Neural and Cardiac Mechanisms in Friedreich's Ataxia with Patient-derived iPSCs

Mariana Burgos Angulo
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Neural and Cardiac Mechanisms in Friedreich's Ataxia with Patient-derived iPSCs

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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ABSTRACT

Friedreich's ataxia (FA) is an autosomal recessive disease caused, in most cases, by a GAA trinucleotide repeat expansion in the first intron of the frataxin (FXN) gene, which results in transcriptional repression of the encoded protein frataxin. FA is a progressive neurodegenerative disorder, but the primary cause of death is hypertrophic cardiomyopathy, which occurs in 60% of the patients. Several functions of frataxin have been proposed, but none of them can fully explain why its deficiency causes the FA phenotypes nor why the most affected cell types are neurons and cardiomyocytes. It is possible that frataxin affects neural and cardiac cells in different ways and that the GAA expansion has a pathological effect independent of frataxin. However, this is also unknown. To fill this gap, I investigated the hypothesis that frataxin deficiency plays a unique role in different FA-affected tissues.

In the first part of this study, I generated induced pluripotent stem cells (iPSC)-derived neurons (iNs) and cardiomyocytes (iCMs) from an FA patient and an unrelated control, and performed RNA-seq and differential gene expression analysis. Results demonstrated that the dysregulated genes of FA iNs were involved in nervous system development, supporting neuronal differentiation, specification, and maturation. On the other hand, the dysregulated genes in FA iCMs play roles in fibrosis, iron overload, and cardiac remodeling.
In the second part of this study, I upregulated FXN expression via lentivirus without altering genomic GAA repeats at the FXN locus in the FA iNs and iCMs. RNA-seq and differential gene expression analyses demonstrated that frataxin deficiency affected the expression of glycolytic pathway genes in neurons and extracellular matrix pathways genes in cardiomyocytes. Genes in these pathways were differentially expressed when compared to a control and restored to control levels when FA cells were supplemented with frataxin.

These results offer novel insight into the specific roles of frataxin deficiency pathogenesis in neurons and cardiomyocytes using patient-specific iPSCs. By exploring the dysregulated genes using FA-derived cells from different patients, generating isogenic cell lines, and obtaining cellular phenotypes to test disease mechanisms, we will be one step closer to understanding and finding a cure for FA.
CHAPTER ONE
INTRODUCTION AND BACKGROUND

Friedreich’s Ataxia (FA)

Friedreich’s Ataxia (FA) is the most frequent hereditary ataxia affecting children, adolescents, and adults, with an incidence of 1 in 50,000 in Caucasians (Aranca et al., 2016; Durr et al., 1996). FA is considered a multisystem disorder that impacts the central and peripheral nervous systems, the musculoskeletal system, the heart, and the pancreas. The neurological disease is characterized by progressive degeneration affecting the dorsal root ganglia, the corticospinal and spinocerebellar tracts, and the deep cerebellar nuclei. Cerebellar atrophy is notable in the dentate nucleus and superior cerebellar peduncles (Koeppen, 2011), the large primary sensory neurons deteriorate early in the disease progression, and the dorsal root ganglia are smaller than in unaffected persons. These alterations lead to axonal peripheral sensory neuropathy (Morral, Davis, Qian, Gelman, & Koeppen, 2010). Magnetic resonance imaging (MRI) studies have shown substantial grey and white matter loss in the deep cerebellar nuclei and brain stem (Della Nave et al., 2008). Recent findings suggest that significant changes in satellite cells and inflammation are important contributors to neuronal death (Koeppen & Mazurkiewicz, 2013; Koeppen et al., 2020). Neuronal degeneration leads to loss of balance and coordination, sensory loss, muscle weakness, and dysarthria (Koeppen & Mazurkiewicz, 2013).
Around 60% of the patients with FA develop Hypertrophic Cardiomyopathy (HCM), with progressive cardiac fibrosis, loss of systolic function, and arrhythmias. In most cases, the Electrocardiogram (ECG) displays repolarization abnormalities that come in the form of T-wave inversion or flattening in lateral or inferior leads and ST-segment elevation or depression. Typical echocardiographic findings involve increased end-diastolic septal and posterior wall thicknesses and left ventricular hypertrophy. Around 20% of the patients present reduced ejection fraction, which usually declines with age (Regner et al., 2012). Heart disease is the patient's leading cause of death (Pousset et al., 2015), and usually develops in the second to third decade of life after the neurological disease is well established, although there is significant variance in range. In a few cases, the hypertrophic heart is the initial finding before the diagnosis of FA, whereas in other cases, the patients may not display cardiac symptoms (Hanson, Sheldon, Pacheco, Alkubeysi, & Raizada, 2019). This disconnection in neurological and cardiac disease onset could suggest differing pathophysiological mechanisms.

Common musculoskeletal features include scoliosis, pes cavus, and talipes equinovarus. Some cohort studies have described depression and anxiety in the FA patient, which may be a consequence of the disease. However, there is also evidence that supports a correlation between depression and frontal grey matter volume loss (Koeppen, 2011; Silva et al., 2013). About half of the FA patients display glucose intolerance, and studies have found that Diabetes Mellitus is more prevalent in these patients, with estimates between 1% and 32% (Cnop et al., 2012; Finocchiaro, Baio, Micossi, Pozza, & di Donato, 1988; Gucev et al., 2009; Isaacs et al., 2016).
FA displays variable phenotypes and age of onset. In early-onset FA, the first symptoms appear at around ten years of age, and there is a rapid progression of the neurologic symptoms and HCM, and average mortality at 39 years of age. In contrast, the symptoms of late-onset FA first appear after 25 years of age. The neurologic symptoms and HCM tend to be less severe, and they have near-average life-span (Burk, 2017; Hanson et al., 2019).

FA is a monogenic autosomal recessive disorder. 96% of cases are caused by a biallelic expansion of a GAA triplet in the first intron of the FXN gene that results in transcriptional repression (Campuzano et al., 1997; Campuzano et al., 1996). This expansion typically ranges between 600 and 900 repeats, but above 66 repeats is considered pathogenic. Clinical severity and disease onset correlate with the size of the GAA expansion of the shorter allele (Durr et al., 1996; Y. Li, Lu, et al., 2015). The remaining 4% of cases are compound heterozygous with point mutations in one FXN allele and a GAA expansion in the other one (Cossee et al., 1999; Galea et al., 2016). Finally, heterozygous carriers are healthy, and their frequency is 1:60 to 1:100 (Bidichandani & Delatycki, 1993).

The molecular mechanism of FXN silencing is still not fully understood, although several studies indicate that epigenetic changes play an essential part in FXN transcriptional repression. Research with transgenic mice demonstrated that the expanded GAA repeat leads to heterochromatin formation near the FXN locus causing the epigenetic silencing effect (Saveliev, Everett, Sharpe, Webster, & Festenstein, 2003). Other studies showed that FA alleles are enriched with post-translational histone modifications that typify heterochromatin, including histone H3 and H4 deacetylation,
histone trimethylation, CpG methylation, and non-coding RNA transcription (Al-Mahdawi et al., 2008; Greene, Mahishi, Entezam, Kumari, & Usdin, 2007; Y. Li, Lu, et al., 2015; Mikaeili, Sandi, Bayot, Al-Mahdawi, & Pook, 2018). There is also evidence of DNA methylation that correlates with the extent of GAA expansion, phenotype severity, and age of disease onset (Al-Mahdawi et al., 2008; Castaldo et al., 2008). However, it is not known if the GAA expansion itself, has a pathological role independent of FXN that could explain disease variability and influence on certain phenotypes.

**Frataxin**

FXN is a 210 amino acid protein synthesized in the cytoplasm, but has a mitochondrial translocation signal in the N-terminal region (Popovic et al., 2015). In the mitochondria, the mitochondrial peptidase processes it into an intermediate form and ultimately into a mature 130 amino acid FXN (Condo et al., 2007; Koutnikova, Campuzano, & Koenig, 1998). This mature form is fully active and capable of improving the survival and phenotypes of FXN deficient cells (Condo et al., 2007). There are at least three different isoforms of FXN (I, II, and III) resulting from alternative splicing (Xia et al., 2012). The canonical isoform is type I, localized in the mitochondria with a molecular weight of 14.2 kDa and 130 amino acids long. Type II has a molecular weight of 14.9 kDa, and it is 135-aminoacid long; it is localized in the cytosol and is abundant in the nervous system. Type III is observed in the nucleus and is abundant in the heart, has 164-residues, and has a molecular mass of 18.2 kDa (Xia et al., 2012). Recent studies have found an additional FXN isoform in the erythrocytes named isoform E (Guo et al., 2018). This isoform is 135- residues long, lacks the mitochondrial translocation signal, and the N-terminal is acetylated in the first methionine amino acid
FXN is highly conserved and critical for life; thus, knockout mice are embryonic-lethal, and homozygous non-GAA mutations have not been found in humans (Adinolfi, Trifuoggi, Politou, Martin, & Pastore, 2002; Cossee et al., 2000; Gibson, Koonin, Musco, Pastore, & Bork, 1996). FXN is ubiquitously expressed, but neurons and CMs are the most susceptible to its deficiency (Koutnikova et al., 1997).

There are multiple proposed functions of FXN. However, none of them can fully explain why its deficiency generates the FA phenotypes. It was initially proposed that FXN might function as an iron-storage protein because yeast FXN can form multimers with ferroxidase activity (Adamec et al., 2000). This property may contribute to protection from iron-mediated oxidative stress resulting from toxic reactive oxygen species (ROS) formed via Fenton chemistry. FA cells are susceptible to oxidative stress, but mature human FXN does not form aggregates, and this function is redundant with the iron-storage protein ferritin. Other studies suggest that FXN may function as an iron donor in iron-dependent biosynthetic reactions such as iron-sulfur cluster (ISC) and heme biosynthesis (Yoon & Cowan, 2003, 2004)). A related hypothesis suggests that FXN functions as a metabolic switch that diverts iron from ISC to heme biosynthesis (Becker, Greer, Ponka, & Richardson, 2002). Lastly, it has been proposed that FXN may act as a negative regulator that inhibits the rate of ISC biosynthesis if iron availability is high and downstream ISC apo receptors are low (Adinolfi et al., 2009). However, ISC and heme deficiency are not observed in several models of FXN deficiency, and anemia is not a common feature of FA.
**Induced pluripotent stem cells (iPSCs)**

Cells derived from FA patients constitute the most relevant FXN-deficient cell model as they carry the complete FXN locus together with GAA repeat expansions and regulatory sequences (Perdomini, Hick, Puccio, & Pook, 2013). Some cell types, such as primary fibroblasts and lymphocytes, are easily accessible. However, these cells do not spontaneously exhibit the complex biochemical phenotype associated with FA, despite having reduced levels of FXN. Neurons and Cardiomyocytes, particularly affected in FA, are not accessible from patients (Schreiber, Misiorek, Napierala, & Napierala, 2019). The poor availability of such cell types can be circumvented by using the technique developed by Yamanaka and Thomson, where somatic cells are reprogrammed into embryonic-like cells, known as induced pluripotent stem cells (iPSCs), that can be differentiated into nearly all cell types of the human body (Takahashi et al., 2007; Yu et al., 2007). Initial reprogramming had a low process efficiency, and viral transduction was necessary to deliver the transcription factors (Oct3/4, c-Myc, Klf4, and Sox 2) to fibroblasts (Takahashi et al., 2007). However, soon after, it was discovered that the reprogramming could be performed by additional transcription factors and small molecules using different somatic cell types such as blood cells and keratinocytes (Malik & Rao, 2013). Presently, iPSC reprogramming has been commercialized, streamlined, is rather inexpensive, and transient expression or non-integrating viruses are conveniently used for transcription factor delivery.

Recently, the standardization of reprogramming techniques has increased the utilization of iPSCs in research. Furthermore, protocols for robust, defined, and reproducible differentiation of iPSCs into various cell types are being developed to
generate disease models. Differentiated cells can be used not only for studying disease mechanisms but also for drug discovery, assessing the efficacy of therapeutic methodologies, and identifying disease biomarkers (Hick et al., 2013). Therefore, much effort has been made in the last years to generate FA-iPSCs and differentiate them into iNs and iCMs.

The first FA fibroblasts that were reprogrammed into iPSCs were performed by Gottesfeld et al. in 2010. They demonstrated that patient-derived iPSCs GAA expansions are unstable, with contractions and expansion of the repeats in succeeding generations (Du et al., 2012; Ku et al., 2010). This instability had been reported in vitro in other patient-derived cells (Ditch, Sammarco, Banerjee, & Grabczyk, 2009) and in different tissues within the same patient (De Biase et al., 2007; Long et al., 2017). iPSCs from FA would allow to study and validate new therapies aiming to target the GAA expansions. With gene editing techniques it is possible to remove or modify the expansion before further differentiation into affected cell types.

Differentiation of FA iPSCs into neurons and cardiomyocytes with phenotypic characteristics of the diseases was first described by Liu et al. (Liu et al., 2011). Since then, we and others have found that these cells retain supressed levels of FXN, present reduced mitochondrial membrane potential, impaired ISC synthesis, disorganized mitochondrial network, and mitochondrial DNA depletion (Codazzi et al., 2016; Hick et al., 2013; Y. K. Lee et al., 2016). Transcriptional profiling of FA iNs and iCMs compared to controls has shown differential expression of genes, indicating a molecular signature of the disease (Lai et al., 2019; J. Li et al., 2019). Current research using FA iPSCs and FA-iPSCs-derived cells is summarized in Table 1.
Since both the peripheral and central nervous systems are affected in FA, most iPSC research focuses on neuron differentiation and characterization (Table 1). Furthermore, methods of neuronal differentiation have been optimized and commercialized. The first FA iNs had the same morphology and physiology as control cells and maintained the GAA expansion and low FXN level (Liu et al., 2011). A following study by Puccio et al. revealed that the FA iNs show indications of mitochondrial dysfunction such as decreased mitochondrial membrane potential and progressive mitochondrial degeneration visualized by electron microscopy (Hick et al., 2013). These initial reports with phenotypic changes supported the concept that FA iNs could be used as disease models and evaluation of drug candidates. These findings were important since undifferentiated iPSC lines did not show any phenotype except for the GAA instability (Hick et al., 2013).

