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Progress in understanding Friedreich's ataxia using human induced pluripotent stem cells

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Abstract

Introduction: Friedreich's ataxia (FRDA) is an autosomal recessive multisystem disease mainly affecting the peripheral and central nervous systems, and heart. FRDA is caused by a GAA repeat expansion in the first intron of the frataxin (FXN) gene, that leads to reduced expression of FXN mRNA and frataxin protein. Neuronal and cardiac cells are primary targets of frataxin deficiency and generating models via differentiation of induced pluripotent stem cells (iPSCs) into these cell types is essential for progress towards developing therapies for FRDA.

Areas covered: This review is focused on modeling FRDA using human iPSCs and various iPSC-differentiated cell types. We emphasized the importance of patient and corrected isogenic cell line pairs to minimize effects caused by biological variability between individuals.

Expert opinion: The versatility of iPSC-derived cellular models of FRDA is advantageous for developing new therapeutic strategies, and rigorous testing in such models will be critical for approval of the first treatment for FRDA. Creating a well-characterized and diverse set of iPSC lines, including appropriate isogenic controls, will facilitate achieving this goal. Also, improvement of differentiation protocols, especially towards proprioceptive sensory neurons and organoid generation, is necessary to utilize the full potential of iPSC technology in the drug discovery process.

Keywords

induced pluripotent stem cells; frataxin; Friedreich's ataxia; GAA repeat expansion; differentiation

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1. Background

Friedreich's ataxia (FRDA), the most common inherited ataxia, is an autosomal recessive multisystem disorder mainly affecting the peripheral and central nervous systems and the heart. Symptoms of FRDA include progressive ataxia, peripheral neuropathy, cardiomyopathy (CMP) and diabetes. A significant fraction of FRDA patients also suffer from hearing and/or vision loss, which dramatically affects their quality of life [1, 2]. FRDA is caused by large expansions of trinucleotide repeats, encompassing frequently several hundreds of copies of the GAA motif, within the first intron of the frataxin gene (*FXN*), located on chromosome 9 [3]. Genetically, the most common form of the disease is caused by homozygous GAA expansions, however compound heterozygotes harboring a point mutation on one *FXN* allele with a GAA expansion on the second allele comprise approximately 4% of FRDA patients [4, 5]. The apparent genetic uniformity of the FRDA patient population is disturbed by interpatient variability in the GAA expansion size ranging from alleles shorter than 100 GAAs up to the largest detected allele containing more than a 1000 GAAs. This fact, in addition to a patient-specific combination of the GAA1 and GAA2 alleles (shorter and longer of the two expanded alleles, respectively), as well as the difficulty to precisely ascertain somatic instability of GAAs in different tissues and organs [6, 7], are responsible for significant heterogeneity in age of onset, symptoms and disease progression in the FRDA population. Variability, both molecular and clinical, makes *in vitro* and animal modeling of FRDA challenging. The *FXN* gene encodes frataxin, which is a small, mitochondrial protein, functioning primarily in biosynthesis of iron sulfur (Fe-S) clusters and iron metabolism [8–10]. Frataxin mRNA and protein levels are drastically reduced in FRDA patient cells compared to healthy individuals or asymptomatic carriers. Localized less than 2,000 bp from the transcription start site, the expanded GAA tracts alter both initiation and elongation of the *FXN* transcription apparatus [11–14]. Effects of the GAA expansions on the transcription process partially explain a recurrently reported inverse correlation between length of the GAA repeats, especially GAA1, and steady state levels of *FXN* mRNA and protein [15–17]. The long polypurine-polypyrimidine tracts are capable of adopting unusual DNA [18] or DNA-RNA conformations [12, 19]. These non-canonical structures are likely to pose a physical impediment to transcription at the mutated *FXN* locus, and are proposed to induce local changes in the chromatin environment contributing to the transcriptional silencing of the *FXN* gene [12]. An increased abundance of heterochromatin histone marks and increased cytosine methylation have been detected in FRDA human samples and various model systems, thus rationalizing the development of “epigenetic” modifying strategies to stimulate synthesis of the *FXN* transcript [20–22].

Generation of reliable cellular models that recapitulate certain disease-related phenotypes is essential not only for mechanistic studies but also for drug screens, testing efficacy of therapeutic approaches and identifying disease biomarkers. Commonly used *in vitro* models of the disease include immortalized lymphoblastoid cell lines, primary FRDA fibroblasts and several different cell lines adapted for FRDA research via downregulation of frataxin expression by RNA interference, gene knock-out or GAA insertion into synthetic constructs [23].

