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Multicellular models of Friedreich ataxia

Abstract Patients with Friedreich ataxia (FRDA) have severely reduced levels of the mitochondrial protein frataxin, which results from a large *GAA* triplet-repeat expansion within the frataxin gene (*FXN*). High evolutionary conservation of frataxin across species has enabled the development of disease models of FRDA in various unicellular and multicellular organisms. Mouse models include classical knockout models, in which the *Fxn* gene is constitutively inactivated, and knock-in models, in which a *GAA* repeat mutation or the conditional allele is inserted into the genome. Recently, "humanised" *GAA* repeat expansion mouse models were obtained by combining the constitutive knockout with the transgenic expression of a yeast artificial chromosome carrying the human FRDA locus. In lower organisms such as *Caenorhabditis elegans* and *Drosophila*, straightforward and conditional RNA interference technology has provided an easy way to knock down frataxin expression. Conditional mouse models have been used for pre-clinical trials of potential therapeutic agents, including idebenone, MnTBAP (a superoxide dismutase mimetic), and iron chelators. Various models of FRDA have shown that different, even opposite, phenotypes can be observed, depending on the level of frataxin expression. Additional studies with animal models will be essential for an enhanced understanding of the disease pathophysiology and for the development of better therapies.

Key words mouse · *C. elegans* · *Drosophila* · frataxin · disease pathophysiology · pre-clinical drug evaluation · iron-sulphur · mitochondria

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Introduction

Friedreich ataxia (FRDA) is caused by severely reduced levels of frataxin that result from a large *GAA* triplet-repeat expansion within the first intron of the frataxin gene (*FXN*). Frataxin is a ubiquitously expressed mitochondrial protein that is highly conserved throughout evolution. Significant efforts have been made over the past 10 years to understand both the physiological functions of frataxin and the pathophysiological process of the disease. Although much progress has been made, the exact functions of frataxin are not completely understood [1].

The high evolutionary conservation of frataxin has enabled the development of disease models in various organisms, from the unicellular eukaryote *Saccharomyces cerevisiae* to the mouse. These models have significantly contributed to our understanding of frataxin function and have allowed us to test potential therapeutic approaches. Initially, the yeast was an important model system for identifying the crucial role of frataxin in the regulation of intracellular iron trafficking, iron-sulphur (Fe-S) cluster and heme biogenesis, and oxidative metabolism [1, 2]. Although lower organism and cell culture models are important, particularly for studying genetic and environmental modifiers of the phenotype and in large-scale, unbiased drug screenings, the eventual development of multi-cellular disease models was essential to our understanding of the pathophysiology of the disease. This review provides a general overview of various multicellular models that have been devel-

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oped for FRDA, with a specific focus on the mouse models. The contribution of these models to our understanding of the pathophysiology of the disease and to the pre-clinical evaluation of therapeutic approaches is discussed.

Murine Models for FRDA

Murine models provide valuable insight into the cascade of events associated with FRDA, particularly the early disease states that only rarely can be studied in humans. The first mouse models were developed with the use of homologous recombination techniques to generate either a classical knockout model, in which the *FXN* gene is constitutively inactivated, or a knock-in model, in which a *GAA* repeat mutation or the conditional allele is inserted into the genome. Recently, "humanised" *GAA* repeat expansion mouse models were obtained by combining the constitutive knockout model with the transgenic expression of a yeast artificial chromosome (YAC) carrying the human locus. These models are complementary, permitting the study of different aspects of the pathophysiological mechanisms involved and the testing of different therapeutic approaches.

Constitutive and conditional knockout

Homozygous deletion of frataxin (KO) causes lethality at embryonic (E) day 6.5 (a few days after implantation), demonstrating an important role for frataxin during early development [3]. However, mice with heterozygous deletion of frataxin, which express around 50 % of wild-type frataxin levels, do not exhibit a pathological phenotype. These results clearly suggest that the residual frataxin expression associated with expansion mutations is critical for survival. No iron accumulation was observed during embryonic resorption, suggesting that the mechanism of cell death might be independent of gross iron accumulation. Furthermore, no evidence of apoptosis was found; thus, the mechanisms that lead to the embryonic death of KO animals remain unclear.