FA iNs were also used to investigate methods to restore the low FXN level. Research has shown that the GAA expansion promotes heterochromatin formation, and histone deacetylases can be found at the repeat’s vicinity. Therefore, FXN was upregulated using histone deacetylase inhibitors (HDACi) (Codazzi et al., 2016)). Treated cells showed corrected molecular phenotypes, including the lower levels of Fe-S and lipoic acid-containing proteins, increased labile iron pool, high expression of superoxide dismutase (SOD2), and increased levels of reactive oxygen species (Codazzi et al., 2016). Finally, the GAA expansion of FA iNs was corrected by zinc finger nuclease excision of one FXN allele, which led to transcription reactivation of FXN (Y. Li, Polak, et al., 2015).
Heart disease is the leading cause of death in 60% of FA patients (Pousset et al., 2015). The proliferation of mitochondria within the cardiomyocytes, loss of contractile fibers, and fibrosis are cellular drivers of patients' hypertrophic cardiomyopathy. Since heart tissue from patients is not easily accessible, two main models have been used to study cardiomyopathy in FA. The first model is a conditional knockout and shRNA mice that show a severe cardiac phenotype; however, the GAA expansion mouse model does not display any cardiac symptoms (Al-Mahdawi et al., 2004; Anjomani Virmouni et al., 2015; Chandran et al., 2017). The second model is necropsy tissue from FA patients (Ramirez et al., 2015). However, the lack of viable human cardiac cells prevents studying the process underlying the development of heart disease. Therefore, there is a need to establish FA cardiac models from human cells that are key to studying disease mechanisms and testing new therapies. Fortunately, methods to differentiate iPSCs into cardiomyocytes are now optimized and stream-lined, and immature beating iCMs are obtained within three weeks of culture.

The first studies in FA iCMs demonstrated that low FXN levels do not prevent cardiac differentiation (Hick et al., 2013; Liu et al., 2011) and that they presented some FA phenotypes. These iCMs exhibit mitochondrial abnormalities, such as proliferation of normal mitochondrial and mitochondrial degeneration (Hick et al., 2013). Then, two other studies described phenotypic changes similar to those encountered in the mouse models and necropsy tissue (Y. K. Lee et al., 2014; Y. K. Lee et al., 2016). Those phenotypes included disorganized mitochondrial networks, mitochondrial DNA depletion, increased iCMs size, and increased brain natriuretic peptide expression. They also found deficits in energy production and calcium handling, and all the
phenotypes were aggravated by iron overload. Subsequently, another study demonstrated an increased variation in beating rates of FA iCMs due to calcium handling defects (Crombie et al., 2017). This investigation was performed in three different FA iCMs lines without iron overload, demonstrating that FA iCMs differ from control even under basal conditions. In collaboration with the Noujaim laboratory, we also demonstrated that FA iCMs display several phenotypes under basal conditions (Chidipi et al., 2021), including a significantly depolarized mitochondrial membrane potential, higher reactive oxygen species, and decreased mitochondrial fission protein dynamin-related protein I. The Napierala group, using zinc finger nuclease excision of GAA repeats in one allele of FA iCMs (J. Li et al., 2019), showed upregulated FXN expression, reduced lipid accumulation observed in patient iCMs, and reversed gene expression phenotype. The transcriptional profile of these cells revealed hypertrophy signatures of FA iCMs and similarities between control and ZFN-corrected FA iCMs.

Although the generation of iPSCs has been one of the most important discoveries in the past decade for permitting studies on drug efficacy, molecular mechanisms, and regenerative medicine, there are still limitations of this technology. Regarding disease modeling, the heterogeneity of iPSCs derived from different individuals and between individual clones makes iPSC research expensive and laborious (Liang & Zhang, 2013). For FA research specifically, this is augmented by the patient heterogeneity, which makes the generation of isogenic cell line pairs essential for further research. An additional issue is the genetic instability of the locus and continuous expansion of the GAA repeats (Ku et al., 2010; Polak, Li, Butler, &
Napierala, 2016), which adds another level of variability, that may change the molecular phenotype of the cells.

Despite reduced FXN levels since embryogenesis (Cook & Giunti, 2017), the first FA symptoms typically appear between 5 and 15 years of age and sometimes much later in life. Although pathological molecular and cellular changes precede the presentation of clinical symptoms, the timeline of FA manifestation is longer than a few weeks of differentiation iPSCs into neural and cardiac cells. Therefore, identifying strong phenotypes in young and immature cells may be difficult or impossible. Methods seeking to accelerate the aging process (Miller et al., 2013) or trans differentiation (where a somatic cell is directly converted into another somatic cell) may help overcome this issue (Mertens, Reid, Lau, Kim, & Gage, 2018).

Finally, it is essential to continue optimizing robust and reproducible protocols to obtain cell types most affected during the disease, like proprioceptive sensory neurons and mature ventricular cardiomyocytes.

**Table 1.** Studies on FA iPSC and FA iNs and ICMs.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Study Objective</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA iPSCs</td>
<td>Generation of FA GAA instability model</td>
<td>(Ku et al., 2010)</td>
</tr>
<tr>
<td>FA iPSCs</td>
<td>The role of mismatch repair enzymes in GAA expansions</td>
<td>(Du et al., 2012)</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Study Objective</td>
<td>References</td>
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<tr>
<td>-----------</td>
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</tr>
<tr>
<td>FA iNs</td>
<td>Generation of patient-specific cells affected in FA</td>
<td>(Liu et al., 2011)</td>
</tr>
<tr>
<td>FA iNs</td>
<td>Evaluation of mitochondrial damage in FA</td>
<td>(Hick et al., 2013)</td>
</tr>
<tr>
<td>FA iNs</td>
<td>Upregulation of FXN with HDAC inhibitors</td>
<td>(Codazzi et al., 2016)</td>
</tr>
<tr>
<td>FA iNs</td>
<td>Upregulation of FXN with zinc finger nuclease GAA repeat excision</td>
<td>(Y. Li, Polak, et al., 2015)</td>
</tr>
<tr>
<td>FA iCMs</td>
<td>Generation of patient-specific cells affected in FA</td>
<td>(Liu et al., 2011)</td>
</tr>
<tr>
<td>FA iCMs</td>
<td>Evaluation of mitochondrial damage in FA</td>
<td>(Hick et al., 2013)</td>
</tr>
<tr>
<td>FA iCMs</td>
<td>studying iron-overload induced cardiomyopathy</td>
<td>(Y. K. Lee et al., 2014)</td>
</tr>
<tr>
<td>FA iCMs</td>
<td>Electrophysiological profile of FA iCMs</td>
<td>(Crombie et al., 2017)</td>
</tr>
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</table>
Table 1. (Continued)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Study Objective</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA iCMs</td>
<td>Investigating the mitochondrial network and fusion and fission machinery in FA iCMs</td>
<td>(Chidipi et al., 2021)</td>
</tr>
<tr>
<td>FA iCMs</td>
<td>Transcriptional profile of FA iCMs and ZFN- GAA excision correction of FA- iCMs</td>
<td>(J. Li et al., 2019)</td>
</tr>
</tbody>
</table>

Hypothesis

FA is a multisystem disease with nerves and myocardium being the most affected tissues. However, the onset and progression of neurological and heart disease vary significantly within a patient and in different patients. Neurological symptoms typically appear during the first decade of life but can also occur after 25 years of age. They are progressive and are the most noticeable of the disease; This variability in disease onset and progression has been correlated with the length of the GAA expansion and the $FXN$ mRNA level. Cardiac disease is highly variable as well. It usually develops in the second to third decade of life after the neurological disease is well established. However, there are reported cases where it precedes neurological dysfunction, and in many cases, the cardiac disease never manifests. This discordance
in neurological and cardiac disease onset might suggest different pathophysiological mechanisms. Despite efforts to understand FXN function (Adamec et al., 2000; Adinolfi et al., 2009; Becker et al., 2002; Yoon & Cowan, 2003, 2004), it is still not clear why its deficiency causes the various FA phenotypes. It is possible that FXN may play different roles in neurons and cardiomyocytes and that the GAA expansion may have a pathological role independent of FXN (Al-Mahdawi et al., 2008; Castaldo et al., 2008).

To gain insight into this possibility, we recruited a patient with FA with a homozygous GAA expansion with established neurological disease and mild cardiac symptoms (Table 2) and generated patient-specific iPSCs using nonintegrated reprogramming methods. We differentiated these cells and unrelated control iPSCs (ATCC -ACS-1026, from a Caucasian male) into iNs and iCMs to evaluate and compare the cellular, genetic, and molecular characteristics.

Additionally, we sought to restore FXN expression level by transducing the FA iNs and iCMs with FXN lentivirus. Transcriptional profile analysis of these cells provides information on the FXN network of FA iNs and iCMs independent of the GAA expansion. A successful conclusion of this study using iPSCs will offer an excellent prospect for mechanism investigation in the most affected cell types in FA.
<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Ethnicity</th>
<th>GAA Expansion</th>
<th>First Neurological Symptom</th>
<th>Age of Onset Neurological Symptom</th>
<th>Neurological Status at Enrollment</th>
<th>First Cardiac Symptom</th>
<th>Age of Onset Cardiac Symptom</th>
<th>ECG</th>
<th>Other Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>34yrs</td>
<td>Caucasian</td>
<td>1092 &amp; 781</td>
<td>Difficulty walking</td>
<td>8</td>
<td>Wheelchair-bound, slurred speech, lack of coordination, Balance problem</td>
<td>Chest pain</td>
<td>28</td>
<td>TWI</td>
<td>Scoliosis, kyphoscoliosis, Osteoarthritis of spine</td>
</tr>
</tbody>
</table>

TWI indicates T wave inversions
Working hypothesis 1: There are differing pathophysiological mechanisms for the heart and neurological disease.

Aim 1: Compare the Molecular Phenotype of FA iPSC-derived Neurons and Cardiomyocytes

To test this hypothesis, I reprogrammed peripheral blood mononuclear cells (PBMCs) from an FA patient into iPSCs and differentiated them into neurons and cardiomyocytes. iPSCs from an unrelated control without the GAA expansion were also differentiated into these cells. Careful evaluation of marker expression, FXN level, and GAA repeats was performed in iNS and iCMs of both patient and control. After one month in culture, transcriptional profiling was carried out in these cells to obtain differences in gene expression and assess the early molecular mechanisms of FA in iNs and iCMs.
**Working hypothesis 2:** *Reduced FXN level has a pathological role independent of the GAA expansion.*

**Aim 2:** Evaluate the pathological role of *FXN* deficiency by supplementing the cells with exogenous FXN.

To evaluate the pathological role of FXN in FA-relevant cells without modifying the GAA expansion, we forced the expression of *FXN* in FA iNs and iCMs by transducing them with *FXN* lentivirus. Careful control of the amount of *FXN* augmentation was performed by modifying a lentivirus transfer plasmid with a weak promoter and determining the lentivirus titer for optimal transduction. Two weeks after *FXN* transduction, we performed RNA-seq analysis in transduced and non-transduced cells to evaluate the expression changes. We also performed enrichment analysis to determine the most enriched process and pathways.
CHAPTER TWO
THE MOLECULAR PHENOTYPE OF FRIEDREICH’S ATAXIA IPSC-DERIVED NEURONS AND CARDIOMYOCYTES

Introduction

The nervous system and the heart are the most affected tissues in FA, even though all somatic cells have exhibit reduced levels of \( FXN \) in FA patients (Koutnikova et al., 1997). FA-HCM usually develops after the neurological disease, but it could be the initial finding or never occur in some cases (Hanson et al., 2019). This discrepancy between cardiac and neurological manifestation may be attributed to different pathophysiological mechanisms. Cells derived from patients are the most relevant \( FXN \)-deficient cell model because they carry the complete \( FXN \) locus with the GAA repeat expansions and regulatory sequences (Perdomini et al., 2013). However, obtaining neurons and CMs from patients is complicated, and accessible cells, such as primary fibroblasts and lymphocytes, do not exhibit the complex biochemical phenotype associated with FA (Schreiber et al., 2019). Therefore, we reprogrammed PBMCS from an FA patient into iPSCs and differentiated them into iNs and CMs. Previous studies on RNA-seq analysis from FA iNs and iCMs compared to controls have shown a differential expression between the two, indicative of a molecular signature of the disease (Lai et al., 2019; J. Li et al., 2019). However, not all the transcriptional profile studies reported the same molecular phenotypes. FA is a complex disease with a wide variability in
patients’ disease onset, severity, clinical phenotypes, and rate of progression (Burk, 2017; Hanson et al., 2019). Therefore, the discrepancy in the results may reflect the variability between patients, controls and that the studies only included iNs or iCMs. Thus, we used the same patient and control to generate iNs and iCMs and performed transcriptional profile in these cells. We hypothesize that there are differing pathophysiological mechanisms for the heart and neurological disease.