Until recently, *in vitro* disease models derived from human cells primarily targeted by the disease (e.g. sensory neurons, cardiomyocytes or pancreatic beta islets) were unattainable. Thus, progress in uncovering the disease mechanism and testing therapeutic approaches in FRDA-relevant cell types relied on animal models. Generation of FRDA mouse models has been successful, but certain aspects of the molecular pathology of FRDA are either difficult to recapitulate in rodents or are mild with a rather late onset, thereby limiting their use [23]. FRDA mouse models can be divided into two categories: (i) non-GAA repeat induced downregulation of FXN levels (e.g. conditional knock-out or shRNA knock-down) [24, 25]; (ii) GAA expansion models (knock-in and humanized transgenic animals) [26–29]. Considering that homozygous deletion of *Fxn* in mouse is early embryonic lethal (~ d7.5) [30], several conditional knock-out models have been developed [23]. The targeted mice typically demonstrate acute and early onset phenotypes making these models excellent for studying gene or protein replacement therapeutic strategies and analyzing defects resulting from ablation of *Fxn* expression in specific tissues and organs. Similarly, shRNA approaches result in strong and progressive phenotypes [25]. However, these models are not appropriate for determining the mechanism of expanded GAA-induced pathology in FRDA or for testing the efficacy of drug candidates targeting the *FXN* transcriptional defect. Two types of mouse models harboring expanded GAAs have been developed – knock-in of an expanded GAA tract into intron 1 of the murine *Fxn* gene [28] and transgenic animals that have the entire human *FXN* locus randomly integrated into the mouse genome [26, 27]. Due to inherent instability of GAA repeats during *in vitro* manipulations necessary to generate the targeting vectors to make these animal models, the original long GAA tracts introduced did not exceed ~250 GAAs, thus representing a short expansion that in humans correlates with a later onset of pathology. As such, the FRDA mouse models with short repeats typically develop delayed and mild molecular and behavioral phenotypes that are sometimes difficult to quantify. A selective breeding approach using animals with naturally occurring GAA expansions events has been undertaken in an effort to improve the current humanized transgenic models with the goal of generating animals with longer GAAs, lower levels of frataxin and more robust phenotypes [26].

Numerous studies, such as those investigating non-coding RNAs, effects of disease modifiers or analyses of three-dimensional (3D) chromatin structure can be only addressed in human cells due to differences with the rodent genome and transcriptome. Thus, cellular and organ-like humanized models are necessary for these types of mechanistic and translational studies. Technological advances in two major areas of research in the past decade opened entirely new possibilities for disease modeling: (i) reprogramming of somatic cells to pluripotency and establishment of induced pluripotent stem cell (iPSC) differentiation protocols, and (ii) genome editing using designer nucleases that enable the introduction of pathologic mutations into wild-type cells or correction of disease-causing mutations in patient-derived lines or animal models. The current status, limitations and future directions of research using FRDA iPSC-derived models will be discussed.

2. The breakthrough of iPSC-technology

The ability of embryonic stem cells (ESCs) to be cultured and differentiated *in vitro* into three germ layers makes them a very attractive source for disease models. ESCs are derived

from an early stage embryo, which is the inner cell mass of a blastocyst [31]. Establishing new ESC lines causes ethical controversies and access to disease-specific ESCs is limited. In 2006, an alternative approach of obtaining cells demonstrating ESC properties via induction of pluripotency by temporary ectopic overexpression of pluripotency transcription factors in somatic cells led to the discovery of iPSCs [32–34]. Initial reprogramming success indicated a low efficiency of the process and required viral transduction delivery of transcription factors, such as Oct3/4, c-Myc, Klf4, and Sox2 to fibroblast cells [33]. The rapidly evolving field soon uncovered that reprogramming could be stimulated by selected additional transcription factors and small molecules and could be performed using a variety of somatic cells, including blood or keratinocytes [35]. Currently, most methods of iPSC generation are commercialized, relatively inexpensive and the iPSCs obtained are footprint-free (i.e. transient expression systems and non-integrating viruses are used). iPSC models representing several neurodegenerative diseases caused by repeat expansions, including but not limited to, Huntington's disease, spinocerebellar ataxias, Fragile X syndrome (FXS), myotonic dystrophy 1 or C9orf72 ALS have been developed in recent years [36]. Some have proved to be essential in deciphering disease mechanisms or testing new therapeutic avenues. Recently, due to standardization of reprogramming techniques, a significant shift towards iPSC utilization in research of other rare genetic diseases has been observed. Furthermore, protocols allowing for robust, defined and reproducible differentiation of iPSCs into various cell types are now being optimized so that disease-relevant models can be made and used in a standardized fashion in drug discovery programs and perhaps in future regenerative medicine approaches [37]. A schematic is shown to illustrate fibroblast reprogramming to iPSCs followed by examples of methods to differentiate iPSCs into FRDA-affected cell types (Figure 1).