Viable mouse models were obtained through conditional gene targeting [4]. Conditional animal models have been crucial in the development of neurological, cardiac, and diabetic models for Friedreich ataxia. Initial models that expressed recombinase under the muscle creatine kinase (MCK) or the neuron-specific enolase (NSE) promoters, which exhibited heart and striated muscle- or neuron-restricted deletion of frataxin, respectively [4], were obtained. The NSE line was a more generalised frataxin-deficient line that included neuronal tissues. Both models are viable and reproduce some morphological and biochemical features observed in patients with FRDA, including cardiac hypertrophy without skeletal muscle involvement, large sensory neuron dysfunction without alteration of small sensory and motor neurons, and deficient activities of complexes I through III of the respiratory chain and of the aconitases.

The murine model of FRDA cardiomyopathy is characterised by left ventricular hypertrophy followed by onset of dilatation consistent with the natural history of the human disease [5]. A detailed time-course experiment in the MCK model revealed that Fe-S enzyme deficiencies begin at the onset of cardiac dysfunction, and intramitochondrial iron accumulation occurs at the end stage of the disease [5]. The NSE mutant animals, which also develop cardiomyopathy, show no iron deposits but have a deficit in Fe-S enzymes [4]. Both models indicate that Fe-S deficiency and cardiomyopathy are independent of gross mitochondrial iron accumulation, providing support for a more subtle abnormality of mitochondrial iron handling in this disease and the need for frataxin in efficient Fe-S cluster synthesis. However, in the cardiac model, even in the absence of detectable frataxin at birth, 50 % of Fe-S enzyme activity is detectable at 4 weeks of age; this observation indicates that frataxin is not essential in Fe-S cluster synthesis [5], as was found in an earlier yeast model [6, 7]. It is interesting to note that extramitochondrial Fe-S cluster proteins also are affected, suggesting that new pathophysiological pathways may be involved in the disease [8]. At the final stage, iron deposits are found in cardiomyocytes of these animals, comparable with findings of FRDA cardiac autopsies [9, 10].

Several reports have suggested that continuous oxidative damage, resulting from hampered signalling of superoxide dismutase(s) (SOD[s]), contributes to mitochondrial deficiency and ultimately to neuronal and cardiac cell death [11]. Indeed, the activity of SODs is abnormally low in the diseased mouse [12]; however, neither overexpression of SOD1 nor treatment with SOD mimetics, in contrast to treatment with idebenone (see below), has any effect on the survival of these animals [10]. Moreover, in a frataxin-deficient *Drosophila* model, hydrogen peroxide (H_2O_2), specifically, and not superoxide, plays an important role in the pathophysiology of the disease [13]. Possibly, H_2O_2-detoxifying enzymes, such as glutathione peroxidase, are protective in frataxin deficiency, rather than SODs, which generate H_2O_2. It is more surprising that no increased oxidative stress (including oxidation of proteins and lipids) was detectable in the conditional mouse models [10]. These results, which contrast with data from simpler organisms and with biomarker findings in human patients, may have been confounded by various factors, including increased mitochondrial Lon protease (C. Bouton and H. Puccio, unpublished results) or the almost complete shutdown of the respiratory chain in frataxin-deficient cells.

The NSE model develops a progressive movement

disorder characterised by gait abnormalities and loss of proprioception [4]. Electrophysiological studies have revealed a specific large sensory nerve conduction defect with normal motor nerve conduction. Although these features mimic neurological symptoms in patients with FRDA, the mouse model is extremely severe, with a life expectancy of 24 days. The severity of this model makes any therapeutic approach very difficult. Furthermore, this model shows lesions not seen in the human disease (liver and spongiform cortical lesions), thus preventing the study of cell-specific degeneration mechanisms.

Pfeiffer and colleagues generated a conditional mouse model with frataxin deficiency exclusively in pancreatic β-cells (Cre under the rat insulin promoter 2), to explore the link between frataxin deficiency and diabetes mellitus [14]. These mice show a slowly progressive reduction in the mass and number of islet cells, causing an impaired insulin secretory response to glucose and carbohydrates and culminating in overt diabetes. Furthermore, insulin secretion from isolated islets is not impaired, suggesting that the stimulus-secretion coupling is intact, which is consistent with findings in patients with FRDA [15]. The exact pathway leading to diabetes in this model is still under investigation, but frataxin-deficient β-cells clearly exhibit increased apoptosis and a reduced proliferation rate.

