Frataxin overexpressing mice

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Abstract Friedreich ataxia, the most common autosomal recessive ataxia, is caused by frataxin deficiency. Reduction of frataxin has been associated with iron accumulation and sensitivity to iron induced oxidative stress. To better understand the function of frataxin, transgenic mice (tgFxn) overexpressing human frataxin were generated. Iron metabolism parameters in tgFxn were normal and no signs of ataxia or other obvious abnormalities were observed, indicating that overexpression of frataxin in mouse is innocuous. Several hypotheses for frataxin function were evaluated in tgFxn mice. In particular, we observed that TgFxn mice show an altered response during hematopoietic differentiation, suggesting that frataxin may directly affect heme synthesis.

Keywords: Friedreich ataxia; Frataxin; Iron; Doxorubicin; Phenylhydrazine; Heme

1. Introduction

Friedreich ataxia (FA) is the most common autosomal recessive ataxia in Caucasians, with an estimated prevalence in this group of 2–4 per 100 000 individuals [1–3]. The major cause of this disease is the presence of a large (GAA)*n* repeat expansion in the first intron of the FA gene, FRDA [4]. When present, this large (GAA)*n* repeat decreases the transcription of the mRNA encoding the protein frataxin [5,6] to levels ranging from 5% to 30% of normal [7].

FA patients develop progressive ataxia around puberty, with gait ataxia and generalized clumsiness being the most common initial symptoms [8]. The main pathological changes occur in the dorsal root ganglia, with loss of large sensory neurons, degeneration of spinocerebellar tracts, and atrophy of the large myelinated sensory fibers of peripheral nerves [8]. A hallmark of the disease is the observed iron accumulation in cardiomyocytes in FA patients [9].

Frataxin is a nuclear-encoded mitochondrial protein expressed in every cell type, but its level of expression varies widely in different tissues and during development. Frataxin mRNA is most abundant in the heart and spinal cord, followed by the liver, skeletal muscle, and pancreas [4]. The distribution of frataxin mRNA in the mouse is in good accordance with the pattern of distribution observed in adult human tissues [10,11]. Northern blot and RNA in situ hybridization studies in mouse embryos showed expression starting at day E10.5, reaching its highest level at day E14.5 and into the postnatal period [10,11]. Embryonic lethality at day E6.5 of homozygous frataxin knockout mice indicates an even earlier expression of frataxin, and suggests an important role during early embryonic development [12].

Although the precise function of frataxin remains unknown, it has been known now for long now that frataxin in yeast plays an important role in the maintenance of mitochondrial iron homeostasis [13–17] and cellular respiration [18,19]. Disruption of the yeast homolog (ΔYFH1) leads to a 10-fold increase in mitochondria iron accumulation compared to wild-type yeast, loss of mitochondrial DNA, and inability to carry out oxidative phosphorylation [13,16]. Importantly, human frataxin complements ΔYFH1, suggesting that frataxin function is conserved [14]. Further studies showed that frataxin deficiency leads to excessive free radical production in mitochondria and dysfunction of iron–sulfur cluster (ISC) containing enzymes (in particular, respiratory complexes I, II and III, and aconitase) [19]. Frataxin's specific function remains, however, still elusive. An early step of ISC synthesis, which takes place on the scaffold protein Isu1, is greatly enhanced by frataxin [15]. It has been proposed that frataxin directly binds iron [16], shielding it from free radicals and making it available for ISC synthesis. It is unclear whether this postulated chaperone function is specific to ISC synthesis. Data in yeast indicate that, at least in this organism, heme synthesis may also be stimulated by frataxin, suggesting a more general role in mitochondrial iron handling [20]. Alternative hypotheses view frataxin as a stabilizer of a complex including Isu1 and the nascent ISC [21], as a protein with a primarily antioxidant function [22], as an activator of the respiratory chain [23].

Cellular and animal models of reduced frataxin expression have been developed to better understand the precise molecular mechanisms that cause FA and find a therapy for this

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Abbreviations: FA, Friedreich ataxia; tgFxn, transgenic mice overexpressing frataxin; DOX, doxorubicin; PHZ, phenylhydrazine; ISC, iron–sulfur cluster

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disabling disorder. However, only a few studies investigated the effects of frataxin overexpression, which may be relevant not only for the understanding of its function, but also to design gene therapy approaches. Some studies were carried out in cultured cells [23,24]. As for animal models, a transgenic mouse line carrying a 370 kb yeast artificial chromosome containing the human frataxin gene was generated [25]. The tissue distribution and levels of expression of frataxin from this transgene resemble those of the endogenous gene, as expected with a construct containing all elements which are known to regulate gene expression, including the original human frataxin promoter, exons, and introns. This transgene also includes an additional gene and part of two other genes, which might influence the results of future experiments. More recently, three independent transgenic mouse lines were generated with the FRDA gene in an 188-kb bacterial artificial chromosome genomic sequence [26]. Three copies of the transgene per diploid mouse genome were integrated in a single site in each mouse line. This construct could rescue the embryonic lethality consequent to the disruption of the mouse frataxin gene (Frda). Also in this model and in all three lines, the relative expression of the human FRDA and mouse Frda genes showed a similar pattern in different tissues.