3. Impact of iPSC research on FRDA studies

The first reprogramming of FRDA fibroblasts into iPSCs was performed in 2010 by Gottesfeld et al. and the results demonstrated an unusual property of patient-derived iPSCs – progressive expansions of the long GAA tracts at a rate of approximately two repeats per replication cycle [38, 39]. Instability of GAAs (defined as coexistence of contractions and expansions of the repeats in subsequent generations) had been detected *in vitro* in other patient-derived cell types, and continuous GAA expansions had been observed when the sequence was incorporated into luciferase reporter constructs expressed in human embryonic kidney cells [40]). However, the phenomenon of persistent expansions had not been previously identified in the context of the endogenous *FXN* locus, as observed in FRDA and carrier (of the expanded allele only) iPSCs, indicating that these cells could serve as an excellent model to study expansion processes (Figure 2). Previous studies in various repeat instability models have demonstrated that the activities of mismatch repair (MMR) enzymes MSH2, MSH3 and MSH6 are critical in the expansion process [41]. Indeed, downregulation of MSH2 and MSH6 expression tempered the progressive expansion of GAAs in FRDA iPSCs, indicating that MMR is an important process responsible for somatic expansions of the GAA tract in human cells [38, 39, 42, 43]. The systematic expansions observed recurrently in FRDA iPSCs could also result from a change in the replication program defined in these cells. Activation of dormant replication origins downstream of the expanded

GAAs, as identified in FRDA iPSCs, could cause a collision between transcription and replication machineries moving in opposite directions at the *FXN* locus [44]. The replication profile in FRDA iPSCs was also characterized by stalling of replication forks as the machinery attempted to traverse the expanded GAA region. Finally, transient stimulation of *FXN* transcription in FRDA iPSCs directed an apparent increase in the rate of GAA expansions, indicating that a collision between the replisome and RNA polymerase II machineries may stimulate GAA expansions [45]. The question can be asked as to why expansion studies are important in a recessive disease in which both expanded alleles are inherited from carrier parents? It has been determined that GAA repeat length and instability, within the same FRDA patient, vary depending on the tissue of sampling [6, 7]. In addition, repeated measurements of GAA size in FRDA blood samples collected over a span of seven years clearly demonstrated a propensity for expansions without a single sample indicating contraction of the GAA tract [6]. As replication potential is highest in hematopoietic stem and progenitor cells, it is tempting to speculate that repeat expansions detected in iPSCs *in vitro* and immature hematopoietic cells *in vivo* share a similar mechanism, thus making iPSCs an attractive model to decipher the continuous *in vivo* expandability of large GAA tracts. This would allow for design and validation of new therapeutic approaches specifically targeting continuous GAA expansions via specific inactivation of MMR components or other pathways that might be involved in GAA expansions [43].

Generation of FRDA iPSCs has been a major step towards creating cellular models that represent tissues affected by the disease. Differentiation of FRDA iPSCs into sensory neurons and cardiomyocytes that demonstrated cellular phenotypes characteristic of the disease was first reported by Liu et al [46]. Since then, iPSCs have been used to advance the field in basic studies regarding the pathological mechanism as well as in translational studies testing various potential therapeutic approaches. Table 1 summarizes all current research using FRDA iPSCs and FRDA iPSC-derived cellular models.

4. Exploring molecular mechanisms of FRDA in differentiated iPSC lines

4.1 Neurons

Several components of both the peripheral and central nervous systems are affected in FRDA with initial signs of neurodegeneration localized to dorsal root ganglia (proprioceptive sensory neurons), dorsal spinocerebellar tracts and the dentate nucleus of the cerebellum [62–64]. Consequently, the majority of FRDA iPSC research focuses on neural cell differentiation and characterization of these cells (Table 1). In addition, methods of neuronal differentiation are relatively straightforward, well developed and recently have been commercialized. The first differentiation of FRDA iPSCs into peripheral sensory neurons was reported by Liu et al. [46]. Differentiated FRDA cells were morphologically and physiologically similar to wild type cells and maintained expanded GAA repeat tracts along with a low level of frataxin expression. Similarly, Puccio et al. uncovered that FRDA iPSC-differentiated neurons demonstrate signs of mitochondrial dysfunction including decreased mitochondrial membrane potential and progressive mitochondrial degeneration as visualized by electron microscopy [48]. These were the first reports to indicate that FRDA-

derived neurons exhibit traceable phenotypic changes that could be utilized as disease markers for evaluation of drug candidates. Importantly, parental iPSC lines did not show any overt biochemical or molecular phenotypes except for a high degree of GAA instability [48]. Neuronal differentiation of control and FRDA iPSCs was also used in an attempt to mimic peripheral neural development [47]. Although the efficiency of iPSC differentiation into sensory neurons was not reported, frataxin expression was upregulated during differentiation of control iPSCs while FRDA cells harboring expanded GAAs failed to upregulate frataxin during that process, thus amplifying the difference in frataxin expression levels between control and FRDA cells observed at the pluripotent stage [47]. These results indicate a possible developmental component in FRDA pathology.