A mouse model that exhibits only the neurological features of FRDA in humans was developed through the use of a tamoxifen-inducible recombinase (Cre-ER^T) under control of the mouse prion protein (Prp) promoter; this approach enabled spatiotemporally controlled somatic mutagenesis of conditional alleles. Two lines were obtained that exhibit the most prominent features of the disease in humans: slowly progressive, mixed cerebellar and sensory ataxia associated with progressive loss of proprioception and absence of motor involvement [16]. These mice also develop histological changes that parallel those seen in humans, including degeneration of posterior columns of the spinal cord and severe lesions of the neurons in Clarke's columns – both hallmarks of FRDA. In addition, one line has specific damage to the large sensory neuron in the dorsal root ganglia (DRG), another distinctive feature of FRDA. The timing of the appearance of these lesions suggests that, as in patients with FRDA, anomalies observed in the DRG neurons are a primary event, whereas the neuronal loss in Clarke's column and the degeneration in the posterior column may be secondary events.

Although oxidative insult to cells cultured from patients with FRDA most often results in apoptosis [17, 18], no *in vivo* evidence of such a cell death mechanism has been observed. In particular, neither the complete frataxin knockout [2] nor the conditional animal models show evidence of apoptosis [4]. Conversely, an autophagic process that led to removal and degradation of damaged cytosolic proteins and organelles was observed in the DRG neurons of the tamoxifen-inducible conditional knockout [16]. Accumulation of lipofuscin, non-degradable intralysosomal substances originating from autophagocytosed cellular components [19], has been reported in both the DRG and the cardiomyocytes of patients with FRDA [20–22], so this cellular response is a consequence of frataxin deficiency. Autophagy may serve as a protective mechanism for the elimination of defective mitochondria with dysfunctional inner membranes [23].

GAA-based mouse models

Although conditional mouse models are excellent tools for studying disease pathophysiology and for evaluating some therapeutic approaches, they do not precisely mimic the human disease. Conditional gene targeting leads to complete loss of frataxin in some cells at a specific time in development, whereas FRDA is characterised by partial frataxin deficiency in all cells throughout life. Furthermore, conditional models do not address the influence of *GAA* repeat dynamics, which most likely contributes to the disease.

A different gene-targeting approach is to use a knock-in strategy. Pandolfo and colleagues generated a mouse model by introducing a $(GAA)_{230}$ repeat within the mouse frataxin gene to mirror the chronically reduced levels of frataxin expression found in the human disease [24]. By breeding these knock-in mice with the FXN knockout, investigators obtained animals that expressed 25–36 % of wild-type frataxin levels (KIKO), similar to the levels found in patients mildly affected with FRDA. However, these mice (up to age 1 year) did not develop similar abnormalities of motor coordination, cardiomyopathy, iron metabolism, or response to iron loading. Thus, 25–30 % of wild-type frataxin levels seem to be compatible with normal neurological function and iron metabolism in mice. The *GAA* repeat is meiotically and mitotically stable in the investigated KIKO mouse strain. Mice homozygous for the knock-in allele containing the $(GAA)_{230}$ repeat (KIKI) have become a valuable tool for evaluating epigenetic changes associated with *GAA* expansion and correlating them with frataxin expression [25]. The exact mechanism by which the *GAA* repeat mutation inhibits frataxin expression remains elusive. However, accumulating evidence highlights the importance of epigenetic changes that lead to heterochromatin formation, with increased trimethylation and decreased acylation on the histone tails, the hallmarks of silent chromatin, found immediately upstream and downstream of the *GAA* repeat in cells from patients with FRDA [26, 27]. KIKI mice have undergone similar changes, indicating that they are a suitable model for *in vivo* evaluation of treatments aimed at altering the epi-

genetic marks of the locus in order to upregulate frataxin [25].

