picked, expanded, and screened for homologous recombinants by Southern blotting using a 5′-external probe and a 3′-internal probe. The 5′-probe was a 900-bp EcoRI-ScaI fragment excised from the Fth proximal promoter region, and the 3′-probe was an 800-bp NsiI-ScaI fragment from the region immediately downstream of exon 4. Two positive ES cell clones among 77 were identified and injected into C57BL/6 blastocysts. Chimeric male mice obtained were then bred to C57BL/6 females to produce F1 heterozygous mice which were then interbred.

Embryo and Mice Genotyping—DNA from mouse tails and 9.5-day post coitus embryos were prepared by overnight lysis in a proteinase K-containing buffer followed by phenol extraction and ethanol precipitation. Genotyping on mouse tails was performed using two independent PCR: one designed to amplify wild-type allele between intron 1 and exon 3 and the other designed to amplify the Neo selection marker in the transgene. Primers used for these PCR were for wild-type: sense primer, 5′-TGGGTGCTTGGCAGTGAGATT-3′; and antisense primer, 5′-ATTGGATCCAGCGCCGCTC-3′; and for Neo transgene: sense primer, 5′-GGTTCCGGCGTTGGCGCTA-3′ and antisense primer, 5′-GTCTTGAGACCGTGCGGCAGC-3′. These PCR results were randomly verified by Southern blot analysis.  

Genotyping of 9.5-day post coitus embryos was performed by Southern blot as described above. For 3.5-day embryo genotyping, blastocysts were flushed out of uterus, washed with water, and then transferred into a 1°Eppendorf cup containing 10 μl of water and 7 μl of phosphate-buffered saline. Cell DNA was released by successive dry freezing and boiling steps followed by a 30-min incubation at 56 °C in the presence of 3 μl of proteinase K (10 mg/ml). A final incubation was done at 95 °C for 10 min, and samples were kept at ~80 °C. The whole lysate was used for multiplex PCR under standard conditions using the following primers: 5′-AGCATGGCGGAAAACGTGAGATGTAACAC-3′ (5′ common
FIG. 1. Targeted disruption of the Fth gene. A, structure of the wild-type allele, targeting vector, and recombined allele are shown together with XbaI (X) restriction sites. Dark boxes represent Fth exons, and dashed lines show area of homology between the vector and the endogenous gene. In recombined allele, transcription from the Fth start site (bent arrow) leads to a bicistronic mRNA containing two open reading frames. The first one is translated from the Fth AUG and corresponds to a truncated nonfunctional H ferritin lacking all amino acids after middle of exon 2 because of a stop codon upstream of the IRES β-geo cassette. The other one is translated from the internal ribosome entry site (IRES) and leads to a fusion β galactosidase reporter/neomycin resistance protein. B, quantification of H and L ferritin mRNAs by RNase protection assay in mouse erythroblastemia cells (MED), mouse duodenum, and ES cells. Five μg total RNA were hybridized with both the L and the H1 probe. The protected fragments were analyzed on 5% denaturing gels, and the dried gel was exposed to autoradiography for 1 h. C, Southern blot analysis of XbaI-digested genomic DNA from ES cell clones using 5' and 3' probes. The size of the XbaI fragment for the targeted allele is 6.4 kb for the 5' probe and 10.5 kb for the 3' probe versus 17 kb for the wild-type allele.

exon 2 sense primer); 3'antisense, 5'-TGAATGAAAACATCGGGTCAAGTC-3' (binding to intron 2 of the wild-type allele); and 3'antisense, 5'-AATTCTTGAGCGCCGACCTA-3' (binding to the selection cassette polylinker of the recombined allele). With the simultaneous addition of these three primers, all possible genotypes can be identified by the size of reaction products (299 bp for wild type allele and 139 bp for mutated sequence) on agarose gel electrophoresis.

RNase Protection Assay—Total RNA from cell pellets and tissues was isolated using RNAZole B (Bioprobe systems, France). For quantification of L-Ft mRNA, a genomic fragment containing exon 1 from the mouse L ferritin gene and 60 bp of promoter region was used to generate a 190-bp antisense RNA probe, using SP6 polymerase in the presence of [32P]UTP. Two different probes were used for quantification of H-Ft mRNA. The first probe, H1, was synthesized from a template consisting in exon 1 and 300 bp of promoter region, as described previously (6). The second probe, H2, was generated from a PCR fragment encompassing the last 116 bp from exon 2 and 17 bp of intron 2. Five μg total RNA were hybridized with 3.10^10 cpm of each probe in 80% formamide-hybridization buffer overnight at 55°C. Following RNase A-T1 and proteinase K digestion, the protected fragments were separated on a denaturing 8% polyacrylamide gel. Radioactivity associated with the bands was quantified using an Instant Imager (Packard Instrs.).

Enzyme-linked Immunosorbent Assay of Tissue H Ferritins—Tissues were homogenized in a 20 mM Tris-HCl, pH 7.4, buffer with protease inhibitors and sonicated three times for 2 min. After centrifugation, the supernatant was diluted in 0.05% Tween 20 in phosphate-buffered saline. The same polyclonal H-Ft specific anti-mouse subunit antibody was used for coating the plate and for labeling with horse-radish peroxidase. Standard curve was made with recombinant mouse H ferritin polymers.