Methods

Reprograming of PBMCs

We recruited a 34-year-old male with FA through the Friedreich Ataxia Neurology Clinic at the University of South Florida, Morsani College of Medicine. The Institutional Review Board of the University of South Florida (IRB: Pro00033948) reviewed and approved this study, and informed consent was obtained. Clinical information about the subject is provided in Table 2. Reprogramming procedures can be found in previous publications (Angulo et al., 2021; Argenziano et al., 2021; Yang, Argenziano, et al., 2021; Yang, Burgos Angulo, et al., 2021; Yang, Samal, Burgos Angulo, Bertalovitz, & McDonald, 2021). Two CPT tubes were used to collect blood (~6 ml per tube) from the patient using a standard aseptic technique. To isolate the PBMCs from the blood, the tubes were centrifuged at room temperature (18-25°C) at 1500 to 1800 RCF for 20-30 minutes shortly after collection. After centrifugation, PBMCs and platelets could be observed in a white layer underneath the plasma. Plasma was discarded, and PBMCs were collected and washed with phosphate-buffered saline (PBS) by centrifugation for 15 min at 300 RCF. Clean PBMCs were counted and separated into cryovials tubes to

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store them for future use or cultured. Complete PBMC medium containing StemPro™-34 SFM Blood Media (Thermo Fisher) with cytokines (100 ng/mL SCF (Peprotech), 100 ng/mL FLT3 (Thermo Fisher), 20 ng/mL IL-3 (Peprotech) and 20 ng/mL IL-6 (Peprotech)) was used to culture PBMCs for 4-7 days. The CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher) uses Non-integration Sendai virus vectors carrying human Yamanaka factors OCT3/4, SOX2, KLF4, and c-MYC was used to transduce the PBMCs according to the manufacturer’s protocol. Briefly, PBMCs were seeded at 5 × 10^5 cells/ml in 24-well plates using the complete PBMC medium. 5 × 10^5 cells in 1 ml of medium were transduced with the reprogramming vectors at an MOI of KOS: c-Myc: Klf4 of 5:5:3. Virus containing PBMCs were then transferred to sterile, round-bottom culture tubes and sealed with Parafilm® laboratory film for centrifugation at 2250 rpm for 90 minutes at room temperature. Then, the cells were transferred to a 12-well plate, and an additional ml of PBMC complete medium was added for overnight incubation at 37°C in a humidified atmosphere of 5% CO2. The following day, PBMCs were collected and centrifuged at 200 x g for 10 minutes, resuspended in 0.5 ml of complete medium, and seeded in a 24-well plate. On the third day, cells were counted to replate 10,000 and 50,000 PBMCs per well of a 6-well plate coated with Matrigel (Corning) in 2 mL of complete StemPro®-34 medium without the cytokines. On days 4 and 6, half of the medium was replaced with fresh StemPro®-34 medium without cytokines. On day 7, half of the medium was removed and replaced with iPSC medium-mTeSR plus medium (STEMCELL Technologies), and the next day all the medium was changed to mTeSR plus medium. The spent medium was changed every two days from then on.
The first iPSCs colonies were observed around day 12 and continued to grow for a few days until they reached an optimum size for picking. The culture plate was examined at 10X magnification with an inverted microscope, and the desired colonies were marked with a cell dotter on the bottom of the plate. A 200 μL pipette was used to scrape each colony. The pieces were transferred to a new 6-well Matrigel-coated culture plate containing mTeSR plus media and ROCK inhibitor Y27632 (Sigma-Aldrich) at 10 μM. Ten distinct colonies were picked and expanded in separate wells of 6-well culture plates. They were allowed to attach to each well for 48 hours before replacing the medium with fresh mTeSR plus medium. Afterward, the medium was replaced every other day. Colonies were manually picked several times until purified iPSCs were obtained and cryopreserved in several aliquots.

**iPSC culture**

Cells were cultured at 37° C in a humidified atmosphere (95% air, 5%CO2). The control iPSC line without the FA GAA expansion was purchased from ATCC company (ACS-1026, Caucasian male). mTeSR plus medium (STEMCELL Technologies) was used to culture iPSCs on 6 or 12-well plates with Matrigel coating (Corning). A solution of 0.5 mM EDTA (Invitrogen) in DPBS was used to detach the cells. The cells were passaged around three to five days using mTeSR plus media with ROCK inhibitor Y27632 (Sigma-Aldrich) at 10 μM. Mycoplasma tests were regularly performed, and to rule out any significant chromosomal abnormalities, a KaryoStat™ assay (Thermo Fisher) was performed after 25 passages. iPSCs between P25 and P30 were used for differentiation.
Trilineage differentiation

To evaluate the differentiation capacity of our FA iPSCs, they were differentiated into the three germ layers using the STEMdiff™ Trilineage Differentiation Kit (Stemcell Technologies). This kit assesses pluripotency by directed differentiation of iPSCs to endoderm, mesoderm, and ectoderm.

Neuron differentiation

For neuron differentiation, a Gibco media system was used (Thermo Fisher). For neural induction, iPSCs were seeded to reach 15%-20% confluency on Matrigel-coated plates after one day. MTESR plus medium was replaced at this confluence for pre-warmed neural induction medium (Thermo Fisher, MA, USA). This medium was changed every two days. By day-7, the iPSCs have differentiated into Neural Stem Cells (NSCs). NSCs were dissociated with StemPro Accutase (Thermo Fisher) and expanded using neural expansion medium (Thermo Fisher). After culturing NSCs to passage 1, they were dissociated and seeded on Matrigel-coated plates with neural differentiation medium (Thermo Fisher) to obtain neurons. This medium was replaced every two days. Neurons were observed seven days after neuron media was added.

Cardiomyocyte differentiation

iPSCs derived from the FA subject and control were differentiated into iCMs with the STEMdiff™ Cardiomyocyte Differentiation Kit (STEMCELL Technologies) per the manufacturer’s protocol (Ye et al., 2020). Briefly, iPSCs were seeded on Matrigel-coated plates with mTESR plus medium until they reached ~ 95% confluency. Then, the medium was changed to STEMdiff™ Cardiomyocyte Differentiation Medium A with
matrigel (1:100) for two days, followed by STEMdiff™ Cardiomyocyte Differentiation Medium B for two days, and STEMdiff™ Cardiomyocyte Differentiation Medium C for four days. Then they were maintained in STEMdiff™ Cardiomyocyte Maintenance Medium, and the medium was changed every other day. iCMs were purified with a metabolic-selection method using glucose starvation (RPMI-glucose + B27) (Thermo Fisher) for five days as previously described (Sharma et al., 2015). For the following experiments, iCMs were dissociated with cardiomyocyte dissociation media (STEMCELL Technologies).

Immunoﬂuorescence analysis

Cells were fixed in 4% PFA (Thermo Fisher) for 15 min at room temperature, permeabilized with 0.2% Triton X-100 (Sigma Aldrich) for 10 min, and blocked for 30 min with 1% of BSA (Sigma-Aldrich) with 22.52 mg/ml glycine (Fisher Scientiﬁc) in PBST (phosphate-buffered saline with 0.1% Tween 20). For staining, antibodies were diluted in 1% BSA in PBST. Samples were incubated overnight at 4°C with primary antibodies. Cells were washed three times with cold PBS and incubated at room temperature for 1 hour with secondary antibodies. Then they were washed three times with cold PBS, counterstained with NucBlue Reagent DAPI (Thermo Fisher), and mounted with ProLong Diamond Antifade Mountant (Thermo Fisher). Samples were imaged with a Keyence BZ-X800 ﬂuorescence microscope and analyzed using Image J. Primary and secondary antibodies are listed in table 3.
Table 3. Antibodies used for immunocytochemistry

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<th>Antibody</th>
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<td><strong>Pluripotency Markers</strong></td>
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<td>Mouse anti-SSEA4</td>
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<td>Rabbit anti-OCT4</td>
<td>1:200</td>
<td>Thermo Fisher Scientific Cat# PA1-16943</td>
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<tr>
<td>Rabbit anti-SOX2</td>
<td>1:200</td>
<td>Thermo Fisher Scientific Cat# PA1-094</td>
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<td>Mouse anti-TRA-1-60</td>
<td>1:200</td>
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<td><strong>Differentiation Markers</strong></td>
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<td></td>
</tr>
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<td>Rabbit anti-PAX6</td>
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<td>Goat anti-Brachyury</td>
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<td>R&amp;D Systems Cat# 963427</td>
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<td><strong>Neuron Stem Cell markers</strong></td>
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<tr>
<td>Rabbit anti-SOX2</td>
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<td><strong>Cardiomyocyte marker</strong></td>
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PCR

Cells were lysed using buffer RLT (Qiagen) and homogenized with QiaShredder (Qiagen). RNA was isolated using the RNAeasy Mini Kit (Qiagen). For the reverse transcription, 2 µg of RNA were mixed with 4ul of Superscript IV VILO Master Mix (Thermo Fisher) and DEPC water to 20 μl. This mix was incubated for 10 minutes at 25°C, then for another 10 minutes at 50°C, and 5 minutes at 85°C. The resulting cDNA was amplified using DreamTaq Polymerase (Thermo Fisher). cDNA samples were run in 2% agarose gels stained with SYBR safe and imaged in an Odyssey-Fc (LI-COR). Primers used are listed in table 4.

Table 4. Primers and TaqMan probes used.

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<th>Primers (5’-3’)</th>
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<tr>
<td>KOS</td>
<td>528 bp</td>
<td>F: ATG CAC CGC TAC GAC GTG AGC GC R: ACC TTG ACA ATC CTG ATG TGG</td>
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<tr>
<td>KLF4</td>
<td>410 bp</td>
<td>F: TTC CTG CAT GCC AGA GGA GCC C R: AAT GTA TCG AAG GTG CTC AA*</td>
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<tr>
<td>c-MYC</td>
<td>532 bp</td>
<td>F: TAA CTG ACT AGC AGG CTT GTC G R: TCC ACA TAC AGT CCT GGA TGA TGA TG</td>
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<td><strong>Pluripotency markers (PCR)</strong></td>
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<tr>
<td>c-MYC</td>
<td>328 bp</td>
<td>F: GCGTCCTGGGAAGGGAG ATCCGGAG C R: TTGAGGGGCACTCGTGCAG GGAGGCT G</td>
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Table 4. (Continued).

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<th>Target</th>
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<td>Pluripotency markers (PCR)</td>
<td>OCT4</td>
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<tr>
<td>Pluripotency markers (PCR)</td>
<td>SOX2</td>
<td>151 bp</td>
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<td>Pluripotency markers (PCR)</td>
<td>KLF4</td>
<td>397 bp</td>
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<tr>
<td>Pluripotency Markers (qPCR)</td>
<td>SOX2</td>
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<td>Pluripotency Markers (qPCR)</td>
<td>LIN28</td>
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<td>FA affected gene</td>
<td>FXN</td>
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<tr>
<td>House-Keeping Genes (qPCR)</td>
<td>GAPDH</td>
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Table 4. (Continued).

<table>
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<th>Target</th>
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<td><strong>Genotyping</strong></td>
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<td>GAA repeat</td>
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<td>FXN intron 1</td>
<td>834 GAA repeats)</td>
<td>GAA_R: 5′–AGGACCATCATGGCCACACTT</td>
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<td></td>
<td>WT: 505 bp (2 GAA repeats)</td>
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**GAA repeat analysis**

Genomic DNA from cells was extracted with the QIAamp DNA Mini and Blood Mini kit (Qiagen, Hilden, Germany). DNA concentration and purity were examined with the Cytation 5 reader (Agilent Technologies, CA, USA).

Amplification of GAA repeat expansion in the FXN gene was performed by PCR as previously described (Long et al., 2017). Reactions utilized the Failsafe PCR System with mix D (Lucigen). The thermal cycler was programmed as follows: 94°C for 3 min, 20 cycles of 94°C for 20 sec, 64°C for 30 sec, and 68°C for 5 min, followed by nine cycles of 94°C for 20 sec, and 68°C for 5 min with each successive elongation step incremented by 15 sec. The last elongation step was 68°C for 7 min. The PCR products were resolved on 1% agarose gels stained with SYBR Safe DNA Gel Stain (Thermo Fisher). The length of an expanded GAA repeat was determined using the base pair size called of Image Lab 6.0 (BioRad). The GAA length was calculated by subtracting the length of the PCR primers and GAA flanking sequences from the number of base
pairs of the PCR product and dividing the difference by three. [Number of GAA repeats
= (length of base pairs of a PCR product - 498)/3]. Primers used are listed in table 4.

**qPCR**

cDNA was obtained as described in PCR methods. For qPCR, the cDNA was amplified on the StepOnePlus™ system (Applied Biosystems) using TaqMan Fast Advanced Master Mix (Thermo Fisher) and Taqman gene expression probes (Thermo Fisher). The expression of mRNAs was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Data were analyzed using the comparative Ct method (Livak & Schmittgen, 2001). The Taqman gene expression probes used are listed in table 4.

**RNA-sequencing sample collection and preparation**

RNA was isolated as described in the PCR method section. The concentration and purity of the RNA were determined using the Cytation 5 reader (Agilent Technologies). RNA from 3 biological replicates of each study group were sequenced at Novogene Co. Ltd for RNA-Seq and differential expression analysis. The detailed procedures are described below.

**RNA quantification and qualification**

RNA was run on 1% agarose gels to evaluate degradation and contamination. To assess the RNA purity, the NanoPhotometer® was used (IMPLEN), and a Nano 6000 Assay Kit was utilized to quantify and examine the RNA integrity (Agilent Technologies).
**Library preparation**

To generate sequencing libraries, the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB) was used, and 1 ug of RNA from each sample was the input. Each sample contained unique index codes for the sequencing. Poly-T oligo-attached magnetic beads were used for mRNA enrichment. Fragmentation was performed utilizing divalent cations at a high temperature in the NEBNext First Strand Synthesis Reaction Buffer (5X). To conduct double-stranded cDNA synthesis, an M-MuLV Reverse Transcriptase, random hexamer primers, Polymerase I, and RNase H. Exonuclease/Polymerase, followed by poly-A adenylation at 3' ends of DNA fragments and adaptor ligation of NEBNext adaptor with hairpin loop structure were utilized for end repair. The selection of the cDNA fragments with a length of 150–200 bp relied on the AMPure XP technology (Beckman Coulter). Samples were incubated with 3 ul USER Enzyme (NEB) for 15 minutes at 37 °C and 5 minutes at 95 °C. Then, PCR was performed with Phusion High-Fidelity DNA Polymerase, Universal PCR primers, and Index (X) Primer. Finally, for PCR product purification and to assess the library quality, the AMPure XP system and Agilent Bioanalyzer 2100 system were used, respectively.

**Clustering and sequencing**

the PE Cluster Kit cBot-HS (Illumina) on a cBot Cluster Generation System was used to cluster the index-coded samples following the manufacturer's recommendations. The library was prepared on an Illumina platform and paired-end reads were acquired following the cluster formation.
Quality control

Clean reads were obtained by removing the adaptor, poly-N sequences, and low-quality reads. Once the clean reads were acquired, the Q20, Q30, and GC content were determined. All the subsequent analyses were constructed on clean, high-quality data. To process the raw reading into the FASTQ format, Fastp was employed.

Mapping to reference genome

The reference genome and gene model annotation files were downloaded from the genome website browser (NCBI/UCSC/Ensembl). The Spliced Transcripts Alignment to a Reference (STAR) software was used to align paired-end clean reads to the reference genome. STAR is based on an RNA-seq alignment algorithm that uses a previously uncharacterized sequential maximum mappable seed search in uncompressed suffix arrays and then seed clustering and stitching. STAR surpasses other RNA-seq aligners in real and simulated data regarding accuracy and sensitivity.

