

**Generation of Induced Pluripotent Stem Cell (iPSCs)-Based Models of
Friedreich's Ataxia (FRDA)**

by

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A Dissertation Submitted to the
Graduate School of Sciences and Engineering
in Partial Fulfillment of the Requirements for
the Degree of

Master of Science

in

Molecular Biology and Genetics



July, 2020

Generation of Induced Pluripotent Stem Cell (iPSCs)-Based Models of Friedreich's

Ataxia (FRDA)

Koç University

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To my family,

ABSTRACT

Friedreich's ataxia (FRDA, OMIM#229300, ORPHA:95) is the most common autosomal recessive ataxia and is characterized by progressive neurological abnormalities, cardiomyopathy, and increased risk of diabetes mellitus. GAA·TTC triplet repeat expansions in the first intron of *FXN* gene are responsible for this disease. These expansions cause a reduction in *FXN* encoded frataxin protein. Frataxin is a small mitochondrial protein involved in the biosynthesis of iron-sulfur clusters (ISCs) and iron storage. The function of frataxin and how GAA·TTC expansions reduce frataxin protein levels are not entirely known. Therefore, to elucidate the molecular mechanisms of FRDA, *in vitro* models are necessary. Induced pluripotent stem cells (iPSCs) can be generated through the reprogramming of patients' somatic cells to an embryonic-like state. iPSCs can be used to model various diseases such as neurodegenerative diseases *in vitro*.

In this study we generated iPSCs-based models of FRDA so that the disease can be studied *in vitro* and new potential therapeutic drugs can be tested. iPSCs lines were generated from fibroblasts of 4 FRDA patients, as well as 2 healthy control individuals. All 6 iPSCs lines were characterized with respect to expression of pluripotency-related genes at the RNA and protein level as well as, embryoid body formation assay.

As hypertrophic cardiomyopathy is the most common cause of death among FRDA patients, the resulting patient-specific and control iPSCs were differentiated into cardiomyocytes to model this disease *in vitro*. Expression of cardiomyocyte-specific genes and beating behavior of the differentiated cardiomyocytes were validated by qPCR analysis, immunofluorescence, and live-cell imaging. Reduced *FXN* levels in fibroblasts collected from FRDA patients were retained in the FRDA iPSCs-derived cardiomyocytes. Taken together, these results showed that the iPSCs derived from both FRDA patient samples and healthy individuals are pluripotent, and FRDA iPSCs-derived cardiomyocytes can be generated to model FRDA *in vitro*. This *in vitro* model can be a useful platform to gain insights into the mechanisms of FRDA and to test new therapeutic strategies for FRDA-related hypertrophic cardiomyopathy.

ÖZETÇE

Friedreich ataksisi (FRDA, OMIM#229300, ORPHA:95) en sık görülen otosomal ataksidir ve nörolojik anormaliteler, kardiyomiyopati ve artmış diyabet riski ile karakterize edilir. *FXN* geninin birinci intronundaki GAA·TTC üçlü baz çifti tekrarlarının artması bu hastalığın nedenidir. Bu tekrar artışları *FXN* kodlu frataksin protein düzeyinde azalmaya neden olmaktadır. Frataksin, hücreiçi demir-kükürt küme üretemine katılan ve demir depolayan küçük bir mitokondriyal proteindir. Frataksinin işlevi ve GAA·TTC tekrarlarının artışının frataksin protein düzeyini nasıl azalttığı tam olarak bilinmemektedir. Bu nedenle, FRDA'nın moleküler mekanizmalarını açıklamak için *in vitro* modeller gereklidir. Uyarılmış pluripotent kök hücreler (uPKH), hastaların somatik hücrelerinin embryonik hücre benzeri bir duruma yeniden programlanması yoluyla üretilir. uPKH'ler, nörodejeneratif hastalıklar gibi birçok hastalığın *in vitro* modellenmesinde kullanılırlar.

Bu çalışmada, bu hastalığı *in vitro* incelemek ve yeni potansiyel terapötik ilaçları test etmek için FRDA'nın uPKH tabanlı modellerini üretmeyi amaçladık. uPKH hatları 4 FRDA hastasının fibroblastlarından ve 2 sağlıklı kontrol bireyden üretildi. uPKH hatlarının tümü, plüripotentiğe bağlı genlerin RNA ve protein seviyesindeki ekspresyonları ve ayrıca embriyoid cisim oluşumu deneyleri ile karakterize edildi.

FRDA hastaları arasında en yaygın ölüm nedeni hipertrofik kardiyomiyopatidir. Bu sebeple oluşturulan hastaya özgü ve kontrol uPKH'leri bu hastalığı *in vitro* modellemek için kardiyomiyositlere farklılaştırılmıştır. Kardiyomiyositlere özgü genlerin ekspresyonu ve farklılaşmış kardiyomiyositlerin atım davranışı; qPCR analizi, immünofloresan boyamaları ve atım bölgelerinin canlı hücre görüntülemesi ile doğrulanmıştır. FRDA hastalarından toplanan fibroblastlardaki düşük *FXN* gen seviyesi, FRDA uPKH'lerinden üretilen kardiyomiyositlerde de korunmuştur. Bunlar bir arada ele alındığında, bu sonuçlar hem FRDA hastalarından hem de sağlıklı bireylerden üretilen uPKH'lerinin pluripotent olduğunu ve FRDA'yı *in vitro* modellemek için FRDA uPKH'lerinden üretilen kardiyomiyositlerin kullanılabileceğini göstermiştir. Bu *in vitro* model, FRDA'nın mekanizmaları hakkında bilgi edinmek ve FRDA ile ilişkili hipertropik kardiyomiyopati için yeni terapötik stratejileri test etmek için faydalı bir platform olabilir.

ACKNOWLEDGMENTS

Before all else, it is my pleasure to express my gratitude to my advisor Assoc. Prof. Dr. Tamer Önder. I believe he is the best advisor with his knowledge, his never-ending curiosity about science, and his support. He always listened to my ideas about the future directions of the project and encouraged me to achieve them. Without his guidance, it would be very hard for me to carry out this project. I feel very lucky to be able to participate in the scientific discussions with him during the journal clubs and lab meetings. I am very grateful to him for shaping me to work independently and for helping me to be the scientists I am today.

I would like to thank Dr. Esra Börklü Yücel. This project belongs to her. She and Dr. Tamer Önder enabled me to study in this project. Particularly, I am grateful to Prof. Dr. Hülya Kayserili and Dr. Esra Börklü Yücel and the team of KUH Diagnostic Centre of Genetic Diseases for their contribution to this thesis. Their contributions in pedigrees, PCR, and karyotype analysis raises this thesis towards a higher point.

I would like to also thank my thesis committee members Prof. Dr. Hülya Kayserili, and Assoc. Prof. Dr. Arzu Çelik Fuss for sparing their precious time for evaluating my works in the thesis. Asst. Prof. Dr. Arzu Çelik is one of the faculties who listened to my poster presentation in detail in the 7th International Congress of the Molecular Biology Association of Turkey. Her encouragement and interest in my project enabled me to better understand how valuable it is to present my project in different congresses and organizations.

This project was funded by TÜBİTAK- The Scientific and Technology Research Council of Turkey (Project no:216S051) and Koc University School of Medicine. I want to thank TÜBİTAK and Koc University School of Medicine.

I am grateful to the past and present members of Onder lab. I am especially grateful to Dr. Burcu Özçimen and Dr. Deniz Uğurlu Çimen for not only their fundamental advice, and endless help but also their friendly and warm attitudes towards me. I want to thank Gülben Gürhan Sevinç, Ata Berk Demir, Ayşe Derya Cavga, Kenan Sevinç, Büşra Bayırbaşı, and H. Dilşad Arabacı. I would like to thank all the members of Koc University School of Medicine (KUSOM) but especially for Bağcı-Onder Lab, Lack Lab, and Ceyda Açılan Ayhan (CAA) Lab. I would like to express my gratitude for each of them for

providing an answer and a solution to my questions when there was no one in my lab. Apart from KUSOM family, I would like to thank KUTTAM Cellular and Molecular Imaging Core. It was an honor to attend the microscopy training organized by Serçin Karahüsyinoğlu. I want to thank Dr. Berna Morova.

I also have to thank my incredible undergraduate students, Abdullah Burak Yıldız, and Mehmet Batuhan Karakuş. They taught me how to teach science and the way that I can improve myself to be a special mentor. I owe a thank for their great contributions to this project.

I would like to thank my friend here from KUTTAM; İpek Bulut, and my life-long friends from METU; Kübra Çelikbaş, Ceren Bilge Çelebi. I consider Kübra and Ceren as my second family. I am very happy to meet them all.

Finally I would like to thank my family, Miyeser Kelekçi and Özge Kelekçi for their unconditional love, infinite support and always trusting me. They always believed in me and saw me through in every possible manner. I hope they will swell with pride about me in the future.

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NOMENCLATURE

AP	antarctic phosphatase
ACO	aconitase
cDNA	complementary DNA
CHIR	CHIR99021
CRISPR	clustered regularly interspaced short palindromic repeats
DAB	3,3 diaminobenzidine
DMEM	dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMTs	DNA methyltransferases
DOX	doxycycline
EB	embryoid body
ESCs	embryonic stem cells
FRDA	Friedreich's ataxia
GFP	green fluorescent protein
gRNA	guide RNA
HDAC	histone deacetylase
HEK293T	human embryonic kidney 293T cells
HRP	horseradish peroxidase
iDOT1L	inhibitor of DOT1L
ISCs	iron-sulphur clusters
iPSCs	induced pluripotent stem cells
iPSC-Cms	iPSCs-derived cardiomyocytes
iPSCs-neurons	iPSCs-derived neurons
KOSR	knock-out serum replacement
LR-PCR	long range polymerase chain reaction
MEF	mouse embryonic fibroblasts
NEAA	non-essential amino acids
NEB	new england Biolabs
PBS	phosphate-buffered saline
PCs	purkinje cells
PSCs	pluripotent stem cells
PFA	paraformaldehyde
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
TALENs	transcription activator-like effector nuclease
s.e.	standard error
SOD2	superoxide dismutase
WT	wild-type

Chapter 1:INTRODUCTION

1.1 Introduction to Friedreich's Ataxia

Friedreich's ataxia (FRDA, OMIM#229300, ORPHA:95) is a hereditary neurodegenerative disease due to the expansion of GAA triplet repeats in the first intron of Frataxin (*FXN*) gene located on chromosome 9 (Koeppen, 2011). This results in decreased protein levels of Frataxin, a small mitochondrial protein. The most affected tissues in the disease are dorsal root ganglia, corticospinal tracts, and the heart. This disease affects approximately 1-2 in 100,000 people and the main disease symptoms include progressive ataxia of limbs, dysarthria, sensory neuropathy, and hypertrophic cardiomyopathy (Delatycki et al., 2000). The mean age onset of this disease is 10-15 years, wheelchair use is generally necessary around 15 years and lifespan is estimated as 36 years (G. De Michele et al., 1996; Delatycki & Bidichandani, 2019).

The most common neurological symptoms include ataxia, which is due to a combination of cerebellar and spinocerebellar degeneration, and sensory neuropathy. Peripheral sensory neuropathy leads to reduced vibration sense along with degeneration in posterior columns of the spinal cord that results in poor proprioception. Axonal neuropathy leads to reduced lower limb reflexes along with degeneration of corticospinal tracts in the spinal cord (Delatycki & Corben, 2012). Dorsal root ganglia are smaller in FRDA patients due to the degeneration of large sensory neurons. It is accompanied by a reduction in the diameters of the spinal cord especially in the thoracic region and a loss of myelin and axons in dorsal columns, spinocerebellar, and corticospinal tracts (Koeppen, 2011). In addition, 90% of FRDA patients develop dysarthria and the severity of this symptom worsens with disease duration (Delatycki & Corben, 2012). There are lesions observed in dentate nuclei of the cerebellum and Purkinje cells in FRDA (Koeppen & Mazurkiewicz, 2013).

59% of the premature death of FRDA patients is due to cardiomyopathy (Lynch et al., 2012; Tsou et al., 2011). Cardiac related death is due to cardiac dysfunction leading to congestive heart failure and arrhythmias (Belbellaa et al., 2019). Generally the type of cardiomyopathy that is seen in FRDA patients is left ventricular (LV) hypertrophy (Jensen & Bundgaard, 2012). Hypertrophy results in a severe reduction in the indexes of

LV systolic function. Later hypertrophy develops into a concentric form. This is accompanied by a gradually developed myocardial fibrosis (Belbellaa et al., 2019). The development of cardiomyopathy or arrhythmias leads to death in FRDA patients (Tsou et al., 2011).

In addition to these, 10 to 30% of FRDA patients have diabetes, while many have impaired glucose tolerance but the reason for the development of diabetes is not known. *In vitro* models of FRDA derived β cells show decreased frataxin levels and functional defects with the sensitivity to apoptosis (Cnop et al., 2012).

1.2 FRDA is a triplet repeat expansion disease

The expansion of GAA triplet repeats varies between 79 and 1700 GAA units in the first intron of *FXN* gene (9q21.11) in FRDA patients whereas 30-40 repeats are seen in normal individuals. These regions are polymorphic in humans (Montermini et al., 1997) (**Figure 1.1**).

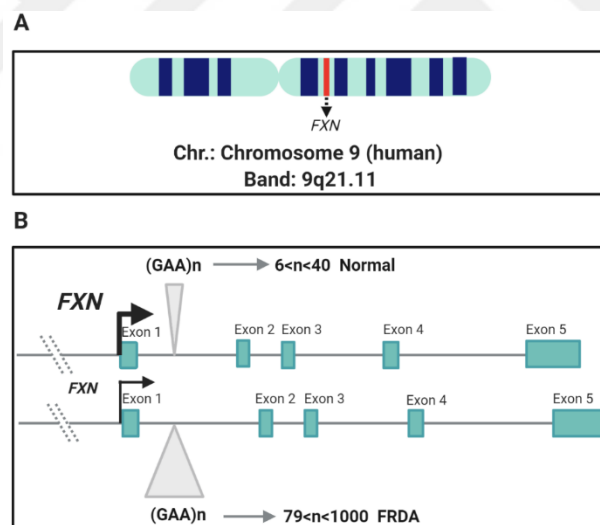


Figure 1.1 Representation of *FXN* gene locus.

(A) The chromosomal location of *FXN* gene is represented as red. It is located on the long arm of chromosome 9 (9q21.11) (B) *FXN* gene is represented. In FRDA, both the length of GAA1 and GAA2 expansion are more than 79. But their lengths could be different. GAA1 is the shorter allele and the length of it determines the age-onset of FRDA. Adapted from (Sandi et. al. 2011).

More than 95% of FRDA patients have homozygous GAA triplet-repeat expansion while the rest carry heterozygous phenotype in which in one allele there is GAA repeat expansion and in the other allele, there may be stop codon, various point mutations, missense mutations, frameshift mutations, deletions or splice-site mutations. Most of the

missense mutations seen in heterozygous individuals are I154F and G130V. Whenever these mutations are in combination with the hyperexpanded allele, the disease phenotype is observed but in a much milder and more slowly progressing manner (Campuzano et al., 1996). In heterozygous cases, the reduction in *FXN* mRNA levels caused by missense mutations is less than that caused by GAA expansion. Most of the *FXN* mRNAs present in these patients code for mutant proteins and this could be the reason for observation of atypical mild FRDA phenotype in those patients (S. I. Bidichandani et al., 1997).

In 1998, Michele et.al demonstrated that GAA repeat length within the shorter allele of the *FXN* gene (GAA1) determined the age-onset and the larger allele, which was named as GAA2, exhibited high intrafamily correlation. He also showed that there is a strong correlation between the severity of the disease and the size of GAA triplet repeat expansion (De Michele et al., 1998).

Campuzano et.al demonstrated that instability of expanded repeats occurs during parent-offspring transmission (Campuzano et al., 1996). GAA repeat length was found to be highly variable during meiosis. Parental gender and sequence length were known to affect repeat length variation. During the transmission of repeats, the length of the repeats either contracts or expands but the contractions outnumber the expansions (Pianese et al., 1997).

During the generation of iPSCs from FRDA fibroblasts, repeat instability in GAA repeats in both alleles, was also observed. The reason for this expansion could be the activation of MMR enzymes (mismatch repair enzymes) such as MSH2, MSH3, and MSH6 because *MSH2* gene expression was found to be higher in FRDA iPSCs compared to control iPSCs (Ku et al., 2010).

GAA expansion causes a severe deficiency of mature *FXN* mRNA, but this deficiency is not due to abnormal alternative splicing (Sanjay I. Bidichandani et al., 1998). Since there are no single mutation in the coding sequence of *FXN* gene, alleviating the transcriptional block due to triplet repeats is applicable to decrease the disease phenotypes. There are three possible mechanisms of transcription inhibition that result from GAA·TTC triplet repeats. The first is due to long GAA·TTC repeats, DNA adopts a non-B-DNA structure by forming triplexes, which are sticky DNAs in this case. The other is the inhibition of transcription due to DNA-RNA hybrid conformations. Finally, epigenetic mechanisms

like DNA methylation, histone deacetylation, and histone methylation may affect *FXN* gene expressions (Al-Mahdawi et al., 2008).

1.3 Possible mechanisms leading to decreased Frataxin protein levels

1.3.1 Formation of NON-B-DNA, R loop, and FAST in the repeat region

The transcriptional deficiencies in FRDA are length-dependent and after 79 GAA repeats, the transcription can be said as impaired. According to Bidichandani and his colleagues, the reason for the transcriptional deficiency is because DNA adopts unusual non-B-triplex DNA structures (Sanjay I. Bidichandani et al., 1998). (GAA-TTC)_n (n=20) can form intramolecular triplexes that are formed when the major groove of the Watson-Crick base-paired double helix supports the binding of a third strand via Hoogsteen base pairing (Sakamoto et al., 1999). In FRDA, however, the novel triplex structure is formed when pyrimidine strands of two RRY triplexes are exchanged with each other. This structure is very stable and called “sticky DNA”. It was shown that this self-associated complex inhibited the transcription (Sakamoto et al., 2001).

By using RT-PCR and ChIP for RNA Polymerase II, it was shown that expanded repeats-triggered transcriptional initiation and elongation defects occur during *FXN* transcription (Kumari et al., 2011). Later, Groh and colleagues hypothesized that these initiation and elongation defects can be due to the presence of R loops. By using DNA immunoprecipitation (DIP) which allows detection of R-loops with the help of S9.6 antibody, they showed that R loops may be seen around GAA repeats in the FRDA lymphoblastoid cells and they are resistant to degradation. In addition, the enrichment of H3K9me2, a repressive histone mark, was observed around the regions that are dominated by R loops. Thus, they speculated that a reduction in Frataxin protein level in FRDA could be a result of R loop formation (Groh et al., 2014).

In addition to the possibility of the formation of sticky DNA and R loops in the GAA-TTC repeat region, a decrease in *FXN* expression seen in patients could be due to the presence of *FXN*- Antisense- Transcript- 1 (FAST-1). The binding site of this antisense transcript was found to be overlapping with the binding site of CTCF (a chromatin insulator protein) (De Biase et al., 2009). FAST-1 expression was detected by 5' and 3' RACE experiments with total RNA and poly(A)+RNA in fibroblast cells. FAST transcript localizes to

nucleotides +164 bp and -359 bp of *FXN* gene and is 523 bp long. When overexpressed in HeLa cells, CTCF occupancy was significantly decreased at the *FXN* 5'UTR region. And this overexpression resulted in decreased acetylation of H3K9 and enrichment of H3K9me3 which suggested the formation of heterochromatin. Importantly, *FXN* gene expression increased upon FAST-1 knockdown (Mikaeili et al., 2018).

1.3.2 Epigenetic mechanisms leading to a reduction in *FXN* gene levels

There are many CpG sites in the 5' region of GAA repeats both in control and FRDA individuals (W. man Liu & Schmid, 1993). Greene and colleagues showed that the DNA methylation status of FRDA patients and control individuals exhibit differences (Greene et al., 2007). In FRDA patient-derived lymphoblastoid cells, the average methylation density is 50% higher in the repeat region compared to the controls. Pyrosequencing data showed that 3 specific CpG sites located on 5' of GAA repeats exhibited more methylation in FRDA patient samples when compared to controls. The hypermethylation of the upstream region of GAA repeats may play a role in the reduction of *FXN* protein level (Greene et al., 2007). There is even a correlation between CpG methylation and triplet expansion size. Although there is no significant correlation between repeat size and hypermethylation in myotonic dystrophy and fragile X; in FRDA, DNA methylation occurs in an expansion size-dependent manner. Moreover, there is a correlation between the CpG methylation seen in the 382 nucleotides previous to the GAA repeats and age-onset (Castaldo et al., 2008).

Apart from DNA methylation, histone deacetylation, and methylation affect gene expression (Kouzarides, 2007). When the FRDA primary lymphocytes were investigated, it was found that histone H3 and H4 acetylation levels are lower in *FXN* locus compared to controls. This was accompanied by trimethylation of histone H3 at Lys9. When histone deacetylase inhibitors were used in FRDA primary lymphocytes, it reversed the silencing of *FXN* gene (Herman et al., 2006). In the FRDA lymphoblast cells, enrichment of hypoacetylation of H4K5 and trimethylation of H3K27 was observed starting from the upstream of GAA repeats spreading to transcription starting sites before exon 1. This showed the presence of repressive chromatin spread. In addition to these, there was a significant reduction in RNAPII and CTCF occupancy and depletion of H2A.Z histone and H3K4me3 marks in the transcription start site of *FXN* (Chutake et al., 2014).

A gene can also be silenced by position effect variegation (PEV). In PEV, heterochromatin spread occurs through a gene that is close to a heterochromatin region. When the triplet-repeat expansion occurs like in FRDA or myotonic dystrophy, it was shown in transgenic animals that triplet repeats confer variegation of expression. And this is due to the recruitment of PEV modifier heterochromatin protein1 (HP1) leading to a decrease in promoter accessibility (Savellev et al., 2003) (**Figure 1.2**).

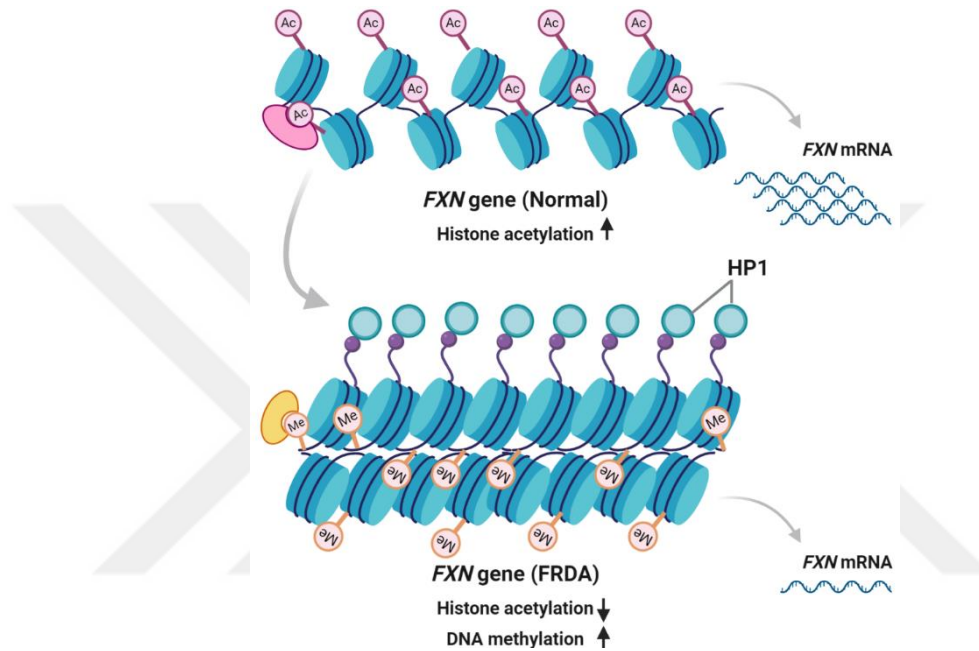


Figure 1.2 Schematic representation of epigenetic silencing seen in FRDA

There are 6-20 GAA triplet repeats in the first intron of the *FXN* gene. However, this number increases up to 1700 repeats in FRDA patients. *FXN* expression in FRDA patients decreases to 10-30% of the normal *FXN* expression. One of the main mechanisms that lead to this decrease is thought to be epigenetic silencing. Adapted from (Gottesfeld, 2019).

1.4 Frataxin protein

The frataxin protein is an essential 23.2 kDa protein of 210-amino acids (Cossee, 2000; Koutnikova et al., 1997; Pastore & Puccio, 2013). It is a conserved protein and the complete absence of frataxin is embryonically lethal (at embryonic day 6.5) (Cossee, 2000). By tagging the frataxin protein with β -galactosidase at the 3' end of frataxin protein, it was found that frataxin protein is a nuclear-encoded mitochondrial protein. Frataxin is encoded in nuclear DNA, expressed in the cytoplasm and imported to the mitochondria (Koutnikova et al., 1997). In yeast cells, frataxin homolog YFH1 (yeast

frataxin homolog) is also localized in mitochondria (Babcock et al., 1997). YFH1 is a 19.5 kDa protein of 174 amino acids (Wilson & Roof, 1997). *YFH1* deletion leads to the accumulation of mitochondrial iron and hypersensitivity to oxidative stress due to iron-catalyzed Fenton-chemistry. The yeast mutant phenotype was found to phenocopy the cells obtained from FRDA patients in terms of high iron accumulation and diminished respiratory chain (Babcock et al., 1997). Wilson and colleagues showed that yeast lacking *YFH1* gene showed respiration deficiency due to the complete absence of mitochondrial DNA. To prove that there is irreversibly mitochondrial DNA damage, they introduced wild-type *YFH1* plasmids to *Δyfh1* strain and investigated the phenotype but they observed the same respiratory deficient phenotype. Then they induced the most common mutation seen in FRDA (which changes isoleucine to phenylalanine within the highly conserved C-terminal domain of frataxin) to the C-terminal domain of yeast homolog YFH1. This resulted in slower growth of yeast cells and a decrease in the respiratory capacity of the cells. Thus, in this way, they showed that the C-terminal of YFH1 protein, which is structurally and functionally similar to frataxin protein, is very important for mitochondrial functionality. And mitochondrial DNA damage could not be reversed back with an introduction of wild type *YFH1* gene to cytoplasm or nucleus (Wilson & Roof, 1997).

The structure of the frataxin protein consists of two N- and C- terminal regions. The N-terminal side of the protein can be said as unfolded and highly flexible whereas highly conserved C-terminal domain folded in an α -(β)₅₋₇- α manner (Pastore & Puccio, 2013).

The function of frataxin is still under investigation but it involves several different processes (Bulteau et al., 2004; Busi & Gomez-Casati, 2012; Chiang et al., 2016; Lill, 2009; Pastore & Puccio, 2013). The first and most important of these processes are due to frataxin's ability to bind to iron (Fe^{2+}). Frataxin acts as an iron chaperone by binding to Fe^{2+} and supplies Fe^{2+} to iron-sulfur clusters (ISCs). These clusters are composed of non-heme iron and inorganic sulfide and are found in the cubane form composed of four iron and four sulfur atoms represented as $[\text{4Fe-4S}]$ (Busi & Gomez-Casati, 2012). ISCs can be defined as inorganic cofactors and are found in mitochondrial respiratory complexes I-III, photosystem I, ferredoxins, and hydrogenases. Their functions are due to their abilities to transfer electrons based on the propensity of iron to switch between

Fe^{3+} and Fe^{2+} (Lill, 2009). Studies also showed that frataxin may involve in the final step of heme biogenesis. Frataxin shows a high affinity towards FECH enzyme which has a role in the insertion of Fe^{+2} into PPIX to form heme. Endogenous yeast *FECH* expression was reduced along with heme levels in the *YFH1* depleted yeast models. In addition to these, frataxin functions as an iron storage protein through the formation of oligomers that owns ferroxidase activity (Chiang et al., 2016) (**Figure 1.3**).

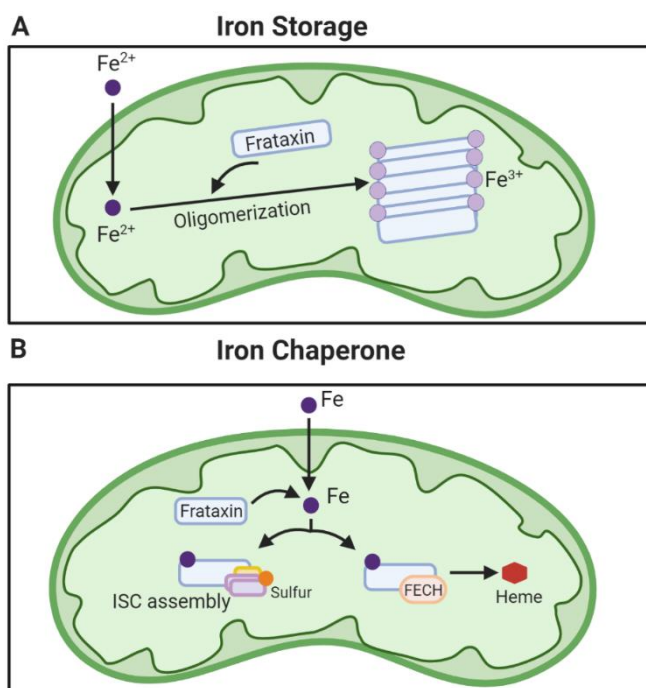


Figure 1.3 Schematic representation of functions of frataxin in the mitochondria.