Hematological Analysis of Heterozygous Mice—Heparinized blood was obtained by direct aortic puncture under anesthesia. Blood cell counts and erythrocyte parameters were determined using an automated Technicon H1 analyzer.

For bone marrow cell count, femoral cavity from adult mice (12- to 26-week-old) were washed with 10 μl of physiological serum. Cell suspensions were spread on slides and stained by May-Grünwald Giemsa coloration, and cell types were scored microscopically according to their morphology. A total of 300 cells was counted for each bone marrow.

Galactosidase Activity in Embryos—Whole mount embryos were fixed in 4% paraformaldehyde in phosphate buffer saline at 4°C for 30 min. They were then rinsed for 45 min in three successive baths of 50 mM phosphate buffer containing 2 mM MgCl2, 0.1% deoxycholate, and 0.02% Nonidet P-40. After a final rinse in phosphate buffered saline, β galactosidase activity was revealed by incubating the embryos in the dark for various periods of time in phosphate buffer saline containing X-gal at 1 mg/ml, 5 mM K3[Fe(CN)6], 5 mM K4[Fe(CN)6], 3H2O, 2 mM MgCl2, and 20 mM Tris-HCl, pH 7.5.

RESULTS

Targeted Deletion of Murine H Ferritin Gene—A null Fth allele was generated by deleting the second half of exon 2 and part of intron 2 with the simultaneous “knock-in” of a promoterless internal ribosome entry site (IRES) β-geo-poly(A) signal cassette (11). This gene-targeting strategy allows the expression of a bi-cistronic mRNA under the control of the target gene promoter region and the IRES of the encephalomyocarditis virus allows cap-independent translation of the β-geo fusion gene containing the β-galactosidase (Lac Z) and the neomycin resistance (Neo+) markers (Fig. 1A). The choice of a promoterless targeting vector was based on the observation made by RNase protection assay (Fig. 1B), that the Fth gene is actively
transcribed in embryonic stem (ES) cells. The amount of H mRNA in ES cells is comparable with that in mouse erythro-leukemia cells, where the Fth gene transcription is high (6, 12). CK35 ES cells were electroporated and selected in G418, and cells carrying the disrupted allele were identified by Southern blotting (Fig. 1C) and used to produce mice heterozygous for the Fth mutation in a mixed (129/sv × C57BL/6) genetic background. Quantification of H and L ferritin mRNAs by RNase protection assay performed on several organs confirmed that the disrupted allele is nonfunctional. There was a 2-fold reduction in H-Ft mRNA in all tissues of Fth+/− mice as compared with their wild type littermates, whereas L-Ft mRNA was not modified (Fig. 2A). Considering that ferritin synthesis is under the control of an iron-mediated translational regulation (reviewed in Ref.13), it was important to confirm that Fth haploinsufficiency resulted in a reduced amount of H-Ft protein. We performed enzyme-linked immunosorbent assay on tissue extracts using H-Ft specific anti mouse ferritin antibodies and recombinant H-Ft homopolymers for calibration. The results demonstrate that the amount of H-Ft subunit which accumulates in mouse tissues is also decreased in Fth+/− mice as compared with wild type littermates.

Phenotype of H-Ft-deficient Mice—Fth+/− mice were generally indistinguishable from their control littermates; they were fertile and grew normally. Furthermore, no gross tissue abnormality nor obvious sign of fibrosis or oxidative stress damage was observed in any tissue, as shown by extensive histopathological studies. These analyses included the liver, spleen, heart, lung, kidney, duodenum, brain, pancreas, ovaries, stomach, and femur bone marrow, at different stages up to six month of age.

A fine regulation of iron homeostasis is also essential for erythropoiesis. H-Ft has been shown to be up-regulated during erythroid cell differentiation (12, 14) and to be necessary to maintain iron bioavailability for hemoglobin synthesis (15). The tight regulation of the H ferritin locus. At 9.5 days of development, X-gal staining was ubiquitous, the strongest transcriptional activity of the H ferritin locus. At 9.5 days of development, X-gal staining was low, but easily detectable after 3 h of incubation, mostly in the developing heart and in the central nervous system (Fig. 4). A longer incubation period did not noticeably change the pattern of expression. At later stages of development, X-gal staining was ubiquitous, the strongest staining being observed in the heart and in brown fat tissue (not shown).