Quantification

FeatureCounts was used to count the read counts mapped for each gene. Then, each gene’s RPKM (Reads Per Kilobase of Exon Model per Million Mapping Reads) was calculated according to the gene length and the number of reads mapped to it. RPKM is the most widely used approach to predict gene expression levels because it considers the impact of sequencing depth and gene length simultaneously (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008).
Differential expression analysis

The DESeq2 R software was used to compare the expression levels of two groups with three biological replicates per condition. DESeq2 is based on a model on the negative binomial distribution to determine differential expression in gene expression data. Benjamini and Hochberg's technique was used to control the False Discovery Rate (FDR). Differentially expressed genes were defined as those found by DESeq2 with an adjusted p-value of 0.05. The threshold for significantly differential expression was established at adjusted p-value < 0.05 and |log2 (Fold Change) | > 1.

Enrichment analysis

For enrichment analysis, the list of differentially expressed genes was submitted to g:Profiler, a web server that interprets and maps genes to the corresponding enriched pathways based on well-established data sources. We included Gene Ontology (GO), which annotates genes to biological processes, molecular functions, and cellular components; Kyoto Encyclopedia of Genes and Genomes (KEGG), which annotates genes to pathways; and Human Phenotype Ontology which provides human disease phenotype associations. The cluster profile R package was used to perform GO, KEGG, and Reactome enrichment analysis of differentially expressed genes, with a corrected P value less than 0.05 considered significant.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical significance was determined by Student’s t-test (two-tailed) for two groups and one-way ANOVA for
multiple groups with post hoc test Bonferroni. Statistical significance was defined as a value of P < 0.05.

Results

Establishment of the iPSC model

The University of South Florida Ataxia Research Center focuses on providing specialized medical care for those people affected with cerebellar ataxia while performing basic and clinical research on ataxia. Therefore, we invited a patient with FA to participate in our study with their collaboration. This study was approved by the Institutional Review Board of the University of South Florida (IRB: Pro00033948), and informed consent was obtained. This patient is 34 year of age Caucasian male presenting a GAA expansion of 1167 and 834 repeats in the intron 1 of FXN. He was first diagnosed with FA at 8-years of age due to walking difficulties. His first cardiac symptom manifested at the age of 28 with chest palpitations. At the time of enrollment in this study, he was a wheelchair user and presented several neurological, cardiac, and other FA symptoms, summarized in table 2.

We collected a blood sample from this patient and isolated PBMCs to reprogram them into iPSCs using the Cytotune Sendai Virus System that delivered the Yamanaka factors KOS, KLF4, and C-MYC into PBMCs. iPSCs colonies were picked into individual clones and passage to at least p20 before any other experiments were performed. We confirmed the homozygous GAA expansion with PCR using primers flanking the GAA repeats (Fig 2. E). The iPSCs derived from the patient exhibit the morphology of embryonic stem cells, with a large nucleus and prominent nucleoli, a high nucleus to
cytoplasmic ratio, and densely packed cells growing in colonies (Fig 2. A).

Immunofluorescence staining confirmed the expression of pluripotency markers SOX2, TRA-1-60, SSEA4, and OCT4 (Fig 2. C). Quantitative real-time PCR demonstrated the relative expression of pluripotency markers OCT4, Nanog, SOX2, and Lin28, and PCR analysis showed the endogenous expression of markers MYC, Nanog, SOX2, and KLF4 in these iPSCs (Fig 2. D). To rule out any primary chromosomal abnormality in the FA iPSCs, we evaluated them with a Karyostat assay and confirmed that they have a normal karyotype (Fig 2.G). Lastly, Functional evaluation of the FA iPSCs was confirmed by their ability to differentiate into the three germ layers (Fig 2.B). The establishment of this iPSC line has been previously described in detail (Yang, Burgos Angulo, et al., 2021).

**Establishment of iPSC-derived neurons**

Neuron differentiation protocols have been optimized and made commercially available, and the companies offer various alternatives. When choosing the protocol for differentiating the iPSCs into neurons, we opted for a Gibco media system (Thermo Fisher) (Fig 3. A). The first step in producing neurons in the induction of iPSCs to NSCs. Most methods of NSC derivation from iPSC require embryoid body formation or co-cultures with stromal cells, leading to time-consuming protocols and variability in the quality of NSCs. The neural induction medium of Gibco differentiates iPSCs into NSCs in one with high efficiency, without the arduous and lengthy process of embryoid body formation and manually isolating the NSCs. As shown in Fig 3. B, we obtained NSCs from both the FA and control iPSCs. Like iPSCs, NSCs continue to divide, but they have a different morphology and do not grow in colonies.
Immunostaining demonstrated the expression of NSC markers NESTIN and SOX2 for both cell lines (Fig 3. B). Mutation analysis was performed with PCR using primers flanking the GAA repeat, and we were able to confirm that patient NSCs preserved the GAA expansion. In contrast, control NSCs did not exhibit GAA expansion (Fig 3. D). NSCs from the FA patient presented significantly lower FXN mRNA levels than controls corresponding to FA pathogenesis, as shown with qPCR (Fig 3. E). The following steps in obtaining iNS involved NSC expansion and differentiation. After NSCs from both FA and control were passage to passage 1 with neural expansion media, they were treated with B27 plus neuronal media to achieve neuron differentiation. After one week of differentiation, the cells displayed a marked change in morphology typical of neurons, with smaller, rounded, cell bodies, and long processes connecting them (Fig 3. C). These cells expressed the neuronal markers DCX and MAP2 shown in immunostaining (Fig 3. C) Like NSCs, FA iNs preserved the GAA expansion, whereas control does not have a GAA repeat expansion (Fig 3. D). The neurons derived from the patient exhibited markedly reduced FXN mRNA than control neurons (Fig 3. E).

**Establishment of iPSC-derived cardiomyocytes**

We compared two published and two commercially available protocols to select the most efficient cardiomyocyte differentiation protocol. The published ones were a chemically defined small molecule differentiation protocol developed by Paul W Burridge et al. (Burridge, Holmstrom, & Wu, 2015; Burridge et al., 2014) and a chemically-defined and albumin-free culture system (S12 medium) for cardiac differentiation (Pei et al., 2017). The commercially available protocols included the PSC Cardiomyocyte Differentiation Kit (Thermo Fisher) and the STEMdiff™ Cardiomyocyte
Differentiation Kit (StemCell Technologies). We were able to differentiate iPSCs from the patient and control into iCMs with each of these four protocols. However, the most efficient and consistent was the commercial differentiation kit from StemCell Technologies (STEMdiff™ Cardiomyocyte Differentiation Kit) (Fig 4.A); therefore, all the iCMs in this study were obtained with this kit. Immunostaining of iCMs from control and patient exhibited spontaneous contractions and showed the expression of cardiomyocytes markers α-actinin and Tropomyosin (Fig 4. B). We reliably produce iCMs in 8-12 days after initiation of the procedure from control and FA subject cell lines. The FA iCMs maintained the GAA expansion and expressed lower levels of FXN mRNA than control iCMs (Fig 4. C and D).

**Differentially expressed genes in iPSC-derived neurons**

To identify gene expression patterns specific to FA iNs, we performed transcriptional profiling using the RNA-seq methodology. We extracted RNA from 30-day-old iNs and performed RT-PCR to evaluate the FXN expression levels. Patient cells had significantly lower FXN levels than control cells (Fig 3. E). A total amount of 1 ug of RNA per sample was used for the RNA-seq analysis. Differential expression analysis between patient and control cell types was performed using the DESeq2R package, and the genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed. 6040 genes were differentially expressed between FA and control iNs, of which 3025 were upregulated, and 3015 were downregulated (Fig 5. A). A heatmap representing the differentially expressed genes showing the three replicates used for the analysis is shown in Fig 5. B. The differentially expressed genes based on adjusted p-value and fold change are illustrated in a volcano plot (Fig 5.C). Noteworthy
upregulated genes in FA iNs include \textit{PTPRT}(\textit{padj.} 1.25E-23, \textit{log2Fold change.} 10.69), \\
\textit{PCDHGA3} (\textit{padj.} 6.24E-41, \textit{log2Fold change.} 9.00668175), \textit{UNC5D} (\textit{padj.} 4.08E-68, \\
\textit{log2Fold change.} 5.18), and \textit{OTP} (\textit{padj.} 1.02E-156, \textit{log2Fold change.} 5.96).

Protein Tyrosine Phosphatase Receptor Type T \textit{(PTPRT)} encodes for a member of
the protein tyrosine phosphatase (PTP) family. PTPs are signaling molecules that
regulate various cellular processes, including cell growth, differentiation, and the mitotic
cycle (Tonks, 2006). \textit{PTPRT} mRNA is expressed in the developing nervous system (J. R. Lee, 2015). In adults, \textit{PTPRT} is expressed in the central nervous system and
localizes in the neuronal synapses, and its activity has also been demonstrated to be
required for neuronal dendrite development (J. R. Lee, 2015; S. H. Lim et al., 2009).

Protocadherin Gamma Subfamily A, 3 \textit{(PCDHGA3)} is a member of the
protocadherin gamma gene cluster. These neural cadherin-like cell adhesion proteins
might play an essential role in the establishment and function of the specific cell-to-cell
connections in the brain (Mancia Leon et al., 2020).

Unc-5 Netrin Receptor D \textit{(UNC5D)} is the receptor for NTN4 and has a role in
promoting neuron cell survival (Takemoto et al., 2011). It plays a role in axon guidance
in the developing nervous system upon ligand binding (Takemoto et al., 2011; Tang et
al., 2008). It functions as a dependence receptor needed for apoptosis induction when it
is not associated with the netrin ligand (Zhu et al., 2013).

Finally, \textit{OTP} encodes a protein of the homeodomain family. These proteins are
transcription factors that play essential roles in the specification of cell fates (Kaji &
Nonogaki, 2013). OTP, in particular, may have a role during brain development (Acampora, Postiglione, Avantaggiato, Di Bonito, & Simeone, 2000).

Some notable downregulated genes in FA iNS are $DMBX1$ (adj. $7.93E-48$, log2Fold change. -6.42), $ZIC4$ (adj. $5.80E-157$, log2Fold change. -5.24), $ZIC5$ (adj. $1.88E-88$, log2Fold change. -5.634172253), and $PHOX2B$ (adj. $1.83E-142$, log2Fold change. -7.73).

Diencephalon/Mesencephalon Homeobox 1 ($DMBX1$) encodes for a transcription factor that may play an important role in brain and sensory organ development (Ohtoshi & Behringer, 2004).

Zic Family Member 4 ($ZIC4$) encodes for a member of the ZIC family of C2H2-type zinc finger proteins. Members of this family are essential during development (Elsen, Choi, Millen, Grinblat, & Prince, 2008).

Zic Family Member 5 ($ZIC5$) is a member of the zinc-finger of the cerebellum protein family. It allows DNA-binding transcription factor activity and DNA binding activity of RNA polymerase II transcription regulatory region (Ishiguro, Inoue, Mikoshiba, & Aruga, 2004) and has a crucial role in cell differentiation, forebrain development, and neural tube closure (Inoue et al., 2004; Nakata, Koyabu, Aruga, & Mikoshiba, 2000).

Lastly, the $PHOX2B$ protein supports the formation and maturation of neurons (Rohrer, 2011). Interestingly, these dysregulated genes in FA iNS have a role in nervous system development, supporting neuronal differentiation, specification, and maturation. These changes may indicate that even though FA iNS appears to have
normal morphology and function, there are differences in the early stages of neuron development.

**Enrichment analysis of DE genes of FA iPSC-derived Neurons**

To elucidate the possible biological function of the DE genes from FA iNs, we carried out a functional enrichment analysis using g:Profiler (Raudvere et al., 2019). The list of dysregulated genes was imputed on this webserver which maps them to well-known functional information sources resulting in statistically significant enriched biological processes and pathways. The top overrepresented processes and pathways are shown in figure (Fig 5. D) The most significant processes involved the development of the nervous system, such as Nervous system development (GO:0007399), Synapse (GO:0045202), Neurogenesis (GO:0022008), and Neuron differentiation (GO:0030182). According to the KEGG database, the most enriched biological pathways included Axon guidance (KEGG:04360), Hippo signaling pathway (KEGG:04390), Glutaminergic synapse (KEGG:04724), and Dopaminergic Synapse (KEGG:04728). These enriched processes and pathways correlate with the roles of the mentioned dysregulated genes, meaning that FA iNs develop differently than control iNS and that the axons, dendrites, and synapses may function in ways that can predispose the FA phenotypes that follow.

**Differentially expressed genes in iPSC-derived cardiomyocytes**

To investigate the difference in gene expression specific to FA iCMs, we used RNA-seq technology. Like with iNS, we collected 30-day-old RNA derived from FA and control iCMS and evaluated the *FXN* mRNA expression with qPCR. Patient cardiomyocytes had significantly reduced *FXN* levels than control cells (Fig 4. D) 1 ug of
RNA per sample and three biological replicates from FA and control iCMs were used for the RNA-seq analysis. Differential expression analysis between patient and control cells was performed using the DESeq2R package. 1053 genes were differentially expressed between FA and control iCMs; 690 were upregulated, and 363 were downregulated (Fig 6. A). A heatmap representing the differentially expressed genes showing the three replicates used for the analysis is shown in Fig 6. B, and a volcano plot illustrates these genes based on adjusted p-value and fold change (Fig 6. C). Notable upregulated genes in FA CMs include \textit{RND1} (padj. 3.18E-10, log2Fold change. 3.26), and \textit{COL22A1} (padj. 0.01, log2Fold change. 1.16).

Rho Family GTPase 1 (\textit{RND1}) encodes a signaling GTPase that belongs to the Rnd subgroup of the Rho family of GTPases. Overexpression of \textit{RND1} induces cellular hypertrophy and proliferation via activation of RhoA-mediated SRF signaling in cardiomyocytes (Kluge et al., 2019).

Collagen Type XXII Alpha 1 Chain (\textit{COL22A1}) is a member of the collagen family produced by muscle cells known to play a role in muscle development and is localized in the myotendinous junction in the heart muscle (Koch et al., 2004). \textit{COL22A1} has been associated with cardiac fibrosis, suggesting that it may play a role in developing pathological cardiac hypertrophy (Q. Li et al., 2016; Rau et al., 2015).