(A) Frataxin is a protein localized in the mitochondria and it captures Fe^{2+} atoms and forming oligomers of frataxin, it holds Fe^{3+} atoms. (B) In addition to the iron storage role, it acts as an iron chaperone by involving in the ISCs assembly pathway. Frataxin also involves in the heme synthesis pathway. Adapted from (Chiang et al., 2016).

In addition to the role of frataxin protein on the formation and protection of 4Fe-4S clusters from disassembly, frataxin may regulate the functionality of aconitase. Aconitase is an iron-sulfur-containing dehydratase which converts citrate to isocitrate in KREBS-cycle. Rötig and colleagues observed that reduction in *FXN* expression results in both mitochondrial Fe-S-dependent respiratory chain enzymes deficiency and total cellular aconitase deficiency in FRDA patient samples (Bulteau et al., 2004).

Lastly, frataxin may play a role in the prevention of ferroptosis dependent cell death. Ferroptosis is a type of cell death triggered by iron accumulation and lipid peroxidation

(Grazia Cotticelli et al., 2019). In 2020, Du and colleagues show that *FXN* depletion increases Erastin-induced ferroptosis as a result of a breakdown of ISC biosynthetic machinery and mitochondrial dysfunction, whereas overexpression of frataxin protein prevents ferroptosis. They also showed that the occurrence of ferroptosis is through the decisive role of frataxin protein in the regulation of cysteine deprivation-induced ferroptosis (Jing Du et al., 2020).

1.5 Induced pluripotent stem cells (iPSCs) and clinical applications

Stem cells are defined by their potential to self-renew and differentiate. They can be derived from embryonic and postnatal animal tissues but show different pluripotency potential and are thus classified differently. Whereas the zygote is totipotent, embryonic stem cells show quintessential pluripotency which enables them to form all tissue types found in the body (De Los Angeles et al., 2015). Somatic cells can be reprogrammed back to an embryonic-like state by nuclear transfer, fusion with embryonic stem cells (ESCs), or by transcription factor transduction. In 2006, Takahashi and Yamanaka discovered that the transient expression of *OCT4*, *SOX2*, *KLF4*, and *c-MYC* transcription factors reprogrammed somatic cells back to a pluripotent state resulting in the generation of human-induced pluripotent stem cells (iPSCs) (Takahashi & Yamanaka, 2006). This technology eliminates the limitations of the use of human ESCs. First, the main constraint for the use of human ESCs is the immune incompatibility between the donor and the recipient. Second is the ethical concerns as embryos are destroyed during the isolation of ESCs (Medvedev et al., 2010).

Due to similarities between human iPSCs and human ESCs, iPSCs can replace ESCs in several research fields (Narsinh et al., 2011). One of the similarities is the ability to generate unlimited amounts of any differentiated cell in the human body. Another similarity is coming from their round-shaped morphology, surface marker expressions, feeder dependence (coculture with MEFs). Like ESCs, iPSCs can form *in vivo* teratomas bearing differentiated cell types of the three main germ layers (Takahashi et al., 2007; Yu et al., 2007). A recent study showed that terminally differentiated cells from iPSCs and ESCs (either generated by somatic cell nuclear transfer or generated after *in vitro* fertilization) exhibit comparable differentiation potential, along with similar

transcriptional, epigenetic, physiologic and pharmacological features (M. T. Zhao et al., 2017).

Reprogramming of somatic cells to pluripotent stem cells enables the generation of disease-specific cell types to study neurodegenerative diseases such as Amyotrophic lateral sclerosis (ALS), Fragile X syndrome, Huntington's disease, Friedreich's ataxia (FRDA), and Parkinson's disease. Through the generation of *in vitro* disease models, molecular mechanisms of diseases can be elucidated and these *in vitro* models can be used in drug screening to reveal possible drugs. But for iPSCs -based models to be meaningful, a clear molecular or cellular phenotypic difference between the control and disease models should be observed (Onder & Daley, 2012). Along with extracellular matrix proteins, organoids can be constructed with different cell types that are differentiated from iPSCs to model diseases that influence many cell types. Organoids can have the potential to meet the rising demand for multi-lineage analysis (Sharma et al., 2020). In addition to the use of iPSCs in the disease modeling *in vitro*, iPSCs fueled expectations for cell-replacement therapy. For instance, the first trial in which iPSCs-derived retinal pigment epithelium cells was carried out in 2014 (Shi et al., 2017; Trounson & DeWitt, 2016). But relatively few clinical trials related to iPSCs-derived cell types have been tested to date due to several concerns. One reason for this is the gene sets used for the induction of pluripotency. Overexpressed exogenous *OCT4*, *SOX2*, *KLF4*, *c-MYC* genes can result in neoplastic development. The expression of these transcription factors is associated with the development of multiple tumors. Another possibility is the formation of teratoma after the clinical transplantation of differentiated-iPSCs. Moreover, to generate iPSCs, scientists use either retro- or lentiviral vectors for the delivery of four transcription factors into somatic cells. This leads to random integration of exogenous DNA into the genome and this may disrupt the functionality of the tumor-suppressor genes leading to tumor formation (Medvedev et al., 2010). However, recent improvements in reprogramming methods such as the use of episomal vectors, Sendai-viral transfections, and mRNA-based delivery of reprogramming factors, minimize these risks (Schlaeger et al., 2015).

1.6 *In vitro* and *in vivo* models of FRDA

1.6.1 iPSCs-based FRDA disease models

The genetic basis of FRDA makes it challenging to study *in vivo*. Although there is 73% similarity in mouse frataxin and human frataxin, there are no organisms other than human and primates which have GAA repeats within the specific genomic context of the first intron of frataxin gene. Thus, no *in vivo* model can be expected to show the disease symptoms perfectly. Only conditional knockout mouse models reproduce important phenotypes for the disease, but they lack the genetic context. Expansion knock-in transgenic mouse models carry GAA repeats but they demonstrate only a very mild phenotype (Perdomini et al., 2013). The perfect model should show a reduced level of frataxin ideally resulting from triplet repeat expansion of GAA in the first intron of frataxin gene. iPSCs-derived cellular *in vitro* models can recapitulate tissue-specific disease phenotypes (Martelli et al., 2012). In early work, FRDA patient skin fibroblast cells were reprogrammed into iPSCs and were able to show pathological GAA repeats and a reduction in frataxin protein level in the iPSCs (Ku et al., 2010).

Hick and colleagues were able to differentiate FRDA iPSCs into both neurons and cardiomyocytes (**Figure 1.4**). They showed that FRDA iPSCs-derived neurons (iPSCs-neurons) and cardiomyocytes (iPSC-Cms) retained pathological GAA repeat expansion. After 34-39 days of post-differentiation, FRDA iPSCs-neurons showed 30% less spontaneous calcium transients. This may be an indication of immature electrophysiological functionality even after 34-39 days of differentiation for FRDA iPSCs-neurons compared to the control neurons that showed full electrophysiological functionality. FRDA iPSCs-neurons also demonstrated a 40% lower mitochondrial membrane potential after 44-49 days of differentiation. In FRDA iPSC-Cms, they observed that *FXN* expression was less than controls. Degenerated mitochondria with a dark matrix and collapsed cristae were observed in FRDA iPSC-Cms (Hick et al., 2013).

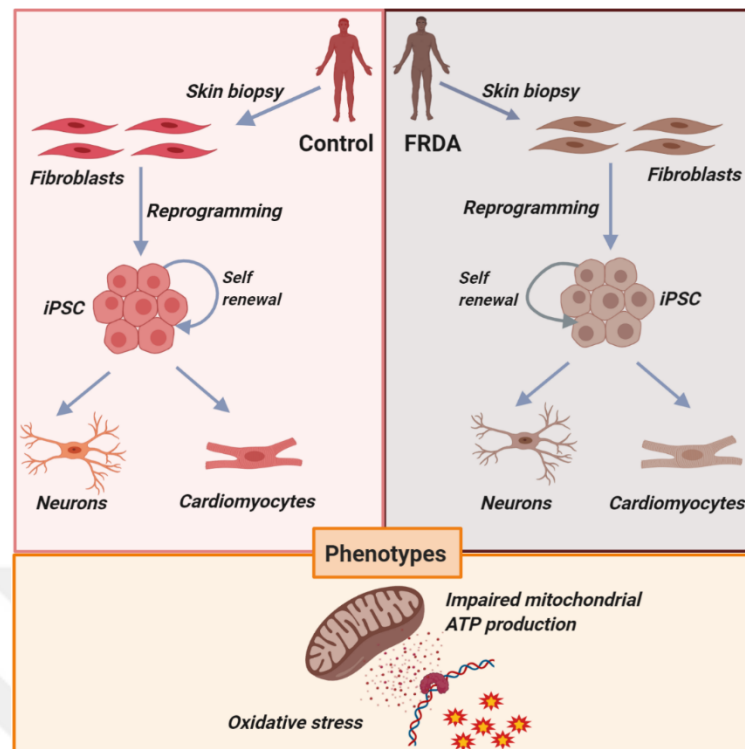


Figure 1.4 The illustration of the *in vitro* disease model of FRDA.

For carrying out iPSCs-based disease modeling, fibroblasts from FRDA can be reprogrammed back to iPSCs and after full characterization, those iPSCs can be differentiated to the targeted cell types. The most damaged cell types in FRDA are neurons and cardiomyocytes. After differentiation and characterizations, disease phenotypes such as oxidative stress accumulation and impaired mitochondrial ATP production can be investigated.

Iron homeostasis in FRDA iPSC-Cms was assessed by fluorescence quenching of calcein-AM. While iron-content in control iPSC-Cms was unchanged, iron content in FRDA iPSC-Cms increased 7,500 folds in the iron-overloading conditions. Under the same conditions, reactive oxygen species (ROS) production and levels of brain natriuretic acid, a cardiac stress marker, was significantly elevated compared to controls. In addition, iron-overloading conditions resulted in reduced ATP production, hypertrophic changes (by measuring cell size), and impaired calcium handling along with an increase in iron uptake due to negative feedback occurring through transferrin-mediated iron uptake in FRDA iPSC-Cms (Lee et al., 2014). In 2016, the same group carried out a drug screen in FRDA iPSC-Cms and showed that iron-induced ROS was significantly reduced by deferiprone rather than idebenone (which is a drug-related with coenzyme Q10) (Lee et al., 2016).

In 2019, RNA-seq results from FRDA iPSC-Cms showed significant upregulation of long-noncoding RNA gene, *MIR22HG* (Jixue Li et al., 2019). This lncRNA codes for *MIR22* gene which functions in cardiac development. Overexpression of *MIR22* is sufficient to induce hypertrophy (Gurha et al., 2013). The upregulation of *MIR22HG* observed in patient cardiomyocytes may lead to an increased level of pro-hypertrophic *MIR22*. RNA-seq data also showed enhanced expression of *NPPA* and *NPPB* (Natriuretic Peptide A and B) in patient iPSC-Cms (Aggarwal et al., 2014). Those genes are repeatedly observed in hypertrophic models. They also showed a reduction in the expressions of those genes by genome editing of the expanded GAA repeats (Jixue Li et al., 2019).

Codazzi and colleagues differentiated neurons from FRDA iPSCs and control iPSCs through treatment with BMP antagonist Noggin. They observed that FRDA neurons retained GAA repeat expansion along with reduced *FXN* levels. Reduction in *FXN* levels was accompanied by a reduction in mitochondrial ISC biogenesis and mitochondrial aconitase (*ACO2*). Additionally, mitochondrial superoxide dismutase (*SOD2*) increased significantly in FRDA neurons compared to control neurons. When they treated cells with H_2O_2 (300 μ M), ROS accumulation was found to be significantly higher in FRDA neurons compared to control neurons. They also showed that when they treated neurons with both H_2O_2 and Fe^{3+} , neuronal death among FRDA neurons after 75 hours of treatment was four times higher than death among control neurons. Interestingly, 2-Aminobenzamide HDAC inhibitor (HDAC-109) treatment was found to reverse the phenotype by increasing *FXN*, *ACO2*, decreasing *SOD2* expressions, and preventing the formation of ROS after H_2O_2 treatment (Codazzi et al., 2016).

In FRDA, degeneration particularly affects sensory neurons (Simon et al., 2004). In 2011, for the first time, FRDA iPSCs were differentiated to sensory neurons (J. Liu et al., 2011). *FXN* level was lower in iPSCs and neuronal crest cells compared to controls. During differentiation towards sensory neurons, there should be upregulation of frataxin. Although this was the case during sensory neuron specification for control cells, this upregulation was not observed for FRDA cells (Eigentler et al., 2013). Later, *FXN* deficiency, deficits in ISC proteins, and abnormal antioxidant responses (such as dysregulation of *SOD2*) were also shown in peripheral sensory neurons derived from FRDA iPSCs. They showed that protein level and mRNA level of NRF2 (a regulator of antioxidant responses) in this FRDA model (Hu et al., 2017). When transcriptional profile

analysis of FRDA sensory neurons and control neurons was compared, 4886 genes were found to be differentially expressed in FRDA sensory neurons. The genes involved in neuronal regulation, transcription, and apoptosis were found to be most affected by the loss of frataxin. Their functions were partially restored via HDACi treatment (Lai et al., 2019).

In addition to sensory neurons, the loss of large primary proprioceptive neurons in dorsal root ganglia is seen in FRDA. Thus, in 2019 FRDA iPSCs were differentiated into primary proprioceptive neurons. They observed that during differentiation, in FRDA samples there were differentiation deficits, and FRDA iPSCs -derived primary proprioceptive neurons show limited survival (Dionisi et al., 2019) (**Table 1.2**).

1.6.2 *In vivo* models of FRDA

Strong ubiquitous RNAi-mediated frataxin inactivation in *Drosophila* significantly reduced lifespan and results in reduced activities of aconitase and respiratory chain (MRC) complexes I, II, III, and IV. Reduction in the frataxin triggered a strong accumulation of iron as well. An important observation in *Drosophila* models was the accumulation of lipid droplets. These accumulations could be associated with neurodegeneration because lipids are extremely prone to Fenton reactions leading to lipid peroxides in FRDA flies (Monnier et al., 2018) (**Figure 1.5**).

Complete loss of frataxin is embryonically lethal (E 6.5 day) (Cossee, 2000). The conditional knockdown model with complete frataxin deletion in cardiac and skeletal muscle (*MCK-Cre-FXN* mice) showed intramitochondrial iron buildup only in late stages of the disease. These muscle creatine kinase conditional *FXN* knockout mice recapitulated most features of FRDA cardiomyopathy (Llorens et al., 2019; Perdomini et al., 2014). Another approach is to generate transgenic mice with the human *FXN* gene having GAA repeats. The entire *FXN* gene with GAA insertions within a human YAC was inserted into the mouse genome. This resulted in the generation of a YG8 line with two GAA sequences of 90 and 190 repeats and a YG22 line with one sequence of 190 repeats. These mice showed a reduction in electron transport chain complexes I, II, III activity along with mitochondrial iron accumulation in their cardiomyocytes but no ROS formation (Perdomini et al., 2013). Later, Ouellet, and colleagues show that the excision of GAA repeats via the CRISPR-Cas9 system is enough to restore the frataxin

transcriptional and protein level in YG8R mice (Ouellet et al., 2017). However, the generation of mice with up to 200 GAA repeats may not represent the disease seen in patients with up to 1000 GAA repeats resulting in only milder symptoms (Crombie et al., 2016) (**Table 1.1**)

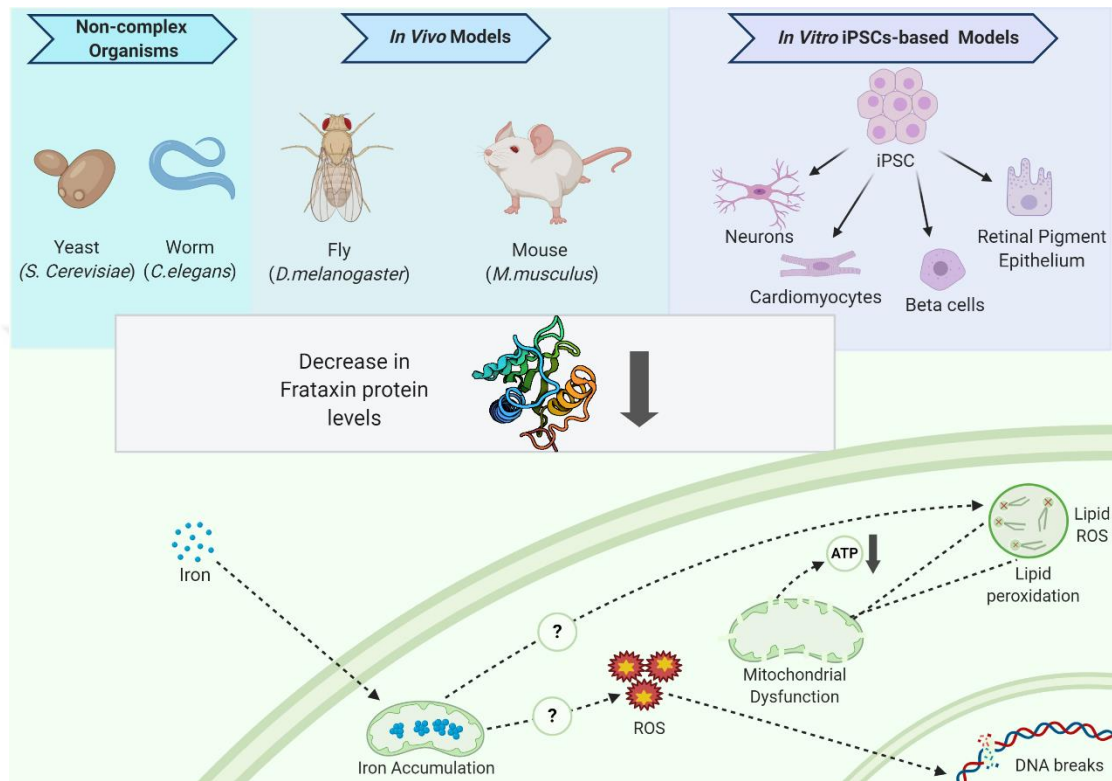


Figure 1.5 Representations of *in vivo* and *in vitro* models of FRDA.

Decrease in frataxin protein level results in FRDA. In the cellular level, this leads to iron accumulation, ROS formation, mitochondrial dysfunction and lipid peroxidation. ROS results in DNA breaks, whereas mitochondrial dysfunction causes a decrease in ATP levels.

Table 1.1 Current mouse models of FRDA.

List of FRDA mouse models		
Name of the model	How they created	References
Conditional <i>FXN</i> knockout mice	Cre inserted into a promoter of <i>MCK</i> (muscle creatine kinase) And Cre inserted into a promoter of <i>NSE</i> (neuron-specific enolase)	(Puccio et al., 2001)
KIKO	Breed knock-in mice ((GAA) ₂₃₀) and heterozygous knockout <i>FXN</i> mice	(Miranda et al., 2002)
KIKI	Mice homozygous for the knock-in allele containing (GAA) ₂₃₀	(Rai et al., 2008)
YG8	Transgenic mice that have expanded entire human <i>FXN</i> gene within a YAC - Have GAA ₉₀ and GAA ₁₉₀ alleles	(Al-Mahdawi et al., 2004)
YG22	Transgenic mice that have expanded entire human <i>FXN</i> gene within a YAC - Have only one GAA ₁₉₀ allele	(Al-Mahdawi et al., 2004)
YG8R and YG22R	YG8 and YG22 transgenic mice were crossed with heterozygous <i>FXN</i> knockout mice and YG8R and YG22R mice were created. They have GAA repeat expansions expressing only human frataxin protein.	(Al-Mahdawi et al., 2006)

Table 1.2 Current iPSCs models of FRDA

List of iPSCs studies to model FRDA		
Types of cells	Phenotypes	References
iPSCs	Intergenerational GAA·TTC Triplet Repeat Instability	(Ku et al., 2010)* First study to generate FRDA iPSCs
	Investigating the role of mismatch repair enzymes in GAA·TTC triplet repeats	(Jintang Du et al., 2012; Polak et al., 2016)
iPSCs-derived neurons	Delayed development of full electrophysiological functionality, reduced mitochondrial potential	(Codazzi et al., 2016; Hick et al., 2013; J. Liu et al., 2011)
	Enhanced cleavage of initiator caspase-9 and effector caspase-3, activation	(Igoillo-Esteve et al., 2015; Shan et al., 2014)
iPSCs-derived neuron progenitors	Able to differentiate to functional neurons <i>in vitro</i> , no susceptibility to cell death, able to differentiate to functional neurons transplanted in rodent brain	(Bird et al., 2014)
iPSC-Cms	Progressive extensive mitochondrial proliferation, characteristics of respiration-compromised mitochondria, mitochondrial iron accumulation	(Hick et al., 2013)* First article to demonstrate a mitochondrial defect in iPSC-Cms
	Disorganized mitochondrial network, mitochondrial DNA depletion, hypertrophic cardiac stress responses, calcium signaling impairment	(Crombie et al., 2017; Lee et al., 2014, 2016)
iPSCs-derived primary sensory neurons	Loss of iron-sulfur proteins, impaired antioxidant responses, restored by HDACi treatment	(Eigentler et al., 2013; Hu et al., 2017)
iPSCs-derived retinal pigment epithelium	No significant phenotypes were observed	(Crombie et al., 2015)* First time to differentiate FRDA iPSCs to other cell types than neurons or cardiomyocytes
iPSCs-derived proprioceptive neurons	Normal appearance but subtle differentiation deficits and more limited survival	(Dionisi et al., 2019)

1.7 Possible treatments for FRDA

Currently there is no established treatment for FRDA but many different approaches have been attempted to increase *FXN* protein levels. One possible approach to increase the protein level could be through genetic correction such as removal of repeats from pathogenic *FXN* alleles. Zinc finger nucleases, transcription activator-like effector nuclease (TALENs), and CRISPR-Cas9 methods are used to excise the repeats (Gottesfeld, 2019). 1.2 kbp GAA repeats in FRDA iPSCs was excised via zinc-finger nucleases followed by differentiation to beating cardiomyocytes (Cms). 3-fold increase in Frataxin protein level was observed in corrected cells compared to parental cells. Since the majority of FRDA patients develop hypertrophic cardiomyopathy, they checked the expression of hypertrophy related genes such as (*NPPA*, *NPPB*, *MYH7*, *ACTA1*, *TAGLN*, *CREB5*, *DUSP4*, *ACTN1*) and showed that excision results in a decrease in those genes compared to patient Cms (Jixue Li et al., 2019). Another approach is to transcriptionally upregulate *FXN* levels by gene activation through TALE-VP64 (Cherif et al., 2018; Gottesfeld, 2019). 19-fold increase in *FXN* gene expression in primary (FRDA) fibroblasts has been achieved with Platinum TALE (pITALE) proteins fused with transcriptional activators such as VP64 or SunTag. They created pITALE_{VP64} targeting the regulatory regions of *FXN* gene. In addition to a 19-fold *FXN* mRNA level increase *in vitro*, they used adeno-associated viruses to deliver pITALE_{VP64} in YG8R mouse model. This resulted in a 10-fold increase in endogenous *FXN* gene in the heart along with a 35% increase in endogenous *FXN* gene in the skeletal muscle (Cherif et al., 2018).

In addition to genetic approaches, epigenetic drugs were tested to investigate their effects on *FXN* expression. Hick and colleagues differentiated FRDA iPSCs to neurons and treated the cells with an HDAC inhibitor (HDACi 109). HDACi 109 treatment reversed the phenotype by increasing expression of *FXN*, aconitase, and decreasing ROS formation after H₂O₂ treatment (Codazzi et al., 2016). Inhibition of HDAC 1 and 3 was sufficient to upregulate *FXN* mRNA levels in YG8R mouse model (Sandi et al., 2011). In addition to HDAC inhibitors, histone methyltransferase inhibitors (HMT) have been used to increase *FXN* expression by reversing histone methylation of H3K9 and H3K27 but common HMT inhibitor BIX-01294 did not show any effect on *FXN* mRNA level. There are no studies that investigated the effect of DNA methylation inhibition (Gottesfeld, 2019).

Other therapeutic drugs also increase the Frataxin protein level. One of them is Interferon Gamma (INF γ). When it is administered to YG8R mouse, frataxin expression in dorsal root ganglia neurons increased and degeneration of neurons decreased. Moreover, erythropoietin (EPO) was tested in clinical trials because recombinant human (rhu-EPO) can cross the blood-brain barrier and is known to increase in Frataxin protein level. Despite early positive results in 8-week studies, rhu-EPO did not have any significant effects on frataxin and neurological function (Codazzi et al., 2016). Since dysregulation of iron and impaired iron homeostasis is the hallmark of this disease, iron chelators such as deferoxamine, and deferiprone were used to reverse the phenotypes of the disease. However, in *in vitro* culture experiments deferoxamine resulted in reduced aconitase protein level. Deferiprone successfully decreased ROS damages as well as iron buildup in the brain but it also decreases the activity of aconitase (Richardson et al., 2013). Lastly, antioxidants can reverse mitochondrial damage decreasing ROS and DNA damage. Coenzyme Q10 derivative idebenone is a widely evaluated compound. Although it improved the marker of ROS damage in both FRDA cellular and murine models, Phase III trials did not improve the life span significantly (Codazzi et al., 2016).

1.8 Cardiomyocyte differentiation protocols in literature

iPSC-Cms are increasingly used for disease modeling, cardiotoxicity screening, and discovery of drugs. There are several ways for the generation of cardiomyocytes from hiPSCs. One initial way is via embryoid body (EB) formation. The yield of this differentiation can be enhanced with the addition of factors such as TGF- β /BMP pathway regulators Activin A and BMP4, and at later stages of differentiation, with the addition of DKK1 which is a WNT pathway antagonist. But this process still yields less than 1% cardiomyocyte (Lian et al., 2013). Another method is to coculture of PSCs with END-2 cells (visceral endoderm-like cells) or exposure of cells with END-2- conditioned medium and this leads to induction of up to 10 % of cardiomyocytes (Kempf et al., 2016). An alternative method to these can be monolayer-based directed differentiation in which WNT pathway activator is firstly added to the cells followed by inhibition of WNT pathway. After 24 hours of WNT pathway activation, an almost pure population of *BRACHYURY*-expressing mesendoderm cells can be observed. Next, WNT pathway was inhibited. For inhibition, porcupine inhibitors IWP2 or IWP4 can be used. In this protocol

cardiac-specific transcription factors, *NKX2.5* and *ISL1*, can get expressed on the fourth day of the differentiation followed by expression of cTnT at day8 (Lian et al., 2013).

Cardiomyocytes can also be induced from somatic cells without the involvement of pluripotent stem cells. In such protocols, key cardiac transcription factors are overexpressed in somatic cells to convert them directly into cardiomyocyte like cells.

The first direct differentiation was done by converting human myocardial scar fibroblasts into induced cardiomyocytes (iCms) *in vitro* via overexpression of cardiac transcription factor-myocardin (*MYOCD*) (Tuyn et al., 2007). But the yield of contractile cells was very low. Later, in 2009, noncardiac mesoderm of mouse embryos was differentiated upon overexpression of *GATA4*, *TBX5*, and *BAF60C* (Takeuchi & Bruneau, 2009). Following these, Ieda and colleagues converted postnatal cardiac fibroblasts to iCms by overexpressing *TBX5*, *MEF2C*, and *GATA4*. After the full characterization of iCms, they analyzed the enrichment of H3K27me3 and H3K4me3. H3K27me3 marks were depleted at the promoters of the genes playing an important role in cardiomyocytes, and H3K4me3 marks increased in the promoter regions of *ACTN2* and *TNNT2*. They showed that after a careful screen of 14 transcription factors, it is possible to efficiently and rapidly induce cardiomyocyte-like cells via a combination of just three factors. iCms showed similar patterns in terms of gene expression profile, and electrophysiologically with the neonatal cardiomyocytes. Also reprogramming by those transcription factors resulted in epigenetic resetting of fibroblast epigenome to cardiomyocyte-like state (Ieda et al., 2010). A chemical cocktail, which is composed of GSK3 inhibitor, TGF β R1 inhibitor, Forskolin, HDAC inhibitor, Parnate, and retinoic acid analog, generated beating clusters from mouse fibroblasts (Fu et al., 2015) and later from human fibroblasts (Cao et al., 2016). Very recently, Testa and colleagues showed that pre-treatment of MEFs and adult (5 weeks old) cardiac fibroblasts with Bmi1 inhibitor PTC-209 increased the yield of chemically-induced direct cardiac reprogramming (Testa et al., 2020).

Chapter 2: MATERIALS AND METHODS

2.1 Cell culture experiments

2.1.1 Medium compositions

Patient and control fibroblasts, and HEK293T cells were maintained in D10 medium (DMEM 1X (Gibco) containing, 10%FBS (Fetal Bovine Serum) (Gibco), 1% Penicillin/Streptomycin (Gibco)) whereas induced pluripotent stem cells (iPSCs) were maintained in human ES medium (hES) (DMEM-F12 (Lonza) containing, %20 KOSR (knockout serum replacement) (Gibco), 1% Penicillin/Streptomycin, 1% Non-essential amino acids (NEAA) (Gibco), 1% L-glutamine (Gibco), 0.1 mM β -mercaptoethanol, 10 ng/ml FGF). iPSCs were passaged in a 1:6 ratio each week. MCM medium was composed of conditioned hES medium collected from MEF feeder layers for 10 days. Before they were used, 10 ng/ml FGF should be added and medium should be filtered. Embryoid body medium (250 ml) composed of 187 ml of DMEM/F12 (Gibco), 50 ml FBS, 1.25 ml of L-Glutamine, 2.5 ml of NEAA, 2.5 ml of P/S, 55 mM β -mercaptoethanol, 32 mg/ml L-ascorbic acid, 10 mg/ml of holotransferrin (Sigma #T0665). For cardiomyocyte differentiation, 2 different mediums were used. RPMI/ B27-Insulin medium composed of RPMI 1640 (Gibco), 1% B27-Insulin supplement (Thermo Fisher Scientific), 1% Penicillin/Streptomycin, 213 μ g/ml L-ascorbic acid (Sigma-Aldrich). RPMI/B27 medium composed of RPMI 1640 (Gibco), 1% B27 supplement (Thermo Fisher Scientific), 1% Penicillin/Streptomycin, 213 μ g/ml L-ascorbic acid (Sigma-Aldrich).