The neuronal cells differentiated from FRDA iPSCs were also used to investigate approaches aimed to relieve transcription silencing of *FXN*. Upregulation of *FXN* expression was achieved using 2-Aminobenzamide histone deacetylase inhibitors (HDACi) [57, 58], which changed the chromatin landscape at the *FXN* locus. Several aspects of the FRDA molecular phenotype, including lower levels of Fe-S and lipoic acid-containing proteins, elevated labile iron pool, upregulated mitochondrial superoxide dismutase (SOD2) expression as well as increased levels of reactive oxygen species and reduced glutathione levels were corrected by treatment with HDACi [58]. More recently, upregulation of *FXN* expression was achieved using GAA-specific synthetic elongation factors (Syn-TEF1) [52]. These designer molecules contain a programmable DNA-binding ligand (polyamide) tethered to a small molecule (JQ1) that serves as a bait to attract endogenous elongation factors (e.g. BRD4) to stimulate transcription elongation specifically within *FXN* loci harboring expanded GAA tracts. The Syn-Tef1 was active in FRDA iPSCs as well as iPSC-derived sensory neurons and cardiomyocytes, demonstrating its potential as a general activator of *FXN* expression. Finally, transcription reactivation of *FXN* was also achieved in FRDA iPSC-derived neuronal cells corrected by zinc finger nuclease (ZFN)-mediated excision of a single expanded GAA tract ([56] and see below).

In addition to evaluating the efficacy of different therapeutic approaches, FRDA iPSC-derived neurons enable studies on cellular mechanisms of neurodegeneration in FRDA. Pandolfo et al. demonstrated activation of the intrinsic apoptosis pathway in iPSC-derived neuronal cells [55]. Finally, while robust MMR-dependent expansions were detected in FRDA iPSCs, differentiation of pluripotent cells to neurospheres abolished further expansions. This result indicates that either pluripotency genes, robust DNA replication or the higher activity of the MMR system observed in iPSCs might be required for progressive GAA expansions, however, influences of other factors cannot be ruled out [39, 48]. Detailed studies focused on the transition from a pluripotent state to committed lineages may shed light on molecular mechanisms underlying GAA expansions observed in many FRDA somatic tissues.

4.2 Cardiomyocytes

Cardiomyopathy is the primary cause of death in around 60% of FRDA patients [65, 66]. Patients with FRDA develop hypertrophic CMP characterized by thickening of the ventricular walls. Proliferation of mitochondria within the cardiomyocytes and a loss of

contractile fibers are thought to drive CMP development at the cellular level. Due to the impracticality of obtaining heart tissue samples from FRDA individuals, two models have been primarily used to study CMP in FRDA: (i) mouse – a cardiac phenotype (sometimes severe) is detected in conditional knockout and shRNA depletion animal models, but not readily observed in GAA expansion models [25, 26, 67]; (ii) necropsy tissue from FRDA patients [68]. One of the critical impediments in deciphering processes underlying the development of CMP in FRDA is the lack of viable human cardiac cells that recapitulate progression of the disease. Thus, establishing new FRDA cardiac models originating from human cells is essential for progress in uncovering disease mechanisms and testing therapeutic strategies. Methods to differentiate iPSCs to cardiomyocytes (CMs) are becoming standardized and commercialized, thus allowing for the generation of immature, beating cardiomyocytes within 2 – 3 weeks of *in vitro* culture.

Initial studies on FRDA iPSC-derived CMs demonstrated that frataxin downregulation does not prohibit cardiac differentiation *in vitro* [46, 48] and that CMs exhibit mitochondrial deficits, including increased proliferation and/or accumulation of structurally normal mitochondria as well as morphological changes of these organelles resembling degeneration [48]. Importantly, Hick et al. noticed clonal differences in beating rhythms within the same CM lines highlighting the necessity to include analyses of multiple lines and clones in experimental design. Subsequently, two detailed studies reported a number of phenotypic changes in FRDA CMs that resembled the pathology observed in mouse models and post-mortem human cardiac samples [49, 50]. The noted anomalies included disorganized mitochondrial networks and mitochondrial DNA depletion, as well as increased CM size and expression of brain natriuretic peptide (BNP). Deficits in energy production and calcium uptake were also observed, and all phenotypes were exacerbated by iron overload conditions. Subsequently, the same group utilized this established model of FRDA CMP to test the FRDA drug candidates idebenone (an antioxidant) and deferiprone (an iron chelator) [50]. Although these two studies demonstrated the great potential of FRDA iPSC derived CMs for drug testing, the data represent only two individual FRDA lines, so corroboration of these findings using lines isolated from different FRDA patients, preferably with established CMP status, is necessary.