A second *GAA* repeat model was generated with use of the human frataxin locus to overcome the embryonic lethality of the FXN knockout. Pook and colleagues generated a transgenic mouse model containing the entire frataxin gene within a human YAC clone onto the null mouse background [28]. Human frataxin, which was expressed in the appropriate tissues at levels comparable to those of endogenous mouse frataxin, was correctly processed and localized to mitochondria. Biochemical analysis of heart tissue demonstrated preservation of mitochondrial respiratory chain function. These investigators subsequently generated two lines of human FXN YAC transgenic mice containing *GAA* repeat expansions derived from FRDA patient DNA. These two lines exhibit the intergenerational and age-dependent, disease tissue-specific, somatic instability of the *GAA* repeat [29, 30]. Humanised *GAA* repeat expansion mutation mouse models were obtained by cross-breeding either line of human FXN YAC transgenic mice with heterozygous FXN knockout mice [31]. These humanised mice, which express only reduced quantities of human-derived FXN from the YAC transgene and completely lack endogenous murine FXN, present a progressive neurodegenerative phenotype with locomotor activity deficiency and without a reduced life span. Histologically, these mice show many abnormalities: vacuoles, chromatolysis, and lipofuscin are present in the DRG; lipofuscin and iron deposits are found in cardiomyocytes without any signs of cardiac hypertrophy; and aconitase activity is decreased in the heart. These mice reproduce the early stages of the pathophysiology identified in the conditional mouse models. Of interest, the vacuolar pathology in the humanised mouse model was progressive from the distal lumbar regions to the more proximal cervical regions, resembling the dying-back phenomenon of neurodegeneration decribed in patients with FRDA. In contrast to the conditional knockout model, these mice present obvious but not severe oxidative stress, which may arise from residual FXN levels, leading to a slowed but not shut-off mitochondrial respiratory chain. Finally, as in KIKI mice, the *GAA* repeat expansion mutation in the humanised mouse induces comparable epigenetic changes in brain and heart tissues [32].

Models in Lower Organisms

Frataxin is essential for all multicellular organisms. As in the mouse, complete deletion of the FXN homologs in *Caenorhabditis elegans* and *Drosophila* leads to developmental arrest. The availability of straightforward and conditional RNA interference technology in *C. elegans* and *Drosophila* provides an easy way to knock down FXN expression.

Caenorhabditis elegans

Several *C. elegans* models with knockdown of the *C. elegans* FXN homolog *frh-1* have been published, with conflicting findings. Ventura and colleagues found that FXN knockdown animals had a significantly extended life span despite a small body size, reduced fertility, and altered response to oxidative stress [33]. *Frh-1*-deficient animals were relatively resistant to H_2O_2 but were more sensitive to juglone, a superoxide generator. Vazquez-Manrique and associates found that FXN knockdown animals presented a consistent pleiotropic phenotype (slow growth, egg-laying defects, abnormal pharyngeal pumping, and defecation defects) associated with an increased sensitivity to oxidative stress [34]. However, these animals had a significantly reduced life span. More recently, Zarse and co-workers reported similar findings of a decrease in life span that results from FXN knockdown [35].

These conflicting findings may be explained by experimental differences [36]. Rea and colleagues developed a novel RNA interference (RNAi) dilution strategy to incrementally reduce the expression level of genes directly implicated in the function of the mitochondrial electron transport chain. Their study showed that mitochondrial electron transport chain RNAi phenotypes in the nematode are dose dependent at a specific period of development – the L3/L4 period of the life cycle; intermediate inhibition leads to an increased life span, and high inhibition leads to a decreased life span [36]. Given the level of RNAi administered, the timing of administration, and analysis of the phenotype threshold, these FXN-deficient *C. elegans* models may prove useful for drug screening in FRDA.

Drosophila

The UAS-GAL4 transgene-based RNAi method has been used to impose downregulation of the *Drosophila* frataxin homolog (*dfh*). This model mimics FRDA by reducing rather than eliminating frataxin (DFH) and produces tissue-specific knockdown. As in *C. elegans*, the suppression of *dfh* is dose dependent and confers distinct phenotypes in larvae and adults. Such studies have shown that the function of DFH is essential during development [37]. A small percentage of *dfh*-suppressed larvae were able to form viable adults if cultured at a more permissive temperature (18 °C), beginning at pupariation. These adult escapees exhibited a greatly reduced life span and a biochemical phenotype consistent with a functionally conserved role of frataxin [37]. It is interesting to note that transgenic overexpression of the anti-oxidant enzymes SOD1, SOD2, and catalase (CAT) did not improve the capacity of DFH-deficient larvae to develop into adults, strongly suggesting that oxidative

stress in larvae or pupae is not a major contributor to the deleterious phenotype [37]. Silencing of *dfh* in the peripheral nervous system (PNS) or in all sensory organs and their precursors allowed larvae to develop into adults. However, these flies exhibited a markedly reduced life span and a slight decline in climbing ability (evaluated only for sensory organ ablation) [37, 38]. Tissue-specific vulnerability of the PNS to frataxin depletion is conserved across a wide spectrum of animal taxa [37].