2.1.2 Primary culture of FRDA and Control skin biopsy samples

4 FRDA and 2 control fibroblast samples were collected by KUH Diagnostic Centre of Genetic Diseases via skin punch biopsies (Ethical Review Board Number: 2016.126.IRB2.080). Samples were transferred to 6-well plates and cut into small pieces (0.5-1 mm) by using sterile razor blades. These pieces were placed under coverslips and maintained in 4 ml of D10 medium for three days in an incubator at 37⁰ C with 5% CO₂. The migration of fibroblasts and keratinocytes was observed under the microscope. Every three days, the medium was changed. When cells reached enough confluency, they were passaged at a 1:3 ratio. For this, they were washed with 2 ml of DPBS, and DPBS was removed and then 2 ml of 0.05%trypsin was added and waited for 5 min at 37⁰ C. After

inactivation of trypsin, cells were split into new plates. Those steps were repeated until the number of fibroblast cells exceeded the number of keratinocytes.

2.1.3 Dissociation, Freezing, and Thawing

For fibroblasts and HEK293T cells, the passage procedure was carried out as described above. For freezing of fibroblasts, HEK293T cells were trypsinized; iPSCs were detached by using ReLeSR and all cells were frozen with freezing medium (90% FBS, 10% DMSO). Then they were transferred to cryovials (Corning) and stored into Mr. Frosty freezing containers at -80°C for 1 to 2 days followed. Next, cryovials were transferred to liquid nitrogen for long-term storage. For culturing of fibroblasts and HEK293T cells, cells were thawed and maintained in D10 medium. But for iPSCs thawing, cells were thawed and maintained in hES medium with addition of 10 μM of Y Compound (Tocris#1254) for 16 hours and replaced with fresh hES medium.

iPSCs were passaged by using 3 different enzymes (ReLeSR, Collagenase IV, and Accutase) in this project. iPSCs were washed with filtered 2ml of DMEM/F12. Then iPSCs were incubated with 700 μl of ReLeSR for 1 minute at room temperature. ReLeSR was removed after this incubation and cells were put back into the incubator for 4 minutes. Then, by using hES medium, iPSCs were detached by pipetting up and down followed by 1:6 splitting of single-cell iPSCs to mito-MEF seeded plates. For ColIV passage, iPSCs were washed with 2 ml of DMEM/F12. 1 ml of Collagenase IV (0.05 g/50 ml F12) was added and waited for 5 minutes at an incubator. After removal of ColIV, 2 ml of F12 was added and iPSCs were collected into a falcon tube by using cell scrapers. Then the cells were centrifuged at 1000 rpm for 2 minutes and after resuspension of cells with fresh hES medium, they were split into mito-MEF feeder layer seeded plates. For Accutase passage, iPSCs were washed with filtered 2 ml of DMEM/F12. Then 500 μl of Accutase (STEMCELL) enzyme was added and cells were incubated with Accutase for 5 min in the incubator. Then, by using 700 μl of DPBS medium, iPSCs were detached by pipetting up and down followed by collection into a falcon tube. Cells were centrifuged and after removal of the supernatant, the pellet can be dissolved in fresh hES medium.

2.1.4 Mitotically Inactivated Mouse Embryonic Fibroblasts Preparation

Balb/C mouse embryonic fibroblasts (MEFs) were harvested from E13.5 mouse embryos, and were frozen previously in our lab. The cryovial containing these isolated Balb/C

MEFs was thawed and split into five already 0.1% gelatin (dissolved in ddH₂O, autoclaved, and filtered) (Sigma)-coated 10 cm cell culture plates and maintained in 12 ml of D10 medium for two days. Then cells in each 10 cm plates were trypsinized and split into two 15 cm plates and maintained in 15 ml D10 medium for two days. After two days, cells in each 15 cm plates were trypsinized and split into ten 15 cm plates and maintained in 15 ml D10 medium for two days. After two days or three days of passage (this is determined according to the confluency of the cells), MEFs were washed with 15 ml of DPBS, and 10 ml D10 fresh medium was added. Then cells were treated with 100 µl of Mitomycin C so that the final concentration was 10 µg/ml. Mitomycin-C (Millipore) was prepared by dissolving it into 10 ml of nuclease-free water and filtered. MEFs were treated with mitomycin-C for two hours at the incubator. Mito-MEFs were washed with DPBS for two times followed by trypsinization with 5 ml of trypsin and collected for counting. Then 1 million, 2 million, 4 million, and 5 million of mito-MEF were aliquoted and frozen with freezing medium.

2.1.5 Reprogramming of FRDA and Control Fibroblast Lines by Episomal Vectors

For reprogramming, episomal vectors containing *OCT4*, *SOX2*, *LIN28*, *L-MYC*, *KLF4* transcription factors and shp53 as well were used. Vectors (pCXLE-hOct3/4-shp53[Addgene#27077], pCXLE-hSK[Addgene#27078], pCXLE-hUL[Addgene#27080], pCXWB-EBNA1[Addgene#37624], pCXLE-EGFP[Addgene#27082]) were ordered from Addgene and after successful diagnostic digestions, they were used in the reprogramming process. Neon transfection system was prepared by adding 3 ml of E2 buffer into the electroporation cuvette. 1.5 µg of each episomal vectors were mixed and R buffer was added to episomal vector mixture until the total volume would be 150 µl. 450,000 fibroblasts were counted and centrifuged at 1200 rpm for 4 minutes. The cell pellet then was resuspended with R buffer+episomal vector mixture (150 µl) and transferred to an Eppendorf tube. 100 µl of R buffer+episomal vector+ cell mixture were taken with Neon 100 µl tip and inserted into electroporation cuvette. Then cells were electroporated with 1400 V, 20 ms, 2 pulse parameters. After that, the tip containing the electroporated cells was removed from cuvette carefully and cells were dispensed onto freshly added D10 without antibiotics medium. After 16 hours, the medium was switched to 3 µM of EPZ4777 added D10

medium, iDOT1L (DOT1L inhibitor) (on the first day of reprogramming). Throughout 14 days of reprogramming, cells were treated with 3 μ M of iDOT1L.

On day 6, cells were transferred to mito-MEF coated plates at a 1:6, 1:8, and 1:10 ratio. The next day, the medium was switched to hES medium. And around 21th day of reprogramming, visible iPSCs colonies were formed and picked manually by using an inverted microscope.

2.1.6 Differentiation to Cardiomyocytes

One hour before starting the experiments, Matrigel-coated 12 well plates were freshly prepared. When iPSCs grown on Matrigel-coated plates and maintained in mTESR medium were >90% confluent, they were detached from Matrigel-coated plates by using Accutase. They were counted and 800×10^3 iPSCs were seeded onto the one well of freshly prepared Matrigel-coated 12 well plates. They were seeded in the mTESR with the addition of 10 μ M Y compound. The next day, the medium was changed to the mTESR medium. iPSCs were grown in mTESR for 3-4 days. Thereafter, the differentiation protocol represented in **Figure 2.1** was used for differentiation.

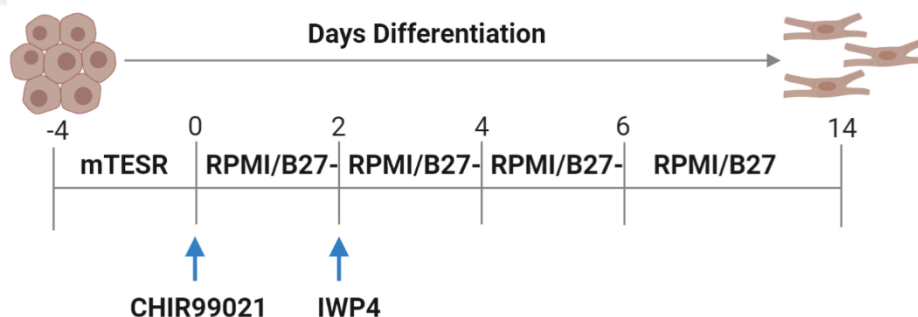


Figure 2.1 Illustration of cardiomyocyte differentiation protocol.

Time-dependent treatments of CHIR99021 and IWP4 small molecules are extremely important.

The cells were treated with varying concentrations of CHIR99021 (from 6 μ M- 10 μ M) (Selleckchem#S1263) in RPMI/B27-insulin medium with addition of 213 μ g/ml of L-ascorbic acid (Sigma-aldrich) (day 0 of the differentiation). 2 ml of CHIR99021 added RPMI/B27-insulin medium was added to the cells for one well of 12-well plates. Cells were placed in the incubator at 37^o C, 5%CO₂. On the 2nd day of the differentiation, cells were washed with 2 ml of RPMI 1640. Following this, 2 ml of 5 μ M IWP4 (Stemgent#04-0036) added RPMI/B27-insulin medium with the addition of 213 μ g/ml of L-ascorbic

acid was added to each well. Cells were placed in the incubator at 37⁰ C, 5%CO₂. On day 4th of the differentiation, the old medium was removed and washed with 1 ml of RPMI1640 medium and replaced with 2 ml of RPMI 1640/B27-insulin with the addition of 213 µg/ml of L-ascorbic acid medium. The culture medium was changed every two days with RPMI 1640/B27 with the addition of 213 µg/ml of L-ascorbic acid medium until beating clusters could be observed on the 9-11th of differentiation.

2.2 Lentiviral Packaging and Concentration of viruses

2.25x10⁶ 293T cells were seeded onto 10 cm cell culture plates and maintained in D10 medium the day before transfection (day -1). On the transfection day, the DNA mix was prepared by following the DNA amounts shown in (**Table 2.1**). Until the volume was 200 µl, 1X DMEM (filtered) added to the DNA mixture. Meanwhile, 180 µl of 1XDMEM and 20 µl of Eugene reagent were mixed and added onto the DNA mixtures. They were incubated for 30 minutes at room temperature. At the end of incubation, the mixture was dropped slowly onto HEK293T (day0). After 16 hours, the old medium was replaced with fresh 8 ml of D10 medium (day 1). The next day, the first viral collection was done (day 2) and fresh 8 ml of D10 medium was added. On the final day, after the second viral collection was done (day 3), the cells were decontaminated.

To concentrate viral solutions collected, all mixed viral supernatants were centrifuged at 1200 rpm for 4 minutes and the supernatant was filtered in 0.45 µm syringe filters (Corning). Onto the filtered viral medium, 5XPEG-8000 (Sigma) was added according to amounts stated in **Table 2.1**. After mixing of viral solution with PEG solution, the mixture should be stored at 4⁰ C overnight. Then the viral solutions were centrifuged at 2000 rpm for 30 minutes at 4⁰ C. After centrifuge, most of the supernatant was removed and decontaminated in a 10% bleach-containing bottle. Then the remaining viral solution was again centrifuged at 2000 rpm for 5 minutes. After the supernatant was again removed, the viral pellet was resuspended in 160 µl of 1X DPBS. Then, this solution was aliquoted in 50 µl of aliquots and stored at -80⁰ C for long term storage.

Table 2.1 Viral packaging instructions

	6-cm Plate (Per Plate)	10-cm Plate (Per Plate)	15-cm Plate (Per Plate)
Number of HEK293T cells seeded	1x10 ⁶ cells	2.25x10 ⁶ cells	6.5x10 ⁶ cells
1X DMEM	92 µl	180 µl	440 µl
Fugene reagent	8 µl	20 µl	60 µl
Plasmid	1000 ng	2500 ng	7500 ng
psPAX2	900 ng	2250 ng	6750 ng
pCMV-VSV-G	100 ng	250 ng	750 ng
Collection medium	3+3=6 ml	8+8=16 ml	15+15=30 ml
Amount of 5XPEG added	1.5 ml	4 ml	10 ml

2.2.1 Transduction of Cells and Puromycin Selection

Before infection day, 2x10⁵ HEK293 cells were seeded per one well of 6 well. The next day, 1500 µl of D10 with 1.5 µl proteamine/sulfate (8mg/ml) and the required amount of viruses were added onto these HEK293T cells. After 16 hours of infection, if there will be doxycycline (DOX) induction, medium was changed with D10+2 µg/ml DOX medium or if not just with D10 medium. The next day, cells could be passaged and thereafter puromycin selection could be started with the addition of 2 µg /ml puromycin to D10 medium for 2-3 days (until there were no cells on the uninfected wells).

2.3 iPSCs characterization experiments

2.3.1 TRA-1-60 Immunostaining of iPSCs

iPSCs can be seeded on MEF-seeded 12 well cell culture plates. After they reached the required confluence, they were washed with 1X DPBS and fixed with 500 µl of 4% paraformaldehyde (PFA) solution for 20 minutes at room temperature on gentle shaking. Then to wash the remaining PFA, cells were washed with 3X DPBS for 5 minutes. Meanwhile, staining solution composed of 3% FBS, 0.3% Triton-X in PBS can be prepared and biotin-conjugated TRA-1-60 antibody (BioLegend #330604) can also be prepared in 1:200 dilution in staining solution. After several washes with DPBS, cells

were incubated in 300 µl of antibody solution overnight at 4⁰ C with gentle shaking. The next day, the cells were washed with 3X DPBS followed by incubation with 300 µl of the secondary antibody (Streptavidin-HRP (BioLegend #405210)) for 1 hour at room temperature. The secondary antibody was dissolved in the staining solution in 1:500 dilution. After incubation, cells were washed 3 times with DPBS. Then cells were incubating with DAB solution containing 1% DAB (3,3 diaminobenzidine), 1% Nickel ammonium sulfate, 0,3% H₂O₂ for 15 minutes at room temperature with gentle shaking. After removal of DAB solution, cells were washed 1X with DPBS solution. The pictures of the stained iPSCs were taken the next day.

2.3.2 Immunofluorescence staining

For iPSCs characterization, iPSCs were passaged onto mito-MEF seeded coverslips. 4-5 days after iPSCs passage, cells can be fixed with 500 µl of 4% PFA solution for 30 minutes at room temperature after washing with 1 ml of DPBS once. Then to remove PFA, cells were washed with DBPS 3 times. Cells were incubated in 500 µl of 0.2% Triton-X (dissolved in DPBS) solution for 30 minutes at room temperature. After 3 times wash with DPBS, cells were incubated with 500 µl of blocking buffer composed of 3% BSA, 5% goat serum (filtered) in DPBS for 2 hours at room temperature. Then coverslips were closed onto 20 µl of 1⁰ antibodies (OCT4, NANOG) (**Table 2.2**) and incubated at 4⁰C overnight. The next day the coverslips were transferred into 12 well plates and washed 3 times with DPBS. Then, the coverslips were closed onto 15 µl of the 2⁰ antibody (Alexaflor488) and incubated at 4⁰C for 3 hours. If cells were costained with OCT4 and SSEA4, Alexaflor488 antibody and SSEA4 antibody solutions were mixed so that Alexaflor488 antibody diluted in 1:500 ratio and SSEA4 antibody diluted in 1:100 ratio. After incubation with secondary antibodies, coverslips were again transferred to 12 well plates and washed with DPBS for 3 times. 5 µl of DAPI mounting medium was added onto the glass slides and coverslips were closed onto this DAPI medium. Then coverslips were enclosed with colorless nail polish to prevent evaporation of DAPI. Until the photos of iPSCs were taken by using LEICA DMI8-SP8 confocal microscope, the slides should be kept in 4⁰C.

For cardiomyocyte characterization, cardiomyocytes were passaged using Accutase onto Matrigel coated-sterile coverslips. For passage, they were washed with 2 ml of DPBS and

then 500 μ l of Accutase was added and incubated for 5 min at 37°C. With 700 μ l of DPBS, all cardiomyocytes were collected into falcon tube and centrifuged for 2 minutes at 1000 rpm. After the removal of the supernatant, cardiomyocytes were resuspended in 2 ml of fresh cardiomyocyte medium with addition of 10 μ M Y compound. And 3 days after this passage, cardiomyocytes were washed with 1 ml of PBS and fixed with 4% PFA for 30 minutes at room temperature. After removal and washing with DPBS for 1 time, cells were incubated in 500 μ l of 0.4% Triton-X (dissolved in DPBS) solution for 30 minutes at room temperature. After 3 times wash with DPBS, cells were incubated with 500 μ l of blocking buffer composed of %3 BSA, %5 goat serum (filtered) in DPBS for 2 hours at room temperature. Then coverslips were closed onto 20 μ l of 1^o antibodies (Cardiac troponin T monoclonal antibody, Anti-alpha actinin[Sarcomeric], Myosin Light Chain 2 Antibody, MLC-2A) (**Table 2.2**) and incubated at 4°C overnight. The next day the coverslips were transferred into 12 well plates and washed 3 times with DPBS. Then, the coverslips were closed onto 15 μ l of the 2^o antibody (Alexafluor488) and incubated at 4°C for 3 hours. After incubation with secondary antibodies, coverslips were again transferred to 12 well plates and washed with DPBS for 3 times. 5 μ l of DAPI mounting medium was added onto the glass slides and coverslips were closed onto this DAPI medium. Then coverslips were enclosed with colorless nail polish to prevent evaporation of DAPI. Until the photos of iPSCs were taken by using LEICA DMI8-SP8 confocal microscope, the slides should be kept in 4°C.

Table 2.2 Antibodies used in the immunofluorescence staining of iPSCs, and cardiomyocytes.

Antibodies	Catalog number	Source	Working concentration
SSEA4	BD560218	Mouse	1:100
NANOG	Abcam ab21624	Rabbit	1:100
OCT4	Abcam ab19857	Rabbit	1:100
Cardiac Troponin T Monoclonal Antibody	Thermo Fischer MA5-12960	Mouse	1:100
Anti-alpha-actinin (Sarcomeric) antibody	Sigma Aldrich A7811	Mouse	1:100
Myosin Light Chain 2 Antibody	Protein Tech Group 10906-1-AP	Rabbit	1:100
Myosin Light Chain 2 - A	Synaptic system- 311011	Rabbit	1:100
Alexa Fluor 488 Anti-Rabbit	Thermo Fischer A27039	Goat	1:500

2.3.3 Analysis of Episomal Vector Integration

Episomal vectors were nucleofected to the fibroblasts. Although nucleofection is an integration-free method, there is a chance of vector integration to the fibroblasts genome. To check whether there is any integration or not, PCR-based method by using primers targeting *EBNA* sequence was utilized. The reason for choosing *EBNA* primers is because all of the episomal vectors contain *EBNA* sequence. For carrying out *EBNA* PCR, genomic DNAs from FRDA and control iPSCs 1-2 passages after colony picking was obtained by using M-N Nucleospin Genomic DNA from tissue kit. And 50 ng genomic DNA was used per PCR reaction with *EBNA* and *GAPDH* primers. *EBNA* and *GAPDH* primer sequences and PCR conditions were the same as shown in **Table 2.3, Table 2.4, Table 2.5.**

Table 2.3 Sequences of PCR primers.

Primers	Sequences
<i>EBNA</i> Forward Primer	GTGGTTTGGAAAGCATCGTGGTCA
<i>EBNA</i> Reverse Primer	ATAGGTGGAAACCAGGGAGGCAAA
<i>GAPDH</i> Forward Primer(NEW)	TCCCTGAGGGTTCTTTGTGC
<i>GAPDH</i> Reverse Primer(NEW)	GGGGCAGAGCATCTACACTC
GAA LR-PCR Forward Primer	GGCTTGAACCTCCACACGTGTT
GAA LR-PCR Reverse Primer	AGGACCATCATGGCCACACTT
<i>FXN</i>-Int1-GAA-F-KUH	AATGGATTTCCTGGCAGGACGC
<i>FXN</i>-Int1-GAA-R-KUH	ATCTTGGCTTAATGCAACCTCTGCC
pENTR1A no ccDB-<i>FXN</i> plasmid sequencing Forward primer	CTACAAACTCTTCCTGTTAGTTAG
pENTR1A no ccDB-<i>FXN</i> plasmid sequencing Reverse primer	ATGGCTCATAACACCCCTTG

Table 2.4 *GAPDH* and *EBNA* PCR conditions.

<i>GAPDH</i>	Temperature	Duration	Cycles	<i>EBNA</i>	Temperature	Duration	Cycles
Cycle1	95 ⁰ C	3 min	1	Cycle1	95 ⁰ C	3 min	1
Cycle2	95 ⁰ C	30 s	35	Cycle2	95 ⁰ C	10 s	30
	64 ⁰ C	30 s			65 ⁰ C	30 s	
	72 ⁰ C	1 min			72 ⁰ C	30 s	
Cycle3	72 ⁰ C	10 min	1	Cycle3	72 ⁰ C	5 min	1
	4 ⁰ C	∞			4 ⁰ C	∞	

Table 2.5 *GAPDH* and *EBNA* PCR protocol.

Reagents (<i>EBNA</i> PCR)	Amounts	Reagents (<i>GAPDH</i> PCR)	Amounts
DreamTaq Green PCR Master Mix	10 μ l	DreamTaq Green PCR Master Mix	25 μ l
<i>EBNA</i> Fwd-Rvs Primer Mix (10 μM)	0.24 μ l	<i>GAPDH</i> Rvs Primer (10 μM)	0.5 μ l
DMSO	1 μ l	<i>GAPDH</i> Fwd Primer (10 μM)	0.5 μ l
Plasmid	200 ng	DNA	200 ng
Water	up to 20 μ l	Water	up to 50 μ l

2.3.4 Karyotype analysis of iPSCs

After *EBNA* integration analysis and pluripotency marker expression analysis were done for each iPSCs clones, 3 clones from iPSCs were sent to karyotype analysis at Koc University Genetic Diagnosis Centre. Before sending the samples there, T25 flasks were coated with Matrigel. 312 μ l of Matrigel was dissolved in 12 ml filtered cold F12 medium and 2 ml of Matrigel dissolved in F12 medium was spread into T25 flasks and waited at room temperature for one hour. The iPSCs clone from one well of 6 well was passaged with ReLeSR and transferred onto one Matrigel-coated T25 flask. The samples were then maintained in the MCM medium.

2.3.5 Embryoid Body Formation Assay

iPSCs grown onto MEF feeder layers were detached by using ColIV from 4 well of one 6 well plates. After the collection of iPSCs into falcon tubes and centrifuged them at 1000 rpm for 2 minutes, iPSCs were carefully resuspended into Embryoid Body (EB) medium. Then they were transferred to one well of 6 well ultra-low attachment plate. The plates were then placed onto a shaker, at 50 rpm, in an incubator at 37⁰ C 5% CO₂. After three days, the medium was changed to fresh EB medium and the medium was changed every 3 days. On day 9, EBs were collected for RNA isolation.

2.3.6 RNA isolation, cDNA Synthesis, and qPCR

If the number of cells seemed not quite enough, a mixture of lysis buffer and β -mercaptoethanol was directly added onto the cells after the removal of the medium. But if the number of cells seemed enough, for RNA isolation, cells were detached from the culture plates via trypsin/or ReLeSR and centrifuged at 1200 rpm for 4 minutes. Then, they were resuspended in cold DPBS and transferred into Eppendorf tubes and centrifuged at 3000 rpm for 5 minutes. After the supernatant was removed, pellets were dissolved in a mixture of lysis buffer and β -mercaptoethanol supplied by MN Nucleospin RNA isolation kit. RNAs were collected by following the instructions of this kit. Then, the concentration of RNA was measured by Nanodrop 200 (Thermo scientific) and 1000 ng of isolated RNA was converted to cDNA by following the cDNA protocol. The volume of starting 1000 ng of RNA for cDNA synthesis was completed to 13 μ l with RNase-free water. Then 2.5 μ l of 2 μ M dNTP mixture (Thermo scientific) and 1 μ l of 50 μ M random hexamer (Jena Biosciences) were added. cDNA melting was performed in the PCR machine at 65⁰ C for 5 minutes which will be immediately placed into ice. After that, a mixture of 5 μ l of 5X first strand buffer (Invitrogen-Y02321), 2 μ l of 0.1 M DTT (Invitrogen-Y00147), 0.5 μ l of RNasin (Promega N251B) was added to each cDNA samples. Then cDNAs were incubated at room temperature for 10 minutes. Following this, 1 μ l of Mmlv Reverse Transcriptase (Invitrogen-28025-013) was added and incubated at 37⁰ C for 1 hour and at 70⁰ C for 15 minutes at the PCR machine. After this incubation finished, 75 μ l of nuclease-free water was added per cDNA sample.

For quantitative PCR (qPCR) analysis, 2 μ l of cDNA was added to a mixture of 2 μ l of 2.5 mM Forward-Reverse primers (listed in table 5), 10 μ l of SYBR Green Master Mix (2X, Roche, Light cycler 480), 6 μ l of nuclease-free water. For every cDNA sample, duplicate qPCR reactions were prepared into 96-well opaque plates (Roche). Then the plate was placed into LightCycler 480 Instrument II(Roche) and the protocol described in **Table 2.7** was run. Expression levels of each of the genes were normalized to endogenous β -actin gene expression levels. Ct values, which stand for threshold cycle, and Cc values were determined and expression values were calculated based on the formula $2^{-(C_t - C_c)}$ by comparing the values with the control samples.

Table 2.6 Sequences of qPCR primers.

qPCR primers	Sequence
<i>β-ACTIN</i> Forward Primer	GGAGGAGCAATGATCTTGAT
<i>β-ACTIN</i> Reverse Primer	TGAAGTGTGACGTGGACATC
<i>OCT4</i> Forward Primer	AGCGAACCAGTATCGAGAAC
<i>OCT4</i> Reverse Primer	TTACAGAACCACACTCGGAC
<i>NANOG</i> Forward Primer	TGATTTGTGGGCCTGAAGAAAA
<i>NANOG</i> Reverse Primer	TGGTGGTAGGAAGAGTAAAG
<i>SOX2</i> Forward Primer	AGCTACAGCATGATGCAGGA
<i>SOX2</i> Reverse Primer	GGTCATGGAGTTGTACTGCA
<i>LIN28A</i> Forward Primer	TGCGGGCATCTGTAAGTGG
<i>LIN28A</i> Reverse Primer	GGAACCCTTCCATGTGCAG
<i>FXN</i> Forward Primer	CAGAGGAAACGCTGGACTCT
<i>FXN</i> Reverse Primer	AGCCAGATTTGCTTGTTTGG
<i>MAP2</i> Forward Primer	CTCAGCACCGCTAACAGAGG
<i>MAP2</i> Reverse Primer	CTCCGCCTTGATCCTTAATCTC
<i>NESTIN</i> Forward Primer	TGGGAGCAAAGATCCAAGAC
<i>NESTIN</i> Reverse Primer	GCGTTGGAACAGAGGTTGGA
<i>AFP</i> Forward Primer	AGC TGGTGGTGGATGAAAC
<i>AFP</i> Reverse Primer	CCCTCTTCAGCAAAGCAGAC
<i>MIXL1</i> Forward Primer	GGTACCCCGACATCCACTTG
<i>MIX1L</i> Reverse Primer	TAATCTCCGGCCTAGCCAAA
<i>MESP1</i> Forward Primer	GAAGTGGTTCCTTGGCAGAC
<i>MESP1</i> Reverse Primer	TCCTGCTTGCCTCAAAGTGT
<i>EOMES</i> Forward Primer	CGGCCTCTGTGGCTCAA
<i>EOMES</i> Reverse Primer	AAGGAAACATGCGCCTGC
<i>BRACHYURY</i> Forward Primer	TGCTTCCCTGAGACCCAGTT
<i>BRACHYURY</i> Reverse Primer	GATCACTTCTTTCCTTTGCATCAAG
<i>GATA4</i> Forward Primer	TCCCTCTTCCCTCCTCAAAT
<i>GATA4</i> Reverse Primer	TCAGCGTGTAAGGCATCTG

<i>ISL1</i> Forward Primer	AGATTATATCAGGTTGTACGGGATCA
<i>ISL1</i> Reverse Primer	ACACAGCGGAAACACTCGAT
<i>MEF2C</i> Forward Primer	ATGGATGAACGTAACAGACAGGT
<i>MEF2C</i> Reverse Primer	CGGCTCGTTGTACTCCGTG
<i>HAND1</i> Forward Primer	GTGCGTCCTTTAATCCTCTTC
<i>HAND1</i> Reverse Primer	GTGAGAGCAAGCGGAAAAG
<i>PDGFR-B</i> Forward Primer	GGTGGGCACACTACAATTTGC
<i>PDGFR-B</i> Reverse Primer	GGTGGGTAGGCCTCGAACA
<i>GATA4</i> Forward Primer	TCCCTCTTCCCTCCTCAAAT
<i>GATA4</i> Reverse Primer	TCAGCGTGTAAGGCATCTG
<i>NKX2.5</i> Forward Primer	GCGATTATGCAGCGTGCAATGAGT
<i>NKX2.5</i> Reverse Primer	AACATAAATACGGGTGGGTGCGTG
<i>TBX5</i> Forward Primer	GAAACCCAGCATAGGAGCTG
<i>TBX5</i> Reverse Primer	CAGCCTCACATCTTACCCTGT
<i>TNNI3</i> Forward Primer	CTGCAGATTGCAAAGCAAGA
<i>TNNI3</i> Reverse Primer	CCTCCTTCTTCACCTGCTTG
<i>MYL2</i> Forward Primer	ACATCATCACCCACGGAGAAGAGA
<i>MYL2</i> Reverse Primer	ATTGGAACATGGCCTCTGGATGGA
<i>MYL7</i> Forward Primer	GAGGAGAATGGCCAGCAGGAA
<i>MYL7</i> Reverse Primer	GCGAACATCTGCTCCACCTCA
<i>TNNT2</i> Forward Primer	TTCACCAAAGATCTGCTCCTCGCT
<i>TNNT2</i> Reverse Primer	TTATTACTGGTGTGGAGTGGGTGTGG
mNGN2 Forward Primer	TACCTCCTCTTCCTCCTTCA
mNGN2 Reverse Primer	GACATTCCCGGACACACAC
β-CATENIN Forward Primer	CCCATAATGTCCAGCGTTT
β-CATENIN Reverse Primer	AACGCATGATAGCGTGTCTG

Table 2.7 qPCR conditions.

qPCR	Temperature	Duration	Cycles	Ramp
Preincubation	95 ⁰ C	5 min	1	4.4 ⁰ C
Amplification	95 ⁰ C	10 s	45	4.4 ⁰ C
	55 ⁰ C	30 s		2.2 ⁰ C
	72 ⁰ C	30 s		4.4 ⁰ C
Melting curve (acquisitions 5 per/⁰ C)	95 ⁰ C	5 s	1	4.4 ⁰ C
	65 ⁰ C	1 min		2.2 ⁰ C
	97 ⁰ C	∞		0.11 ⁰ C
Cooling	40 ⁰ C	30 sec	1	2.2 ⁰ C

2.4 GAA-PCR and Cloning Experiments

2.4.1 GAA-PCR

For GAA repeat PCR, genomic DNAs from fibroblasts and iPSCs were collected by using MN Nucleospin Tissue kit. The concentrations of gDNAs were measured using Nanodrop. For DNA concentration optimization PCR, those DNAs were used but in different concentrations (ranging from 20-250 ng). In addition to this, a gradient PCR from 64⁰ C to 70⁰ C was done. All PCR conditions and protocol in **Table 2.8**, **Table 2.9** were adapted from PCR protocols from Scripps Institute (Jintang Du et al., 2012). The agarose gel was prepared as 1% agarose. The sequences of primers that were used for this PCR was represented in **Table 2.3** as GAA PCR Forward and Reverse primers.