For the present study, we generated transgenic mice with ubiquitous and constitutive overexpression of frataxin by using an artificial promoter and frataxin cDNA, which might be a more realistic gene structure for a potential gene therapy than the entire gene. Iron metabolism, response to iron challenge, iron-related oxidative damage, and erythropoietic stress were all evaluated in this animal model.

2. Materials and methods

2.1. Generation of transgenic mice overexpressing frataxin

The Institutional Animal Care Committee reviewed and approved all procedures, which were performed in accordance with the Canadian Council on Animal Care guidelines. To generate transgenic mice overexpressing frataxin (tgFxn), the full-length human frataxin cDNA was inserted in the *Eco*RI site of the pCI-neo vector (Promega, Madison, WI, USA) that carries the human immediate-early enhancer/promoter cytomegalovirus (CMV) promoter. To prepare the transgene (MP214), the vector was digested with the enzymes *Alw*NI and *Dra*III, generating an approximately 3-kb fragment. The MP214 transgene was then microinjected into pronuclei of fertilized mouse oocytes (C57Bl/6J × C3H), which were then transplanted into foster mothers. Tail DNA from the resulting 29 offspring was analyzed by Southern blot and PCR. For Southern blot analysis, 10 μg of DNA was digested with *Hin*fI, run overnight in a 0.8% agarose gel, and transferred to Hybond-N^+ nylon membrane (Amersham – Life Science, Arlington Heights, IL, USA) using standard procedures [27]. After UV cross-linking, blots were hybridized with the MP214 *Eco*RI digested fragment that was labeled by random priming using the Red prime Labeling System (Amersham Biosciences). Membranes were exposed to X-ray film at −80 °C for 24–48 h. For PCR analysis, primers PCI-1206F (5′-GTGGTTTGTCCAAACTCATCAATG-3′) and Exon4-939R (5′-CTAGGAACCTATGTGATCAACAAG-3′) were used to identify the presence of the transgene. As an internal control for the PCR, the endogenous locus *Dystonia musculorum* (*dt*) [28] was amplified using primers RAS-57 (5′-GGCAGCTGTCTTTGCTTCTGTGCACCC-GAAG-3′) and RAS-58 (5′-GGGCGCGCTCTGCACCAGCTG-TTACAGTAC-3′). Three transgenic founders were identified and backcrossed into the C57Bl/6 background.

2.2. Quantification of the number of inserted copies

Genomic DNA (10 μg) from the F1 progeny of transgenic positive mice was digested with *Sac*I and analyzed by Southern blot. Blots were hybridized sequentially to a 0.9 kb *Eco*RI fragment from the MP214 transgene and a 1.3 kb *Sac*I fragment from the *dt locus* [29]. The inserted copy number was determined by analyzing autoradiograms with Scion image software (Scion Corporation, MD, USA). The intensity of each frataxin band was corrected according to the intensity of the endogenous *dt* band.

2.3. Western blot analysis

Tissues were homogenized in 10 volumes of Laemmli buffer and adjusted to a final protein concentration of 2 μg/μl, using the Sigma protein assay following the manufacturer's protocol (Sigma, St. Louis, MO); 15 μg of each homogenate was loaded onto a 12% Tris–glycine SDS–polyacrylamide gel, run by electrophoresis and then blotted onto a Hybond-C super membrane (Amersham). Blots were probed with a monoclonal antibody to frataxin (Chemicon, Temecula, CA, USA) that recognizes both human and mouse frataxin, as well as with a monoclonal antibody to β-tubulin (Calbiochem, S. Diego, CA, USA) or to β-actin (Abcam, Cambridge, UK) to control for protein loading. Specific signals were visualized by chemiluminescence. Quantification of frataxin expression was determined by analyzing autoradiograms with Scion image software. The intensity of each frataxin band was corrected according to the intensity of the β-tubulin (or β-actin) band for that same lane.

2.4. Transferrin saturation and erythroid parameters

Hemoglobin (Hb), hematocrit (HCT), red blood cells (RBC), and mean corpuscular volume (MCV) were determined in EDTA-treated blood samples using an automated blood counter (ABX hematology, Montpellier, France). Serum iron (SI) and total iron-binding capacity (TIBC) were measured using a Kodak Ektachem DT60 instrument (Johnson & Johnson, Clinical Diagnostics, NY, USA). Transferrin saturation (TS) was calculated from the TIBC and SI values.

2.5. Dietary iron-loading

Dietary iron-loading was achieved by placing six-weeks-old mice on an iron-enriched diet containing 3% (wt/wt) carbonyl iron (Sigma) for a period of one month.