To parallel more closely the situation in patients, Llorens and colleagues generated a *Drosophila* line with ubiquitous moderate reduction of *dfh* (a three-fold decrease) [38]. The 30 % residual FXN was compatible with normal embryonic development but resulted in a shortened life span and a reduced climbing ability in adulthood. No differences were observed in the activity of aconitase or succinate dehydrogenase (SDH) between control and *dfh*-RNAi flies [38]. The lack of specific biochemical hallmarks of FRDA is reminiscent of results in KIKI mice, further demonstrating the threshold dependence associated with FXN deficiency. The moderately *dfh*-deficient flies had a hypersensitive response to hyperoxia, which supports a causative role of oxidative stress in FRDA [38]. Under hyperoxia, the animals showed a reduction in aconitase activity with normal SDH, leading the authors to propose that regular, oxidatively mediated inactivation of aconitase, which occurs normally during aging, is accelerated in FXN deficiency. These results are consistent with the recent proposal that the yeast FXN homolog (*Yfh1*) is an iron chaperone involved in protection of aconitase against reactive oxygen species [39, 40].

Recently, Anderson and colleagues [13] investigated the role of H_2O_2 in the pathophysiology of FRDA, as susceptibility to H_2O_2 challenge is typical in yeast and cell culture models of FXN deficiency [18, 41]. Indeed, ectopic expression of enzymes that scavenge H_2O_2 (i.e., peroxisomal and mitochondrial catalases [CATs] and a mitochondrial peroxiredoxin) suppresses some of the deleterious phenotypes associated with FXN deficiency [13]. Overexpression of enzymes that scavenge superoxides (SOD1 and SOD2) had no effect, supporting the results obtained in the conditional mouse models.

Potential of Disease Models to Evaluate Experimental Treatments

The conditional models have been used for pre-clinical trials of potential therapeutic agents, including idebenone, MnTBAP (a superoxide dismutase mimetic), and iron chelators. Idebenone effectively delayed progressive cardiac hypertrophy and dilatation, and it preserved ventricular contractility by 1 week, thus increasing the life span of the animal by 10 % [5]. However, in contrast to the results of a heart biopsy from a patient with FRDA, idebenone did not restore Fe-S enzyme activities in the animal model. Idebenone had no effect on the neurological phenotype of the inducible conditional model (unpublished results), but this disease model is more severe than FRDA.

Restoration of FXN expression represents an important step toward an effective treatment for FRDA, and the two *GAA*-based models represent powerful tools for evaluating therapies that target epigenetic modifications of the *FXN* gene to increase FXN expression. Histone deacetylase inhibitors (HDACi) can de-condense the chromatin structure, allowing gene expression [42]. Indeed, a substantial increase in FXN in lymphoblastoid cells from patients with FRDA was obtained with the use of a specific class of HDACi [27]. A recently published study in the KIKI mouse demonstrated the *in vivo* feasibility of a therapeutic approach to activating the *FXN* gene [25]. After 3 days of treatment, KIKI mice exhibited increased levels of FXN and normalisation of the transcriptional profile associated with FXN deficiency.

Conclusion

The many different models that have been developed for FRDA have provided important insight into the pathophysiology of the disease and are powerful tools for evaluating potential therapies for FRDA. Moreover, these diverse models have clearly shown that different, even opposite, phenotypes can be observed, depending on the level of FXN expression. This finding has contributed to the controversy surrounding the role of oxidative stress in the pathophysiology of the disease. Additional studies with animal models will continue to provide insight into this disease and will help us further understand each component of the pathophysiology of FRDA, which will be essential for the development of better therapies.

Conflict of interest Dr. Puccio reports no conflicts.

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