Table 2.8 GAA-PCR conditions.

GAA	Temperature	Duration	Cycles
Cycle 1	98 ⁰ C	5 min	1
Cycle 2	98 ⁰ C	5 s	40
	67.7 ⁰ C	15 s	
	72 ⁰ C	1.5 min	
Cycle 3	72 ⁰ C	5 min	1
	4 ⁰ C	∞	

Table 2.9 GAA-PCR protocol.

Reagents (GAA PCR)	Amounts
5X Phusion HF buffer	4 µl
GAA Forward primer (10 µM)	0.2 µl
GAA Reverse primer (10 µM)	0.2 µl
dNTP	0.4 µl
gDNA	50 ng
MgCl ₂	0.2 µl
DMSO	1 µl
Phusion DNA Polymerase	0.2 µl
Water	Up to 20 µl

2.4.2 Cloning for *FXN* overexpression

pBabe puro human Frataxin (Addgene #11505) plasmid was ordered from Addgene. First, pBabe puro human Frataxin plasmid was cut with EcoRI enzyme (NEB) and

digested products were run on 1% agarose gel and inserted product, which is frataxin cDNA, was purified from the gel (Gel extraction kit, MN). Then, pENTR1A no ccDB plasmid (Addgene #17398) was cut with EcoRI enzyme (NEB) for 2 hours at 37⁰ C and treated with antratic phosphatase (AP) enzyme for 30 min at 37⁰ C. The purified product from pBabe puro human Frataxin were ligated into digested and dephosphorylated pENTR1A no ccDB plasmid. The ligation was done by using T4 DNA ligase (NEB) overnight at 16⁰C. Later, ligated products were transformed into Stbl3 competent bacteria and spread onto bacterial plates. The next day, colonies were picked and plasmids were purified (Nucleospin plasmid kit MN). The plasmids can be named as pENTR1A no ccDB-*FXN* plasmids and send to sequencing. In the final step of the cloning, pENTR1A no ccDB-*FXN* plasmid was recombined with pLEX-307 (Addgene #41392) via LR cloning (Invitrogen). After transformation of the recombined plasmids to Stbl3 bacteria and spread those bacteria to bacterial plates, colonies were picked and this time plasmids were isolated using MN midi kits and send to sequencing.

2.4.3 Sequencing of cloning samples

Samples for sequencing were prepared by mixing 25 pmole sequencing primers (**Table 2.3**) and 500 ng of the plasmid (in this case pENTR1A no ccDB-*FXN* plasmids). Shipping was done to Macrogen Europe Laboratories with UN3373 regulations for category B. Sequencing was done there by using Sanger sequencing (EZ-Seq).

Chapter 3:RESULTS

3.1 Collection of fibroblast samples

Pedigrees can be said as the charts that show the presence or absence of a trait (Bonney & Thompson, 1986). It includes searches for a trait within a family across generations by giving ideas about the pattern of inheritance of that trait. In **Figure 3.1, Figure 3.2, Figure 3.3, Figure 3.4, Figure 3.5, Figure 3.6** one can see the pedigrees of healthy and FRDA individuals.

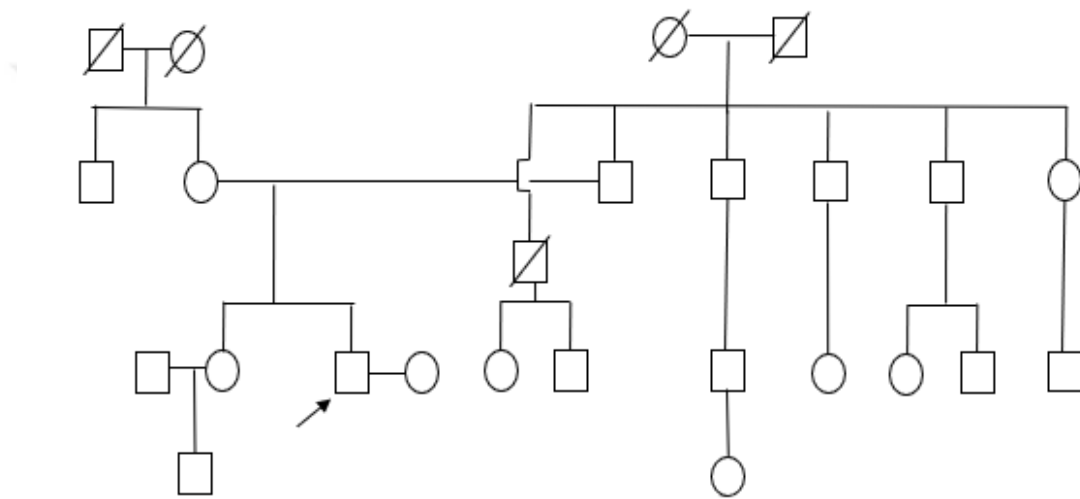


Figure 3.1 Pedigree of the individual who donates skin biopsy for this research.

Skin biopsy samples were taken from the one that is represented with an arrow. This individual is named as WT 1.

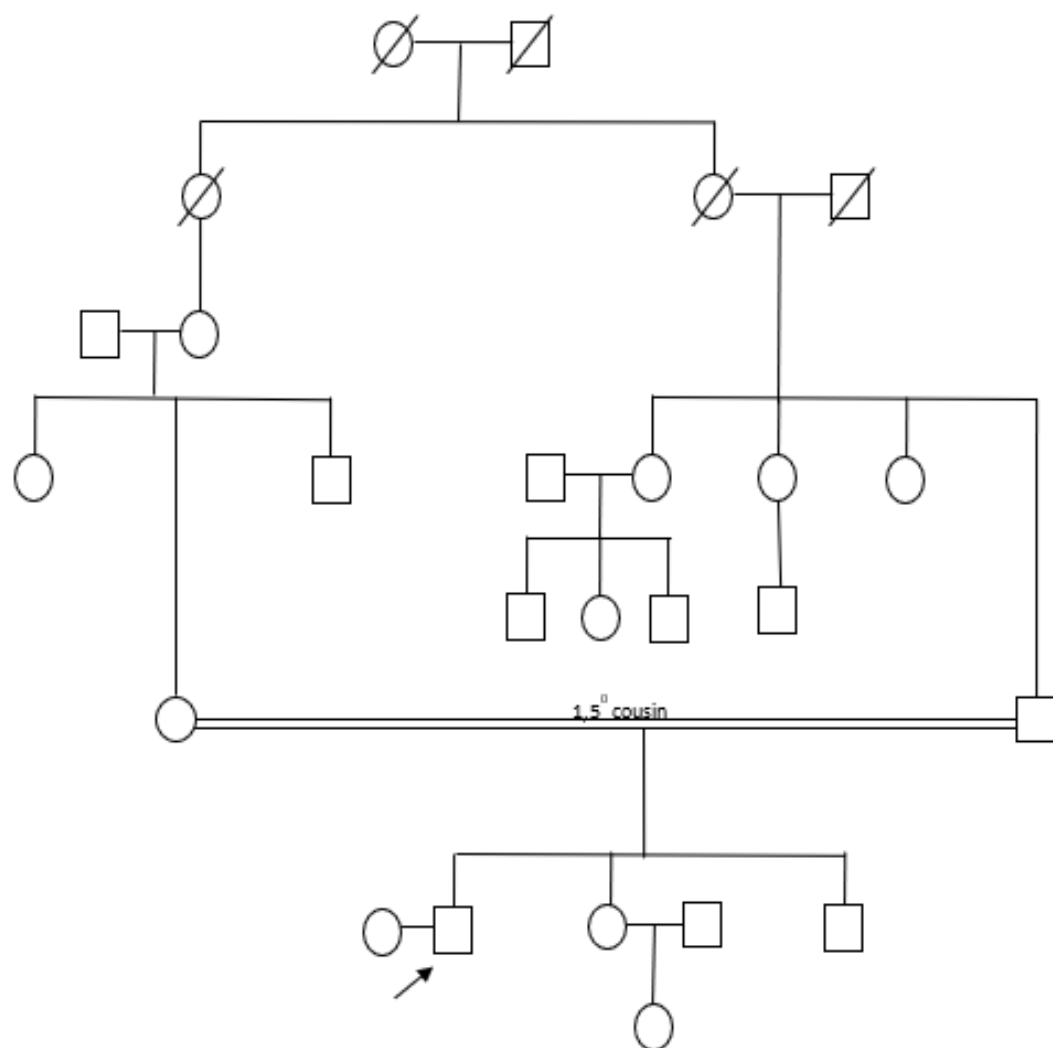


Figure 3.2 Pedigree of the individual who donates skin biopsy for this research.

Skin biopsy samples were taken from the one that is represented with an arrow. This individual is named as WT 2.

Volunteers were confirmed to be healthy via physical examinations. In addition, there is no such individual with muscle disease in their families. Therefore, fibroblasts and blood samples were collected from those voluntary control individuals (WT 1 and WT 2).

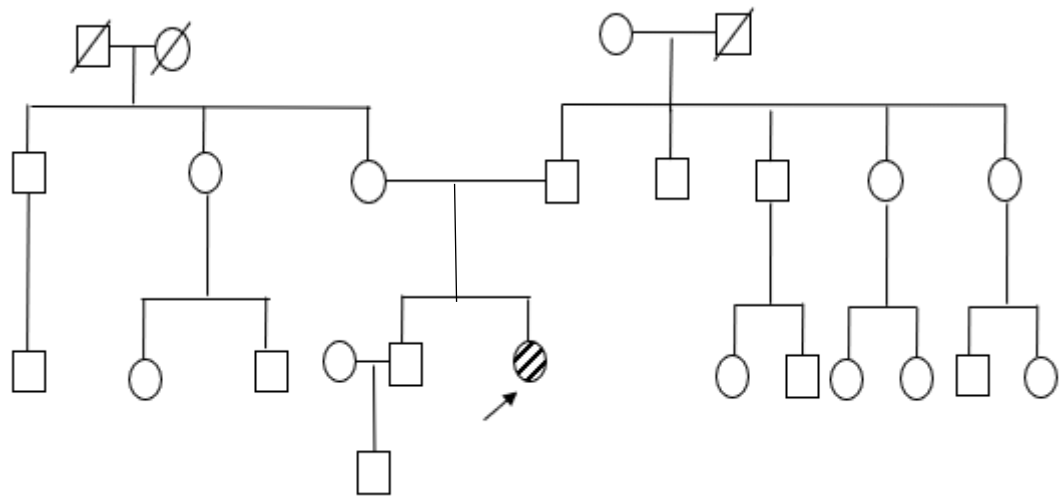


Figure 3.3 Pedigree of the individual who donates skin biopsy for this research.

Skin biopsy samples were taken from the one that is represented with an arrow. This individual is named as FRDA 3.

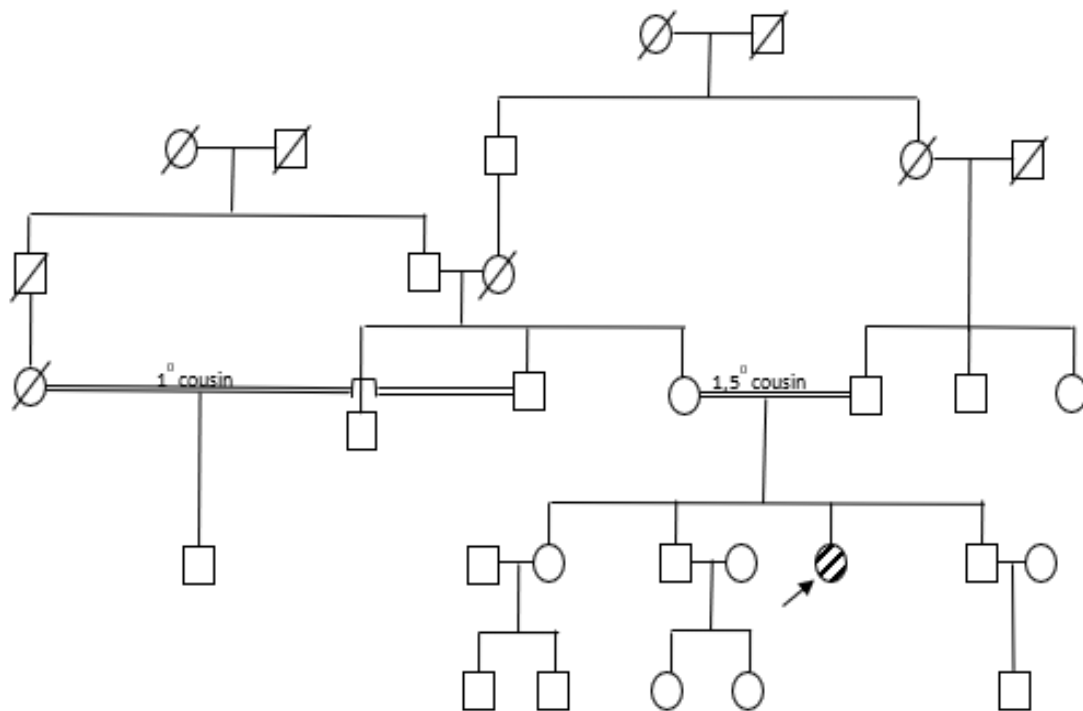


Figure 3.4 Pedigree of the individual who donates skin biopsy for this research.

Skin biopsy samples were taken from the one that is represented with an arrow. This individual is named as FRDA 4.

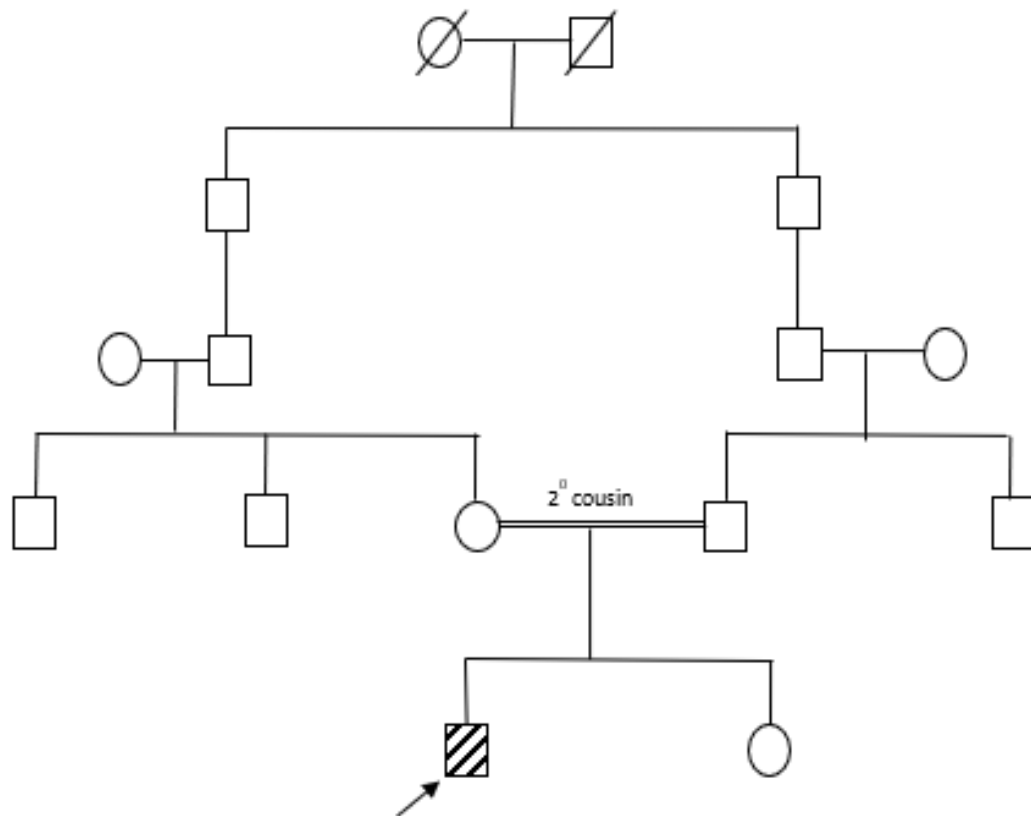


Figure 3.5 Pedigree of the individual who donates skin biopsy for this research.

Skin biopsy samples were taken from the one that is represented with an arrow. This individual is named as FRDA 5.

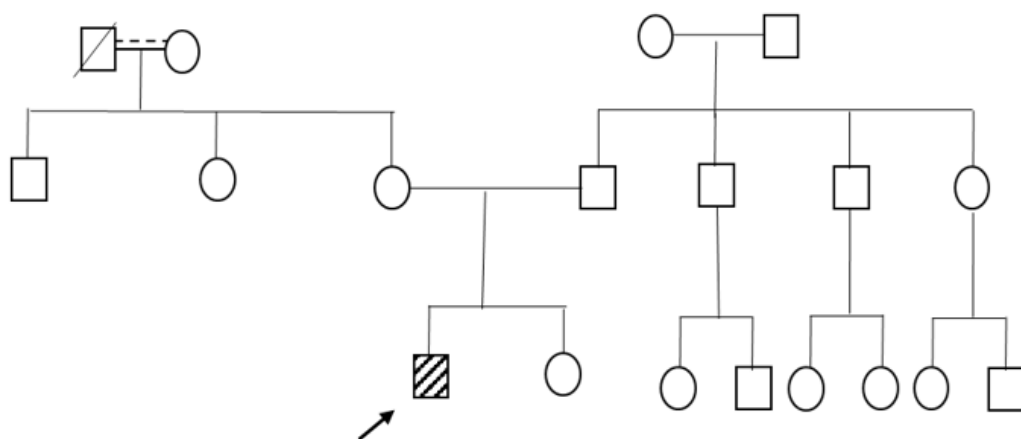


Figure 3.6 Pedigree of the individual who donates skin biopsy for this research.

Skin biopsy samples were taken from the one that is represented with an arrow. This individual is named as FRDA 7.

The circles or squares with dashed lines represent an individual affected by the traits (in our case $(GAA)_n$ expansion mutation in chromosome 9q21.11). The first complaints of

the patient, that is named as FRDA 3 in this project, started at the age of 15 and these were falling and occasional dizziness. At the age of 25, she diagnosed with FRDA. She has been living in a wheelchair for the last 2.5 years. The first complaints of the patient, that is named as FRDA 4 in this project, started at the age of 17 and these were fatigue and falling while walking. The patient, who is now 35, has had hearing loss accompanied by dysarthria. The first complaints of the patient, that is represented as FRDA 5 in this project, started at the age of 10 and these were difficulty in walking, speech disorder, and impaired coordination in the hands. The patient, who is now 15, was diagnosed as FRDA at the age of 12. The first complaints of patient, that is represented as FRDA 7 in this project, began in childhood and these were fatigue, and balance problems. The patient is now at the age of 12 and hypertrophic left ventricular cardiomyopathy was detected.

Whereas in this project, WT 1, 2 cell lines were used as control cell lines as they do not have GAA triplet repeat hyperexpansion mutation in the first intron of *FXN* gene, FRDA 3,4,5,7 cell lines represent the affected cell lines.

Fibroblasts were collected by using skin biopsy and the affected individuals were validated by long-range PCR (LR-PCR). LR-PCR was done by KUH-Diagnostic Centre for Genetic Diseases. The length of the bands obtained from fibroblasts and peripheral blood samples were calculated by using ImageLab 5.2. It is known that the primers amplify 290 bp (UCSC browser) (**Table 3.1**).

Table 3.1 Amplified region after GAA LR-PCR.

Primers	Amplified region
<i>FXN</i> -Int1-GAA-F-KUH	>chr9:71652046+71652335 ggtggctcatgccataatctcagcacttgggaggcctaggaaggtggatcacctgaggtccggagttcaagactaacct ggccaacatggtgaaccagatctactataaaaaatacaaaaaaaaaaaaaa <u>gaagaagaagaagaaga</u> aata aagaaaagttagccggcggtgtgtcgcgcctgtaatccagctactccagaggtcggcaggagaatcgcttgagc ccggaGGCAGAGGTTGATTAAGCCAAGAT
<i>FXN</i> -Int1-GAA-R-KUH	

The underlined region is GAA·TTC repeat region (**Table 3.1**). Since there are 6 repeats in the reference genome, the length of the amplified region from each individual (apart from repeats) should be calculated as 290-18=272 bp. And then the length of the repeats (n) can be calculated as the following formula:

$$\frac{\text{length of the PCR bands} - 272 \text{ bp}}{3} = n$$

The results of this calculation are shown in **Table 3.2**. The sequence of the primers can be found in **Table 2.3**. The gel image for this PCR can be found in **Appendix Figure 5.6**.

Table 3.2 GAA repeat lengths of samples.

Sample	Base pair (bp) Peripheral blood	# of GAA repeats	Base pair (bp) Fibroblasts	# of GAA repeats
WT 1	320	16	332	20
	285	6	397	42
WT 2	283	4	320	16
FRDA 3	1500	409	1198	309
	2054	594	1400	376
FRDA 4	2600	776	1839	522
	3066	931	2561	763
FRDA 5	2700	809	1981	570
	3428	1052	3500	1076

FRDA 7 is not included in this table because LR-PCR did not result in any bands for this sample. But another kit for amplifying this region in FRDA 7 was used (by KUH-Diagnostic Centre for Genetic Diseases) and GAA expansion was detected in FRDA 7 (**Appendix Figure 5.1**).

Bidichandani and his colleagues discovered contractions and expansions in GAA·TTC region of the genomic DNAs of peripheral blood cells, and fibroblasts samples of FRDA patients (Bidichandani et. al., 2002). In addition, Long and his colleagues found that due to somatic instability in GAA·TTC region, differences in the length of the GAA triplet repeats were detected among different tissues of FRDA patients (Long et. al, 2017). Similarly, **Table 3.2** represents the differences in the length of GAA repeats in fibroblasts and peripheral blood samples. There is a difference in GAA triplet repeats in those samples, although they were from the same individuals. However, this was expected and the reason could be somatic cell instability.

3.2 Generation of iPSCs via episomal vector nucleofection

Human iPSCs can be generated by using different types of cells such as fibroblasts, keratinocytes, and peripheral blood mononuclear cells. The induction of iPSCs from these tissues can be carried out by using viral gene delivery but this protocol disrupts the endogenous genomic organization. Nonintegrating episomal plasmids can be used to generate transgene-free iPSCs (Okita et al., 2013).

The important component is *EBNA1* gene in the combination of plasmids encoding *OCT3/4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and short hairpin RNA for *p53*. *EBNA1* is a factor for the amplification of episomal plasmids. The efficiency of iPSCs induction can be increased after the nucleofection of the factors mentioned above.

To generate iPSCs from 6 fibroblasts derived from 4 fibroblasts from FRDA individuals and 2 fibroblasts from healthy individuals, episomal vectors were nucleofected by using Neon Nucleofector System. At passage number 4, fibroblasts were counted and the protocol for the generation of iPSCs adapted from Yamanaka's group (Okita et al., 2013) was carried out as described in **Figure 3.7**.

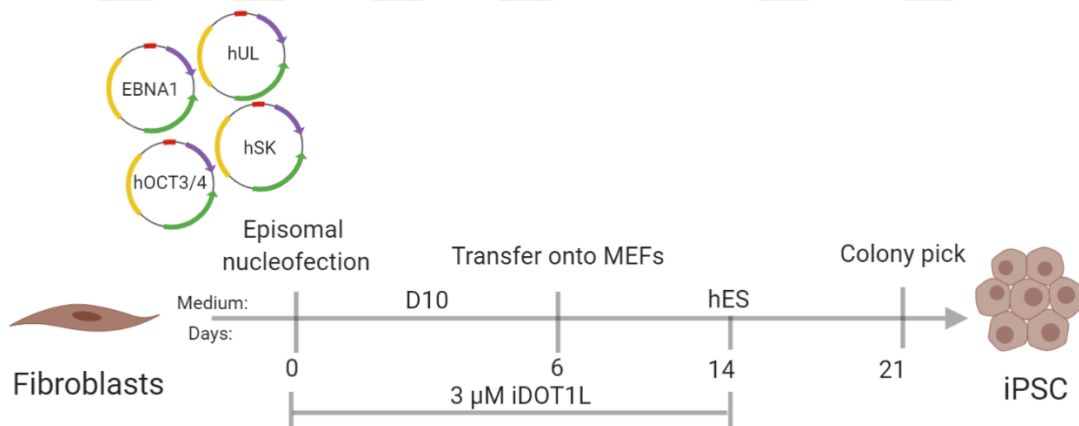


Figure 3.7 Representation of reprogramming protocol from fibroblasts.

After 16 hours of nucleofection, the medium was switched to D10 medium with 3 μM of EPZ4777, iDOT1L (DOT1L inhibitor) (on the first day of reprogramming). Throughout 14 days of reprogramming, cells were treated with 3 μM of iDOT1L because it is known that DOT1L inhibition increases reprogramming efficiency during the first two weeks of reprogramming (Onder et al., 2012).

The next day after nucleofection, the cells were checked for cell viability under a microscope along with a check for GFP signals. In addition to Yamanaka's factors, pCXLE-EGFP plasmid (Addgene#27082) was introduced during nucleofection. The percentage of GFP-positive cells was also calculated from the photos of fibroblasts represented in **Figure 3.8**. To calculate this percentage, 3 different 10x images of nucleofected fibroblasts were capture from 3 different sides of the one well of 6 well cell culture plates. The percentage was calculated when the number of fibroblasts with GFP signals was divided into the number of fibroblasts without GFP signals in those 3 different sites.

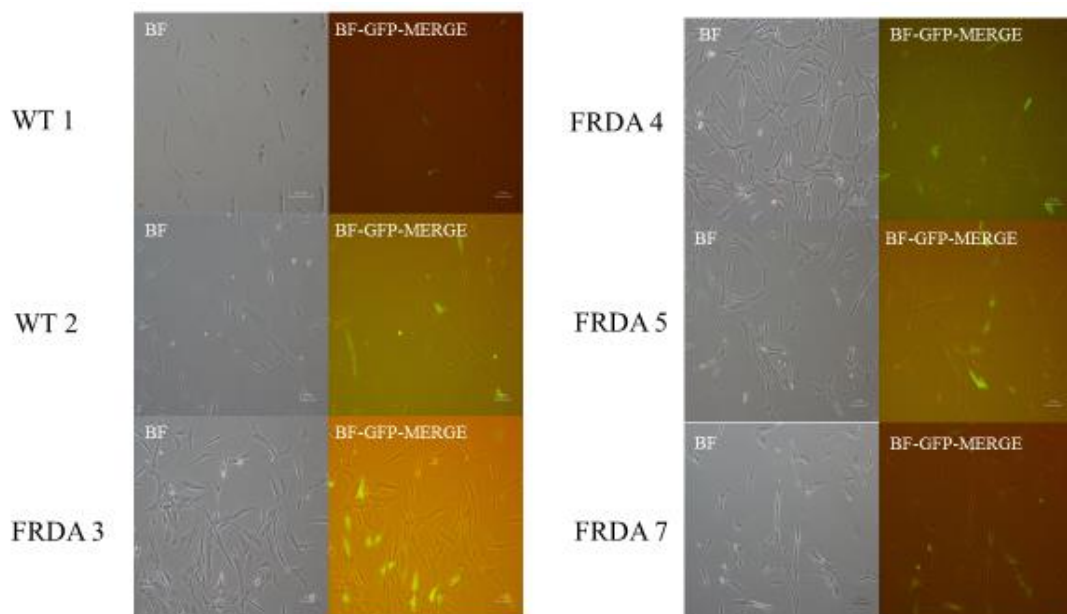


Figure 3.8 Representation of cell culture images of fibroblasts two days after nucleofection.