2.6. Measurement of tissue iron levels

Organ samples were weighted wet, dried overnight at 106 °C and then weighted again. Dried samples were ashed in an oven at 500 °C for 17 h, solubilized in 6 N HCl, and the final solution was adjusted with demineralized water to a final concentration of 1.2 mol/L HCl. Iron concentration was determined by flame atomic absorption spectrometry (AAS).

2.7. Histology

Tissue samples from liver, spleen, heart and pancreas were fixed in buffered 4% formaldehyde and embedded in paraffin. Tissue sections were stained with hematoxylin/eosin and trichrome blue for detection of fibrosis. Ferric iron (Fe(III)) was detected by Prussian blue staining.

2.8. Doxorubicin treatment

Doxorubicin (DOX) can bind to iron forming a highly reactive complex that ultimately leads to an increase in oxidative stress. The major site of iron–DOX interaction has been identified within mitochondria and the heart has been shown to be the organ most severely affected [30–33]. Since frataxin can possibly act as a detoxifying agent of reactive-oxygen species [34–36], or as a sequester of iron excess in the mitochondria [15], the possible protective role of frataxin in DOX-induced cardiotoxicity was analyzed. Eight-weeks-old tgFxn line #3 and normal controls were injected i.p. with DOX hydrochloride (2 mg/ml in saline, Sigma) at 25 mg/kg. At day 4, animals were anaesthetized with 65 mg/kg pentobarbital and blood was collected by cardiac puncture. Serum creatine kinase (CK) and lactate dehydrogenase (LDH) activity were determined by colorimetric assay using a Kodak Ektachem DT60 instrument (Johnson & Johnson).

2.9. Phenylhydrazine treatment

Acute treatment of mice with phenylhydrazine (PHZ) causes hemolytic anemia, triggering active erythropoiesis in the spleen. In order to assess whether frataxin expression changes with erythropoiesis in vivo, wild-type mice were treated with PHZ (i.p. 60 mg/kg body weight) for two consecutive days. Spleens were collected for protein extracts and flow cytometry analysis at day 4 and 6 after treatment and from mice without treatment as controls. In addition, to determinate if

the increase in frataxin levels was correlated with a specific cell population, flow cytometry was performed on the same samples used to prepare the protein extracts. Spleens of mice treated with saline were used as controls. Cell suspensions were obtained by passing the tissue through a 40 μm filter followed by centrifugation. Cells were resuspended in PBS and stained with anti-rat Ter119 and CD71 mAbs (PharMingen, San Diego, CA), and analyzed by flow cytometry with a Coulter Epics (Coulter). The transferrin receptor (CD71), expressed at high levels in early erythroid precursors, and the cell-surface erythroid-specific Ter119 antigen, expressed in terminally differentiating erythroblasts were used to discriminate between the different erythroid cell populations [37]. In the spleen, pro-erythroblasts express high levels of CD71 and medium levels of Ter119 ($CD71^{high}/Ter119^{med}$), basophilic erythroblasts highly express both markers ($CD71^{high}/Ter119^{high}$), while late basophilic and polychromatophilic erythroblasts continue to express high levels of Ter119 but show a reduction on CD71 expression ($CD71^{med}/Ter119^{high}$) [37].

Additionally, response to hemolytic anemia of transgenic mice as well wild-type mice was determined by hematological parameters. Eight-weeks-old tgFxn lines #1 and #2, and normal controls were injected i.p. with 6 mg/Kg PHZ (total of 60 mg/kg body weight/day, Sigma), for two consecutive days. Blood was collected for hematological analyses at day 4, 6 and 11.

2.10. Statistical analysis

Results are presented as means ± S.D. or means S.E.M., as indicated. Student's *t* test was used for comparison between the control and knockout mouse groups and between treatments. The level of significance was pre-set at $P = 0.05$.

3. Results

3.1. Generation of tgFxn mice

TgFxn mice were generated by introducing full-length human frataxin cDNA, encompassing exons 1–5a, into the *Eco*RI cloning site of the PCI-neo vector (Fig. 1(a)). This vector contains the CMV promoter, which drives constitutive expression of cloned DNA inserts in mammalian cells. Out of the 29 offspring born after microinjection, 5 were positive for the insertion of the transgene, as determined by Southern blotting (Fig. 1(b)) and PCR analysis (Fig. 1(c)). Germ line transmission was observed in 3 independent mouse lines (referred as tgFxn lines #1, #2, and #3). The number of copies inserted was variable among the different lines, ranging from 1 to 6 (Fig. 2). Each generation of tgFxn mice has reproduced effectively to give normal-sized litters.