Hemojuvelin BMP Co-Receptor (\textit{HFE2}) (padj. 0.002, log2Fold change. -2.87) and \textit{CDON} (padj. 0.001441188, log2Fold change. -1.16) are two significant downregulated genes in FA iCMs. \textit{HFE2} is a membrane-bound and soluble protein involved in iron metabolism. (Piperno, Pelucchi, & Mariani, 2020). This protein is expressed in the liver, heart, and skeletal muscles and plays a role in maintaining adequate iron levels in the
body by regulating the expression of hepcidin. Hepcidin is necessary for maintaining iron homeostasis (Hernandez et al., 2021; Piperno et al., 2020).

Cell Adhesion Associated, Oncogene Regulated (CDON) encodes a cell surface receptor member of the immunoglobulin superfamily. It mediates cell-to-cell interactions, positively regulating myogenesis (Okumura et al., 2021). CDON has a crucial role in preventing cardiac remodeling by suppressing Wnt signaling (Jeong et al., 2017). Mice with CDON deficiency develop cardiac fibrosis and altered expression of remodeling genes (Jeong et al., 2017).

These dysregulated genes are involved in developing fibrosis, iron overload, and cardiac remodeling, leading to pathologic cardiac hypertrophy. These are features of cardiac disease in FA, and therefore it would be important to understand the pathologic role of these genes in the context of FA.

**Enrichment analysis of DE genes of FA iPSC-derived CMs**

To understand the possible biological function of the FA iCMs DE genes, we also performed a functional enrichment analysis with g:Profiler (Raudvere et al., 2019) (Fig 6. D). The most significant processes involved genes from the cell periphery (GO:0071944) and the extracellular matrix (GO:0031012). Significant pathways involved extracellular matrix-receptor interaction (KEGG:04512) and focal adhesion (KEGG:04510). Cardiac remodeling involves the interaction of extracellular matrix and cell proteins that leads to fibrosis and eventually the development of hypertrophy and heart failure. Research has found that FA patients can develop fibrosis prior the hypertrophy; therefore, these gene changes may be early signs of heart disease.
Discussion

In this part of the study, we reprogrammed PBMCs into iPSCs from an FA patient at the USF Ataxia Research Center. This 34-year-old Caucasian male had a GAA expansion of 1167 and 834 repeats in the first intron of FXN. He displayed neurological symptoms first, in his first decade of life, and in his late twenties started to show mild cardiac symptoms (Table 2). The iPSCs generated from this patient maintained the homozygous GAA expansion, exhibited the morphology of a typical embryonic stem cell, and expressed the typical pluripotency markers. FA iPSCs can differentiate into the three germ layers and present a normal karyotype. FA iPSCs and control iPSCs (ACS-1026, from a Caucasian male from ATCC) were differentiated into iNS and iCMs. FA-derived cells kept the GAA expansion and displayed lower levels of FXN compared to control iNs and iCMs. Therefore, after 30 days of differentiation, iNs and iCMs from FA and control were subject to transcriptional profiling. DE-seq analysis showed the differentially expressed genes from both iNS and iCMs, showing a molecular signature of the disease. Interestingly, there were more dysregulated genes from the iNs than from the iCMs (6040 vs. 1053), which may reflect the predominantly neurological disease of this patient. Enrichment analysis and careful examination of the DE genes showed that there are different enriched pathways and processes and dysregulated genes between iNs and iCMs, meaning that there are differing pathophysiological mechanisms for the heart and neurological disease.

For the iNs, the most significant processes and pathways are involved in the development of the nervous system, and significantly dysregulated genes have roles in supporting neuronal differentiation, specification, and maturation. These changes may
indicate that neuron development is altered in FA and may lead to the neuron symptoms of the disease.

On the other hand, iCMs' most significant processes and pathways involved genes from the cell periphery and extracellular matrix and extracellular matrix-receptor interaction. Significantly dysregulated genes are involved in fibrosis development, iron overload, and cardiac remodeling. Cardiac remodeling requires extracellular matrix and cell protein interaction leading to fibrosis and eventually the development of hypertrophy and heart failure, and iron overload is a feature of FA. Therefore, these gene changes may be an early sign of developing heart disease.

Interestingly, few genes and pathways overlap when comparing this study to other RNA-seq studies in FA iNs and iCMs (Lai et al., 2019; J. Li et al., 2019). This could reflect the different techniques used to generate iNs and iCMs, the patient-to-patient variability, and control variability.

By comparing FA-cells and controls we are observing differences that may be due to FXN-deficiency and differences that may be due to epigenetic effects of the GAA repeat expansion. The limitations include the fact that the control line is not isogenic and therefore, some of the differences in gene expression will be due to inherent genetic background differences.
Fig 2. Characterization of iPSCs derived from a FA patient. (A) Bright-field image of iPSC colony generated from the patient. (B) Immunostaining representing the differentiation of the patient iPSCs into the three germ layers, SOX17 (red) was used as a marker for endoderm, BRACHYURY (red) was used as a marker for mesoderm, and PAX6 was used as a marker for ectoderm. Nuclei were stained with DAPI (blue). Scale bar 100 μM. (C) Immunostaining of patient iPSCs pluripotency markers SOX2 (green), TRA-1-60 (red), SSEA4 (green), and OCT4 (red). Nuclei were stained with DAPI (blue). Scale bar 100 μM. (D) PCR analysis showing the loss of the episomal Sendai virus and exogenous reprogramming factors SeV, KOS, KLF4, and MYC and the endogenous expression of markers MYC, Nanog, SOX2, and KLF4 in these iPSCs. (E) PCR analysis showing the FXN GAA expansion in patient iPSCs and no expansion in control iPSCs. (F) qPCR analysis showing the relative expression of pluripotency markers OCT4, Nanog, SOX2, and Lin28. (G) Karyostat assay of patient iPSCs showing a normal Karyotype.
Fig 3. iNSCs and iNs characterization. (A) Diagram of the protocol for neuron differentiation. (B) Immunostaining of control and patient iNSCs showing expression of the markers NESTIN (green) and SOX2 (red). Nuclei were stained with DAPI (blue). Scale bar 50 um. (C) Immunostaining of control and patient iNs showing expression of neuron markers of DCX (green) and MAP2 (red). Nuclei were stained with DAPI (blue). Scale bar 50 um. (D) PCR analysis of the GAA repeat region in the FXN gene using genomic DNA extracted from iNSCs and iNs from control and FA patient. (E) qPCR quantification of FXN mRNA levels in iNSCs and iNs from control and FA patient. Data are expressed as means ± S.D. of three independent experiments. *p < 0.05. NSC_Ctrl indicates iNSCs from control, NSC_FA indicates iNSC from patient, N_Ctrl indicates iNs from control, and N_FA indicates iNs from patient.
Fig 4. iCMs Characterization. (A) Diagram of the cardiomyocyte differentiation protocol. (B) Immunostaining of control and patient iCMs showing expression markers α-actinin (green) and Tropomyosin (TPM) (red). Nuclei were stained with DAPI (blue). Scale bar 50 um. (C) PCR analysis of the GAA repeat region in the FXN gene using genomic DNA extracted from iCMs from control and patient. (D) qPCR quantification of FXN mRNA levels in iCMs from control and FA patient. Data are expressed as means ± S.D. of three independent experiments. *p < 0.05. C_Ctrl indicates iCMs from control, and C_FA indicates iCMs from patient.
Fig 5. Transcriptional profile of FA iNs and control iNs. (A) Venn diagram illustrating the DE genes between FA iNs and control iNs. (B) A heatmap showing the expression of 6040 genes DE in both groups (FA and control iNs). Three independent replicates are shown per group. (C) Volcano plot of DE genes between FA and control iNs based on adjusted p-value and \(\log_2(\text{Fold Change})\) \(\leq 0.05\) and \(|\log_2(\text{Fold Change})| > 1\). Significant genes discussed in results are labeled. (D) Enrichment analysis of DE genes in FA and control iNs using g:Profiler. We included results from the Gene Ontology and KEGG databases.
Fig 6. Transcriptional profile of FA iCMs and control iCMs. (A) Venn diagram illustrating the DE genes between FA iCMs and control iCMs. (B) A heatmap showing the expression of 1053 genes DE in both groups (FA and control iCMs). Three independent replicates are shown per group. (C) Volcano plot of DE genes between FA and control iCMs based on adjusted p-value < 0.05 and |log2 (Fold Change)| > 1. Significant genes discussed in results are labeled. (D) Enrichment analysis of DE genes in FA and control iCMs using g:Profiler. We included results from the Gene Ontology and KEGG databases.
CHAPTER THREE

CONTRIBUTION OF FXN DEFICIENCY IN FRIEDREICH’S ATAXIA NEURAL AND CARDIAC DISEASE

Introduction

By performing transcriptional profiling in the FA iNs and iCMs compared to an unrelated control without the GAA expansion, we obtained insight into the molecular signature of disease and how neurons and cardiomyocytes are affected in different ways. The FA iNS display developmental differences, whereas the iCMs are prone to have changes in genes that lead to fibrosis, iron overload, cardiac remodeling, and hypertrophy.

However, there are some caveats with this approach. Comparing the FA cells to those of a genetically distinct control may show differences related to the genetic background and mask some actual disease signatures. Our differential gene expression analysis may show other dysregulated genes if compared to an alternative control cell line, explaining why other RNA-seq studies do not always agree.

Another important aspect to consider is the proposed functions of FXN (in iron homeostasis, cellular redox reaction regulation, iron-sulfur cluster biogenesis, and heme synthesis) (Adamec et al., 2000; Adinolfi et al., 2009; Becker et al., 2002; Yoon & Cowan, 2003, 2004) do not sufficiently explain why its deficiency generates the various FA phenotypes and affects only certain cell types when it is ubiquitously expressed.
(Koutnikova et al., 1997). One possibility is that the GAA expansion itself may have a pathological role independent of FXN expression (Al-Mahdawi et al., 2008; Castaldo et al., 2008).

Therefore, to consider these aspects, we upregulated FXN expression by transducing an FA patient’s iNs and iCMs with FXN lentivirus to approximate normal levels and analyzed subsequent changes in gene expression with RNA-seq. Using this approach, we focus on the early molecular mechanisms of FXN deficiency independent of the GAA expansion, and since this is performed in the same patient, the genetic background is identical.

We hypothesized that the FXN deficiency has a pathological role independent of the GAA expansion.

**Methods**

**Generation of FXN lentivirus transfer plasmid**

The pcDNA3.1-hFXN-HA plasmid was acquired from Addgene. The primers (forward) 5’-GCTCGCTAGCGCCACCATGTGGACTCTCGGGCG-3’ and (reverse) 5’-GCCCGGATCCTCAAGCATCTTTTCCGGAATAGGCC-3’ were produced (Sigma-Aldrich) to generate a FXN-containing PCR product without Human influenza hemagglutinin (HA)-tag and flanked by NHEI (5’ end) and BAMHI (3’ end) restrictive sites. This PCR product was digested with restriction enzymes NHEI and BAMHI and gel purified with the DNA Gel Extraction Kit (NEB). Then, the Lentiviral NanoLuc control expression vector plasmid was acquired from Addgene, digested with restriction enzymes NHEI and BamHI to remove the NanoLuc insert and the amino-terminal MYC
epitope tag, and gel purified using the Monarch® DNA Gel Extraction Kit (NEB). To insert the gel-purified insert into the gel-purified backbone of the plasmid, T4 ligase was used. Sanger sequencing was performed to verify the final expression plasmids were verified by Sanger Sequencing (Eurofins Genomics).

**Frataxin lentivirus production**

HEK293T cells were seeded in 60 mm dishes coated with Poly-d-lysine (Thermo Fisher) in 5ml media without antibiotics (RPMI-1640 supplemented with FBS, Thermo Fisher). The following day when cells were 70% confluent, they were transfected using the Lipofectamine 3000 transfection reagent (Thermo Fisher) with the following plasmids: 1.25 ug of envelope plasmid pCMV-VSV-G (8454, Addgene), 1.25 ug of packaging plasmid psPAX2 (12260, Addgene), and 2.5 ug of FXN lentivirus transfer plasmid. The following day, the media was exchanged for 5 ml of media with antibiotics (Thermo Fisher). Two days later, the media, which now contains lentivirus, was collected and filtered through a 0.45 um filter (Genesee Scientific) and stored at 4°C. 5 ml of new media with antibiotics was added to the transfected cells. After two days, the media was collected and filtered through a 0.45 um filter and mixed with previously collected media. For further analysis and transduction, samples were aliquoted and frozen at -80°C.

**Frataxin lentivirus titer and transduction**

According to the manufacturer's instructions, we used the Lenti-X GoStix Plus (Takara) to determine the lentivirus titer. The GoStix Plus app was downloaded on a mobile device, and the lot number of the kit was entered. To ensure that our
unconcentrated lentivirus sample had a reading within the standard curve range, we tested the sample undiluted and in a 1:2 dilution. Following the app, 20 ul of the lentiviral supernatant (diluted and undiluted) were added to the Lenti-X GoStix Plus cassette sample well. Then, 80 ul of Chase Buffer were added to the sample well and allowed to appear in the cassette window. The lateral flow test was run for 10 min, and the cassette was scanned with the app. The app included the standard curve, which automatically calculated the titer (i.e., a GoStix Value [GV] equivalent to ng/ml p24) based on the band's intensity.

To transduce the FA iNs and iCMs with the FXN lentivirus, the optimal concentration of lentiviral supernatant was determined by assessing several dilutions. The cells were transduced on day 15 after differentiation, and the media was changed the next day to remove residual lentivirus. On day 30, iNs and iCMs were lysed, and the RNA was extracted. RT-PCR was carried out to evaluate the FXN expression level, comparing it to control cell line and non-transduced FA cells. The Lentiviral dilution for transduction was chosen based on that producing the closest FXN mRNA to control cells.

Cytotoxicity assay

We used the Cell Counting Kit-8 (CCK8)(Sigma-Aldrich) to determine whether forced expression of FXN resulted in cell toxicity. 5000 cells/well (iNs and iCMs) were seeded in a 96-well plate and transduced with FXN lentivirus. After two weeks, the cytotoxicity was assessed with the assay. 10 μl of the CCK8 solution was added to each
well of the plate containing transduced, non-transduced cells, and control without cells. 2 hours after incubation at 37°C, the absorbance was measured at 450 nm with the Cytation 5 reader (Agilent Technologies). This assay was performed three times, using 6 wells per condition.