The images were taken in 10x magnifications.

According to **Figure 3.8**, the percentages of GFP were calculated as 27% for WT1, 30% for WT2, 28% for FRDA 3, 35% for FRDA 4, 40% for FRDA5, 48% for FRDA 7. These percentages indicated the approximate efficiency of nucleofection.

After 21 days of nucleofection, iPSCs colonies, which resemble embryonic stem cell colony morphology, were observed (**Figure 3.9**). Those iPSCs colonies were picked, passaged, and expanded on MEFs-feeders.

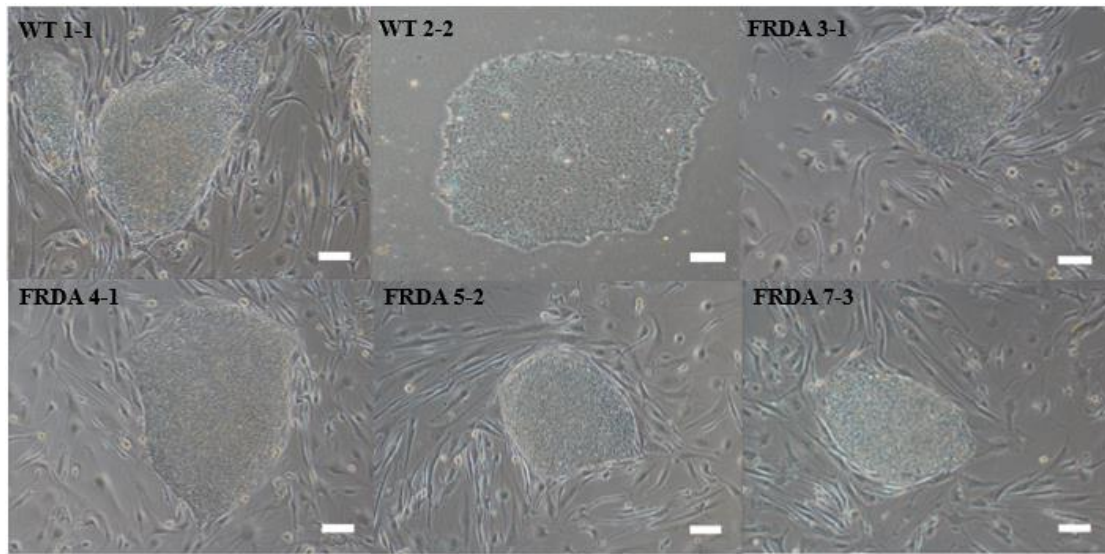


Figure 3.9 Representation of cell culture images of iPSCs.

Only WT 2-2 iPSCs were grown onto Matrigel-coated plates. The rest were grown on MEFs. The pictures were taken under 10x magnification. The scale bars represent 100 μ m.

WT 1-1 is the iPSCs cell line derived from WT 1 fibroblast. WT 2-2 is the iPSCs cell line derived from WT 2 fibroblast. Both WT 1-1, WT 2-2 are used as control iPSCs cell lines. FRDA 3-1 is the iPSCs cell line derived from FRDA 3 fibroblast. FRDA 4-1 is the iPSCs cell line derived from FRDA 4 fibroblasts. FRDA 5-2 is the iPSCs cell line derived from FRDA 5 fibroblast. FRDA 7-3 is the iPSCs cell line derived from FRDA 7 fibroblasts. FRDA 3-1, 4-1, 5-2, 7-3 are iPSCs samples derived from fibroblasts of FRDA patients.

After expansion, the iPSCs were used for characterization and differentiation experiments and they were frozen in -160° nitrogen tanks for storage. From **Figure 3.9**, we can understand that the generated iPSCs lines display embryonic stem cell-like morphology. Later in this project, all these iPSCs lines were maintained with hES medium on MEF-feeders. But for DNA and RNA collections of iPSCs (**Figure 3.10**, **Figure 3.15**, **Figure 3.17**, **Figure 3.22**), they were maintained with mTESR medium on Matrigel-coated plates.

3.3 Fibroblasts-derived iPSCs fully express pluripotency markers

Pluripotent stem cells can be said as cells that are self-replicating and can differentiate into tissues of all three germ layers of endoderm, mesoderm, ectoderm. A select set of core transcription factors both governs and defines pluripotency are *OCT4 (POU5F1)*,

SOX2, and *NANOG*. Thus, human iPSCs are expected to express *OCT4*, *SOX2*, *NANOG* endogenously. These pluripotency markers can be validated by quantitative PCR (qPCR) (Jaworska et al., 2016). Therefore, mRNAs collected from both control and FRDA iPSCs were collected and converted to cDNAs. Following this, qPCR using primers for pluripotency related genes (*OCT4*, *SOX2*, *NANOG*, *LIN28A*) were performed.

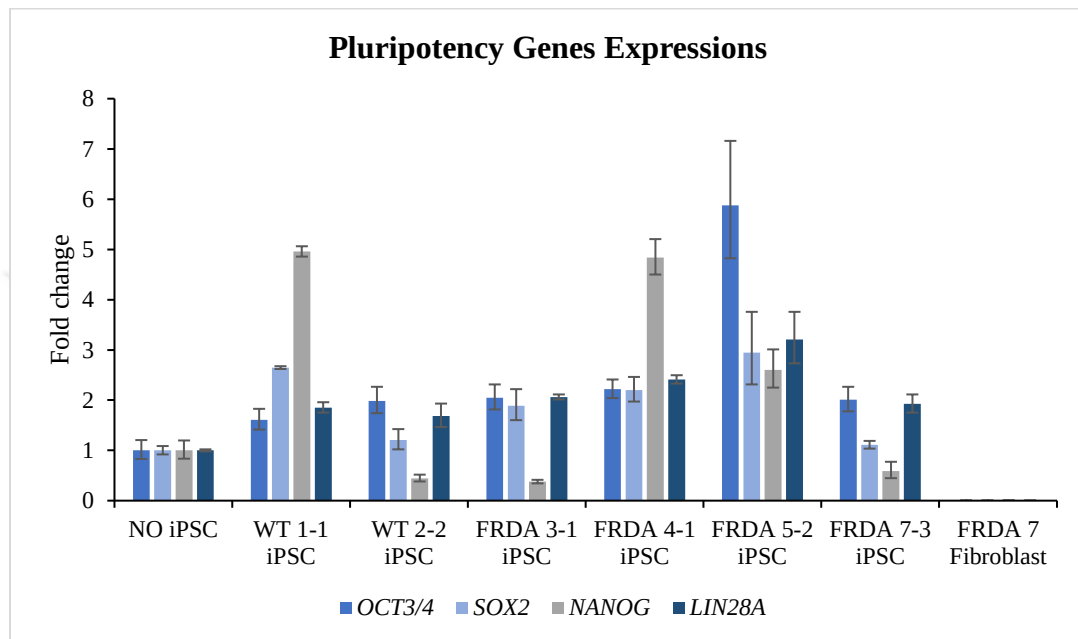


Figure 3.10 mRNA expression results of pluripotency related genes.

β-actin was used as an internal control gene. (n=1, error bars represent $\pm$ s.e.). All the *OCT4*, *SOX2*, *NANOG*, *LIN28A* expression levels in WT 1-1, WT2-2, FRDA 3-1, FRDA 4-1, FRDA 5-2, FRDA 7-3 were normalized to those expressed in NO-iPSCs.

The result in **Figure 3.10** shows that all five iPSCs lines express *OCT4*, *SOX2*, *NANOG*, and *LIN28A* genes at a similar level to NO iPSCs. NO iPSCs cell line is a fully characterized iPSCs cell line. Thus, this cell line was used as a control cell line in terms of pluripotency gene expressions.

This result shows us that our iPSCs express pluripotency marker genes whereas fibroblast cells (represented as FRDA 7 Fibroblasts) do not express those genes.

Like embryonic stem cells, somatic cell-derived iPSCs can grow indefinitely and maintain pluripotency. Embryonic stem cells express SSEA3 and SSEA4, TRA-1-81 and TRA-1-60. Those markers were used to mark human pluripotent stem cells (PSCs). Like ES cells, iPSCs cells express surface markers SSEA3, SSEA4, TRA1-60, and TRA-1-81

as an indication of pluripotency and maintenance of growth (Adewumi et al., 2007; Natunen et al., 2011; Wright & Andrews, 2009; W. Zhao et al., 2012).

To examine the expression of TRA-1-60 surface marker protein, TRA-1-60 staining was carried out and pictures of iPSCs colonies were captured on 10x magnification by using a Nikon microscope.

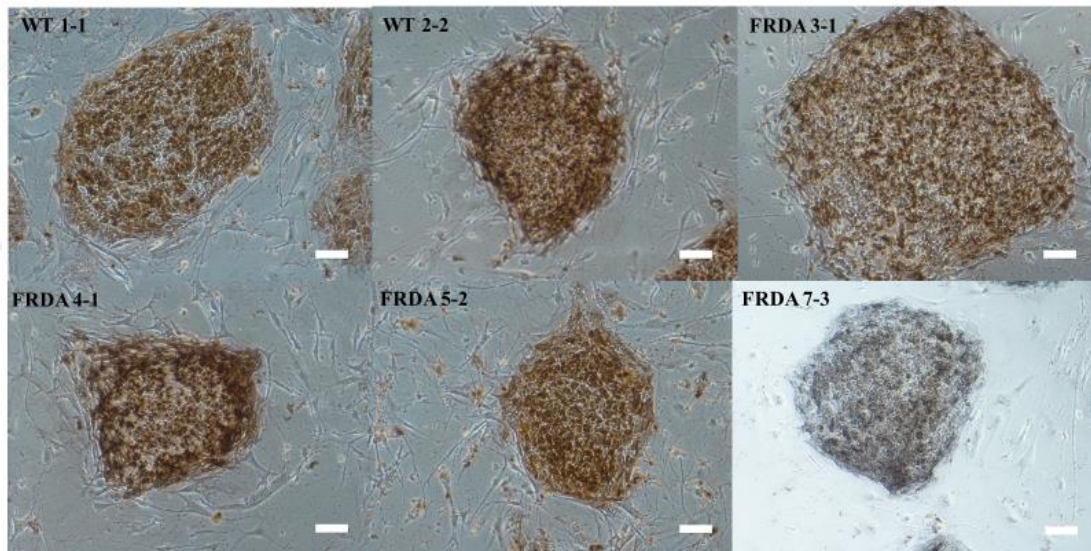


Figure 3.11 TRA-1-60 staining of WT and FRDA colonies.

iPSCs were grown onto MEF feeder layers. The scale bars represent 100 μ m.

DAB stained blackish-brownish iPSCs colonies with embryonic stem cell-like shape can be seen in **Figure 3.11**. However, due to the specificity of anti-TRA1-60 antibody MEF feeder layer cells were not stained while iPSCs colonies have brownish-blackish color.

Until now pluripotency related genes expressions were found to be higher in our iPSCs clones at m-RNA level. Additionally, iPSCs colonies showed higher staining for TRA-1-60. Next, whether those mRNAs are translated to proteins at similar levels were assessed. Thus, WT and FRDA iPSCs were stained with primary antibodies specific for OCT4, NANOG, and SSEA4 (**Figure 3.12**, **Figure 3.13**). The pictures were taken by using LEICA DMI8 SP8 confocal microscope. Secondary only images of iPSCs can be seen in **Appendix Figure 5.2**.

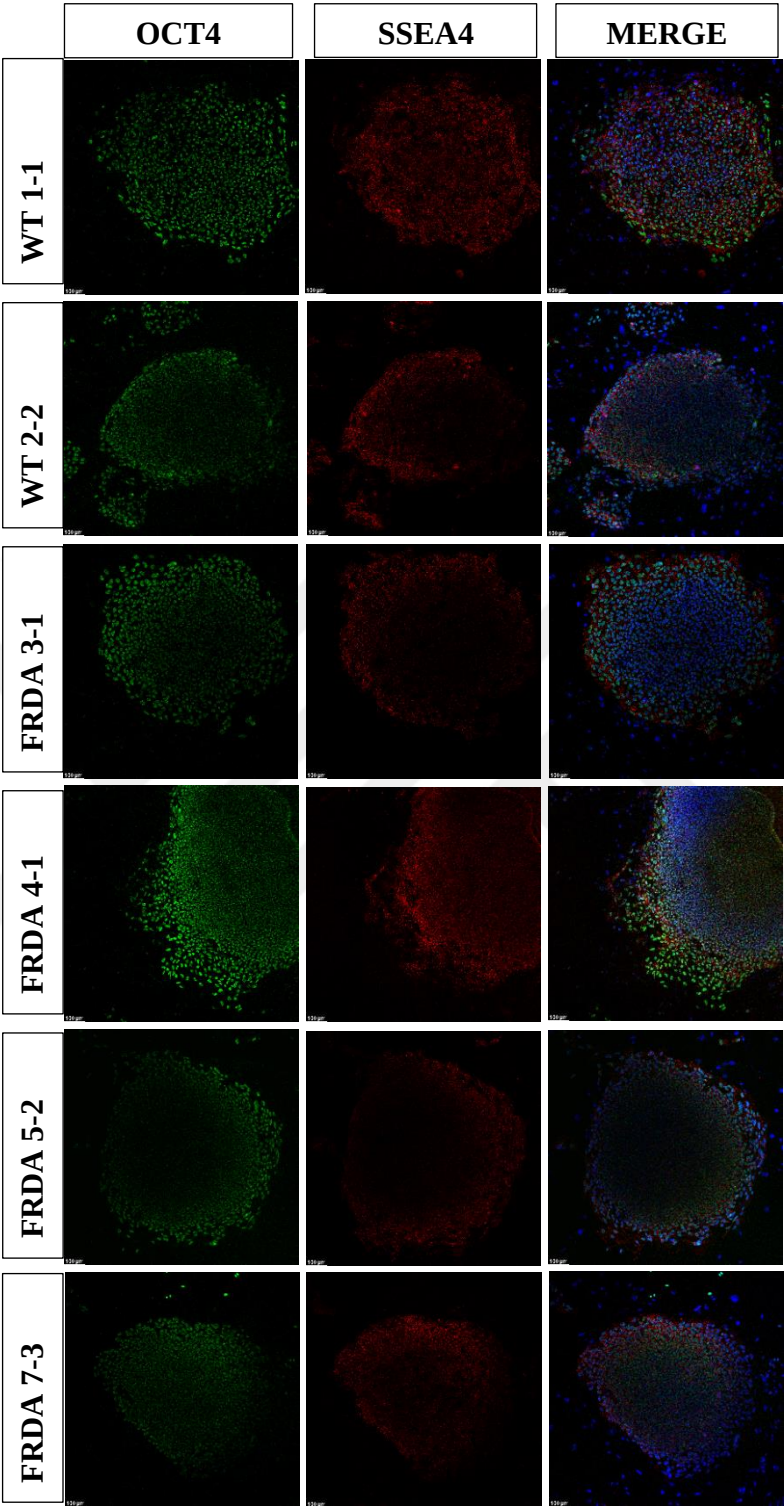


Figure 3.12 Immunofluorescence staining for SSEA4 and OCT4 proteins of WT and FRDA iPSCs.

iPSCs were grown onto MEFs. DAPI stained the nuclei. Images were taken with 10x magnification, scale bar 100 μm.

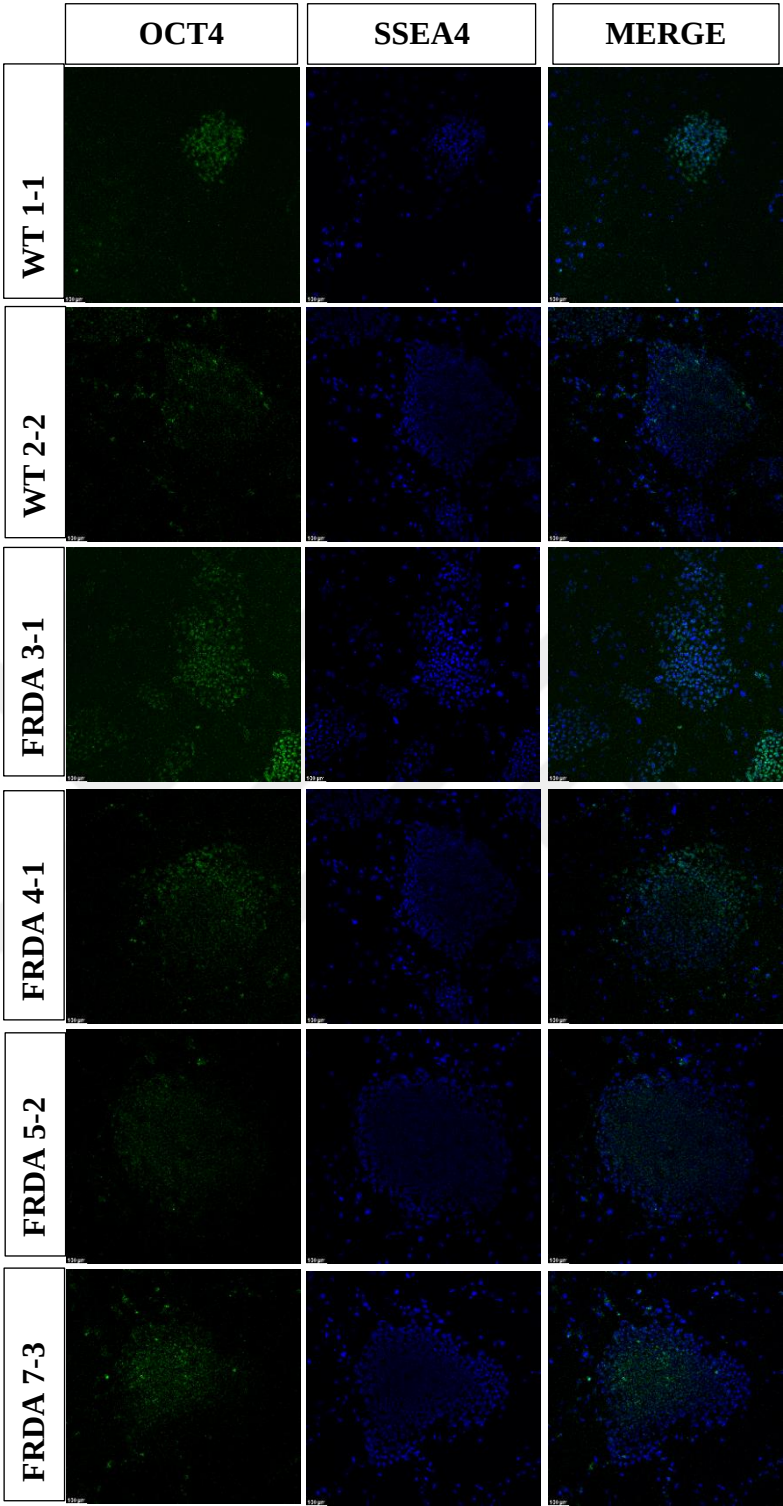


Figure 3.13 Immunofluorescence staining for NANOG of WT and FRDA iPSCs.

iPSCs were grown onto MEFs. DAPI stained the nuclei. Images were taken with 10x magnification, scale bar 100 μ m.

OCT4 and NANOG are transcription factors and SSEA4 belongs to cell surface proteins. In **Figure 3.12** and **Figure 3.13**, the localization of OCT4, and NANOG in the nucleus and SSEA4 on the surface of iPSCs colonies can be seen. In addition to WT and FRDA iPSCs expressions of TRA-1-60 antigens, WT, and FRDA iPSCs were shown to express pluripotency-related genes both in mRNA and protein level.

During the generation of iPSCs and their subsequent prolonged culture, karyotypic abnormalities may occur. One of the most common abnormalities reported in iPSCs is aneuploidy. According to the study of International Stem Cell Initiative, there are karyotype abnormalities approximately one in three hESC or hiPSCs clones (Amps et al., 2011). It was estimated by Taapken et al. that around 13% of the pluripotent stem cells (PSCs) clones have aberrant karyotypes (Taapken et al., 2011). Those abnormalities may potentially affect the quality of PSCs decreasing their utility in downstream studies (Liang & Zhang, 2013). Thus, karyotype analysis of all iPSCs clones were done and the results are shown in **Figure 3.14**.

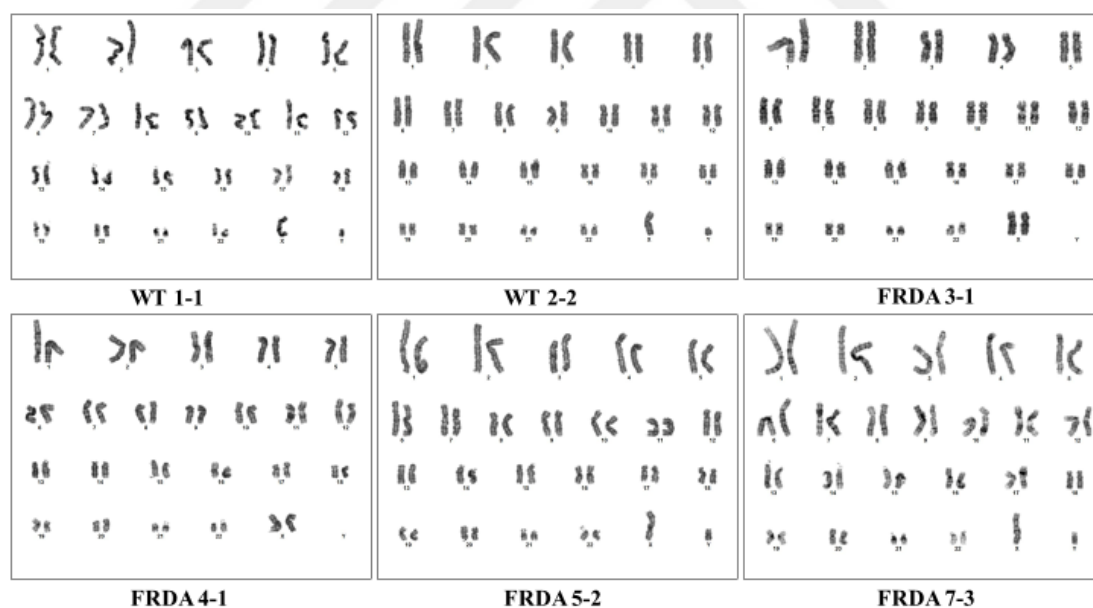


Figure 3.14 Karyotype pictures of WT and FRDA iPSCs.

Table 3.3 Results of the karyotypes.

iPSCs	iPSCs clones	Results
WT 1	WT 1-1	Normal
	WT 1-2	Normal
	WT 1-4	Normal
WT 2	WT 2-1	Contamination
	WT 2-2	Normal
	WT 2-3	Normal
FRDA 3	FRDA 3-1	Normal
	FRDA 3-4	Normal
FRDA 4	FRDA 4-1	Normal
	FRDA 4-2	Normal
	FRDA 4-4	Translocation (t(3;4))
FRDA 5	FRDA 5-1	Translocation (t(6;17))
	FRDA 5-2	Normal
	FRDA 5-3	Normal
	FRDA 5-4	Metaphase cells can not be obtained.
WT 6	WT 6-1	Abnormality in X chr.
	WT 6-2	Abnormality in X chr.
	WT 6-3	Abnormality in X chr.
	WT 6-6	Normal
FRDA 7	FRDA 7-2	Normal
	FRDA 7-3	Normal
	FRDA 7-4	Normal

For the analysis, 3 clones from each iPSCs cell lines were sent to Koc University Hospital Genetics Diagnosis Centre. For example, for WT 1-1, the clones that were sent to karyotype analysis are WT 1-1, WT 1-2, and WT 1-4 (**Table 3.3**). After analysis, some of the clones show translocations (FRDA 5-1, and FRDA 4-4), or abnormality on X chromosomes (WT 6-1, 6-2, 6-3). Thus, they were eliminated from the project. The

frequency of observing karyotypic abnormalities in literature is 13% as mentioned above. We had 5 abnormal iPSCs clones in 21 iPSCs clones having 24% abnormality.

Figure 3.14 shows the clones of iPSCs showing a normal karyotype with a normal number of chromosomes which is 44+XX/XY. Also, those clones were bolded in **Table 3.3**. Later in the whole project, I continued with these clones.

3.4 FRDA and Control iPSCs can differentiate into 3 germ layers

One of the qualitative methods to measure pluripotency is through an *in vitro* approach which involves the generation of embryoid bodies (EBs) from iPSCs cells. In this approach, iPSCs can be maintained in suspension and via switching the medium to differentiation medium/EB medium, spontaneous differentiation can be induced. There are several methods to create embryoid bodies such as liquid suspension culture, stirred flask culture, and hanging drop culture (Lin, 2014). To elucidate the pluripotency ability of WT and FRDA iPSCs, EB formation assay was performed. And I chose the liquid suspension culture method. Via qPCR analysis, EBs' abilities to differentiate *in vitro* to three germ layers were assessed (**Figure 3.15**).

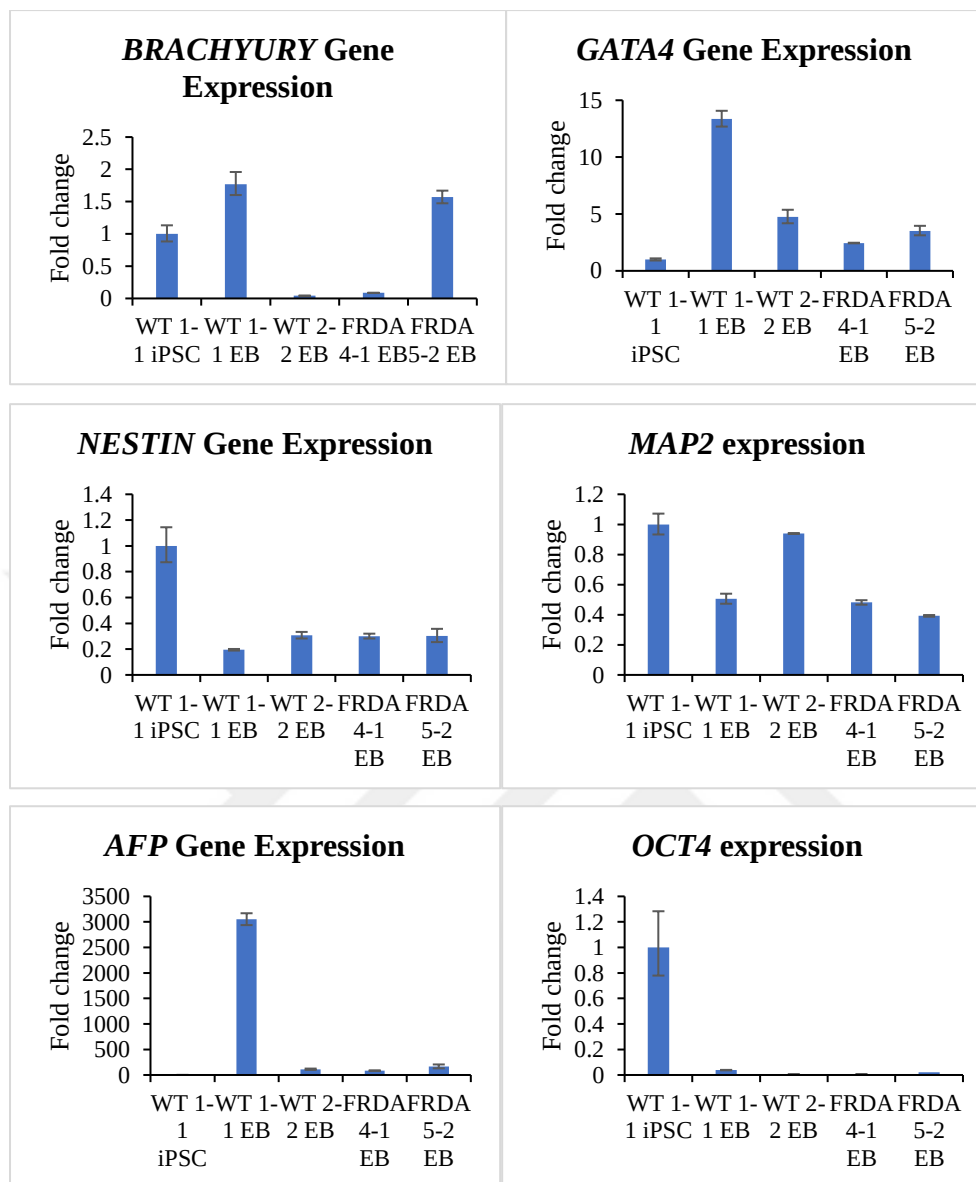


Figure 3.15 mRNA expression results of endoderm, mesoderm, ectoderm genes.

β-actin was used as an internal control gene. (n=1, error bars represent $\pm$ s.e.).

BRACHYURY and *GATA4* are transcription factors that are typical markers for the mesoderm layer. *NESTIN* and *MAP2* are typical markers for the ectoderm layer whereas *AFP* is a typical marker for the endoderm layer (Itskovitz-Eldor et al., 2000).

The pluripotency related gene expressions should decrease after EB formation assay. From **Figure 3.15**, one can observe that *OCT4* expression of WT iPSCs-derived EBs and FRDA iPSCs- derived EBs decrease. When the expressions of these genes were compared to WT iPSCs, it was found that WT and FRDA EBs were differentiating to 3 germ layers.

Thus, one can understand from this result that WT iPSCs and FRDA iPSCs can attain pluripotency because they were differentiating to all three germ layer cells (endoderm, mesoderm, and ectoderm) via EB formation.