3.2. Protein levels and motor coordination in tgFxn mice

Frataxin protein levels were estimated by Western blot analysis in the heart, brain, pancreas, skeletal muscle, spleen, kidney, lung and liver. In these experiments, the transgene-encoded human frataxin band overlaps the endogenous mouse frataxin band, also recognized by the antibody, so the levels of transgene expression had to be estimated by comparison with wild-type, non-transgenic animals. Not unexpectedly, each line showed a different pattern of frataxin overexpression, which is likely to be related to position effects consequent to the random insertion of copies of the transgene in the mouse genome. However, with the exception of brain in line 2, increased frataxin expression could be obtained in all organs where mouse frataxin mRNA is normally more abundant [10,11], such as heart, brain, muscle and pancreas (Fig. 3). These are the organs affected in FA patients and therefore represent targets for gene replacement therapy.

In order to determine whether overexpression of frataxin would affect motor coordination and balance in mice, rotarod analysis was performed. Similar performance on the rotarod was observed in wild-type and in tgFxn mice up to one-year of age (data not show), well above the corresponding age of onset in humans (one-year-old mouse is comparable in terms of age to a 40–50-years-old human). These results indicate that increased frataxin levels are not associated with any obvious neurological phenotype, at least up to the age studied.

3.3. Iron metabolism in tgFxn mice

In order to study the effect of frataxin overexpression on iron metabolism, several hematological and iron parameters were analyzed. Values of Hb, HCT, red blood cells, and MCV were all within the normal range in tgFxn mice compared with wild-type mice (Table 1). Levels of SI and TS were also determined, and similar values were found in transgenic mice and in their wild-type littermates (Table 1). Similar levels of tissue

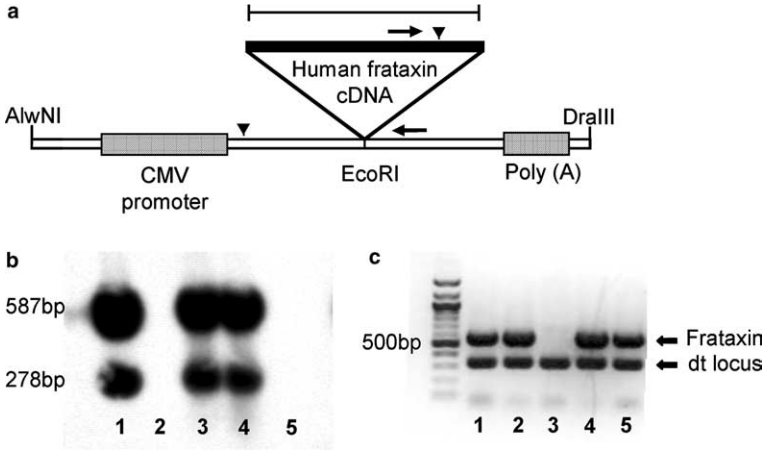
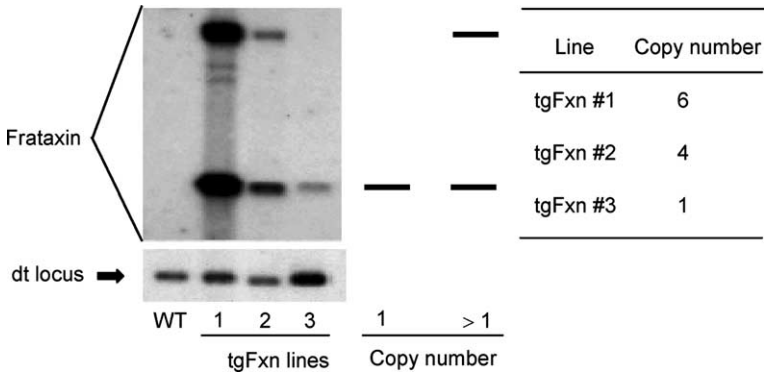
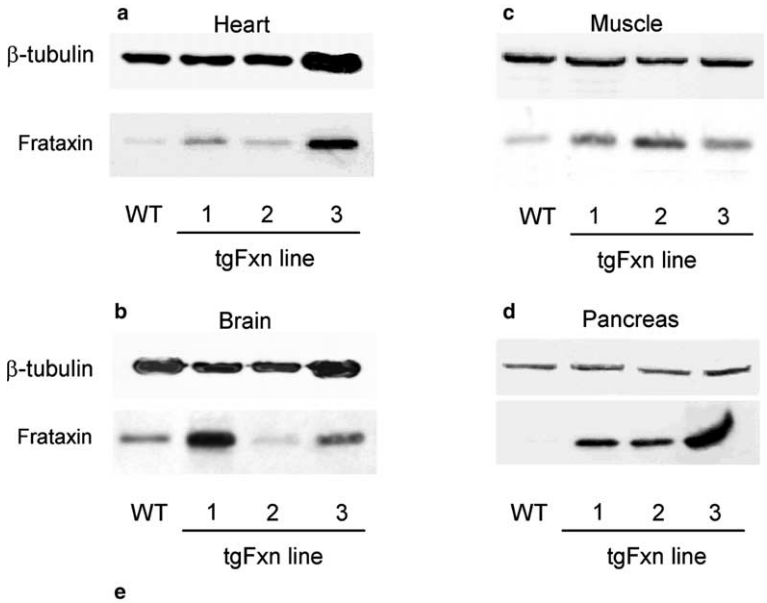