Recently, Pebay et al. group demonstrated increased variation in beating rates of FRDA CMs caused by defective calcium handling [51]. This study was performed using three unique FRDA iPSC-derived CM lines without *in vitro* induction of the hypertrophy phenotype by iron overload, showing that even under basal conditions immature CMs differ from control cells that express higher levels of frataxin.

Although iPSC-derived CMs show great potential of becoming essential models to elucidate underlying mechanisms or evaluate treatment strategies for FRDA CMP, some considerations in the experimental design employing these cells are necessary. First, analyses should be conducted on numerous patient and control lines, preferably obtained from iPSC lines established from patients with clinically diagnosed CMP. Careful classification of CM identity in a population of cells or directed differentiation towards atrial, nodal or ventricular CMs should also be included as part of the experimental design. Moreover, FRDA CMP typically manifests between the 3rd and 5th decade of life, thus

directing culture conditions to mature the CMs *in vitro* could facilitate identification of robust cellular phenotypes. The iPSC-derived CMs structurally and functionally resemble early embryonic CMs [69], thus creating higher order 3D cultures, such as cardiac organoids and cardiac patches, could allow studies in more mature, organized cardiac tissues *in vitro*.

4.3 Retinal pigment epithelium cells

As mentioned above, pathology of the nervous system and heart are focus areas of FRDA research. Although vision and hearing loss affects the minority of FRDA patients (8 – 18 %), these problems bear tremendous impact on independence and quality of life for affected individuals [4, 70]. Most of our knowledge regarding loss of visual and sound perception comes from clinical tests, electrophysiological analyses and imaging studies [71, 72]. Development of iPSC technology and protocols allowing for differentiation of pluripotent cells into retinal pigment epithelium (RPE) cells, retinal ganglion cells and even otic epithelial progenitors will soon open the door for more studies to define mechanisms affecting vision and hearing in FRDA patients. To date, only a single study attempted to address this unmet need by differentiating FRDA iPSCs into RPE cells [61]. Crombie et al. compared retina histology in humanized transgenic FRDA mouse models with eye samples from FRDA patients collected post-mortem. Functional analyses of iPSC-derived FRDA RPE cells showed no abnormalities when compared to those derived from control iPSCs when grown under optimal conditions. Although no pathological changes were observed in this study, the prospect of obtaining any cell type from iPSCs reveals new perspectives for testing therapeutic approaches on the specific cell types as well as evaluating drug candidates that selectively target specific organs or tissues in FRDA.

5. Generation of isogenic cell lines

Both FRDA-intrinsic (GAA size variability and frataxin expression) and FRDA-extrinsic (confounding genetic and environmental factors) characteristics pose challenges when deriving *in vitro* models from individuals that are intended to recapitulate a disease state that is highly heterogeneous within the affected population. To overcome these hurdles and distinguish actual FRDA hallmarks from noise, analyses of numerous, unique samples should be performed in parallel. However, considering costs incurred for iPSC derivation, culture and differentiation, along with the time and workload requirements to establish these lines, simultaneous analyses of numerous lines and multiple clones highly depend on availability of personnel and funds. An alternative is the use of carefully characterized isogenic lines where a mutation is either introduced (to healthy cells) or corrected (in patient cells). The use of such lines would decrease effects of individual variability and reduce the number of samples necessary to conduct well-controlled experiments. In fact, such “pseudo-isogenic” models in the form of siRNA/shRNA knockdown of frataxin have already been extensively used to elucidate molecular mechanisms of FRDA [23, 73]. These artificial models carry some additional concerns posed by potential random integration of shRNA-encoding viral vectors, differential efficiency of delivery (e.g. siRNAs) and reliance on additional cellular processes to achieve downregulation of FXN mRNA and protein (e.g. RNAi machinery). An alternative strategy for correcting the underlying genetic defect is to simply overexpress *FXN* in patient cells. This option is being explored predominantly by

gene therapy approaches. Current gene therapy procedures have minimal risk of vector integration, however potential deleterious consequences of excessive frataxin expression need to be considered and carefully monitored [74].