3.5 Generation of iPSCs is integration-free

To generate iPSCs from fibroblasts, episomal vectors were utilized. These vectors encode reprogramming factors without integration into the genome. By avoiding viral transduction of reprogramming factors, genomic disruption is prevented. But still, episomal vectors are reported to have a small probability of genomic integration (Okita et al., 2013). To test for any integration possibility of episomal vectors, PCR-based integration assay with WT iPSCs and FRDA iPSCs was performed. In this PCR assay, primers targeting *EBNA* and *GAPDH* sequences were used. *GAPDH* was used as it is expressed in all cells.

Gradient PCR for *GAPDH* primers pair was carried out to select the optimal annealing temperature (**Figure 3.16**). *EBNA* primers were optimized previously (Fidan et al., 2016).

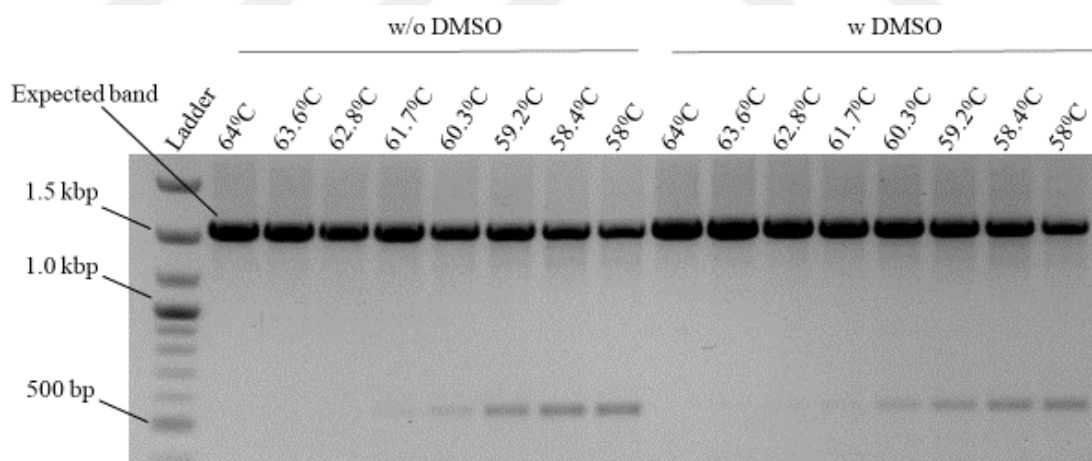


Figure 3.16 Gradient PCR for optimization of *GAPDH* PCR with a new primer pair.

GAPDH PCR products were run on 1% agarose gels.

According to the T_m temperatures of *GAPDH* primer pairs, temperature ranges of annealing temperatures of gradient PCR were determined as from 58°C to 64°C. Gradient PCR was repeated for the case without DMSO to select the optimal condition for *GAPDH* PCR. From the gel image in **Figure 3.16**, for future *GAPDH* PCRs, 64°C was chosen as

the optimal annealing temperature in which a product of 1596 bp long was obtained. Additionally, conditions without DMSO was chosen. In **Figure 3.17**, these conditions were used.

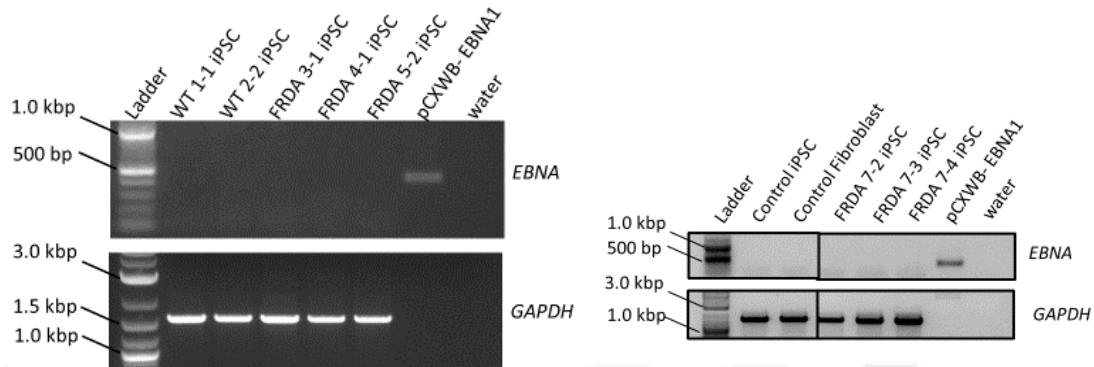


Figure 3.17 Integration analysis result of all iPSCs.

EBNA PCR and *GAPDH* PCR products were run on 1% agarose gels.

Genomic DNAs from WT iPSCs and FRDA iPSCs were collected and *EBNA* PCR and *GAPDH* PCR were used to assess whether iPSCs were integration-free (**Figure 3.17**). Only the control sample, which is pCXWB-EBNA plasmid, was positive for *EBNA* sequence. There were no PCR products produced from all iPSCs after *EBNA* PCR. But, after *GAPDH* PCR, clear bands for all iPSCs clones were obtained. Thus, it is possible to say that episomal vectors are not integrated into the genomic DNAs of all generated iPSCs during the generation of iPSCs from fibroblasts.

3.6 Hyperexpansion of triplet GAA repeats in FRDA fibroblasts and during reprogramming

GAA·TTC triplet repeat hyperexpansions in FRDA occur in the first intron of *FXN* gene (**Figure 3.18**). Long GAA·TTC triplet repeats cause heterochromatin-mediated gene silencing and reduced frataxin levels in FRDA individuals (Ku et al., 2010). Thus, demonstrating the presence of expanded regions in the FRDA samples (either in FRDA fibroblasts or in FRDA iPSCs) is very important. For this, LR-PCR can be used.

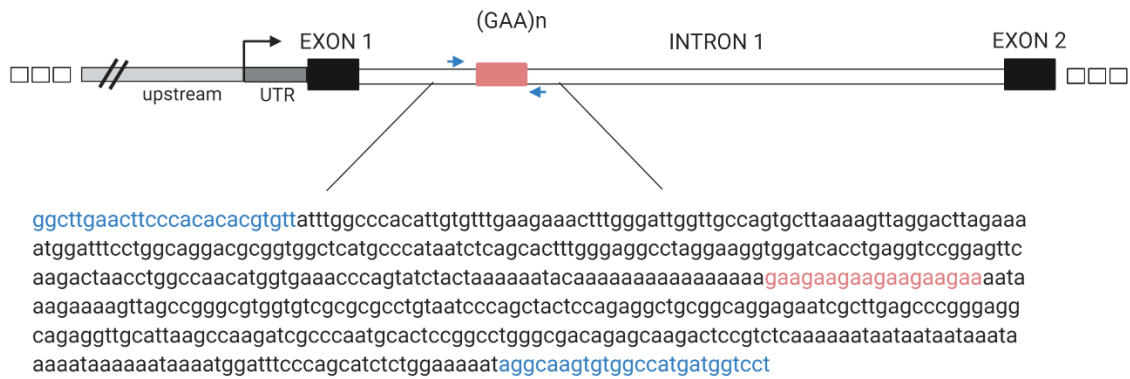


Figure 3.18 Representation of GAA·TTC triplet repeat hyperexpansions in *FXN* gene locus.

The location of primers for amplification of GAA repeats is represented as blue arrows. GAA PCR primers' binding the sequences are represented as blue. GAA expansions are represented as red.

The lengths of the GAA triplet repeats in the fibroblasts collected from FRDA individuals were determined (**Table 3.2**) previously by KUH Diagnostic Centre of Genetic Diseases. The gel image of that PCR can be found in **Appendix Figure 5.6**. But we used another PCR protocol which is an adapted protocol from Gottesfeld group and primers were used (Jintang Du et al., 2012) to amplify the repeat regions (**Figure 3.19**, **Figure 3.20**, **Figure 21**).

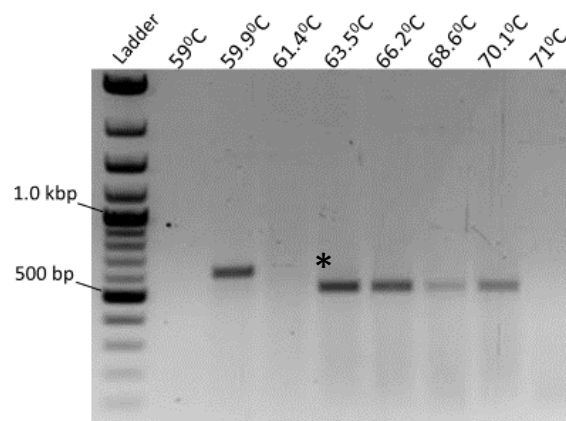


Figure 3.19 Gel image of gradient PCR of amplification for GAA repeats for WT 2.

The products were run into 1% gel agarose gel. The expected bands were represented with asterisks (*).

The primer pairs should produce bands with 516 bp lengths in the representative genomes (from healthy individuals). Firstly, gradient PCR was done by using genomic DNAs collected from WT 2 fibroblasts because it is a control line and the expected PCR product was obtained for this cell line (**Figure 3.19**).

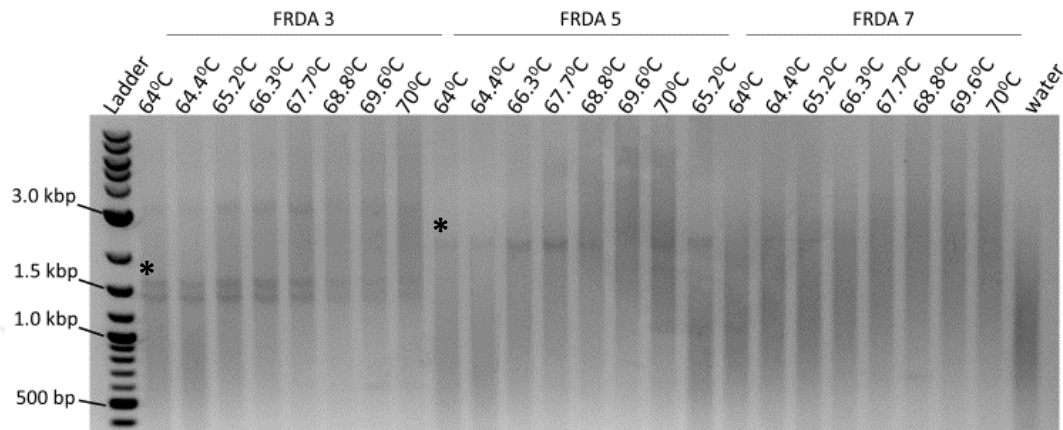


Figure 3.20 Gel image of gradient PCR of amplification for GAA repeats.

The products were run into 1% gel agarose gel. The expected bands were represented with asterisks (*).

This gradient PCR was done for the optimization of GAA PCR for amplifying the expanded region found in FRDA 3, 5, 7 samples. According to the T_m temperatures of GAA primer pairs, temperature ranges of annealing temperatures of the gradient PCR were chosen to be from 64°C to 70°C (**Figure 3.20**). According to the length of the bands obtained, 67.7 °C was chosen as the optimal annealing temperature for further GAA PCR analysis.

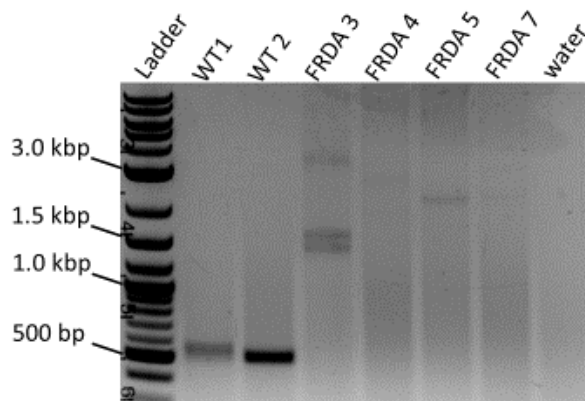


Figure 3.21 GAA PCR gel image for FRDA fibroblasts.

The products were run into 1% gel agarose gel.

After optimizations, GAA PCR was carried out with the determined annealing temperatures and DNA concentrations. GAA PCR carried out with genomic DNAs from WT 1 and WT 2 produced the expected bands (516 bp). One can see two expanded alleles in FRDA 3 fibroblasts bearing around 1500 bp lengths. The top band on the FRDA 3 column could be a non-specific band. In FRDA 5, one band having a length of around 2500 bp can be seen. However, no band was obtained for FRDA 7 and FRDA 4. The length of the bands found in this PCR (**Figure 3.21**) can be compared to the repeat numbers in individuals (**Table 3.2**).

This result shows that FRDA cells maintained GAA repeat expansions of a pathological size compared to the control (WT) cells.

3.7 FRDA-like phenotype maintained in FRDA fibroblasts and FRDA iPSCs

Friedreich's ataxia is a progressive neurodegenerative disorder resulting from decreased levels of frataxin. The reduction in homozygous cases is 5 to 35% of the normal level whereas it is 50% of the normal levels in heterozygous cases (Bürk, 2017; Campuzano et al., 1997). Thus, it is essential to show the reduction in *FXN* mRNA level in fibroblasts collected from individuals affected by FRDA.

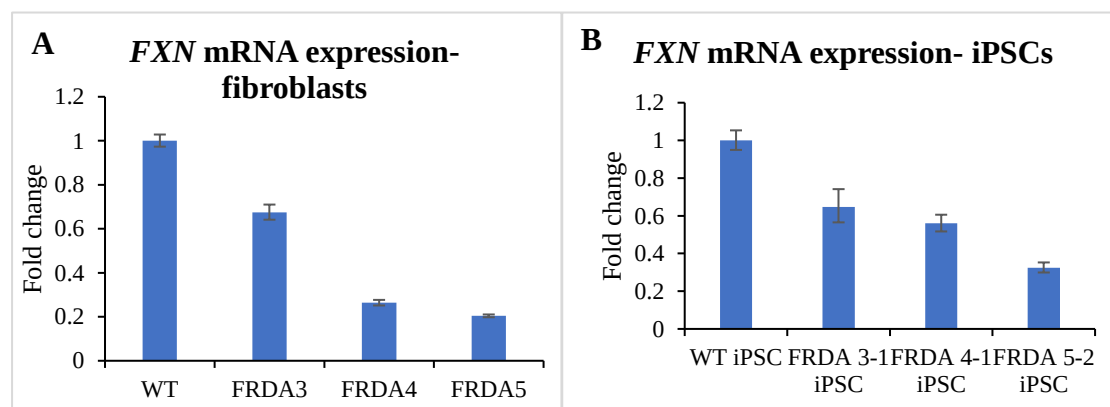


Figure 3.22 mRNA expression results of *FXN* gene (fibroblasts and iPSCs).

β-actin was used as an internal control gene. (n=1, error bars represent $\pm$ s.e.)

WT represents the average *FXN* levels found in WT 1, 2, and NO fibroblasts, three independently derived healthy control lines. qPCR analysis showed that *FXN* levels in FRDA 3, FRDA 4, FRDA 5 fibroblasts are lower compared to WT fibroblasts (**Figure 3.22.A**). *FXN* levels in iPSCs were found to be lower when Ku and colleagues generated

iPSCs from FRDA individuals (Ku et al., 2010). Thus, via qPCR analysis, *FXN* expressions in iPSCs derived from fibroblasts of FRDA patients were compared to the *FXN* expressions in control iPSCs. As can be seen from **Figure 3.22.B**, *FXN* levels were lower in FRDA 3-1, FRDA 4-1, and in FRDA 5-2 iPSCs.

3.8 FRDA iPSCs and control iPSCs can differentiate into cardiomyocytes

Cardiac disease can said as one of the pathologic findings of FRDA, which can lead to arrhythmia, and cardiac failure (Pousset et al., 2015; Tsou et al., 2011). FRDA cardiomyopathy in individuals is usually observed to include commonly reported abnormalities such as electrical abnormalities in cardiac cells, an increase in Left Ventricle (LV) wall thickness and reduction in LV chamber size (Dutka et al., 1999; Isnard et al., 1997; Meyer et al., 2007; Mottram et al., 2011; Peverill et al., 2018; Regner et al., 2012; St John Sutton et al., 2014; Weidemann et al., 2012). To model cardiomyopathy observed in FRDA, iPSCs derived from fibroblasts of FRDA individuals can be differentiated to cardiomyocytes. Thus, in this project both WT and FRDA iPSCs were converted to cardiomyocytes using the protocol described in **Figure 3.23**.

But cardiomyocyte differentiation protocol requires optimizations. For example, the concentration of WNT pathway activator, CHIR99021, was optimized according to iPSCs lines used. For this optimization, 75.000 cells of WT 1-1 iPSCs were counted and seed onto freshly coated Matrigel-coated plates and treated with CHIR99021 in different concentrations (6 μ M, 7.5 μ M, 10 μ M). Then the differentiation optimization protocols were performed as described in **Figure 3.23**.

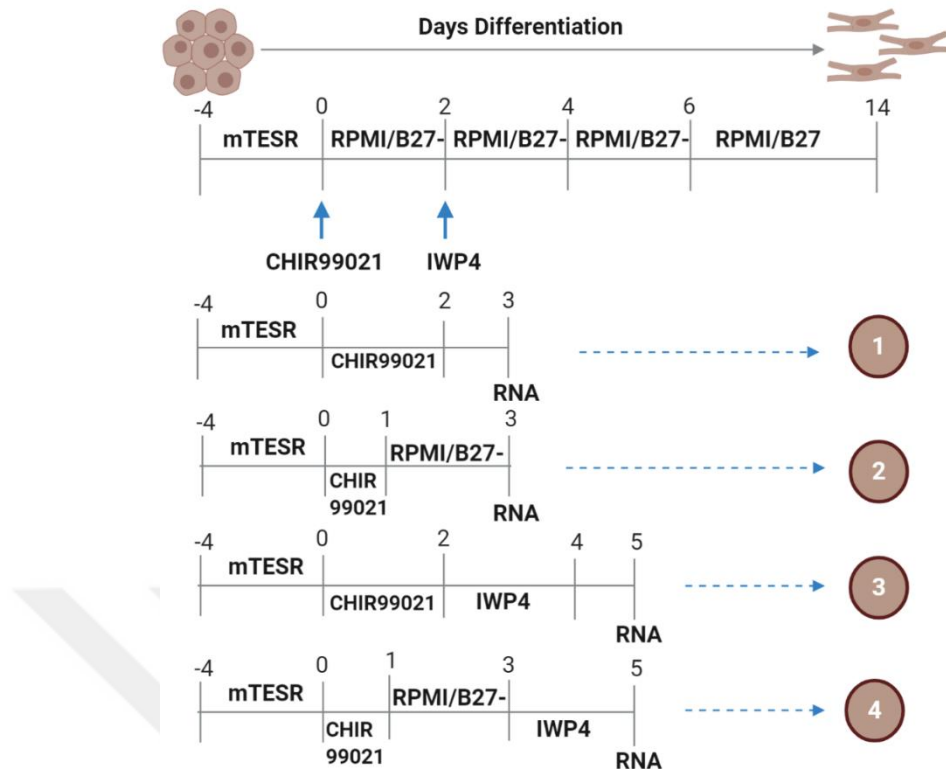


Figure 3.23 Representation of optimization protocols for cardiomyocyte differentiation.

RNAs were extracted from the cells at different time points of differentiation. RNAs were collected on the 3rd day of differentiation for 1 and 2 protocols whereas RNAs were collected on the 5th day of differentiation (**Figure 3.23**). RNAs were extracted by using MN Nucleospin kits. Then to assess which protocol suits best for iPSCs used in this project, qPCR analysis for mesoderm, cardiac mesoderm, and cardiomyocyte-specific genes were carried out (**Figure 3.24**).

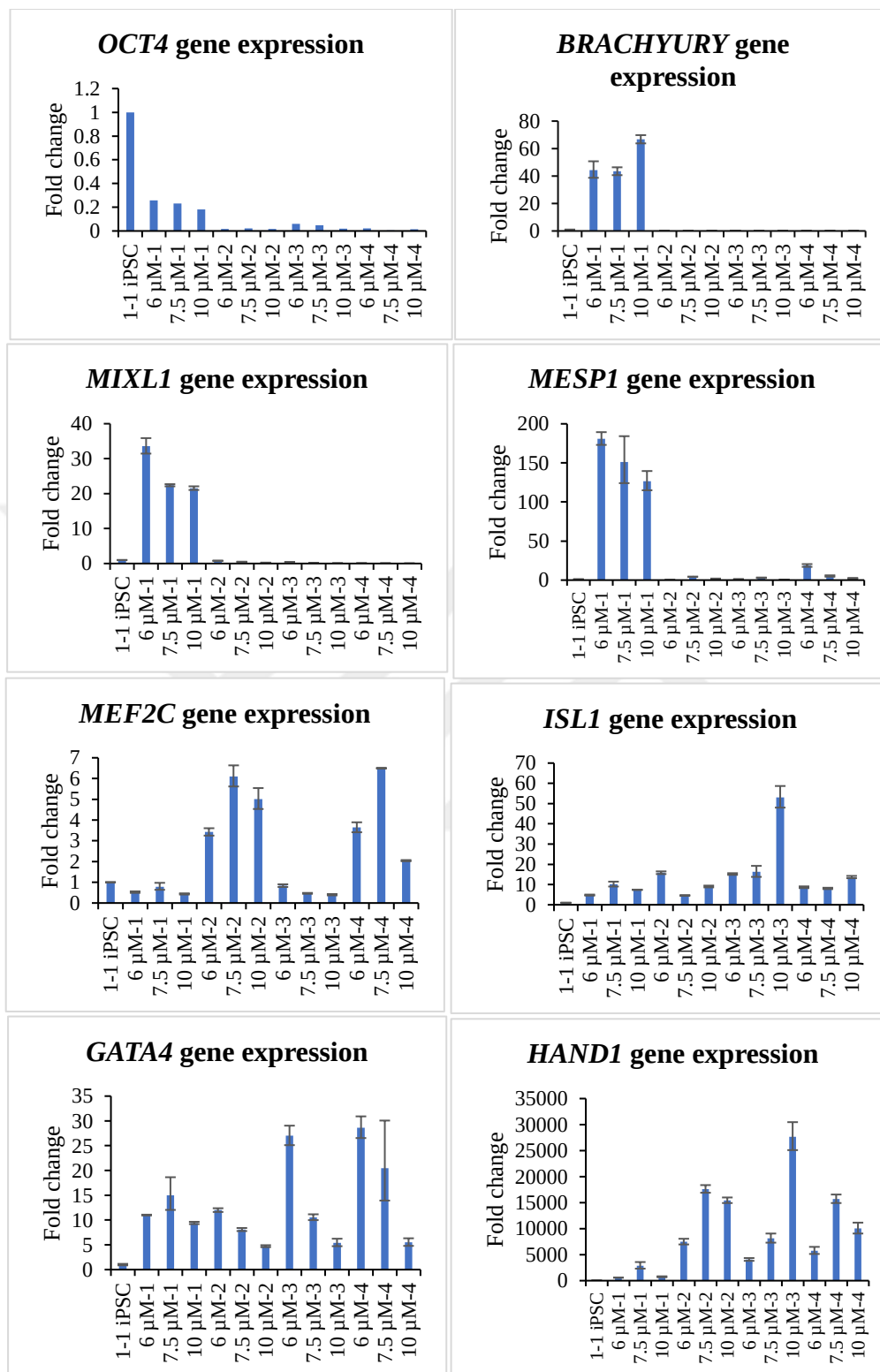


Figure 3.24 mRNA expression results of mesoderm, cardiac mesoderm, and cardiomyocyte-specific genes. β -actin was used as an internal control gene. (n=1, error bars represent $\pm$ s.e.)

mRNA level for each gene was compared to mRNA levels in WT 1-1 iPSCs because this optimization was carried out by using this iPSCs line. This qPCR was carried out to understand the best cardiomyocyte differentiation protocol. The result of this figure showed that protocol 1 and its proceeding protocol, protocol 3, along with 6 μ M of CHIR99021 treatment could be used to differentiate iPSCs to cardiomyocytes.

3.9 iPSCs -derived cardiomyocytes can express cardiomyocyte-specific markers and FRDA iPSC-Cms maintain FRDA-like phenotype

In the previous result, protocol 1 and 3 (with 6 μ M of CHIR99021 treatment) were chosen as the best protocol. Thus, WT 1-1 and FRDA 3-1 iPSCs lines were differentiated to beating cardiomyocytes by using this protocol beginning with 800.000 iPSCs seeded. At the end of day 10 after differentiation, beating cardiomyocytes were observed.

Cardiomyocyte characterization can be done via flow cytometry with troponin T (TNNT2) antibodies, immunofluorescence staining, and gene expression profile of differentiated beating cardiomyocytes (Burrige et al., 2015). Thus, at the end of day 15, RNAs from cardiomyocytes were extracted from WT 1-1 iPSC-Cms and FRDA 3-1 iPSC-Cms. qPCR analysis for cardiomyocyte-specific genes was done to characterize the beating iPSC-Cms.

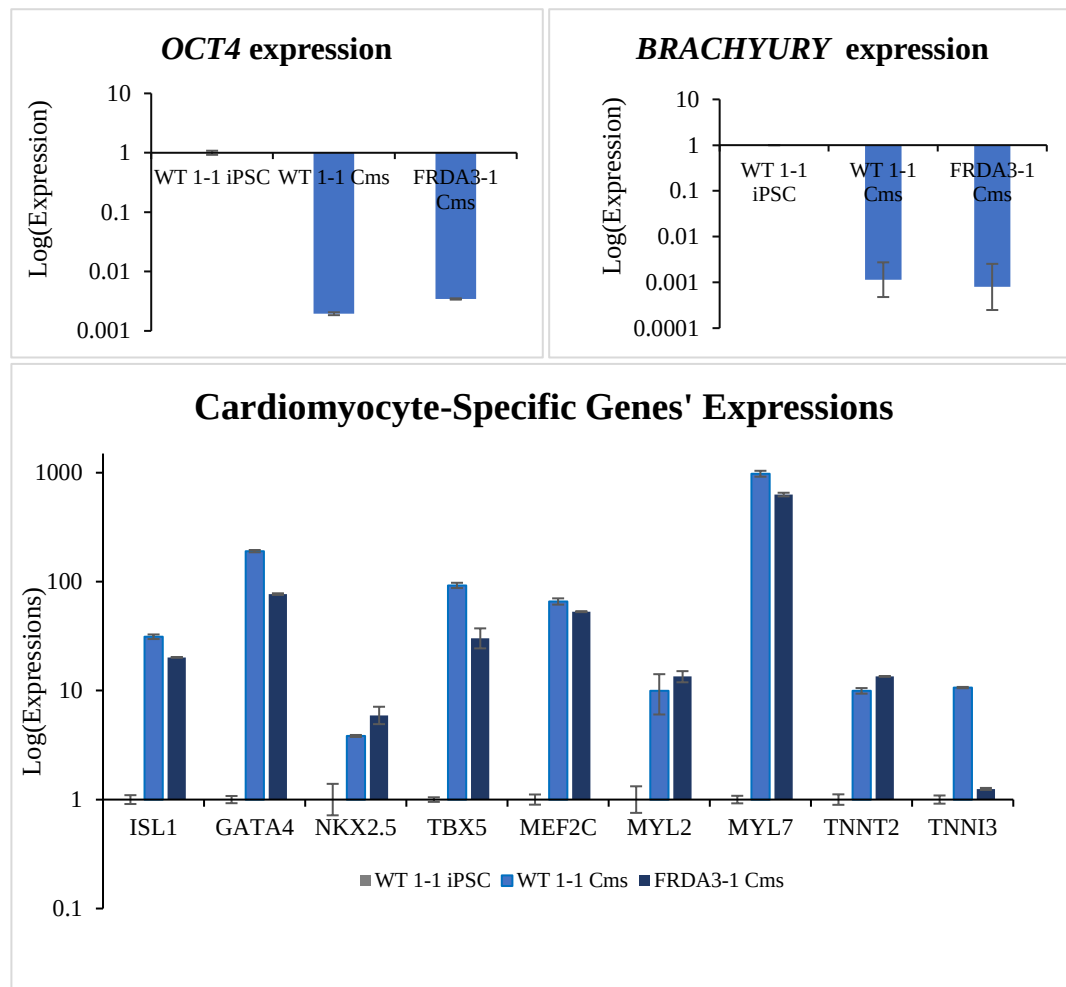


Figure 3.25 mRNA expression results of cardiomyocyte-specific genes.

Cms is the abbreviation of cardiomyocytes. β -actin was used as an internal control gene. (n=1, error bars represent $\pm$ s.e.)

While quantifying the results, mRNA level of each gene was compared to WT 1-1 iPSCs. And the expression level changes were represented on a log scale. This result shows that while *OCT4* expression decreases in the beating cardiomyocytes, cardiomyocytes specific genes (such as a transcription factor, *GATA4*) increases compared to the WT 1-1 iPSCs (**Figure 3.25**). As a result, WT 1-1 iPSC-Cms and FRDA 3-1 iPSC-Cms were found to express cardiomyocyte-specific genes in mRNA level. After qPCR analysis, to confirm that the iPSC-Cms synthesize cardiomyocyte-specific genes in protein level as well, immunofluorescence staining was done.