**Capillary western blot**

Cells were lysed with RIPA buffer (Thermo Fisher) containing protease and phosphatase inhibitors (78430, Thermo Fisher, MA, USA). Cellular lysates were sonicated and centrifuged for 15 min at 15,000 rpm, and a Bradford assay (Thermo Fisher) was used to determine the protein concentration. Capillary western analyses were carried out in the ProteinSimple Wes® System. Samples were diluted with 0.1XSample Buffer. Then four parts of the diluted sample were combined with 1 part of 5X Fluorescent Master Mix (which contains 5X sample buffer, 5X fluorescent standard, and 200 mM DTT) and incubated at 95 °C for 5 min. Then, the samples, blocking reagent, primary, HRP-conjugated secondary antibodies, and chemiluminescent substrate were added to assigned wells in a 13-well assay plate. Once the plate was loaded, the separation electrophoresis and immunodetection occurred in the fully automated capillary system (Protein Simple). The Primary antibodies and secondary antibodies are listed in table 5.
Table 5. Capillary western blot antibodies

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Results

**Forced expression of FXN in iPSC-derived neurons and cardiomyocytes**

To study the role of FXN deficiency in iPSC-derived iNs and iCMs from the FA-patient, we sought to normalize its expression level by transducing them with *FXN* lentivirus (Fig 7. A). Previous studies have demonstrated that induction of high *FXN* expression levels are toxic to cells (Belbellaa, Reutenauer, Messaddeq, Monassier, & Puccio, 2020; Huichalaf et al., 2022; Vannocci et al., 2018). Therefore, we modified an expression vector using a relatively weaker constitutive promoter (UbC) (113450, Addgene, MA, USA) (Qin et al., 2010) to better control *FXN* overexpression (Fig 7. A). To test this *FXN* lentivirus, we started by transducing iPSC iNs and iCMs from control. We subsequently collected the cells for analysis two weeks post-transduction to ensure that *FXN* overexpression was not detecting an early transient event. To assess the mRNA level of *FXN*, we performed RT-PCR and obtained a higher than 10-fold increment in transduced iNs and iCMs compared to non-transduced cells (Fig 7. B). To determine that the *FXN* mRNA was being translated into protein, we performed capillary western blot and confirmed an increased FXN protein in both iNs and iCMs (Fig 7. C).
To corroborate that this FXN overexpression was evenly distributed among the cells, we performed immunostaining of FXN, co-stained with a neuron or cardiac marker (DCX and cTnT, respectively) on transduced and non-transduced iNs and iCMs. Staining showed that FXN is overexpressed broadly in transduced iNs and iCMs (Fig 7. D and E.) Therefore, we concluded that transduction with this FXN lentivirus is appropriate for FXN overexpression in iPSC-derived iNs and iCMs and can be used to restore the FXN level in FA patient cells.

**Determination of FXN lentivirus titer and optimal concentration to transduce patient's iNs and iCMs**

Previous studies have demonstrated that restoring the levels of FXN to normal levels improves phenotypes of FA (Chandran et al., 2017; F. Lim et al., 2007; Sacca et al., 2011). Before examining if our introduction of FTX-lentivirus could achieve corrective changes, we established that our FXN overexpression method would result in FXN levels in FA-iNs and iCMs as close as possible to those levels observed in control cell lines. To maintain reproducibility in all the experiments, we determined the lentivirus titer using the Lenti-X GoStix Plus (Takara). This assay quantifies the amount of p24 capsid protein present in lentiviral supernatants, which correlates directly with infectious viral titer. We obtained 270 ng/ml of p24 for our FXN lentivirus. Then we transduced the FA patient iNs and iCMs with different dilutions and assessed the FXN expression with RT-PCR relative to control cells to optimize the desired amount for transduction (Fig 8. and 9. A). We opted for a lentivirus dilution of 1/50 (5.4 ng/ml of p24) to transduce the FA iNs and 1/10 (27 ng/ml of p24) to transduce the FA iCMs. A cytotoxicity assay was performed to evaluate the toxicity of this chosen amount to iNs and iCMs. We did not
observe any difference in the number of living cells between transduced and non-transduced iNs and iCMs (Fig 8. B, and 9. B).

**Transcriptome profiling identifies DE genes in FXN transduced and FA iPSC-derived neurons and cardiomyocytes**

To identify gene expression patterns for FA and specifically which genes are regulated exclusively by FXN levels in iNs and iCMs, we performed transcriptional profiling using RNA-seq in FXN transduced and non-transduced cells derived from an FA patient. Total RNA from 3 biological replicates was collected two weeks after transduction when iNs and iCMs were 30-days old (from differentiation time). For non-transduced cells, the RNA from 3 replicates was collected on the same day, (30-days after differentiation). Differential expression analysis between FA transduced and non-transduced cells was performed using the DESeq2R package, and the genes with an adjusted P-value <0.05 found by DESeq2 were assigned as DE. 127 genes were DE between transduced and non-transduced FA-derived iNs, of which 37 were upregulated, and 90 were downregulated (Fig 8.C). For iCMs, 417 genes were DE, 320 were upregulated with lentivirus transduction, and 97 were downregulated (Fig 9. C). Heatmaps representing the differentially expressed genes showing the 3 replicates are shown in Fig. 8. D and 9.D.

**Enrichment analysis of DE genes of FA iPSC-derived neurons and cardiomyocytes**

To elucidate the potential biological function of the differentially expressed genes of iNs and iCMs derived from the FA patient, we performed functional enrichment
analysis using g:Profiler (Raudvere et al., 2019). By providing the list of differentially expressed genes from both cell types, the webserver maps them to known functional information sources, resulting in statistically significant enriched biological processes and pathways. The top overrepresented biological processes and pathways for iPSC-derived iNs and iCMs from the patient are shown in Fig 8. E and 9. E. For iPSC-derived iNs, the most significant processes were related to carbohydrate metabolism, such as Glycolysis (GO:0006096), ATP generation (GO:0006757), and ADP and Pyruvate metabolic processes (GO:0046031, GO:0006090). According to prior studies, FA patients have abnormal glucose metabolism and exhibit a higher risk for diabetes (Finocchiaro et al., 1988; Gucev et al., 2009; Isaacs et al., 2016). Furthermore, lower levels of ATP that are restored with increased FXN levels have been shown in FA patients and models of FA (Bolinches-Amoros, Molla, Pla-Martin, Palau, & Gonzalez-Cabo, 2014; Heidari, Houshmand, Hosseinkhani, Nafissi, & Khatami, 2009; Y. Li, Polak, et al., 2015; Lodi et al., 1999). Regarding the biological pathways based on the KEGG database, Glycolysis and Gluconeogenesis (KEGG:00010) is the most significant, but HIF-1 (KEGG:04066) and Carbon metabolism (KEGG:01200) are also enriched, among other related pathways. Finally, from human phenotype ontology which annotates genes to phenotypic abnormalities encountered in human disease, we obtained features of FA such as sensory dysfunction (HP:0003474), muscle weakness (HP:0001324), and peripheral neuropathy (HP:0009830).

From iPSC-derived iCMs, the most significantly enriched terms were related to the processes and components of the extracellular matrix, such as extracellular matrix organization (GO:0030198) and collagen fibril organization (GO:0030199). Cardiac
Fibrosis, which is the consequence of extracellular matrix remodeling from pathological processes, has been observed in FA patients even before the overt cardiac disease has developed (Hanson et al., 2019). There is also enrichment for extracellular matrix pathways, including extracellular matrix-receptor interaction (KEGG:04512) and focal adhesion (KEGG:04510).

**DE genes in FA vs. control iPSC-derived neurons and cardiomyocytes rescued with forced FXN expression**

To further understand the role of FXN deficiency in FA, we investigated the DE genes of FA vs. control that are restored to control levels when patient cells are transduced with FXN. To assess this, we performed RNA-seq and DEA of FA iPSC-iNs and iCMs compared to control iPSC-derived cells. Then we analyzed the FA genes that were corrected to control transcription levels after FXN lentivirus transduction. The corrected genes of both iNs and iCMs are shown in the heatmaps in Fig 10. A. and 11. A. We performed functional enrichment analysis using these corrected genes to determine the overrepresented biological pathways and processes. The most significant processes and pathways for iPSC-iNs are Glycolysis (KEGG:00010, GO:0006096), ATP generation (GO:0006757), and HIF-1 signaling (KEGG:04066). These were enriched in our previous analysis of FA vs. FXN-transduced FA, meaning that the FXN expression level is directly involved in these pathways and processes.

For iPSC-iCMs, the enriched processes in these corrected genes are related to the extracellular matrix, as found in the previous analysis. This means that FXN deficiency may play a role in these processes. IPA from the most enriched pathways for
the corrected genes in iNs and iCMs showed network diagrams with the most plausible connections to each other and genes in the IPA knowledge base (Kramer, Green, Pollard, & Tugendreich, 2014) (Fig 10. C and 11. C). For iNs, the most relevant genes belonging to glycolysis and gluconeogenesis included $GPI$ (padj.5.79E-06, log2Fold change. -0.37), $HK2$ (padj. 4.33E-09, log2Fold change. -0.65), $TPI1$ (padj. 0.02, log2Fold change. -0.22), $ALDOA$ (padj. 2.71E-11, log2Fold change. -0.43), $ENO1$ (padj. 3.41E-07, log2Fold change. -0.37), $LDHA$ (padj. 2.52E-07, log2Fold change. -0.39), $PFKP$ (padj. 0.01, log2Fold change. -0.25), and $PKM$ (padj. 0.0003, log2Fold change. -0.26). The most relevant genes belonging to extracellular space in iCMs are $GDF15$ (padj. 0.01, log2Fold change. 1.53), $HMOX1$ (padj. 0.002, log2Fold change. 1.76), and $HSPG2$ (padj. 0.02, log2Fold change. 1.10).

Discussion

It is accepted that the loss of FXN is the cause of FA, although there is recognition that there may be some genetic modifiers that influence disease severity. Despite knowing the genetic cause FA, the mechanisms of disease pathogenesis are still not fully understood. FXN has been implicated in iron ion homeostasis, redox reaction control, iron-sulfur cluster biogenesis and heme synthesis (Adamec et al., 2000; Adinolfi et al., 2009; Becker et al., 2002; Yoon & Cowan, 2003, 2004). However, none of these functions can fully explain why its deficiency generates the various FA phenotypes. Furthermore, it is unknown why FXN deficiency particularly affects certain cell types despite being ubiquitously expressed. Whether FA leads to nerve and heart muscle dysfunction by the same or different routes is also uncertain. GAA repeat expansion in FA leads to heterochromatin formation and DNA methylation that
correlates with the extent of the expansion, phenotype severity, and age of disease onset, possibly independent of FXN deficiency (Al-Mahdawi et al., 2008; Castaldo et al., 2008).

Here, we sought to further explore disease mechanisms by studying gene expression consequences of FXN deficiency and interactions in relevant affected cells. Therefore, we performed transcription analysis to investigate the FXN network in iPSC-derived iNs and iCMs from an FA patient. FA is a disease that progresses with time; thus, iPSC-derived cells provide information on the early molecular mechanism of disease.

Conceptually, methods that would increase FXN mRNA and protein levels to some specific degree are the most straightforward therapy for FA. FA carriers are asymptomatic despite expressing about half the level of FXN as unaffected persons (S. Zhang, Napierala, & Napierala, 2019). On the other hand, high FXN levels are toxic to cells (Belbellaa et al., 2020; Huichalaf et al., 2022; Vannocci et al., 2018). Therefore, we modified a lentivirus transfer plasmid with a weak promoter to better control FXN expression. After determining the Lentiviral titer, and optimal concentration for transduction, we transduced both iNs and iCMs from the FA patient with FXN lentivirus. Using this method, we obtained a modest increase in FXN mRNA and protein close to control amounts that was sustained for more than four weeks.

Restoring FXN was associated with the expression changes of 127 genes in iNs, primarily related to processes and pathways involved in Glycolysis, ATP generation, and HIF-1. We also found some genes annotated to phenotypic abnormalities associated with FA, such as sensory dysfunction, muscle weakness, and peripheral
neuropathy (Fig 8. E). Some of the genes belonging to these processes and pathways
were also differentially expressed when FA cells were compared to an unrelated control. Furthermore, they were restored to control levels when FA-iNs were supplemented with FXN. The DE genes from the most enriched pathways in corrected iNs were GPI, HK2, TPI1, ALDOA, ENO1, LDHA, PFKP, and PKM (Fig 10. B). Interestingly, all these genes
were downregulated in FA-derived iNs.

Glucose-6-Phosphate Isomerase (GPI) encodes for a member of the glucose phosphate isomerase protein family. This protein performs distinct functions as a glycolytic enzyme in the cytoplasm. However, extracellularly, it operates as a neurotrophic factor promoting motor and sensory neurons’ survival (Faik, Walker, Redmill, & Morgan, 1988). Interestingly, these are the most affected neurons in FA. Although this gene has not been associated with FA, defects in this gene are associated with neurological impairment (Romagnoli et al., 2003).

Hexokinase 2 (HK2) catalyzes the first essential step of glycolysis, converting D-glucose to D-glucose 6-phosphate. However, it also plays a crucial role in maintaining the integrity of the outer mitochondrial membrane by preventing the release of apoptogenic proteins from the intermembrane space and consequent cell death (Chiara et al., 2008). Loss of mitochondrial membrane potential and mitochondrial defects are phenotypes observed in FA cells and are a sign of bioenergetic stress 9 (Codazzi et al., 2016; Hick et al., 2013; Y. K. Lee et al., 2016). This means that HK2 may be playing a role in affecting the mitochondrial integrity in FA patients.

Triosephosphate Isomerase 1 (TPI1) encodes for a metabolic enzyme that catalyzes the interconversion between dihydroxyacetone phosphate (DHAP) and D-
glyceraldehyde-3-phosphate (G3P) in glycolysis and gluconeogenesis. Deficiency of this enzyme causes anemia, movement problems, increased susceptibility to infection, and muscle weakness. The movement problems are caused by impairment in motor neurons, which causes muscle weakness (Orosz, Olah, & Ovadi, 2006). Motor neurons are affected in FA, and one of the symptoms they experience is muscle weakness.