Tremendous progress has been made in recent years in genome editing approaches that can be used to introduce or remove mutations with constantly improving efficiency and specificity. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 systems use different molecular mechanisms to elicit the same effect – introduction of a specific cleavage or nick into DNA that can be repaired via non-homologous end joining (NHEJ) or homology-directed repair (HDR) [75, 76]. In the case of intronic GAA repeat expansion, genome editing could be utilized in at least three different ways to generate isogenic corrected lines from FRDA iPSCs (Figure 3). A simple excision of the GAA tract using a pair of nucleases flanking the repeats results in removal of the entire tract together with a certain amount of flanking sequence. Our group employed this approach by using a pair of ZFNs targeting regions upstream and downstream of the GAA tract to generate isogenic, FRDA corrected lines [56]. We were able to obtain heterozygous correction (single allele correction) associated with approximately three-fold increase in *FXN* expression. Neuronal cells differentiated from corrected iPSCs demonstrated increased cellular levels of ATP and aconitase activity, indicating reversal of these FRDA phenotypes. The low efficiency of simultaneous ZFN cleavage precluded homozygous correction of both expanded alleles at that time. Another approach (Figure 3) relies on homologous recombination to stimulate the replacement of the expanded repeats with a shorter non-pathogenic tract. Nuclease cleavage in the vicinity of the repeats facilitates the recombination process by inducing double-strand breaks or nicks in the DNA strand [77, 78]. This approach has the potential to generate a perfect isogenic line with no or minimal sequence changes to the flanking region. A pitfall of this strategy is the potential for nonspecific genome integration of the exogenous DNA donor sequence, which is typically introduced in excess to serve as a template during recombination. Lastly, nucleases can be designed to target repeat sequences themselves and, via introduction of DNA nicks, stimulate DNA repair mechanisms to induce contractions [79]. This method would generate multiple lines with gradual changes in repeat number, and in doing so, is associated with a greater risk of off-target effects due to numerous short repeat sequences scattered throughout the human genome that could become unintended targets. However, experience from studies on CAG repeats indicates that using a particular type of Cas9 nickase (D10A mutant) stimulates repeat contractions preferentially in hyperexpanded alleles [79]. Finally, in principle, the complement to the above-mentioned approaches is to generate FRDA isogenic iPSC pairs by introducing expanded GAAs into the unaffected allele containing short GAAs. This approach is possible, however, feasibility is limited due to inherent difficulties of cloning and propagating expanded GAAs in plasmids.

In conclusion, thus far only a single study has reported generation of isogenic lines by correction of the endogenous *FXN* locus in patient cells, and this direction of FRDA research certainly deserves more attention. In addition, the continuous and rapid advancement of engineered nuclease technology helps to place gene editing approaches in contention to become an *in vivo* therapy of FRDA and other repeat expansion diseases [80].

6. Expert opinion

Although the generation of patient-specific iPSCs has been one of the most important breakthroughs of the past decade for allowing studies of molecular mechanisms, testing efficacy of novel therapies in numerous human disease states and holding the promise for future regenerative medicine, the limitations of this technology need to be mentioned. One important issue to address in terms of disease modeling is heterogeneity of iPSC lines derived from different individuals and even between individual cell line clones [48, 81], which makes research with iPSC-based models expensive and highly labor-intensive. For FRDA research, this is certainly magnified by heterogeneity within the patient population making generation of well-characterized isogenic cell line pairs essential for further progress of the field. An additional variable in FRDA iPSC studies is genetic instability of the locus and continuous expansions of the GAA tract [38, 39, 45]. On one hand, this feature reinforces the utility of iPSCs as a model to study the GAA expansion process. On the other hand, it adds another level of variability considering that with any extension of the GAA tract FXN mRNA and protein levels are reduced, thus potentially changing the molecular phenotype of the cells.

The first symptoms of FRDA typically present between the ages of 5 – 15 years, however patient cells have had to cope with a reduced level of frataxin since embryogenesis [63, 64]. Although clinical presentation of symptoms is presumably preceded by pathological changes at the molecular and cellular levels, the timeline of FRDA manifestation is certainly longer than a few weeks of differentiation of iPSC lines into neuronal or cardiac cells. Therefore, identifying robust phenotypes in relatively young and immature cells may be difficult if not impossible. Perhaps accelerating the aging process by exogenous expression of progerin as demonstrated in Parkinson's disease iPSC studies [82] would result in early manifestation of some of the more severe, typically late-onset anomalies detected in FRDA. Another potential solution could be a transdifferentiation approach whereby one type of somatic cell (e.g. fibroblasts) are directly converted into another type of somatic cell (e.g. induced neurons, iNs) using specific growth factors and expression of lineage specific transcription factors. This method would bypass possible “rejuvenation” introduced at the pluripotent cell stage [83–85].