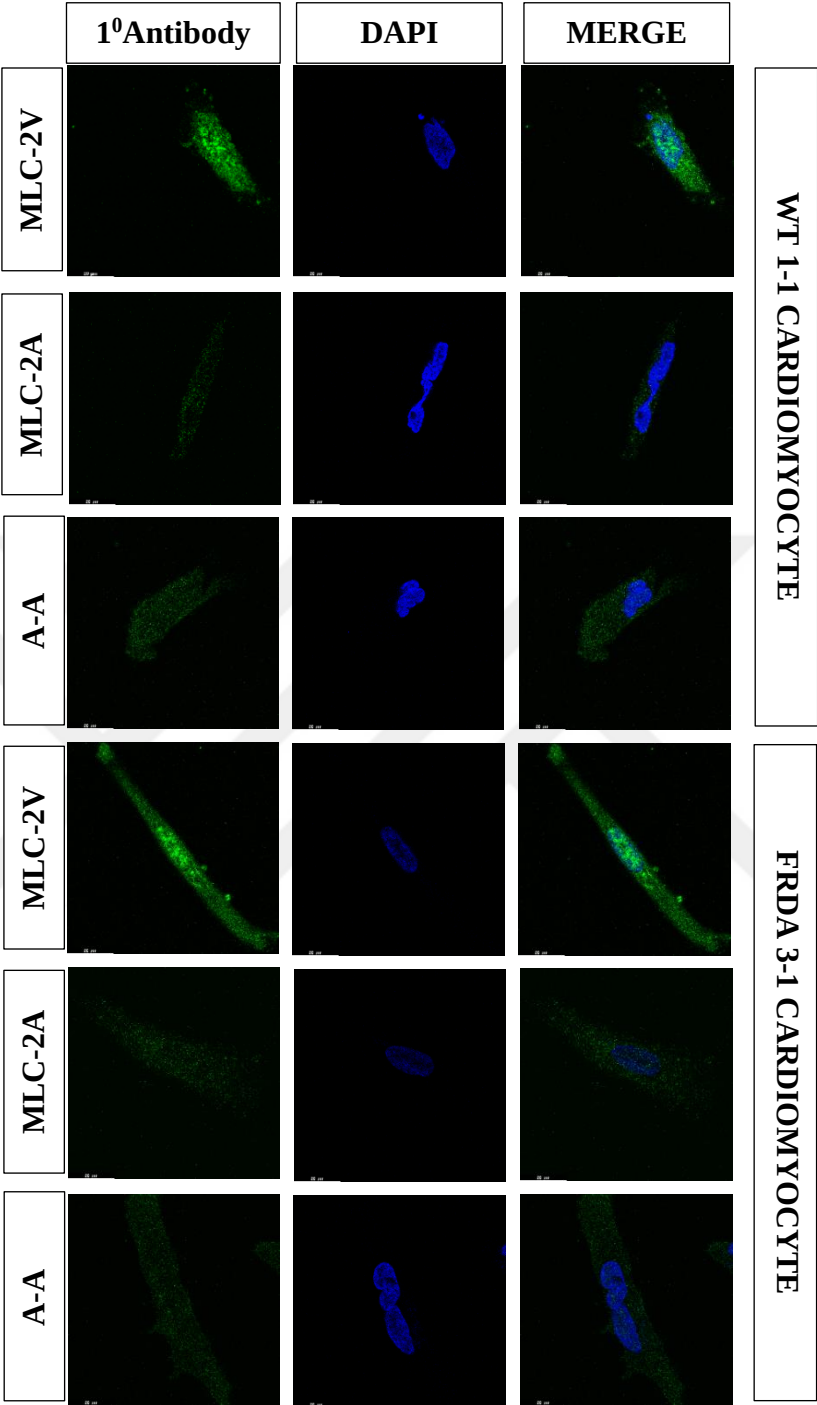


Figure 3.26 Immunofluorescence staining of WT 1-1 and FRDA 3-1 iPSC-Cms is represented.

DAPI stained the nuclei. Images were taken with 63x magnification, scale bar 20 μ m.

Primary antibody, MLC-2V, stains myosin light chain 2 ventricular form. Primary antibody, MLC-2A, stains myosin light chain 2 atrial form. Those belong to EF-hand family members. Primary antibody, ALPHA-ACTININ (represented as A-A), stains the

microfilament proteins found in cardiomyocytes. Both light chains and alpha-actinin should stain cytoplasm. WT 1-1 iPSC-Cms and FRDA 3-1 iPSC-Cms were positively stained for all those antibodies (**Figure 3.26**). This figure indicates the existence of the expression of cardiomyocyte-specific proteins.

Secondary only staining of iPSC-Cms and undifferentiated iPSCs staining using MLC-2V, MLC-2A and AA antibodies can be found in **Appendix Figure 5.4, Figure 5.5**. WT 1-1 iPSC-Cms were also positively stained for cardiac-troponin (**Appendix Figure 5.4**).

To examine *FXN* mRNA expression in iPSC-Cms, RNAs were collected from WT 1-1 cardiomyocytes and FRDA 3-1 cardiomyocytes at day 15 and qPCR analysis were done for *FXN* gene.

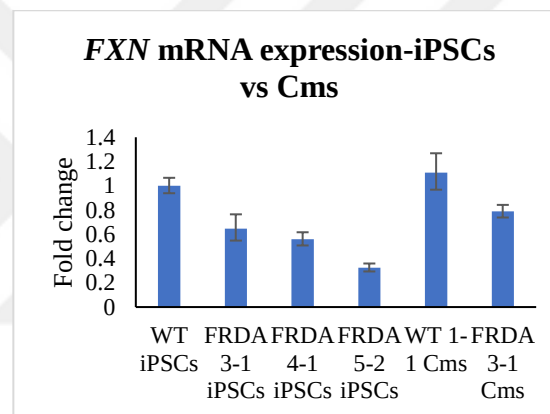


Figure 3.27 mRNA expression results of *FXN* gene (iPSC-Cms).

β-actin was used as an internal control gene. (n=1, error bars represent $\pm$ s.e.)

In this experiment, I investigated whether the decrease in *FXN* mRNA observed in the FRDA fibroblast and FRDA iPSCs is maintained after differentiation (**Figure 3.27**). And *FXN* mRNA level was found to be lower in FRDA 3-1 iPSC-Cms when compared to WT 1-1 iPSC-Cms. In addition, when *FXN* expressions in iPSCs and *FXN* expressions in iPSC-Cms were compared, a slight increase in *FXN* mRNA level after differentiation was observed.

3.10 Overexpression of *FXN* gene in HEK293T cells increases *FXN* expression

Until now, FRDA-like phenotype (decrease *FXN* mRNA level) was shown to maintain in FRDA iPSCs and FRDA iPSC-Cms. Later, *FXN* gene can be overexpressed in FRDA iPSC-Cms so that *FXN* expressions become almost equal to the normal levels in those cells. For overexpression, subcloning followed by LR cloning was performed (**Figure 3.28**). Then, the infection ability (**Figure 3.29**) of the cloned pLEX-307-*FXN* plasmid was tested in HEK293T cells (**Figure 3.30**).

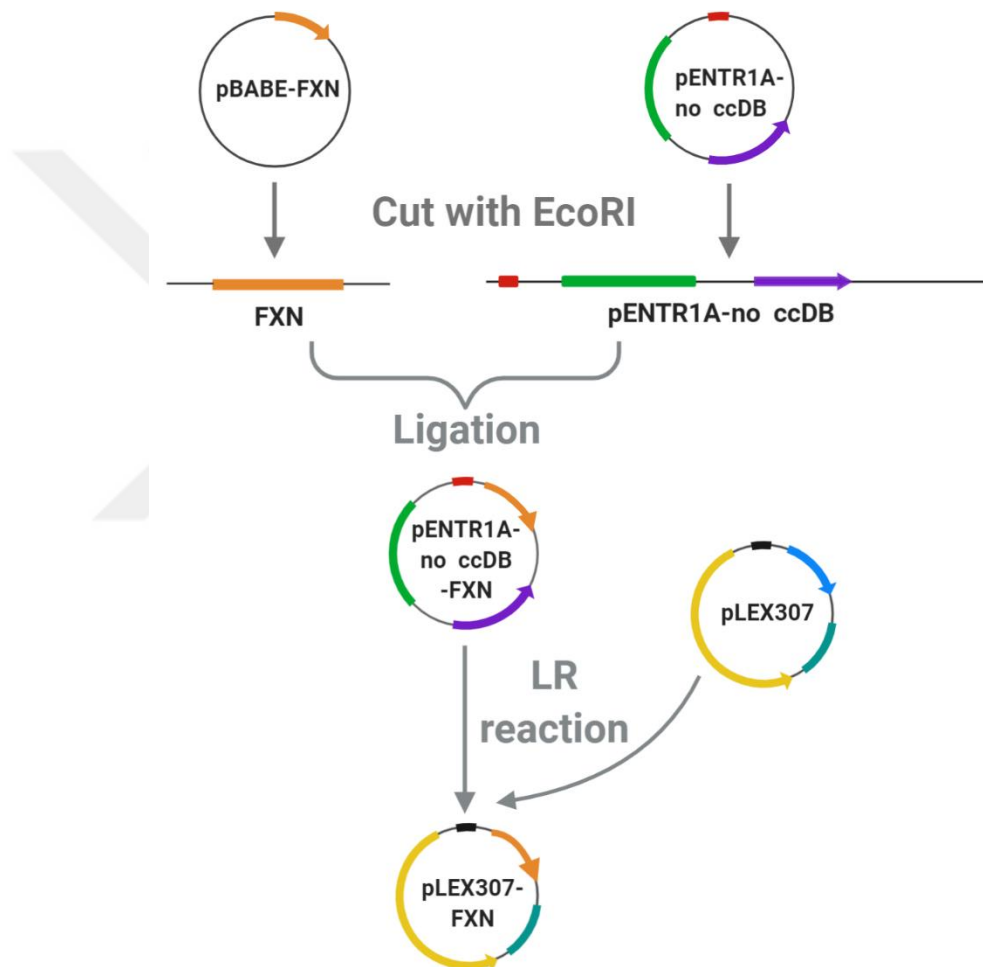


Figure 3.28 Representation of cloning for *FXN* overexpression experiments.

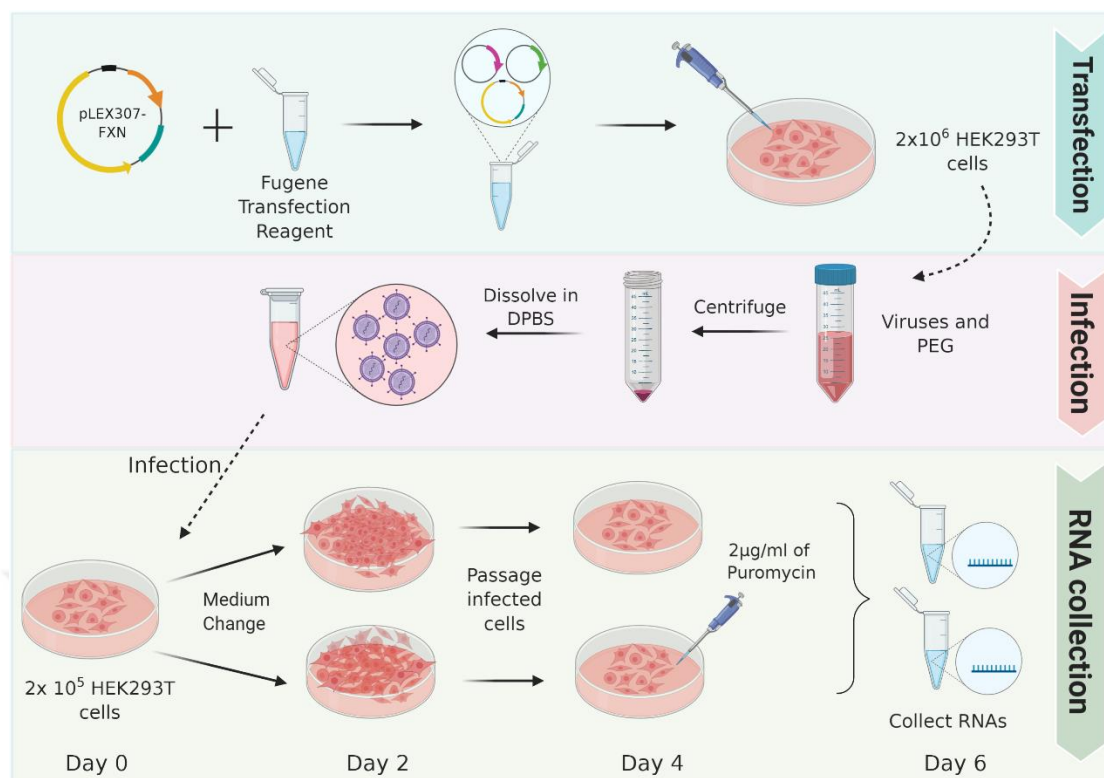


Figure 3.29 Representation of infection for *FXN* overexpression experiments.

For the generation of lentiviruses for *FXN* overexpression experiments, 2×10^6 HEK293T cells were seeded and along with lentiviral vectors and pLEX-307-*FXN* plasmid, Fugene transfection reagent was used. D10 medium containing lentiviruses were collected and viruses were concentrated using PEG. Later, 2×10^5 HEK293T cells were infected with different concentrations of lentiviruses and puromycin selection was performed on the 4th day of infection (Figure 3.29). At 6th day, RNAs were collected.

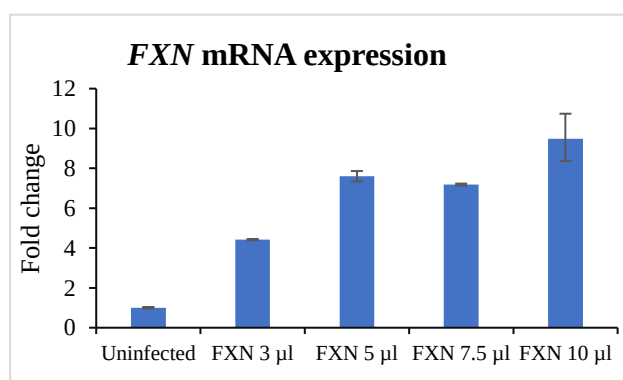


Figure 3.30 mRNA expression results of *FXN* gene (HEK293T). *β-actin* was used as an internal control gene. (n=1, error bars represent $\pm$ s.e.)

In **Figure 3.30**, + sign is for puromycin addition. Puromycin was used to select cells that were infected with our pLEX-307-*FXN* plasmids. The viruses were used with increasing volumes (from 3 μ l virus to 10 μ l virus volumes). Thus, it is possible to see an increase in *FXN* mRNA level with an increase in virus volumes. To sum up, with this experiment, it is possible to say that these lentiviruses can be used to increase *FXN* mRNA levels in FRDA iPSC-Cms to make them equal to control iPSC-Cms in terms of *FXN* expression.



Chapter 4:DISCUSSION

FRDA is a disease that results from GAA repeat expansions in the first intron of the *FXN* gene (Sanjay I. Bidichandani et al., 1998; Campuzano et al., 1997). This expansion results in transcriptional silencing of *FXN* gene (Ohshima et al., 1998). However, whether this silencing is due to transcriptional initiation or transcriptional elongation defect or both is an unsolved question (Gottesfeld, 2019). The symptoms of FRDA involve neurodegeneration of large sensory neurons and cardiomyopathy (Bürk, 2017; Delatycki et al., 2000; Delatycki & Corben, 2012; Tsou et al., 2011). However, why certain types of neurons are more-strongly affected in FRDA (Eigentler et al., 2013) and molecular pathology behind cardiomyopathy is not well-studied (Crombie et al., 2017).

In addition, there is currently no effective treatment for FRDA and there is no FDA-approved therapeutics to correct genetic defects or cellular deficiencies observed in FRDA (Aranca et al., 2016). Considering the unknowns in this disease and the unavailability of effective treatment, it is very important to model FRDA to elucidate the molecular mechanisms and carry out drug screens. Traditionally, genetically modified mice strains have served as best models to study human diseases. However, the genetic complexity of FRDA makes it challenging to study in mice (Perdomini et al., 2013). Thus, current efforts in the FRDA field focus on the generation of better models that could recapitulate the disease phenotypes mainly via generation of the most damaged cell types by the use of iPSCs technology (Martelli et al., 2012).

Therefore, in this thesis, we aimed to generate iPSCs-based models of FRDA to study this disease *in vitro* and find a potential drug through drug screens. In this thesis, three main findings have been presented: (1) WT and FRDA iPSCs have been generated from fibroblasts via integration-free delivery of Yamanaka's factors. (2) The generated WT and FRDA iPSCs have been shown to maintain pluripotency and differentiation capacity. (3) *FXN* levels in fibroblasts, iPSCs, and cardiomyocytes were observed to be lower compared to WT samples. These models can therefore be used to investigate disease mechanisms and to identify the possible drug targets for FRDA.

4.1 WT and FRDA iPSCs have been generated from fibroblasts via integration-free delivery of Yamanaka's factors

The first iPSCs from FRDA patients was generated by Gottesfeld group (Ku et al., 2010). In that study, they generated two iPSCs lines from primary fibroblasts of two FRDA patients through transcription factor overexpression. GAA·TTC repeats in iPSCs were shown to exhibit repeat instability and this was accompanied by the repression of *FXN* gene. They hypothesized that the instability is a result of mismatch repair enzyme (MSH2) because it was found to be highly expressed in pluripotent cells and occupies *FXN* intron 1 (Ku et al., 2010). In our study, however, we focused on the generation and characterization of 4 iPSCs from 4 different FRDA patients. Additionally, we generated iPSCs lines using an integration-free method (**Figure 3.7, Figure 3.9**). The reason for this is that lentiviral delivery causes insertions resulting in disruption of the genes. Moreover, constitutive expressions of transcription factors may cause changes in the differentiation potential of the iPSCs lines (Doss & Sachinidis, 2019). Integration-free FRDA iPSCs were also generated to investigate calcium flux changes in FRDA iPSC-Cms (Crombie et al., 2017) and to investigate the sensitivity of FRDA iPSCs-derived neurons towards drugs being used in clinics (Georges et al., 2019).

4.2 The generated WT and FRDA iPSCs have been shown to maintain pluripotency and differentiation capacity

WT 1-1, WT 2-2, FRDA 3-1, FRDA 4-1, FRDA 5-2, and FRDA 7-3 iPSCs lines were generated successfully. However several assays are needed to be carried out to show their pluripotency (Park, Arora, et al., 2008). The first assay evaluates pluripotency-related gene expressions via qPCR analysis. Regardless of the genetic background of the parental somatic cells, all iPSCs lines expressed pluripotency-related genes (*OCT4*, *SOX2*, *NANOG*, *LIN28A*) at the mRNA level (**Figure 3.10**).

Then to check whether the transcripts of pluripotency-related genes were translated into protein, immunofluorescence stainings were carried out by using anti-OCT4, anti-SSEA4, and anti-NANOG antibodies (**Figure 3.12, Figure 3.13**). In the case of NANOG, the expression was detectable but slightly lower compared to the OCT4, and SSEA4 expressions. The reason for this could be due to the primary antibody incubation step.

Increasing the concentration of the NANOG antibody and/or increasing the time of incubation could solve the problem.

After confirming that there were no chromosomal abnormalities (**Figure 3.14**) in the selected iPSCs clones, EB formation assay was performed. EB formation assay followed by qPCR for markers of all three embryonic germ layers (endoderm, mesoderm, ectoderm) was performed to assess iPSCs' potential to develop along specific lineages. When the results were analyzed (**Figure 3.15**), it was found that all EB lines were not able to effectively differentiate into ectoderm lineage (represented as NESTIN and MAP2 qPCR results) while they were able to express mesoderm and endoderm markers higher than iPSCs. When Matilla and colleagues investigated the spatial and temporal expressions in EBs, they observed that the EBs with best cardiac differentiation potentials (mesoderm lineage) exhibited less SOX1 (ectoderm marker). They also observed abundant SOX1 expression at day 20. Thus, they concluded that certain circumstances such as EB medium composition, the time when RNAs collected, EB size may support ectoderm and cardiomesoderm formation exclusively (Pekkanen-Mattila et al., 2010). In addition, to increase the potential differentiation of EBs towards ectoderm, Khoo and colleagues added FGF-2 to the medium (Khoo et al., 2005). In this way, they were able to detect earlier NESTIN expression. Therefore, by changing the medium composition or growing EBs more and collecting RNAs at later stages of differentiation may help us to detect ectoderm lineages specific genes expressions.

Apart from *in vitro* differentiation assays, teratoma formation can be said as a powerful method to show the differentiation abilities of iPSCs (Park, Zhao, et al., 2008). Thus, later in this study, iPSCs lines will be injected into SCID mice intramuscularly and tested for teratoma formation.

Gene expression analysis, immunofluorescence stainings, and EB formation assays showed all iPSCs lines have self-renewal and differentiation abilities. In addition, all iPSCs were found to be integration-free via a PCR-based assay (**Figure 3.17**).

Although several studies used fibroblasts and lymphoblastoid cells collected from FRDA patients, they may not optimally represent the disease. The reason for that is that fibroblasts or lymphoblastoid cells are not the primarily affected cell types in FRDA. Thus, the generation of iPSCs-based FRDA models plays a crucial role. Until now in this

study, WT and FRDA iPSCs were fully characterized in terms of pluripotency. Later those iPSCs can be used to differentiate to the affected cell types (cardiomyocytes and neurons) to study FRDA as Liu and colleagues did in 2011 (J. Liu et al., 2011).

Apart from differentiation, generated iPSCs can also be used to study repeat instability, because it was shown that GAA repeats can contract or expand during reprogramming (Ku et al., 2010; J. Liu et al., 2011). In the preparation for this experiment, GAA PCR was optimized in FRDA fibroblasts which can then be repeated in the FRDA iPSCs. Similarly, in a pioneering study the genetic excision of GAA repeats in FRDA iPSCs have shown to impact frataxin levels. Zing-finger endonuclease-based excision of GAA repeats (1230 bp) located 1339 bp away from the exon1/intron1 resulted in an increase in *FXN* mRNA and frataxin protein levels (Y. Li et al., 2015). Therefore, in the future experiments, GAA repeats in FRDA iPSCs can be removed via CRISPR-based dual excision and the change in frataxin mRNA and protein expression can be studied both in iPSCs and in iPSCs-derived models.

4.3 *FXN* levels in fibroblasts, iPSCs, and cardiomyocytes were observed to be lower compared to WT samples

FRDA patients have low *FXN* mRNA levels (Punga & Bühler, 2010). Thus, qPCR analysis for *FXN* gene in iPSCs and parental somatic cells was carried out. The reduction in *FXN* mRNA levels were demonstrated both in patient-derived fibroblasts and iPSCs-derived from them (**Figure 3.22**). However, data for FRDA 7-3 iPSCs and its parental somatic cell is missing.

Several research groups performed *in vitro* differentiation of iPSCs to primarily affected cell types in FRDA, such as cardiomyocytes, and studied the phenotypes. Hick group in 2013 showed mitochondrial damage in FRDA-iPSC-Cms for the first time (Hick et al., 2013). Lee and colleagues investigated iron overloading and its effects on cardiomyopathy observed in FRDA iPSC-Cms (Lee et al., 2014). Later, the same group screened iron chelators on iPSCs- Cms (Lee et al., 2016). In addition, FRDA iPSC-Cms exhibited electrophysiological differences compared to controls (Crombie et al., 2017). Finally, in 2019, the excision of GAA triplet repeats in FRDA iPSC-Cms corrected cardiomyopathy phenotypes (Jixue Li et al., 2019). Inspired by all these, we aimed to differentiate iPSCs lines to cardiomyocytes and study the disease phenotypes.

Following the optimizations of cardiomyocyte differentiation (**Figure 3.23, Figure 3.24**), we successfully differentiated one control iPSCs and one FRDA iPSCs. Next, we characterized differentiated iPSC-Cms by live-cell imaging of beating areas and by cardiac gene analysis with qPCR and immunofluorescence staining (**Figure 3.25, Figure 3.26**). We showed that *FXN* level in FRDA 3-1 cardiomyocytes is lower than WT 1-1 cardiomyocytes (**Figure 3.27**).

FRDA 5-2 iPSCs is a good candidate line to be differentiated to cardiomyocytes because it has the lowest *FXN* level and has the potentiality to show phenotypes more severely than other FRDA iPSCs lines.

The enrichment of iPSCs-Cms can be performed through FACS targeting to SIRPA (signal-regulatory alpha protein). SIRPA protein was found after a large screen analysis of CD antibodies. SIRPA are expressed specifically in human pluripotent stem cells derived cardiomyocytes. If cells can be sorted using this protein, populations yielding up to 98% cardiac troponin positive cells can be purified (Dubois et al., 2011; Talkhabi et al., 2016). Similarly VCAM-1 which is vascular cell adhesion molecule 1 can be used to purify Cms from a mixed population (Mummery et al., 2012). CD77⁺/CD200⁻ cell-surface signature can also be used to purify at least 98% cardiac troponin positive cells (A. et al., 2019; Veevers et al., 2018). As an another enrichment protocol, iPSCs-Cms can be cultured with glucose-free medium with an addition of lactate. By this way, pluripotent stem cells derived Cms population was enriched from 4.5% to 98% alpha-actinin positive cells (BurrIDGE et al., 2014).

In the future work described below, these fully-characterized cardiomyocytes will be used to investigate the disease phenotypes such as iron accumulation, reactive oxygen species formation, lipid droplet formation, and ferroptosis. After the validation of the phenotypes, further experiments will be carried out to correct the mutation via excision or upregulate *FXN* expressions, and to screen for drugs to reverse FRDA-specific phenotypes (**Figure 4.1**).

4.4 Future Work on Cardiomyocytes

4.4.1 Imbalance Iron Homeostasis Phenotype

The relation between FRDA and iron was first considered when myocardial iron deposits were observed in FRDA patients (Sanchez-Casis et al., 1976). Later in 1999, significantly higher mitochondrial iron deposits were shown in 4 fibroblasts from FRDA patients (Delatycki et al., 1999). In the heart of *MCK* conditional *FXN* mice, intramitochondrial iron-deposits were found (Puccio et al., 2001). Disease-specific-iPSCs recapitulated FRDA-associated iron accumulation *in vitro* under iron-overloaded conditions (Lee et al., 2014). Therefore, impaired iron homeostasis in FRDA 3-1 iPSC-Cms, and FRDA 5-2 iPSC-Cms can be investigated. After treating cardiomyocytes with 100 μ M iron(II)sulfate for 48 hours and measuring fluorescence quenching of calcein by iron (Schoenfeld et al., 2017), iron accumulation in FRDA iPSC-Cms can be compared to WT 1-1 cardiomyocytes. A drastic increase in iron content in FRDA 3-1 and 5-2 cardiomyocytes compared to WT 1-1 cardiomyocytes is expected (**Figure 4.1**).

In addition, it was hypothesized that cardiomyopathy seen in FRDA could be due to iron deposits (Lee et al., 2014). Thus, in the future, the effect of iron overloading on cardiomyopathy phenotypes can be investigated. For that, any change in the cell size of cardiomyocytes (via detection of α -actinin-positive cell sizes in FACS) and expression of brain natriuretic peptide (a cardiac stress marker) can be analyzed in FRDA iPSC-Cms.

In recent years, Zucchini and colleagues investigated iron homeostasis in iPSC-Cms of FRDA patients. They investigated the expression levels of transferrin receptor 1 (TFRC- a transmembrane protein that plays an important role in cellular uptake of iron), and ferroportin (FPN- an iron-exporting protein) mRNA levels. They observed that the expression of TFRC was significantly increased in FRDA cardiomyocytes because of the

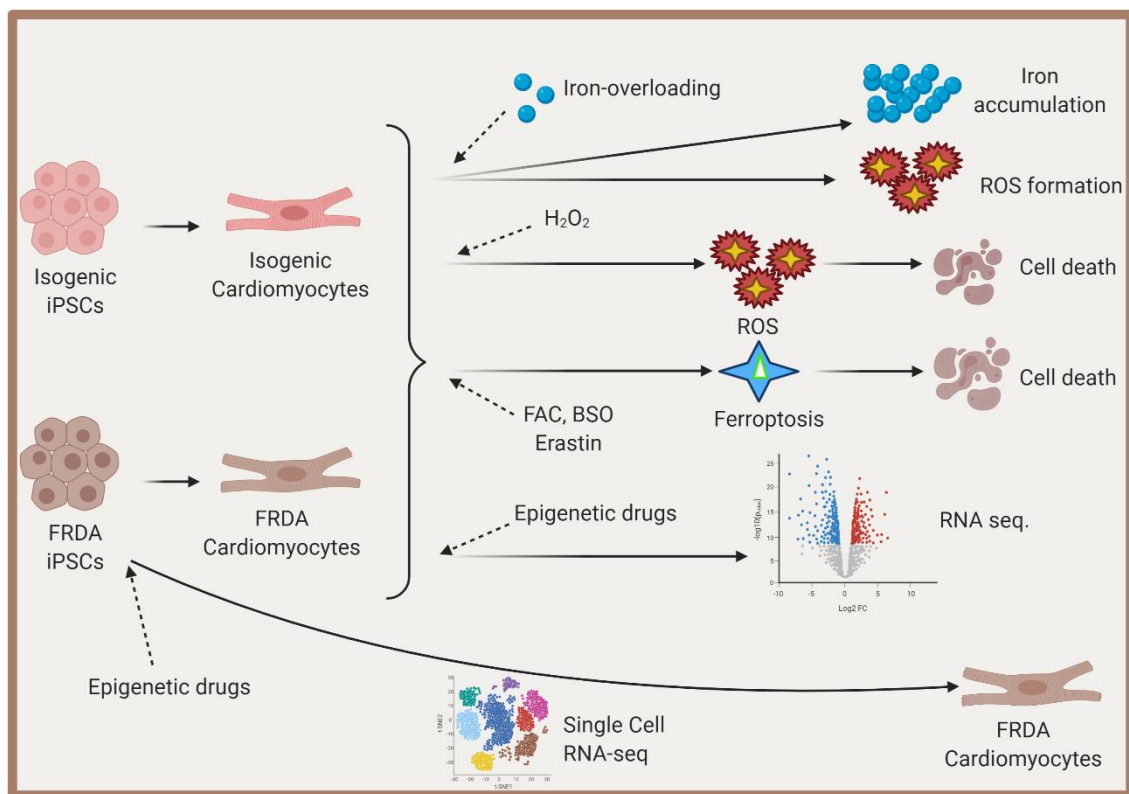


Figure 4.1 Representation of future directions in this project.