Other downregulated genes that are part of energy metabolism include ALDOA, ENO1, LDHA, PFKP, and PKM. Cells with high energy demands, such as nerve cells and heart muscle cells, are susceptible to cell death due to reduced energy caused by impaired glycolysis, and these are the most affected cells in FA. In addition to their role in metabolism, these proteins have other functions that may play a part in FA pathophysiology. ALDOA can regulate cell shape and motility (Mamczur, Gamian, Kolodziej, Dziegiel, & Rakus, 2013; Merkulova et al., 2011), ENO1 can associate with mitochondrial membrane, and it is essential for membrane stability (Didiasova, Schaefer, & Wygrecka, 2019), and LDHA deficiency is associated with neuronal damage in Alzheimer’s Disease (M. Zhang et al., 2018). The IPA shows how these genes are connected in a signaling network (Fig 10. C), demonstrating the possibility that FXN is involved in glycolysis and metabolic energy in affected FA neurons.

Other studies in iPSC-derived iNs from FA have shown lower ATP levels, decreased aconitase activity, and reduced mitochondrial membrane potential (Codazzi et al., 2016; Hick et al., 2013; Y. Li, Polak, et al., 2015). Therefore, these phenotypes are consistent with our findings. Interestingly, when compared to another transcriptional profile study in FA iPSC-derived iNs, the only shared process is the one related to extracellular matrix (Lai et al., 2019). This discrepancy could reflect the variability
between patients, controls, and techniques to overexpress FXN. In the study by Lai et al, a combination of HDAC inhibition and gene editing was used with iNs, and these methods focused on the GAA expansion instead of just correcting the FXN level.

Restoring FXN in iCMs led to changes in 417 genes related to processes and components of the extracellular matrix. Pathway analyses indicated the most significant enrichment for extracellular matrix (Fig 9. E). Our FA patient has mild cardiac symptoms, suggesting that cardiac involvement was in an early phase. Cardiac disease in FA is characterized by mitochondrial proliferation, inhibited mitochondrial fission, elevated reactive oxygen species, loss of contractile proteins, and the development of cardiac fibrosis (Hanson et al., 2019). That the extracellular matrix, collagen-containing extracellular matrix, and other related processes are enriched, suggest that cardiac fibrosis is a consistent finding. MRI studies have detected subclinical fibrosis in the left ventricle and remodeling before the hypertrophy in FA (Hanson et al., 2019). Therefore, FXN may play a role in developing the cardiac disease by regulating processes related to fibrosis.

The genes that are differentially expressed when we compared the FA iCMs to an unrelated control, and their expression level were corrected when FXN was added, are also related to the extracellular matrix and the plasma membrane. Some possible relevant genes include GDF15, HMOX1, and HSPG2, which were upregulated in FA Cms.

Growth Differentiation Factor 15 (GDF15) encodes a secreted ligand of TGF-beta. Several studies have shown associations between GDF15 and cardiac fibrosis, and some demonstrated a correlation between circulating GDF15 and the severity of
fibrosis, which means *GDF15* is involved in this process. In addition to this, *GDF15* is also increased in response to cellular and mitochondrial dysfunction. It acts as an inflammatory marker and is a player in the pathogenesis of cardiovascular diseases, metabolic disorders, and neurodegenerative processes (Rochette, Dogon, Zeller, Cottin, & Vergely, 2021; Yatsuga et al., 2015). The fibrosis and mitochondrial dysfunction are hallmarks of FA, as well as inflammation and neurodegeneration, which could explain the elevated levels of *GDF15* in FA.

Heme Oxygenase 1 (*HMOX1*) encodes for the heme oxygenase 1 enzyme, which mediates the first step of heme catabolism. HMOX upregulation has been implicated in increased ferroptosis in diabetic atherosclerosis (Meng et al., 2021). FXN is involved in Iron regulation and heme synthesis, ferroptosis is a newly proposed mechanism in FA (Turchi, Faraonio, Lettieri-Barbato, & Aquilano, 2020), and 10-30% of FA patients develop diabetes. This means that *HMOX1* may play a role if FA, and FXN influence it.

The *HSPG2* gene encodes for perlecan protein found in the extracellular matrix. This protein is a heparan sulfate proteoglycan; these proteins interact with many other proteins and have different functions. Perlecan has been implicated in various pathologies associated with extracellular matrix remodeling, including diabetes, cardiovascular disease, and Alzheimer’s disease (Martinez, Dhawan, & Farach-Carson, 2018). Perlecan may be involved in FA by contributing to the extracellular matrix remodeling that leads to cardiac disease.
The IPA shows how these genes may connect in a signaling network to other DE genes and genes of the IPA knowledge base (Fig 11. C). This illustrates the possibility that FXN may be involved in remodeling the extracellular matrix in FA CMs.

When comparing the differentially expressed genes of FA iPSC-derived iCMs from this study with another study in iCMs derived from an FA patient (J. Li et al., 2019), we found that the shared dysregulated genes belong to processes related to the extracellular matrix. Some of the processes are supramolecular collagen fibril organization, collagen fibril organization, extracellular space, and extracellular matrix. This further supports the evidence that extracellular matrix remodeling plays a role in developing cardiac disease in FA and that FXN dysregulation is involved in this process.

In this study, we overexpressed FXN in a controlled fashion in iPSC-derived iNs and iCMs from an FA patient. We used a modified FXN lentivirus to control the expression level and determine the long-term stability and even distribution of the overexpression over the duration of the cell culture. Other studies have used drugs or removal of GAA repeats to increase FXN levels which are valuable methods. However, the goal of our approach was to directly evaluate the role of the FXN level independent of the GAA expansion and side effects of drugs. By doing this, we identified key players of FXN network that may contribute to FA pathogenesis. Genes involved in glycolysis seem to have an essential role in the neurological aspect of FA, whereas genes related to the extracellular matrix are fundamental for the development of cardiac disease. This difference between iNs and iCMs may offer insights into different effects and susceptibilities of the neurons and the heart in FA. The heart and neurons differ significantly in their energy sources, metabolism, and susceptibility to nutrient
deprivation. In the healthy human heart greater than 95% of the ATP is produced from oxidative phosphorylation in the mitochondria. The preferred substrates in the heart are fatty acids (FA) accounting for 70-90% of ATP. Approximately 10% to 25% of myocardial ATP comes from oxidation of glucose, lactate, amino acids, and ketone bodies. Glycolysis and citric acid cycle account for about 5% of myocardial ATP (Stanley, Recchia, & Lopaschuk, 2005; Taegtmeyer, 1994). Most cardiac disease states (e.g., ischemia, heart failure, hypertrophy) exhibit reduced myocardial energy production with a shift away from FA-oxidation towards more glucose-dependence (Neubauer, 2007). Conversely, neurons in the central nervous system rely almost exclusively on glycolysis with full oxidation of glycolytic products by oxidative phosphorylation in the mitochondria (Dalsgaard, 2006; Yellen, 2018). Thus, the perturbation of glycolytic pathway gene expression in FA neurons may explain why the earliest manifestation of disease is usually neurological while cardiac pathology is later and more variable.

It is interesting to observe that few genes and pathways overlap when comparing this study to other RNA-seq studies in FA-relevant cells (Lai et al., 2019; J. Li et al., 2019). This could reflect the different techniques used to generate iNs and iCMs to upregulate FXN and the patient-to-patient variability. Additionally, the dysregulated genes and enriched pathways obtained with this approach are different to the ones in our previous study comparing the FA cells to control cells. As mentioned before, by using the same patient we have controlled for differences in genetic background. And by restoring FXN, we have exclusively addressed FXN-deficiency effects. Potential epigenetic effects of homozygous GAA expansion however, would still be expected to exist. Therefore, future studies are required to resolve these outstanding issues. These
would include the generation of isogenic cell lines containing genome-edited GAA repeats combined with and without FXN supplementation to specically distinguish which genetic mechanisms are operational in different tissues. Furthermore, studies with cells from multiple patients is vital in resolving which biological pathway pertubations predict disease progression. To further determine if pathological epigenetic effects of GAA repeats play a role in FA, genome-wide epigenetic assays in these systems is warranted. Another important factor to consider in future studies is the ability to mature the iPSC-derived cells to generate reliable phenotypes that could be corrected with FXN replacement. Understanding the early molecular mechanisms is important but aging the cells to recapitulate the neurodegeneration or hypertrophy will be key in obtaining the complete picture of how FA develops.
Fig 7. FXN lentivirus validation. (A) Diagram of the modified FXN lentiviral transfer plasmid. (B) qPCR quantification of FXN mRNA levels in FXN lentivirus transduced and non-transduced control iNs and iCMs. Data are expressed as means ± SD of three independent experiments. *p < 0.05. (C) Detection of FXN in capillary western blot (Wes) assay in transduced and non-transduced control iNs and iCMs. (D) Immunostaining of control transduced and non-transduced iNs showing expression of FXN (green) and neuronal marker DCX (red) Nuclei were stained with DAPI (blue). Scale bar 50 um. (E) Immunostaining of control transduced and non-transduced iCMs showing expression of FXN (green) and cardiomyocyte marker cTnT (red). Nuclei were stained with DAPI (blue). Scale bar 50 um. N_Ctrl indicates iNs from control, N_Ctrl_LT indicates iNs from control transduced with FXN lentivirus, C_Ctrl indicates iCMs from control, and C_Ctrl_LT indicates iCMs from control transduced with FXN lentivirus.
Fig 8. FXN lentivirus transduction in FA iNs and RNA-seq analysis. (A) qPCR quantification of FXN mRNA levels of transduced FA iNs with different lentivirus dilutions to determine the optimal concentration for transduction. The chosen dilution was 1/50, equivalent to 5.4 ng/ml of p24. Data are expressed as means ± SD of three independent experiments. *p < 0.05. (B) Cytotoxicity assay to evaluate the toxicity of the chosen concentration for transduction. Data are expressed as means ± SD of three independent experiments. *p < 0.05. (C) Venn diagram illustrating the differentially expressed genes between transduced and non-transduced FA iNs. (D) A heatmap illustrating the expression of 127 genes DE in both groups (transduced and non-transduced FA iNs). Three independent replicates are shown per group (E) Enrichment analysis of DE genes in transduced and non-transduced Ns using g:Profiler. We included results from the Gene Ontology, KEGG, and Human Phenotype Ontology databases. N_Ctrl indicates iNs from contro, N_FA indicates iNs from patient, N_FA_Lt 1/20, 1/50, 1/100 indicates iNs from patient transduced with FXN lentivirus with the designated dilution.
Fig 9. *FXN* lentivirus transduction in FA iCMs and RNA-seq analysis. (A) qPCR quantification of *FXN* mRNA levels of transduced FA iCMs with different lentivirus dilutions to determine the optimal concentration for transduction. The chosen dilution was 1/10, equivalent to 27 ng/ml of p24. Data are expressed as means ± SD of three independent experiments. *p* < 0.05. (B) Cytotoxicity assay to evaluate if the chosen concentration for transduction was toxic to iCMs. Data are expressed as means ± SD of three independent experiments. *p* < 0.05. (C) Venn diagram illustrating the DE genes between transduced and non-transduced FA iCMs. (D) A heatmap is illustrating the expression of 417 genes DE in both groups. Three independent replicates are shown per group (E) Enrichment analysis of DE genes in transduced and non-transduced iCMs using g:Profiler. We included results from the Gene Ontology and KEGG databases. C_Ctrl indicates iCMs from control, C_FA indicates iCMs from patient, C_FA_Lt 1/5, 1/10, 1/20 indicates iCMs from patient transduced with *FXN* lentivirus with the designated dilution.
Fig 10. Differentially expressed genes in FA vs. control iNs corrected by FXN transduction. (A) Heatmap illustrating the expression of DE genes in FA and control iNs that are restored to control level when transduced with FXN lentivirus. Three independent replicates are shown per group. (B) Enrichment analysis of corrected genes using g:Profiler. We included results from the Gene Ontology and KEGG databases. (C) Ingenuity pathway analysis (IPA) of the genes from the most enriched pathway of corrected iNs (Glycolysis/Gluconeogenesis). IPA generated a network diagram with the most probable connections to each other and to additional genes in the IPA knowledge base.
Fig 11. Differentially expressed genes in FA vs. control iCMs corrected by FXN transduction. (A) Heatmap illustrating the expression of DE genes in FA and control iCMs that are restored to control level when transduced with FXN lentivirus. Three independent replicates are shown per group. (B) Enrichment analysis of corrected genes using g:Profiler. We obtained results from the Gene Ontology database. (C) Ingenuity pathway analysis (IPA) of the genes from the most enriched pathway of corrected iCMs (Extracellular space). IPA generated a network diagram with the most possible connections to each other and to additional genes in the IPA knowledge base.
CHAPTER FOUR
CONCLUSIONS AND PERSPECTIVES

To investigate how FXN deficiency leads to the various phenotypes of the neural and cardiac cells in FA, we successfully generated iPSC-derived neurons and cardiomyocytes from a patient to use as a model. A complete characterization of the iPSCs and derived iNs and iCMs, is shown in chapter 2, in figures 2, 3 and 4. Neurons derived from the FA patient displayed a normal neuron morphology and expressed the typical neuronal markers. Similarly, iCMs expressed cardiomyocyte markers, showed a typical morphology, and beat on the dish.

In the first part of this project, we aimed to decipher the molecular mechanism of FA iNs and iCMs, by comparing them to an unrelated control. We were interested in understanding if FXN was affecting these cell types by similar or distinct mechanisms. To answer this question, transcriptional profile was performed in 30-day-old iNs and iCMs from the FA patient and a control. Differential expression and enrichment analyses demonstrated that the dysregulated genes in FA iNs belonged to processes and pathways involved in neuron system development (Fig 5). Interestingly, a prior study demonstrated that FA iNs at 34-39 days of differentiation have not developed full electrophysiological functionality (Hick et al., 2013). This means that FA iNs, have a delayed maturation that could influence the phenotypes that develop later in the course of the disease.
In contrast, differential expression and enrichment analysis in iCMs demonstrated that the FA dysregulated genes play a role in fibrosis, iron overload, and cardiac remodeling (Fig 6), which are all features of the cardiac disease in FA. Histopathological studies in FA hearts have found evidence of primary fibrosis, which means that this may be part of the heart disease pathogenesis (Koeppen et al., 2020).

According to these results, the molecular mechanisms of neural and cardiac disease are different and specific to each cell type. Therefore, FXN is affecting neurons and cardiomyocytes through distinct routes.

In the second part of this project, we wanted to investigate the role of FXN deficiency independent from the GAA expansion in neurons and cardiomyocytes. Therefore, we forced expression of $FXN$ in these cells with a modified $FXN$ lentivirus to bring the level of $FXN$ as closed to control levels. With this approach we were only focusing on the FXN function without modifying the causing mutation.