Fig. 1. (a) Structure of the MP214 construct. Arrows represent primers Exon4-939F and PCI1206R, used for PCR genotyping. Arrowheads represent *Sac*I restriction sites, used to determine the inserted copy number by Southern blot. The bracketed area represents the fragment used as a probe for Southern blot analysis. (b) Identification of mice carrying the human frataxin transgene, by Southern blot analysis of *Hin*fI digested genomic DNA. Lanes 1, 3, and 4 are transgenic mice ($tgFxn^{+/-}$); lanes 2 and 5 are wild-type littermates. (c) PCR analysis of the $tgFxn^{+/-}$ offspring. Only transgenic positive mice show a band of 568 bp (lanes 1, 2, 4 and 5). The 300 bp band is a control band for the PCR, resulting from the amplification of the endogenous *dt* locus. Lane 3 is a wild-type littermate.



Line	Copy number
tgFxn #1	6
tgFxn #2	4
tgFxn #3	1

Fig. 2. Quantification of inserted copy number in tgFxn$^{+/-}$ mice: Southern blot analysis of genomic DNA, digested with *Sac*I and probed with human frataxin cDNA (upper blot). Quantification of endogenous *D. musculorum* was used as control (lower blot). WT, wild-type; 1, transgenic line #1; 2, transgenic line #2; 3, transgenic line #3. The inserted copy number was obtained after comparing the intensity of each frataxin band and correcting according to the intensity of the control endogenous band.



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Line	Increase in frataxin compared with wild type mice			
	Heart	Brain	Pancreas	Muscle
tgFxn #1	4x	3x	>10x	3x
tgFxn #2	3x	0	>10x	6x
tgFxn #3	6x	2x	>10x	3x

Fig. 3. Frataxin levels are increased in frataxin tgFxn$^{+/-}$ mice. (a) Heart; (b) brain; (c) muscle and (d) pancreas. Crude protein extracts were probed with antibodies to frataxin and β-tubulin. WT, wild-type; 1, transgenic line #1; 2 transgenic line #2; 3, transgenic line #3. (e) Quantification of frataxin expression levels.

iron were found in heart, liver, pancreas and spleen of wild-type and tgFxn$^{+/-}$ mice (Table 2). Histological analysis did not reveal iron or collagen deposition in any of the examined organs (data not shown).

Next, the response of tgFxn$^{+/-}$ mice to iron challenge was studied. Six-weeks-old tgFxn$^{+/-}$ mice were placed on a 3% (wt/wt) carbonyl-iron-supplementation diet for a period of one month. Analysis of tissue iron concentration clearly shows that iron loading was achieved in both animal groups. When comparing mice on a regular diet to mice receiving the iron-supplemented diet, both tgFxn$^{+/-}$ and wild-type mice showed a statistically significant increase in iron concentration in all organs with the exception of the heart (Table 2). Taken together, these results demonstrate that higher frataxin levels in different organs do not alter systemic iron metabolism in mice.

Table 1
Erythroid parameters, SI and TS

Mouse	*n*	Hb (g/L)	MCV (fl)	HCT (%)	RBC ($\times 10^{12}$/L)	SI (μmol/L)	TS (%)
Wild-type	13	134 ± 22	47 ± 2	43 ± 6	9.1 ± 1.3	23 ± 8	42 ± 14
tgFxn, line #1	8	136 ± 19	45 ± 1*	41 ± 6	9.0 ± 1.3	25 ± 5	50 ± 11
tgFxn, line #2	5	114 ± 23	45 ± 1	38 ± 7	8.4 ± 1.4	17 ± 3	41 ± 5
tgFxn, line #3	5	133 ± 8	49 ± 3	43 ± 3	8.7 ± 0.3	23 ± 3	38 ± 7

Data are presented as means ± SD. n, number of animals; Hb, hemoglobin; MCV, mean corpuscular volume; HCT, hematocrit; RBC, red blood cells; SI, serum iron; TS, transferrin saturation. Mice were all three-months-old.
* $P < 0.05$ compared with wild-type.