Besides the capacity of stem cells to differentiate into multiple cell types, they can also self-organize and differentiate *in vitro* into 3D aggregates, called organoids [86]. These 3D cultures more closely mimic *in vivo* conditions of tissues and organs [87]. The advantage of organoids over 2D cultures is the ability to explore tissue-like organization and coexistence of different cell types, for example neurons and astrocytes, in an *ex vivo* setting [88, 89]. Modelling of FRDA using organoids has not yet been reported.

In vitro, pluripotent stem cells exist in two major states of pluripotency: naïve (representing the pre-implantation blastocyst inner cell mass) and primed (representing post-implantation epiblast cells). Besides the developmental timing equivalent, these two states differ in several aspects, including morphology and epigenome and X-inactivation status [90, 91]. An overwhelming majority of iPSC studies, including all analyses of FRDA pluripotent cells have been conducted in primed iPSCs (and referred to simply as iPSCs). Recently a

technology was developed to reprogram somatic cells to naïve iPSCs as well as converting existing primed iPSCs to a naïve state [92]. Why introduce yet another variable to FRDA research? Studies on naïve iPSCs derived from patients with Fragile X syndrome (FXS; a neurological disorder caused by hyperexpansions of CGG repeats that silence transcription of the *FMR1* gene) demonstrated that although transcriptional silencing found in patient somatic cells persisted in primed iPSC lines, it can be alleviated by converting them to naïve cells [93]. Thus, naïve FXR iPSCs, despite having large CGG expansions, maintain expression of FMR1 mRNA and protein. It would be extremely valuable, from the perspective of transcription silencing induced by expanded GAAs, to compare frataxin expression and chromatin modification status between primed and naïve iPSC lines derived from the same individual. Creating pluripotent lines with expanded GAAs that maintain high levels of frataxin could stimulate discovery of new therapeutics capable of reactivating the *FXN* gene in patient cells.

Where to go after ~ 8 years since the initial report of FRDA iPSC creation and ~ 20 publications utilizing iPSCs in FRDA studies? One issue hampering advancement of the field is availability of uniformly generated, well-characterized iPSCs from different FRDA patients along with paired isogenic lines with corrected expansions and reactivated *FXN* expression. This resource would help reduce technical variability and allow multiple research sites to use standardized, yet diverse, patient-derived models to define molecular events that underlie specific features of FRDA as well as for testing therapeutic candidates.

Also, attention should be focused on continuing to optimize robust and reproducible protocols to obtain cell types most often affected during the course of the disease, such as proprioceptive sensory neurons or mature ventricular cardiomyocytes. Thus far, no research has been published in pancreatic cells differentiated from FRDA iPSCs to define mechanisms underlying glucose intolerance and diabetes frequently observed among FRDA patients. Methods of generating and differentiating human iPSCs are still relatively new and require further development, however the advantages of iPSC-based approaches in terms of modeling FRDA are undeniable. Generation of well-characterized isogenic iPSC-derived FRDA models will likely be critical not only for discovery of new therapeutics but also for validation and final FDA approvals of the first therapy for FRDA.

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Abbreviations

FRDA	Friedreich's ataxia
GAA	guanine – adenine - adenine
FXN	frataxin

iPSC	human induced pluripotent stem cells
ESC	embryonic stem cells
RPE	retinal pigment epithelium cells
ZFN	zinc-finger engineered nucleases
TALEN	transcription activator-like effector nucleases
CRISPR	clustered regularly interspaced short palindromic repeats
CM	cardiomyocyte
CMP	cardiomyopathy

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Article highlights

- Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disorder affecting primarily neurons and cardiac cells, with symptoms including peripheral neuropathy, cardiomyopathy, diabetes, vision impairment and hearing loss
- Because heterogeneity between patients is high, generation of representative and defined disease models to study FRDA pathology is in great demand
- FRDA is caused by expansion of a GAA trinucleotide sequence within the first intron of the *Frataxin* gene (*FXN*), which leads to its transcriptional repression
- Reduction of FXN protein levels impairs iron metabolism, iron-sulfur cluster formation and heme synthesis
- FRDA iPSCs can serve as a model to study GAA expansion mechanisms
- Development of somatic cell reprogramming technology and robust iPSC differentiation methods make it possible to generate neuronal and cardiac cells, which represent tissues affected by FRDA
- Improved genome editing approaches are allowing development of paired FRDA and GAA-excised isogenic cell lines

Figure 1.