Previous studies have demonstrated that high levels of FXN are toxic to cells (Belbellaa et al., 2020; Huichalaf et al., 2022; Vannocci et al., 2018). To solve this issue, we modified a transfer plasmid with a weak promoter, and performed several transductions to optimize the adequate viral titer for iNs and iCMs. We also carried out a cytotoxicity assay to determine that the chosen concentration was not toxic to the cells. The details of the $FXN$ transfer plasmid and its optimization for transduction are shown in figures 7 and 8.

FA iNs and iCMs were collected 2 weeks post transduction when they were 30 days old. Transcriptional profile was performed in transduced and non-transduced cells,
and differential expression and enrichment analysis used to determine the dysregulated genes and pathways that are affected by FXN deficiency.

As expected from the previous analysis, FXN is affecting iNs and iCMs through different molecular mechanisms. However, since we are comparing the same patient cells with different FXN levels instead of to a control, we obtained some differences in the results. A detailed discussion of these analysis can be found in chapter 3.

To summarize, the dysregulated genes in FA iNs belong to processes and pathways related to carbohydrate metabolism, such as glycolysis and ATP generation. Whereas for iCMs, they were related to the processes and components of the extracellular matrix. Furthermore, the genes that were dysregulated in FA iNs and iCMs compared to control that are restored to control levels when FXN was added are also related to glycolysis and extracellular matrix. Previous studies have shown that FA patients have abnormal glucose and exhibit a higher risk for diabetes (Finocchiaro et al., 1988; Gucev et al., 2009; Isaacs et al., 2016), and that lower levels of ATP are restored with increased FXN levels in patients and models of FA (Bolinches-Amoros et al., 2014; Heidari et al., 2009; Y. Li, Polak, et al., 2015; Lodi et al., 1999). As mentioned before, fibrosis (a consequence of extracellular matrix remodeling from pathological processes) has been observed in FA patients even before the evident cardiac disease has developed (Hanson et al., 2019).

The difference between iNs and iCMs may offer a perspective into different effects and susceptibilities of the neurons and the heart in FA. The neurons and heart differed significantly in their energy sources and susceptibility to nutrient deprivation. In
the hearth most of the ATP is produced from oxidative phosphorylation in the mitochondria, and the preferred substrates are fatty acids. (Stanley et al., 2005; Taegtmeyer, 1994). In the majority of cardiac diseases there is a reduced myocardial energy production that becomes more glucose-dependence (Neubauer, 2007). On the other hand, neurons in the central nervous system depend almost exclusively on glycolysis with complete oxidation of glycolytic products by oxidative phosphorylation in the mitochondria (Dalsgaard, 2006; Yellen, 2018). Therefore, the perturbation of glycolysis pathways in FA iNs may explain why the first manifestation of disease is neurological whereas the cardiac disease occurs later and is more variable.

As discussed in previous chapters it is notable that few genes and pathways overlap when comparing this study to other RNA-seq studies in FA-relevant cells (Lai et al., 2019; J. Li et al., 2019). This could be the result of different techniques used to generate iNs and iCMs, to upregulate FXN, and the patient and control variability. Therefore, there is a lot that remains to be done to continue this research and address these issues.

FA is a disease with variable phenotypes, age of onset, and severity. Therefore, trying to find a cure that fits all the patients may not be suitable. The advantage, of using iPSCs as a model, is that it can lead to personalized medicine. By obtaining patient specific iPSC-derived cells we could evaluate the molecular mechanisms on each patient, and test potential treatments accordingly. Therefore, generating iNs and iCMs from several FA patients, upregulating FXN, and performing transcriptional profile on each one, is one of the first steps to continue this research. It will be interesting to see if some of the molecular mechanisms that we obtained are shared by all the patients.
Another important aspect to consider is the control variability. A more suitable control will be an isogenic cell line from each patient. While this is expensive and time consuming, it will be an ideal way to eliminate dysregulated genes that are related to differences with the control. One recent paper successfully used the CRISPR-Cas9 method to excise the GAA expansion in dorsal root ganglia sensory neurons (Mazzara et al., 2020).

While some neurons from the CNS are affected in FA, neurons from the peripheral nervous system like the sensory neurons from the dorsal root ganglia are also affected. Therefore, generating these types of neurons will also be important in addressing the full FA pathology.

iPSC-derived neurons and cardiomyocytes are immature in nature, and FA is a progressive disease that manifests later in life. Therefore, it is of key importance to find ways to mature the cells to obtain reliable phenotypes to assess the disease mechanisms. Some of the proposed methodologies to aged iNs has been prolonged culture, the used of progerin protein to accelerate the aging process (Miller et al., 2013), and trans differentiation, where a somatic cell is directly converted into iNs. Likewise, continuous electrical stimulation, 3D culture, and prolonged culture can be applied to accelerate the process of iCM maturation (Ahmed, Anzai, Chanthra, & Uosaki, 2020).

Lastly, although neurons and cardiomyocytes are the most affected cells in FA, most cells in the nervous system are glial cells (Barres, 2008), and CMs constitute only about 50% of the cells in the heart (Banerjee, Fuseler, Price, Borg, & Baudino, 2007). The GAA expansion is present in every cell type, and while it may not affect other cells directly, they may influence the disease phenotypes. The iPSC technology allows us
also to study other cell types, and with co culture and the generation of organoids it is possible to assess to what extent they are affecting neurons and cardiomyocytes in FA.
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APPENDIX I

LIST OF ABBREVIATIONS

FA ............. Friedreich’s Ataxia
FXN ............. Frataxin
iPSCs .......... Induced pluripotent stem cells
iNs ............ iPSC-derived neurons
iCMs .......... iPSC-derived cardiomyocytes
ROS .......... Reactive oxygen species
ISC .......... Iron-sulfur cluster
MRI .......... Magnetic resonance imaging
ECG .......... Electrocardiogram
PBMCs ...... Peripheral blood mononuclear cells
HDACi ........ Histone deacetylase inhibitors
STAR ........ Spliced transcripts alignment to a reference
RPKM .......... Reads Per Kilobase of Exon Model per Million Mapping Reads
FDR .......... False discovery rate
DE .......... Differentially expressed
GO .......... Gene ontology
BP .......... Biological process
CC .......... Cellular component
MF .......... Molecular function
KEGG-------Kyoto encyclopedia of genes and genomes

PTPRT ------Protein Tyrosine Phosphatase Receptor Type T

PCDHGA3--Protocadherin Gamma Subfamily A, 3

UNC5D------Unc-5 Netrin Receptor D

OTP--------Orthopedia Homeobox

DMBX1------Diencephalon/Mesencephalon Homeobox 1

ZIC4--------Zic Family Member 4

ZIC5--------Zic Family Member 5

PHOX2B-----Paired Like Homeobox 2B

RND1-------Rho Family GTPase 1

COL22A1---Collagen Type XXII Alpha 1 Chain

HFE2-------Hemojuvelin BMP Co-Receptor

CDON------Cell Adhesion Associated, Oncogene Regulated

GPI--------Glucose-6-Phosphate Isomerase

HK2--------Hexokinase 2

TPI1-------Triosephosphate Isomerase 1

ALDOA------Aldolase, Fructose-Bisphosphate A

ENO1-------Enolase 1

LDHA-------Lactate Dehydrogenase A

PFKP-------Phosphofructokinase, Platelet

PKM--------Pyruvate Kinase M1/2

GDF15------Growth Differentiation Factor 15

HMOX1------Heme oxygenase 1
HSPG2 ⋯⋯⋯ Heparan sulfate proteoglycan 2
MEA ⋯⋯⋯⋯ Microelectrode array
APPENDIX II

FUNCTIONAL EXPERIMENTS ON IPSC-DERIVED NEURONS AND CARDIOMYOCYTES FROM PATIENT AND CONTROL

Microelectrode array (MEA)

To determine the electrical network behavior of the iNs, Axion’s microelectrode array (MEA) technology was used (Axion Biosystems). Three parameters to determine neural network function were measured: neural activity, which indicates the frequency of action potentials being fired (mean firing rate); synchrony, which reflects the strength of synaptic connections (burst frequency); and oscillation, defined by alternating periods of high and low activity, which is a measure of how the spikes from all the neurons in a well are organized in time (network burst frequency).

To perform these experiments, 24-well MEA plates were coated with 50 μg/ml PDL in 5 μl drops in the center of the wells at room Temp for 1 hour. Then, the PDL was washed away with PBS 3 times, and the plates were coated with laminin (cat. 23017-015) for 1 hour at 37°C. Neurons were dissociated into single cells, plated (20,000 cells per well) in a volume of 5 μl per well, and allowed to attach for 1 hour at 37°C. Then 500 μl of neuron media is added to each well. MEA recordings were performed three times per week before media changes by loading the MEA plate into the Maestro MEA plate reader. After allowing 5 minutes of equilibration, the plates were recorded for 10
minutes. Since iPSC-derived neurons require three weeks in the MEA plates to reach complete neural network functions, the recordings were performed until day 25.

To measure the electrical activity of iCMs, MEA plates were also used. The following parameters were determined to assess functional cardiac performance: field potential, which measures the spontaneous action potential from a group of cells, can detect depolarizations, repolarizations, and possible arrhythmias. Propagation, which measures the cardiac beat propagation pattern and conduction velocity across the well, is key to detecting irregular heartbeats. Finally, contractility, detected as an increase or decrease of impedance, can obtain information on beat amplitude, beat timing, and excitation-contraction coupling.

To do these experiments, iCMs were dissociated into single cells and plated (50,000 cells per well) into fibronectin-coated MEA plates in a volume of 5 ul per well and allowed to attach for 1 hour at 37°C. Then 500 ul of cardiomyocyte support media (STEM cell technologies) was added to each well. MEA recordings were performed three times per week before media changes by loading the MEA plate into the Maestro MEA plate reader. After allowing five minutes of equilibration, the plates were recorded for 10 minutes for contractility, and extracellular field potential and propagation were recorded for 10 min from spontaneously beating cells at 37°C/5% CO2 with Axis Navigator software (Axion Biosystems). Patient cells were compared relative to control. Independent experiments were repeated three times in 3 wells/ cell lines.
Fig 1. Analysis of electrophysiological characteristics of iNs on MEA. Graphs show the longitudinal changes in (A) mean firing rate, (B) burst frequency, and (C) network burst frequency for iNs from FA and control up to 25 days; n = 3 wells per group. Data are expressed as means ± SEM of three independent experiments. *p < 0.05.
Fig 2. Analysis of electrophysiological characteristics of iCMs on MEA. Histograms showing the distribution of recorded (A) Beat period, (B) Corrected field potential duration (FPDc), (C) Conduction velocity, and (D) Excitation-contraction delay (E-C delay); n = 3 wells per group. Data are expressed as means ± SEM of three independent experiments. *p < 0.05.
**ROS-Glo™ H2O2 Assay**

To determine the Reactive Oxygen Species (ROS) generated in iNs and iCMs from patient and control, we used the ROS-Glo™ H2O2 Assay (Promega). This assay employs an H2O2 substrate that reacts directly with H2O2. Then, a detection reagent that contains recombinant luciferase and D-cysteine is added. The D-cysteine converts the precursor to luciferin, which reacts with the recombinant luciferase to generate a luminescent signal proportional to H2O2 concentration. A 96 well-opaque plate was coated with Matrigel. iCMs, and iNs, were dissociated with cardiomyocyte dissociation media (STEM Cell Technologies) or StemPro Accutase (Thermo Fisher), respectively, and seeded into the 96 well-plate at a 95% confluency. iCMs were plated with 80 ul of cardiomyocyte support media (STEM Cell Technologies) and iNs with neural differentiation media (Thermo Fisher). The following day, the media was changed to cardiomyocyte maintenance media (STEM Cell Technologies) for iCMs and more differentiation media for iNs. Cells were allowed to attach and recover for a week before proceeding with the assay. 20 ul of H2O2 substrate solution was added to each test well, including a blank control, and was allowed to incubate for 2 hours in the incubator (37°C, 5% CO2). Then, 100 ul of ROS-GLO detection solution was added to each well and incubated for 20 min at room temperature. The relative luminescence unit was recorded with the Cytation 5 plate reader (Agilent Technologies). Patient cells were compared relative to control. Independent experiments were repeated three times in 3 wells/ cell line.
Fig 3. Reactive Oxygen Species (H$_2$O$_2$) generated by the patient, control, and patient transduced with FXN in (A) iNs and (B) iCMs. Patient and transduced patient cells were compared relative to control; n = 3 wells per group. Data are expressed as means ± SD of three independent experiments. *p < 0.05.
**ATP detection assay**

We used an ATP detection assay kit (Cayman) to measure total ATP levels in iNs and iCMs. This assay utilizes firefly luciferase to convert ATP and luciferin into oxyluciferin and light. This light is directly proportional to the concentration of ATP in the cells. The kit includes an ATP detection standard to measure the ATP quantitatively. Cells in 12 well plates were lysed with 500 ul of 1x ATP detection sample buffer, and the lysate was transferred to a pre-chilled polypropylene tube. To prepare the standard, 20 ul of the 100uM ATP detection standard were mixed with 180 ul of ice-cold 1x ATP detection sample buffer to yield a 10 uM stock. Serial dilutions were performed to yield the following concentrations: 1 uM, 325 nM, 105.6 nM, 34.3 nM, 11.2 nM, 3.6 nM, and 1.2 nM. All dilutions were kept on ice. A reaction mixture containing 1X ATP detection assay buffer, D-luciferin, and luciferase was prepared according to the manufacturer's instructions. 100 ul of the reaction mixture and 10 ul of ATP detection standards were added to designated wells on the plate. For blanks, 100 ul of the reaction mixture and 10 ul of 1X ATP detection sample buffer were added, and for samples, 100 ul of the reaction mixture and 10 ul of sample lysates were added to designated wells in the plate. The plate was covered from light and incubated at room temperature for 20 minutes. Luminescence was recorded using the Cytation 5 plate reader (Agilent Technologies). Patient cells were compared relative to control. Independent experiments were repeated three times in 3 wells/ cell line.
Fig 4. Total ATP levels produced by (A) iNS and (B) iCMs of FA and control. Patient cells were compared relative to control; n = 3 wells per group. Data are expressed as means ± SD of three independent experiments. *p < 0.05.