Table 2
Effects of iron-challenge on tissue iron concentration

Mice	Treatment	*n*	Liver	Spleen	Heart	Pancreas
			μg Fe/g dry weight			
Wild-type	–	13	203 ± 50	1028 ± 294	327 ± 30	75 ± 12
tgFxn, line #1	–	8	197 ± 34	913 ± 236	346 ± 42	79 ± 5
tgFxn, line #2	–	5	215 ± 45	630 ± 145*	360 ± 20	79 ± 14
tgFxn, line #3	–	8	189 ± 28	1247 ± 387	379 ± 40**	74 ± 11
Wild-type	CI	6	842 ± 144	2840 ± 867	333 ± 11	149 ± 43
tgFxn, line #1	CI	7	703 ± 159	2412 ± 837	322 ± 45	121 ± 23
tgFxn, line #2	CI	6	758 ± 166	2412 ± 386	355 ± 45	129 ± 16
tgFxn, line #3	CI	4	1301 ± 194	2887 ± 927	319 ± 9	146 ± 22

CI, carbonyl-iron-supplemented diet. Data are presented as means ± S.D. *n*, number of animals. Mice were three-months-old.
* $P < 0.05$ compared with wild-type.
** $P < 0.01$ compared with wild-type.

3.4. Iron-related doxorubicin cardiotoxicity in tgFxn mice

Transgenic mice from line #3 (with the highest levels of frataxin in the heart) and wild-type mice were treated i.p. with 5 mg/kg of DOX (total dose of 25 mg/kg body weight). Four days after treatment, levels of serum CK and LDH, both markers for heart and skeletal muscle damage, were measured. DOX treatment significantly increased serum levels of CK and LDH, demonstrating muscle damage both in tgFxn$^{+/-}$ and wild-type mice (Fig. 4); however, similar levels of serum CK and LDH were found in tgFxn$^{+/-}$ and wild-type mice treated with DOX (Fig. 4). These results show that overexpression of frataxin does not protect from the cardiotoxicity induced by DOX treatment.

3.5. Erythropoietic stress in tgFxn$^{+/-}$ mice

Treatment with PHZ resulted in a dramatic increase in spleen size (up to 6 times, data not shown), accompanied by an increased number of all cells from the erythroid lineage (Table 3). Protein extracts obtained from wild-type mice treated with PHZ ($n = 2$ per time point) showed a variable frataxin expression at day 4, followed by a 6-fold increase at day 6 (Fig. 5(b)). Flow cytometry analysis of spleen cellular extracts from mice treated with PHZ showed an increase in cell populations expressing CD71 or Ter119. Modest increase was observed in cells expressing CD71$^{\text{med or low}}$/Ter119$^{\text{high}}$ (4-fold at day 6) and cells expressing CD71$^{\text{high}}$/Ter119$^{\text{med or low}}$ (9-fold at day 6), and a remarkable increase in CD71$^{\text{high}}$/

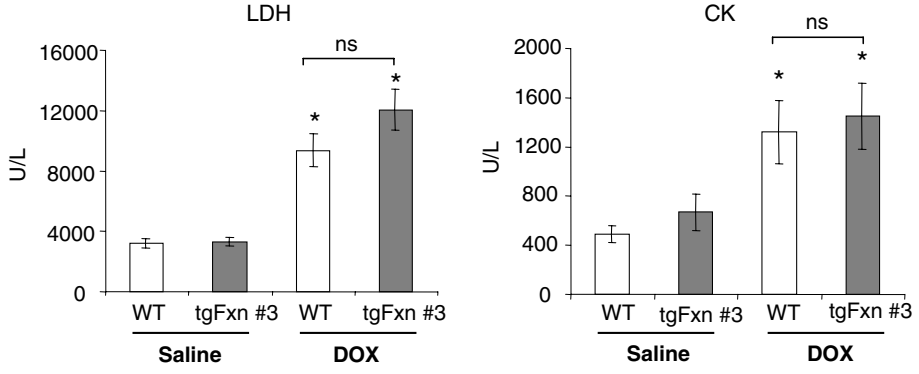

Fig. 4. DOX-induced increase in LDH and CK activity. While DOX induced a statistically significant increase of both enzymes compared to saline-injected mice, no difference was observed in the response of wild-type and tgFxn mice. WT (saline), wild-type mice injected with saline solution; WT (DOX), wild-type mice injected with 25 mg/kg body weight of DOX; TG (saline), transgenic mice from line #3 injected with saline solution; TG (DOX), transgenic mice from line 3 injected with DOX. Values represent means ± S.E.M. from 8 mice for each treatment group. Student's *t* test was used for comparison between saline and DOX treated mice (* $P < 0.05$). ns, Not significant.