**Table I**

| Genotype | Blood parameters | Bone marrow differential counts |
|----------|------------------|--------------------------------|
|          | RBC | WBC | Hb | HBC | Granulocytes | Erythroid cells | Lymphocytes | Monocytes |
| Wild type | 6.8 ± 0.3 | 1.9 ± 0.6 | 13.8 ± 0.4 | 42.8 ± 1.7 | 66 ± 10 | 18.5 ± 3.8 | 13 ± 9 | 1 ± 1 |
| Fth+/−    | 8.8 ± 0.3 | 2.8 ± 0.7 | 13.9 ± 0.6 | 43.7 ± 1.5 | 73 ± 6.6 | 15.5 ± 6.4 | 8.6 ± 1.5 | 2.4 ± 0.6 |

Values are mean ± S.E. We determined blood cell indices on 6- to 26-week-old wild type (n = 14) and Fth+/− (n = 20) animals. RBC, red blood cells number (×10⁶/ml); WBC, white blood cells (×10⁷/ml); Hb, hemoglobin (g/dl); HBC, hematocrit (%). Bone marrow differential counts are expressed as % of total bone marrow cells ± S.E., after counting 300 cells for wild type (n = 7) and Fth+/− (n = 8) mice.

**FIG. 3.** Embryonic lethality of a homozygous null Fth allele. A, genotypes of offspring from intercrosses of Fth+/− mice. Genotypes were determined by PCR and/or Southern blotting as described in the legend to Fig. 1 B, multiplex PCR of genomic DNA from 3.5-day blastocysts. The results of one representative experiment are shown with the 299-bp fragment corresponding to the wild-type allele and the 139-bp fragment corresponding to the recombined allele. Lane 1, negative control (no DNA added); lane 2, positive control (tail DNA from heterozygous animal); lane 3, 100-bp ladder; lanes 4–9, amplification products from blastocysts DNA. Resulting genotypes are given below each lane.

**DISCUSSION**

This paper demonstrates that H ferritin subunit is nonindispensable for embryonic development because a complete lack of this protein leads to early embryonic death. In addition, there is no redundancy between the H ferritin biological function and either the L subunit or any other protein.

In Fth+/− mice, there was no evidence that the remaining wild type allele was up-regulated either at the transcriptional or translational level, to compensate for the disrupted allele. The tight regulation of the Fth gene transcription which operates in multiple conditions is assumed to protect cells against iron-mediated oxidative injury (16), through rapid chelation of the labile iron pool (17). We therefore expected that H ferritin deficit might result in increased intracellular labile iron pool and tissue damage. The lack of obvious phenotype of Fth+/− mice could be because of the synthesis of H subunit in amounts sufficient to ensure the formation of functional ferritins. In *in vitro* studies indicated that the ferroxidase activity conferred by 1–2 H subunits per ferritin molecule is sufficient to make it functional, and the ferroxidase activity conferred by 8–9 H...
subunits per molecule is enough to reach a maximum rate of iron incorporation (18). This implies that the physiological amount of H subunits in tissues is not limiting.

Death of Fth−/− embryos could result from a modification of intracellular iron availability during the critical period, which spreads developmental stages of implantation, gastrulation, and early organogenesis. The autonomous growth of the embryo from fertilization to the 3.5 day blastocysts may rely on the use of maternal ferritin-iron present in the oocyte. Ferritin is a very stable molecule, and rat liver ferritin has a half-life of 1–2 days (19). Because Fth−/− embryos survive at least to the 62-cell stage, they should have enough iron to ensure DNA replication and heme synthesis. Indeed, disruption of uroporphyrinogen III synthase, an intermediate enzyme in the heme biosynthetic pathway, is lethal between 2- and 4-cell stage, suggesting that heme synthesis takes place at very early stages (20).

However, after degradation of all maternal ferritin, the newly synthesized ferritins will lack the H-linked ferroxidase activity and will consist in L subunit homopolymers which are not competent for iron incorporation (21). Thus, the absence of a functional intracellular ferritin compartment is lethal to embryonic cells, possibly because iron entering into the cells cannot be sequestered and detoxified, leading to the catalytic Fenton-driven overproduction of reactive oxygen species which are deleterious for all cell components including membranes and DNA (16). In addition, the H-containing ferritins may be essential for making iron available to essential enzymes and to DNA replication. These mechanisms are not mutually exclusive because ferritin-deficient mutants of Campylobacter jejuni have impaired growth because of iron deficiency and are more sensitive to killing by H₂O₂ (22). During organogenesis, the absence of functional ferritins will also impair iron storage. Early in development, the primitive embryonic erythroid cells in the yolk-sac are the site of iron deposition and contain high amounts of ferritins (23, 24), before the liver becomes the major site of iron storage at a later stage. However, our observation that the highest level of H ferritin gene expression at 9.5 days of gestation is found in the heart suggests that the embryos could also die of heart failure because of massive iron deposition.

To date, from the various mouse models with disrupted genes of iron metabolism, embryonic lethality seems to result from iron deficiency associated with impaired iron storage (Fth) or transport because transferrin receptor (TfR) knock-out embryos die between 8.5 and 12.5 days of development, mostly from defective erythropoiesis and neurological abnormalities (25).

Taken together with the fact that H-type ferritins are present in plants and throughout the animal kingdom, our results demonstrate that the ferroxidase activity of the ferritin molecule, which is the only known cytoplasmic activity which can transform the more toxic Fe(II) into a less toxic Fe(III), is essential for detoxification of iron and/or for maintaining its bioavailability.

The benefit of having two different H and L subunits, as it is the case in humans and other vertebrates, remains to be analyzed, and the study of L ferritin knock-out mice might bring some information on this point.

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