Control iPSCs will be either WT iPSCs or *FXN*-overexpressed iPSCs. Control and FRDA iPSC-Cms and neurons will treated with chemicals and phenotypes will be investigated.

imbalance in iron homeostasis. They also showed for the first time that intracellular mislocalization of FPN in FRDA iPSC-Cms (Bolotta et al., 2019). Therefore, in future work, FPN and TFRC expressions can be investigated under normal, iron-overloading, and iron chelator-treated conditions in FRDA iPSC-Cms.

In 2019, hypoxic conditions were found to elevate bioavailable iron, activate iron sulfur clusters synthesis, and increase frataxin expression. In ambient 1% O₂ conditions, *FXN* mutant yeast, murine cells, and *FXN* knockout human cells were viable but breathing 11% elevates ataxia in mice (Ast et al., 2019). Therefore, it would be interesting if we

investigate the role of HIF-pathway or HIF-independent pathways on frataxin expressions in *in vitro* models.

4.4.2 Reactive Oxygen Species Formation Phenotype

All types of cells found in the heart are susceptible to reactive oxygen species (ROS) induced damage. Most importantly, cardiomyocytes are more susceptible to ROS due to the presence of fewer anti-oxidant enzymes, and higher consumption of oxygen. ROS production can be due to iron-catalyzed Haber-Weiss and Fenton reactions (Gammella et al., 2015). The iron-mediated generation of ROS may contribute to the development of cardiomyopathy. When Lee and colleagues treated iPSC-Cms with iron sulfate, they observed a significant increase in the levels of ROS in FRDA iPSC-Cms (Lee et al., 2014). Thus, ROS formation can be assessed in FRDA iPSC-Cms via Muse® Oxidative Stress Kit which is a kit that is based on quantitative measurement of superoxide radicals. A significant difference in ROS levels between FRDA iPSC-Cms and control iPSC-Cms is expected. Moreover, in the iron-overloading conditions (with addition of 100 μ M iron(II)sulfate), a further significant increase in ROS production is expected in FRDA iPSC-Cms.

Similarly, the sensitivity towards ROS generators such as H_2O_2 could be investigated in FRDA iPSC-Cms, because FRDA fibroblasts were shown to be hypersensitive to H_2O_2 (Wong, 1999).

In addition, downregulation of NRF2 pathway and downregulation of NRF2-targeted proteins are expected in FRDA iPSC-Cms because NRF2 antioxidant pathway is one of oxidant scavengers pathways and it is reduced in FRDA fibroblasts and other models (Abeti et al., 2018).

Apart from ROS formation, apoptosis can be induced by a reduction of antiapoptotic proteins. Therefore, in addition to *NRF2*, *HAX1.v1* expressions will be investigated. It is an antiapoptotic protein that modulates cardiac cell survival and death. Recently, Tiano and her colleagues observed a positive correlation between *FXN*, *HAX1.v1*, and *NRF2* expressions in *FXN*-silenced cardiomyocytes (Tiano et al., 2020). Therefore, in the future, the correlations between those genes can be investigated in FRDA iPSC-Cms.

4.4.3 Lipid Droplet Accumulation Phenotype

In glial cells of frataxin RNAi-knockdown *Drosophila melanogaster*, disruption of lipid metabolism was demonstrated. This was accompanied by the formation of multiple lipid droplets (Navarro et al., 2010). Similarly, FRDA iPSC-Cms accumulated lipid droplets compared to control iPSC-Cms (Jixue Li et al., 2019). Thus, in the future, the amount of lipid droplet structures can be investigated via Oil Red O staining experiments in FRDA iPSC-Cms.

In addition, recently Utkan Demirci's lab at Stanford University established a protocol to sort hiPSC-Cms according to the lipid content. After separation of cells in terms of their lipid contents, sorted cells maintain viability and function (Demirci et al., 2020). Therefore, the levitation-based sorting of FRDA hiPSC-Cms can be performed to select Cms with more lipid accumulation. Later, those sorted cells can be used for further experiments because they will be viable after sorting.

4.4.4 Ferroptosis Phenotype

The iron can generate ROS through Fenton reactions leading to the initiation of detrimental lipid peroxidations (Jie Li et al., 2020). Mitochondria is more susceptible to lipid peroxidation due to high polyunsaturated fatty acid content in the inner membrane and this may play a role in neurodegenerative diseases (Reed, 2011). Ferroptosis can be described as a type of iron-dependent and lipid-mediated programmed cell death (Jie Li et al., 2020). Primary patient-derived fibroblasts and murine models of FRDA showed susceptibility to this process (Grazia Cotticelli et al., 2019). Therefore, in the future, the sensitivity of our FRDA iPSC-Cms towards ferroptosis inducers (such as Erastin) can be investigated. In addition, instead of erastin, cells can be treated with ferric ammonium citrate (FAC) and buthionine sulfoximine (BSO) to activate ferroptosis pathways. Then, ferroptosis pathways can be investigated through measurements of glutathione-dependent peroxidase activities at 340 nm. After FAC/BSO treatment, significant decrease in glutathione-dependent peroxidase activities in FRDA iPSC-Cms compared to control iPSC-Cms is expected. In addition, we also expect to observe a decrease in protein GPX4 and NRF2 levels by using western blot.

Recently, class I HDAC inhibitors were found to selectively protect primary cortical neurons against ferroptosis (Zille et al., 2019). In the future, investigating the effects of epigenetic drug treatment on ferroptosis could be very promising for this project.

Once FRDA iPSC-Cms are demonstrated to exhibit FRDA phenotypes such as iron accumulation, ROS formation, lipid droplet accumulation, and ferroptosis; therapeutic approaches described below can be investigated.

4.5 *FXN* overexpression on FRDA iPSCs-based models

Transcription factor-based reprogramming has been used to generate human iPSC- based models of several diseases. However, the lack of genetically matched nondiseased controls is a challenge. This challenge must be overcome in order to show that the phenotypes observed in iPSC-based models are not the results of line-to-line variations, or differences in the genetic background (Ambasudhan et al., 2013). Therefore, isogenic controls generated by genetic manipulations or controls obtained from family members can be used. Family members may not carry the disease-causing mutations. Thus, isogenic controls can be used as reliable controls in iPSCs based disease modeling (Hinz et al., 2019). For FRDA disease modeling, isogenic controls can be generated either through dual excisions of GAA repeats or through *FXN* overexpression in FRDA iPSCs lines. Therefore cloning of *FXN* cDNA into expression plasmids was performed in this thesis work and lentiviruses for overexpression of *FXN* gene were generated (**Figure 3.29**). Therefore, overexpression of *FXN* gene (**Figure 3.30**) in FRDA iPSC-Cms can be carried out, and changes in the phenotypes such as iron accumulation, lipid droplet accumulation can be investigated.

4.6 CRISPR-Cas9 dual excision and dCas9-based *FXN* activation on FRDA iPSCs-based models

Current therapeutic approaches for FRDA are through upregulation of *FXN* gene expression, and amelioration of metabolic impairment, cellular toxicity phenotypes. However, in FRDA, irreversible cell damage and cell death are observed and those therapeutic approaches become less useful. As a result of this, cell replacement therapy by using CRISPR-Cas9 corrected or dCas9-rescued FRDA iPSC-Cms has a stronger therapeutic potential.

FRDA is caused by hyperexpansion of GAA triplet repeats in the first intron of *FXN* gene (Campuzano et al., 1996, 1997; Montermini et al., 1997). Zing finger nucleases-based (ZFN) excision of GAA repeat regions from the *FXN* gene restored expression of frataxin in FRDA patient-derived fibroblasts, lymphoblasts, iPSCs, iPSCs-derived neurons (Y. Li et al., 2015), and recently in FRDA iPSC-Cms (Jixue Li et al., 2019).

Although Li and colleagues corrected the expansions in iPSCs, iPSC-Cms, and iPSCs-neurons via ZFN-based correction; the percentage of gene editing varies, and the efficiency of correction was too low. Therefore, it could be better to use CRISPR-Cas9 based dual excision. In this method, two guide RNA flanking the repeat region is delivered into cells along with Cas9 endonuclease. Recently, CRISPR-Cas9 dual excision has been performed to remove the expansions in hematopoietic stem cells and progenitor cells (HSPCs) isolated from FRDA patients (Rocca et al., 2020). A similar strategy can be used for CRISPR-Cas9 based dual excision of GAA triplet repeats in FRDA iPSC-Cms. The resulting cells can be assayed for *FXN* expression as well as for the rescue of the phenotypes such as iron accumulation, lipid droplet accumulation, and ferroptosis dependent death.

Tremblay and colleagues used platinum TALE (Transcription Activator-Like Effector) (pITALE) proteins fused with transcription activators (VP64 or p300). They found that pITALE_{VP64} targeting *FXN* gene promoter resulted in a 19 fold increase in *FXN* expression in FRDA primary fibroblasts. They also validated their results *in vivo* (using YG8R mouse model) by using adeno-associated virus delivery of pITALE_{VP64} (Cherif et al., 2018). Similarly, a possible further step in this project can be to target the promoter of *FXN* gene with a gRNA that will form a complex with an activator such as Cas9-p300 or Cas9-VP64 leading to upregulation in *FXN* gene followed by a reduction in FRDA-like phenotypes in FRDA iPSC-Cms.

Although DNA methylation seems to play a role in the silencing of *FXN* gene, there are no studies that tested DNA methylation inhibitors (DNMT inhibitors) or the effect of Tet1 (DNA demethylase) enzymes on FRDA cells (Gottesfeld, 2019). In 2018, Liu and colleagues targeted the dCas9-Tet1 fusion demethylation system to hypermethylated CGG expansions in Fragile X syndrome iPSCs-neurons and observed rescue of the phenotypes (X. S. Liu et al., 2018). Similarly, it would be possible to design a dCas9-

Tet1 fusion system that targets the CpG sites found around the first intron of *FXN* gene and investigate the changes in FRDA phenotypes.

4.7 Drug Screening on FRDA iPSC-Cms and FRDA iPSCs-neurons

The reversal of epigenetic silencing in the *FXN* gene is an appealing strategy in the treatment of FRDA. Fentenstein and colleagues demonstrated an upregulation of *FXN* gene in FRDA primary lymphoblastoid cells, fibroblasts, and YGR8 mouse model cells upon treatment with a class III HDAC inhibitor or sirtuin inhibitor vitamin B3 (nicotinamide). A reduction in H3K9me3 and H3K27me3 and an increase in H3/H4 acetylation levels was hypothesized to be operative with these treatments (Gottesfeld, 2019). Similarly, 2-Aminobenzamide HDAC inhibitor 109 was shown to upregulate *FXN* expression and protect FRDA iPSC-neurons from oxidative stress-mediated cell death (Herman et al., 2006). Very recently, inhibition of SUV4-20 histone methyltransferase reduced H4K20me2/me3 and also increased *FXN* expression (Wade-Martins et.al., 2020). In addition, Resveratrol (which was tested in yeast cells under this TUBITAK-1003-216S051 project) is a drug that is used for FRDA treatment clinically. It is an activator of SIRT1, which is known to involve in epigenetic silencing (Clay et al., 2019). Taking all these into account, carrying out an epigenetic drug screen as an extension of this project may be beneficial. The drug screens should include HDAC inhibitors, histone methyltransferase (HMT) inhibitors especially against H3K9me3 and H3K27me3, and DNA methyltransferase (DMT) inhibitors. The effects of those epigenetic drugs can be analyzed on FRDA iPSC-Cms and iPSCs-neurons through *FXN* qPCR analysis and in parallel with the assays for FRDA phenotypes.

When H3K36me3 ChIP was performed in FRDA cells, the levels of H3K36me3 on the downstream region of GAA repeats, and the *FXN* exon 2 was found to be lower (Kumari et al., 2011). Deferasirox 3a was found to be a *bona fide* active site-binding inhibitor of (JMJD2A-KDM4A), which is one of Fe²⁺- and 2-oxoglutarate (2OG)-dependent JumonjiC domain-containing histone demethylases. KDM4A is an H3K36me3 demethylase (Roatsch et al., 2019). Although deferasirox is an iron chelator, treatment of FRDA cells with this drug for the first time as both an epigenetic drug and an iron chelator could be very interesting. Further investigation of the effect of this drug on trimethylation of H3K36 via ChIP in FRDA iPSC-Cms could be performed.

In addition to epigenetic drugs, iron chelators such as deferiprone are potential drugs for this disease. Deferiprone can cross the blood-brain barrier and over the past years, it has been used in patients 7-35 years of age with FRDA (Clay et al., 2019). This drug has already tested in yeast cells under this TUBITAK-1003-216S051 project and results were promising. Therefore, our FRDA iPSC-Cms can be used as a drug-testing platform for iron chelators such as deferiprone, deferasirox 3a, and deferoxamine 1.

Metformin is a drug used for lowering blood sugar levels. But this drug is also known to suppress the inflammation, reduce, and eliminate inflammatory factors at the cellular level (Dehkordi et al., 2019). Metformin may be used to reduce the levels of oxidative stress in FRDA. In addition, Manrique and his colleagues treated the mouse model of Huntington disease with metformin and observed alleviation of motor and neuropsychiatric phenotypes (Sanchis et al., 2019). Considering all these, testing metformin as a potential therapeutic drug for FRDA in FRDA iPSC-Cms could be very interesting for this project.

In 2019, RNA-seq-based transcriptomic analysis of FRDA iPSCs-neurons and isogenic neurons was performed to understand expression changes due to GAA triplet repeat expansion and expression changes after HDACi treatment (Lai et al., 2019). Similarly, transcriptomic analysis of FRDA iPSC-Cms can be performed to determine total expression changes after the treatment of DNA methyltransferase inhibitors and/or HMT inhibitors. Or single cell-based transcriptomic analysis during differentiation towards cardiomyocytes can be carried out to determine total expression changes after the treatment of DNA methyltransferase inhibitors and/or HMT inhibitors (**Figure 4.1**).

In short, phenotypical changes such as ROS formation, iron accumulation in FRDA iPSC-Cms can be compared with either WT iPSC-Cms or isogenic iPSC-Cms that are derived after overexpression of *FXN*. Therapeutic strategies either via genetic manipulations or drug screening for reversal of the FRDA phenotypes can be followed.

Chapter 5:APPENDIX

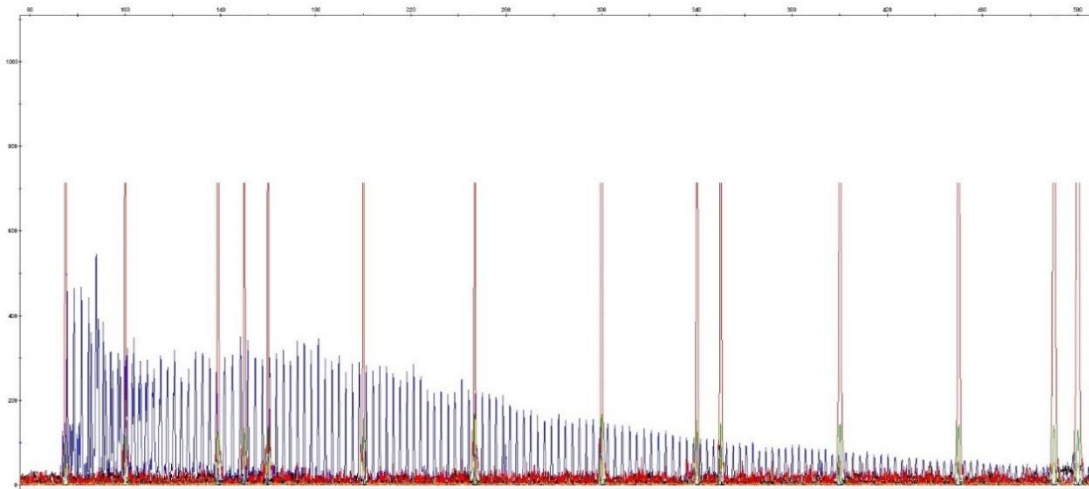


Figure 5.1 Figure of triplet-repeat PCR of FRDA 7-1 iPSCs.

This figure represents the result of triplet-repeat PCR done by KUH Diagnostic Centre of Genetic Diseases. Although from this figure, one can understand that the region that was amplified has triplet repeat expansion, it is not possible to calculate how much repeats there are in that region.

Secondary antibody stainings should be done in immunofluorescence stainings experiments. Whether secondary antibodies that are used to stain WT and FRDA iPSCs bind unspecifically or not was assessed in appendix **Figure 5.2**.

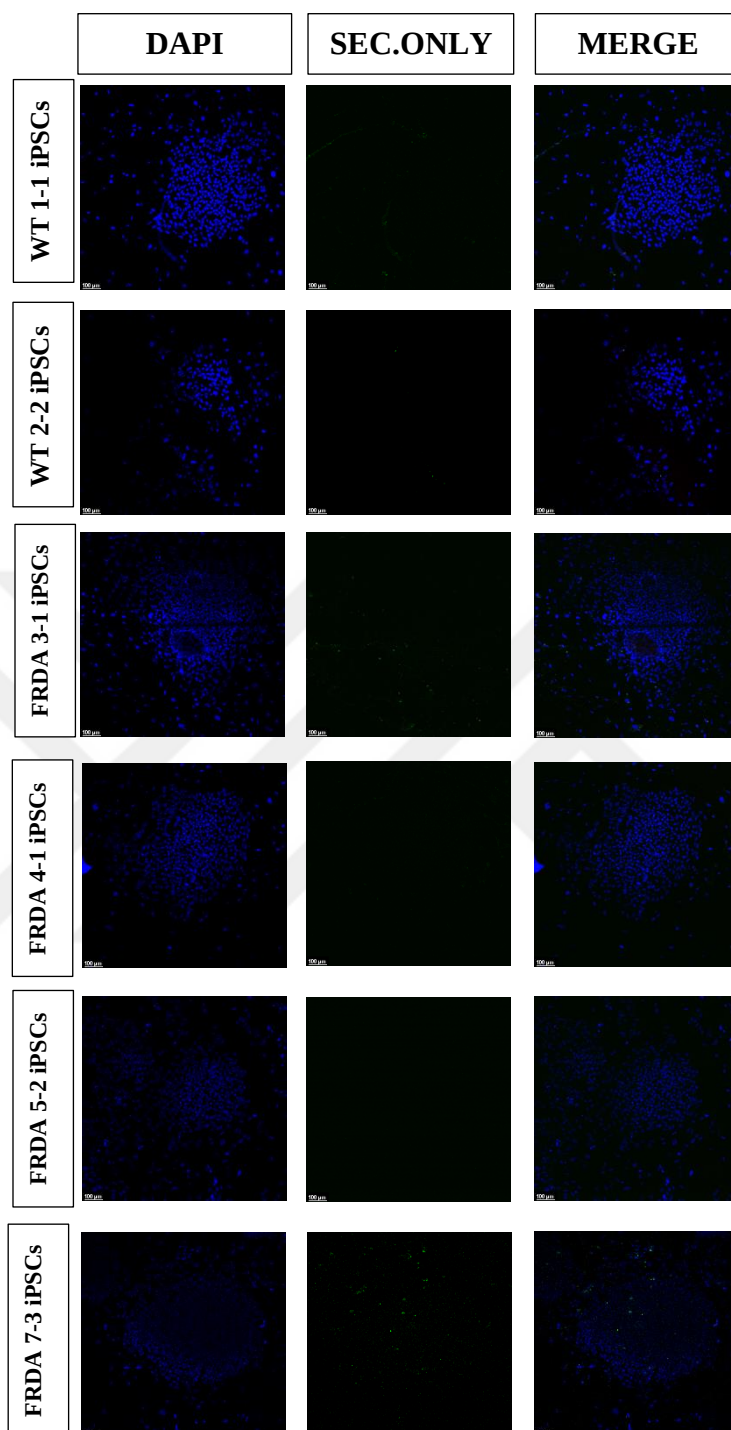


Figure 5.2 Immunofluorescence staining of WT and FRDA iPSCs is represented.

DAPI stained the nuclei. Images were taken with 10x magnification, scale bar 100 μm . SEC.ONLY (abbreviation of secondary only) means that the cells were incubated with secondary antibody which is Alexafluor488 but not with the primary antibodies.

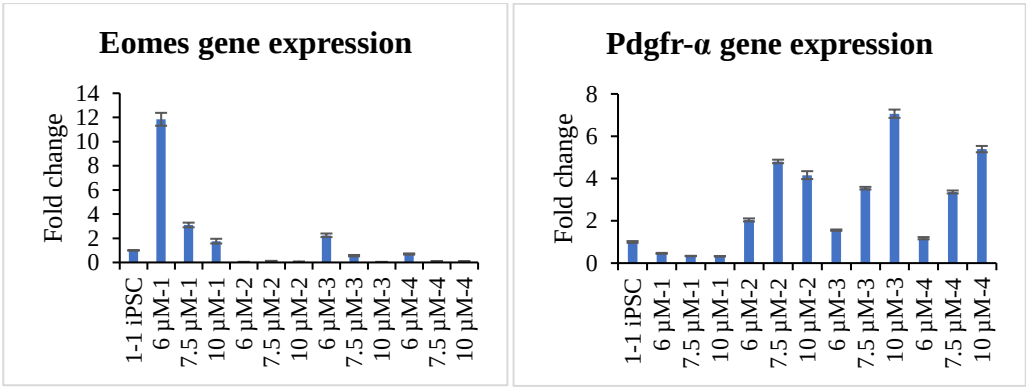


Figure 5.3 mRNA expression results of cardiomyocyte-specific genes.

Cms is the abbreviation of cardiomyocytes. *β-actin* was used as an internal control gene. (n=1, error bars represent ± s.e.)

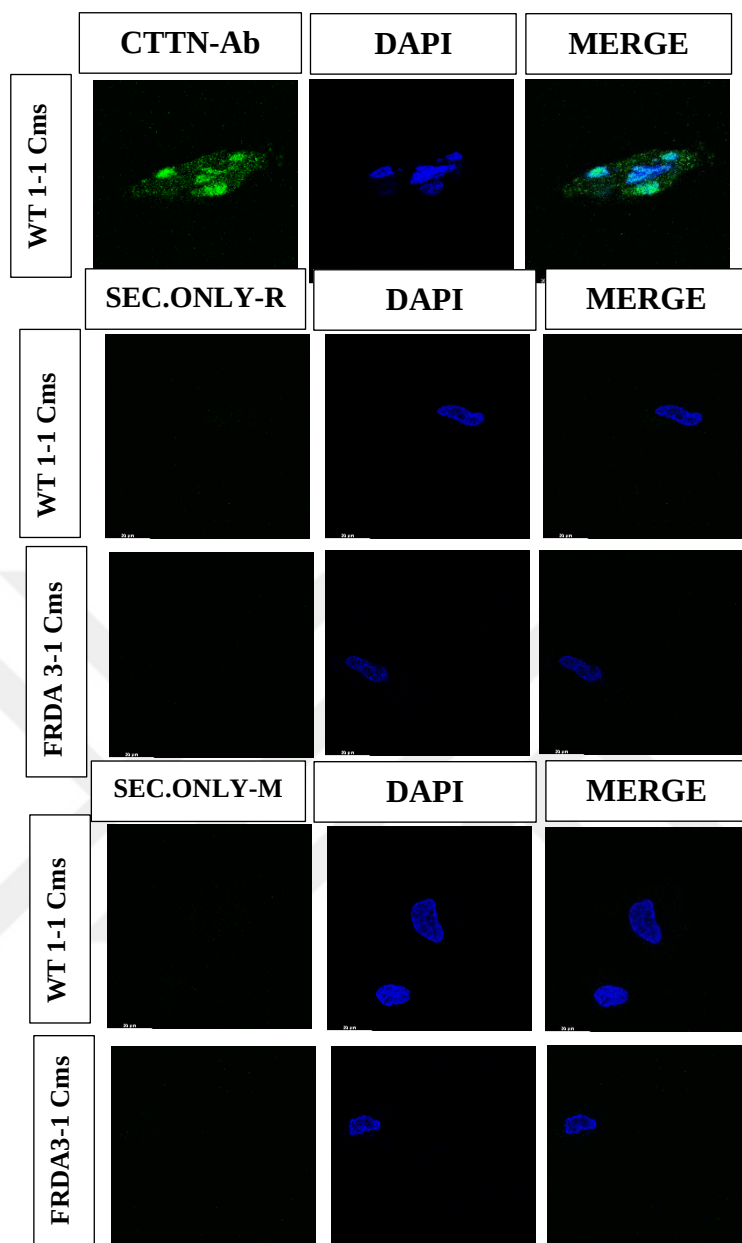


Figure 5.4 Immunofluorescence images of Cms.

Cms is the abbreviation of cardiomyocytes. Images were taken with 63x magnification, scale bar 20 μ m. SEC.ONLY (abbreviation of secondary only) means that the cells were incubated with secondary antibody which is Alexafluor488 but not with the primary antibodies.

CTTN-Ab is the abbreviation of cardiac-troponin primary antibody. From figure 31, we can see that both secondary rabbit (SEC.ONLY R) and secondary mouse (SEC.ONLY M) did not stain Cms unspecifically. In **Figure 5.5**, the same immunofluorescence staining for iPSC-Cms were applied to 6-1 iPSCs in which 6-1 iPSCs were incubated with primary

antibodies that were used to characterize iPSC-Cms. This was done to validate that primary antibodies are specific.

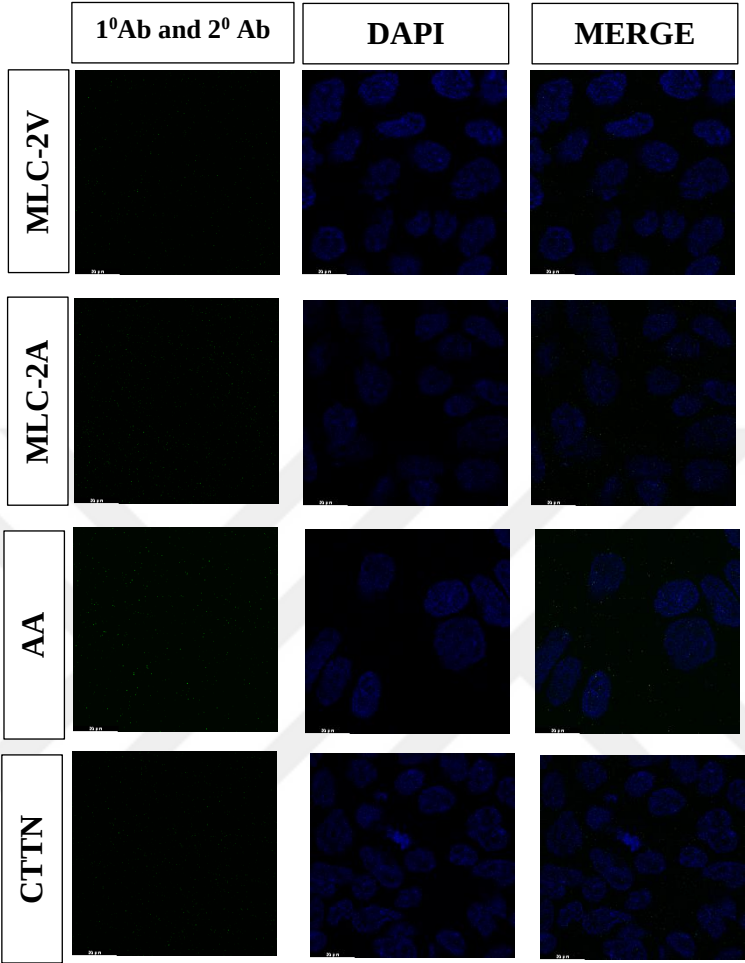


Figure 5.5 Immunofluorescence images of iPSCs.
Images were taken with 63x magnification, scale bar 20 μ m.

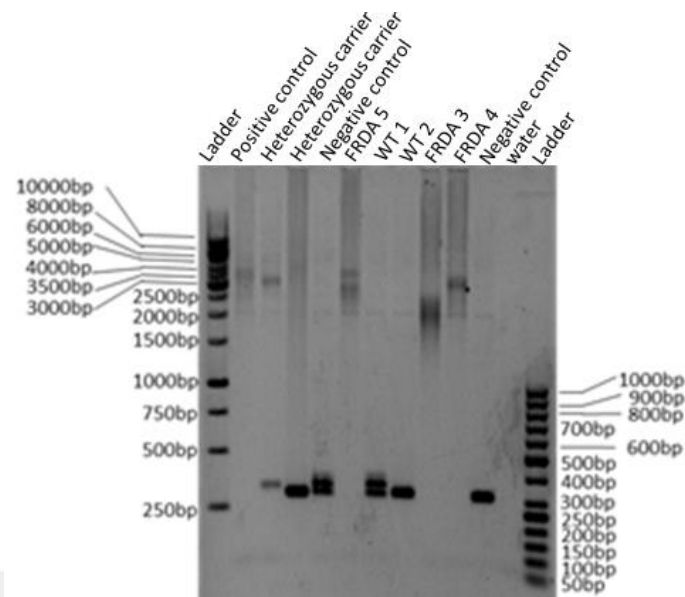


Figure 5.6 LR-PCR of blood samples

The products were run into 1% gel agarose gel.

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