Table 3
Frataxin expression and erythroid differentiation in the spleen of wild-type mice, after treatment with PHZ

Day	Mouse #	(%)				
		Frataxin expression	CD71^{neg}/ Ter119^{neg}	CD71^{pos}/ Ter119^{neg}	CD71^{pos}/ Ter119 ^{pos}	CD71^{neg}/ Ter1119^{pos}
D0	1	100	93.8	4.1	0.1	1.9
	2	100	93.7	2.2	0.1	4.0
D4	3	50	86.7	1.4	2.4	9.5
	4	200	64.6	4.0	17.8	13.8
D6	5	600	40.4	23.3	28.3	8.0
	6	700	28.2	32.6	25.3	13.9

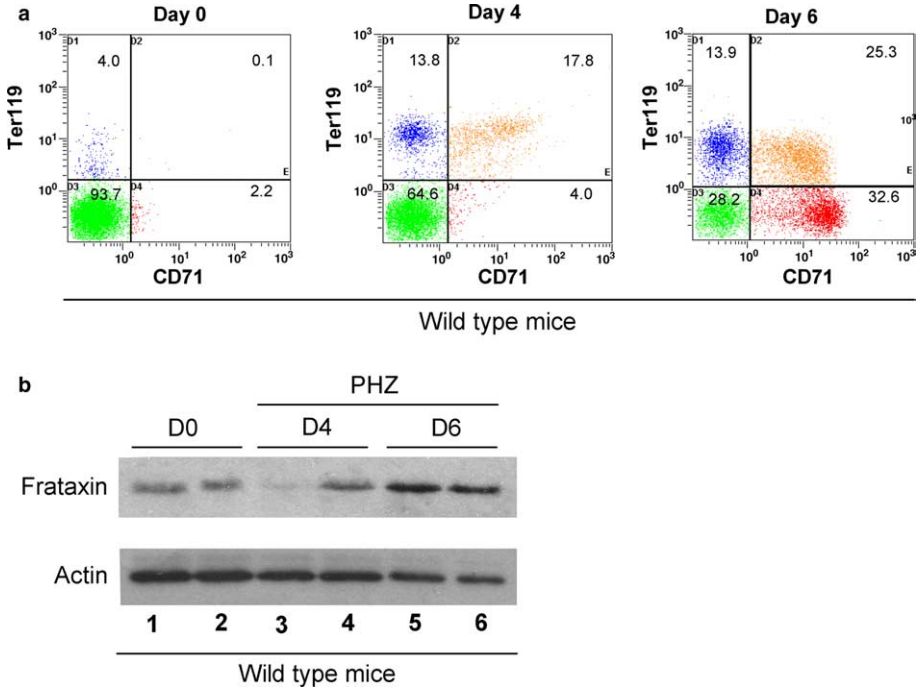

Fig. 5. Effect of PHZ treatment on frataxin levels and erythroid differentiation in the spleen of wild-type mice. (a) Flow cytometry assessment of spleen erythroid differentiation. PHZ treatment causes an increase in proerythroblasts (CD71^{high}/Ter119^{med}, blue), erythroblasts (CD71^{high}/Ter119^{high}, yellow) and reticulocytes/erythrocytes (CD71^{med}/Ter119^{high}, red). (b) Western blot analysis of frataxin expression in the spleen of wild-type mice treated with PHZ. After PHZ treatment, frataxin levels increased at day 6. Frataxin expression at day 0 was normalized to 100% and used as a baseline for all comparisons. Actin was used as a loading control.

Ter119^{high} (~250-fold at day 6), corresponding to basophilic erythroblast cells (Fig. 5(a), Table 3). When the data from Western blotting were reviewed according to the flow cytometry analysis, the increase in frataxin expression after PHZ treatment, as clearly seen by day 6, was lower than expected, if the dramatic increases CD71^{high}Ter119^{high} cells would be taken in account. Western blot analysis revealed only a 6- to 7-fold increase in frataxin expression, at day 6, as compared to the 250-folds increase expected given the size of the CD71^{high}/Ter119^{high} cell population (Fig. 5(b), Table 3). The downregulation of frataxin expression during erythropoiesis was particularly evident in one wild-type mouse at day 4 (mouse #3), when Western blot analysis showed a 2-fold decrease in frataxin expression (Fig. 5(b)), while CD71^{high}/Ter119^{high} cells increased 24-fold (data not shown). These results are therefore in agreement with previously published observations using our in vivo system.

Additionally, PHZ was administered to wild-type and tgFxn$^{+/-}$ mice (lines 1 and 2) and blood samples were collected at days 4, 6 and 11 for hematological analysis (Fig. 6). Different values of Hb and RBC counts were observed in tgFxn and wild-type mice, at days 4 and 6 after PHZ treatment, reaching statistical significance at day 4. These results showed an initially delayed response of tgFxn mice to PHZ treatment, although by the end of the time points both wild-type and tgFxn were shown to recover from PHZ treatment, with Hb and RBC levels reaching values similar to pre-treatment levels.

4. Discussion

Up to this date several attempts have been made to find an efficient therapy for FA, a lethal and very disabling disorder.