Generation of human iPSCs and further differentiation schemes to create FRDA affected cell types. The iPSCs can be obtained from different somatic cell types including blood cells, keratinocytes, and fibroblasts. In addition to the classic “Yamanaka” transcription factors (Oct3/4, Sox2, c-Myc and Klf4), many variants of transcription factors have been reported to be both sufficient and necessary to increase the efficiency of the reprogramming process, including additional auxiliary factors or small molecules. Only cell types that are the most relevant to FRDA pathology are depicted.

Figure 2.

Continuous expansions observed in FRDA iPSCs. A. The FRDA fibroblast line GM04078 (Coriell Cell Repositories) harboring ~ 340/450 GAA repeats (GAA1/GAA2 alleles, lane F) was reprogrammed to iPSCs using retroviral transduction of pluripotency transcription factors. Somatic reprogramming was associated with expansion to 410/540 GAAs in passage 0 of the iPSC culture (p0). Subsequent passaging resulted in continuous expansions reaching ~700/860 GAAs after passage 27 (p27). Approximately 200 days of culture (reprogramming followed by 27 passages of the iPSCs) was sufficient to double the number of GAAs observed in the parental fibroblasts. B. Expansion of the GAA repeats is observed for both GAA1 and GAA2 alleles. An increase of 10 – 11 GAAs per passage was detected for each allele.

Figure 3.

Use of genome editing to generate isogenic pairs of FRDA and corrected iPSC lines. For simplicity correction of a single allele harboring the expanded repeats is shown. Expanded GAAs are depicted as blue triangles while short, nonpathogenic GAAs appear as blue rectangles. Only exons 1 and 2 and intron 1 of *FXN* are shown. Also, only the Cas9/gRNA system is depicted as the DNA DSB or nick inducer. A. Excision of the expanded GAAs using a pair of nucleases cleaving upstream and downstream of the GAAs. A fragment of intron 1 is removed together with the entire GAA tract. B. Replacement of the expanded

GAA repeats with a homologous intron 1 DNA fragment containing short GAAs via HDR. No or minimal changes to the nucleotide composition of intron 1 are introduced. C. Generation of GAA tract contractions using a repeat targeting Cas9 nickase. Several different products can be expected, which contain shorter pathogenic GAA tracts or a stretch of the repeats within the unaffected range. Two distinct possible products of editing are depicted: “improved” and corrected.

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Table 1.

Current status of FRDA iPSC research. Some studies utilized FRDA iPSCs as well as iPSC-derived neurons and/or cardiomyocytes.

Cell type	Study purpose	References
FRDA iPSCs	- Generation of FRDA GAA instability model	[38]
	- Investigation of the role of mismatch repair enzymes in GAA repeats expansion	[39]
	- <i>In vitro</i> study of FXN dynamic change in expression during differentiation	[47]
	- Reversing transcriptional repression caused by elongated GAA tracks by altering specific epigenetic modifications	[45]
	- Studies on DNA replication at the FXN locus	[44]
FRDA iPSC-derived cardiomyocytes	- Generation of patient specific cells affected in FRDA	[46]
	- Investigation of mitochondrial damage in FRDA	[48]
	- Modeling iron-overload induced cardiomyopathy	[49]
	- Drug screening platform for studying iron-overload cardiomyopathy in FRDA	[50]
	- Cardiac-specific electrophysiological profile of FRDA	[51]
	- Activating FXN expression by synthetic transcription elongation factors	[52]
FRDA iPSC-derived peripheral sensory neurons	- Generation of patient specific cells affected in FRDA	[46]
	- <i>In vitro</i> study of FXN dynamic change in expression	[47]
	- Reversing oxidative stress and loss of Fe-S proteins with 2-Aminobenzamide HDAC inhibitors	[53]
FRDA iPSC-derived neural stem cells	- Investigation of the role of mismatch repair enzymes in GAA repeats expansion	[39]
	- Proteomic study of targets and pathways of 2-Aminobenzamide HDAC inhibitors	[54]
FRDA iPSC - derived neurons	- Investigation of mitochondrial damage in FRDA	[48]
	- Studying mechanism of apoptosis in FRDA	[55]
	- FXN expression upregulation via zinc finger nuclease-mediated GAA tract excision	[56]
	- FXN expression upregulation via 2-Aminobenzamide HDAC inhibitors	[57]
	- FXN deficiency alleviation via HDACi treatment	[58]
	- Activating FXN expression by synthetic transcription elongation factors	[52]
FRDA iPSC - derived neurons	- FXN expression upregulation via 2-Aminobenzamide HDAC inhibitors	[59]
	- Functional characterization and brain integration	[60]
FRDA iPSC-derived retinal pigment epithelium cells	- Function and characterization of retinal pigment epithelium in FRDA patients	[61]