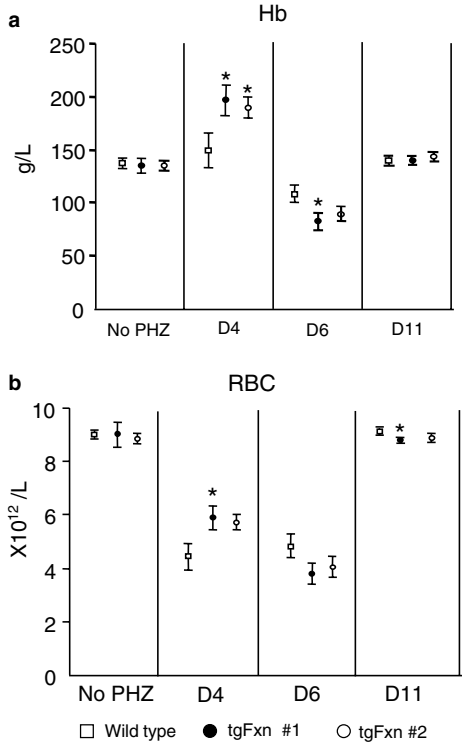

Fig. 6. Frataxin overexpressing mice have a slower recovery from erythropoietic stress. Alteration in wild-type, tgFxn line 1 and tgFxn line 2 mice of (a) hemoglobin and (b) RBC counts, after PHZ treatment. TgFxn mice show an altered response to PHZ treatment, as observed by a delayed response normalization of the hematological parameters. 6–7-weeks-old animals were used. Data are presented as means ± S.E.M. ($^{*}P < 0.05$).

So far, idebenone, a short chain analog of coenzyme Q_{10}, has shown a positive impact on cardiac hypertrophy in the majority of patients, although with no improvement in the neurological condition [38–41]. A combined therapy involving long term treatment with high doses of vitamin E and coenzyme Q10 has shown rapid and sustained increase in the energy generated by the heart and skeletal muscle [42]. However, although significant, this improvement corresponded only to a partial amelioration of symptoms. Clearly, more effective drug treatments are needed.

Since FA results from a reduction of frataxin levels, with patients having 5–30% of control levels [7], boosting its expression through gene therapy may be another option. In this work, we generated mice that overexpress frataxin primarily as additional tools to study its function, but also as a way to check whether such overexpression can be toxic, a case that would affect the design of gene replacement therapy. We obtained three lines of transgenic mice showing high levels of frataxin in all major organs affected in FA, including pancreas, brain, skeletal muscle and heart. The specific pattern of expression was different in each line, likely reflecting position effects related to the sites of insertion of the transgene. TgFxn$^{+/-}$ mice are born healthy and have no obvious behavioral, neurological, metabolic or pathological phenotype up to the age of one year. Therefore, a first conclusion of our study is that overexpression of frataxin does not have toxic effects, suggesting that this need not be a concern when designing a gene therapy treatment for FA.

Overexpression of frataxin does not seem to grossly affect iron metabolism, that is normal in all three transgenic lines as assessed by erythroid parameters analysis, SI levels and TS. Tissue iron concentration is similar in tgFxn$^{+/-}$ and in wild-type mice when kept on a standard diet, as well as after iron loading with a 3% carbonyl-iron supplemented diet for one month.

We then wished to use these models to further investigate frataxin function, in particular the hypothesis that it might prevent oxidative stress [43,44,34]. In a first experiment, we compared TgFxn and wild-type mice for their vulnerability to DOX toxicity. DOX is a very effective antibiotic used to treat a large variety of tumors, but whose clinical application has been limited by the development of dose-dependent cardiotoxicity. Iron is believed to play a major role in the development of DOX-induced cardiotoxicity [30,31,33]. Excess iron in the heart leads to increased susceptibility to DOX-induced cardiotoxicity, as observed in He-deficient mice [32], and can be attenuated by the use of iron chelators [45], or by the overexpression of antioxidant enzymes [46–49]. Since mitochondria have been identified as the major site of iron–DOX interaction, if frataxin exerts an antioxidant function in this compartment and can prevent iron-mediated free radical formation, it may be protective against DOX toxicity. However, TgFxn and wild-type mice show similar susceptibility to DOX toxicity, indicating that any antioxidant property of frataxin does not affect the pathway activated by DOX.

We performed a second experiment to test whether frataxin may be directly involved in mitochondrial iron metabolism. The final step of heme synthesis, i.e., the insertion of ferrous iron into the protoporphyrin IX ring, takes place in mitochondria and is catalyzed by the enzyme ferrochelatase. Though heme biosynthesis and development of blood, bone marrow and red cells appear to be grossly normal in patients with FA [50], frataxin mRNA and protein levels have been shown to decrease during erythroid development, suggesting that frataxin down-regulation may promote heme biosynthesis [51]. Constitutive overexpression of frataxin could therefore interfere with heme biosynthesis. In our experiment, we confirmed a decrease of frataxin expression during the erythropoietic response triggered by PHZ-induced hemolytic anemia and also detected a delayed response of TgFxn mice compared to wild-type mice. Taken together, these results suggest a direct involvement of frataxin in mitochondrial iron metabolism that may not be limited to ISC synthesis and that deserves further study.

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