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# Generation of Induced Pluripotent Stem Cell Lines from Friedreich Ataxia Patients

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**Abstract** Friedreich ataxia (FRDA) is an autosomal recessive disorder characterised by neurodegeneration and cardiomyopathy. It is caused by a trinucleotide (GAA) repeat expansion in the first intron of the *FXN* gene that results in reduced synthesis of *FXN* mRNA and its protein product, frataxin. We report the generation of induced pluripotent stem (iPS) cell lines derived from skin fibroblasts from two FRDA patients. Each of the patient-derived iPS (FA-iPS) cell lines maintain the GAA repeat expansion and the reduced *FXN* mRNA expression that are characteristic of the patient. The FA-iPS cells are pluripotent and form teratomas when injected into nude mice. We demonstrate that following *in vitro* differentiation the FA-iPS cells give rise to the two cell types primarily affected in FRDA, peripheral neurons and cardiomyocytes. The FA-iPS cell lines have the potential to provide valuable models to study the cellular pathology of FRDA and to develop high-throughput drug screening assays. We have previously demonstrated that stable insertion of a functional human BAC containing the intact *FXN* gene into stem cells results

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in the expression of frataxin protein in differentiated neurons. As such, iPS cell lines derived from FRDA patients, following correction of the mutated gene, could provide a useful source of immunocompatible cells for transplantation therapy.

**Keywords** Induced pluripotent stem cells · Friedreich ataxia · Frataxin

## Introduction

Human embryonic stem cells (hES) cells are derived from the inner cell mass of the human blastocyst and are able to differentiate into every cell type found in the body [1, 2], while induced pluripotent stem (iPS) cells are obtained by forced expression of a cocktail of transcription factors (such as OCT-4, SOX2, c-MYC and KLF4; or OCT-4, SOX2, NANOG and LIN28) into adult somatic cells resulting in reprogramming to a pluripotent state [3–8]. iPS cells are similar to hES cells in their ability to differentiate into all cell lineages, but can differ from hES cells in many characteristics of cell growth and efficiency of differentiation to give specific lineages [9].

iPS cells can be generated from individuals with genetically inherited diseases [10]. To circumvent the anticipated immune rejection resulting from unmatched cell transplantation [11], it would be ideal to use stem cells derived from patients for stem cell therapy, particularly for a disease such as Friedreich ataxia (FRDA) where there is a reduced level of normal protein produced in affected individuals.

FRDA, the most common inherited ataxia, is an autosomal recessive disease characterised by onset of neurodegeneration and cardiomyopathy, generally late in childhood. FRDA patients present symptoms including ataxia, loss of coordination, abnormal speech, heart disease, muscle weakness, diabetes, scoliosis, hearing and vision loss. In most affected individuals, the disease is caused by the presence of an expanded trinucleotide repeat sequence (GAA) in the first intron of both copies of the *FXN* gene, causing reduced levels of *FXN* transcript and lower synthesis of the protein frataxin, encoded by nuclear DNA but localised to the mitochondrion. There is an inverse correlation between GAA expansion length and transcript levels, the amount of residual frataxin synthesised, and the age of onset and severity of the disease [12–14]. The GAA expansion does not alter the coding sequence of the gene or the amino acid sequence of the resultant protein, but only affects the level of mRNA and protein synthesis. Up-regulation of *FXN* gene expression would be therapeutic, and even a relatively small increase could restore cellular frataxin to a level that may provide clinical benefit to patients.

## Materials and Methods

*Ethics Statement* This research was approved by the Human Research Ethics Committee of the University of Melbourne (HREC 0829937) and Monash Institutional Bioethics Committee (PC2-N39/08). Written informed consent was obtained from all participants. All animal experiments were approved by the Monash University Animal Ethics Committee (SOBSA/MIS/2006/40) in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes.

*Skin Biopsies/Fibroblasts* Fibroblast epithelial cells were obtained by non-keratinised skin biopsy of individuals with FRDA and control subjects over the age of 18 years (Table 1). The biopsy measured approximately 2–3 mm in diameter and was taken via a needle from the inside of the forearm by a qualified clinician. The risks associated with a skin biopsy are small, however the risks of bleeding and infection were minimised through careful technique, the use of antiseptics and sterile instruments. Minimal pain was experienced at the site once the anaesthetic has worn off and none of the individuals reported any complications following this procedure. Cells were maintained in basal Eagles medium supplemented with 10% fetal bovine serum, 25 U/mL penicillin, 25 μg/mL streptomycin and fungizon.

*Retroviral Production, iPS Cell Generation and Maintenance* Moloney-based retroviral vectors (pMXs) containing the human complementary DNAs (cDNAs) coding for OCT-4, SOX2, KLF4 and c-MYC were obtained from Addgene. Nine μg of each plasmid was transfected into viral packaging Plat-A cells by Fugene 6 (Roche). Virus-containing supernatants were collected 48 h and 72 h post-transfection, filtered through a 0.45 μm pore-size filter and supplemented with 4 μg/mL of polybrene (Sigma-Aldrich, Castle Hill, NSW, Australia). Equal parts of the four transcription factor-containing retroviral supernatants were added to the fibroblast cell lines, which had been plated 24 h before infection at density of $1\times10^4$ cells/cm$^2$. Two rounds of infection were performed over 48 h. At day four after infection, the culture medium was changed to hES/iPS cell medium, consisting of Dulbecco's Modified Eagle Medium (DMEM, without sodium pyruvate, glucose 4500 mg/L), supplemented with 1% insulin/transferrin/selenium, 0.1 mM 2-mercaptoethanol, 1% Non Essential Amino Acids (NEAA), 2 mM glutamine,25 U/mL penicillin, 25 μg/mLstreptomycin (all from Invitrogen), 20% fetal calf serum (Hi-Clone) and 10 ng/mL human recombinant basic fibroblast growth factor (bFGF, Peprotech, Rocky Hill, NJ). The medium was changed every day for three to four weeks. To establish iPS cell lines, iPS cell colonies were picked at three to four weeks post-infection, based on

**Table 1** Information on patients used for generation of FA3-iPS and FA4-iPS cell lines

| Code | Age | Gender | FARS | GAA1 | GAA2 | Age of onset | Disease Duration |
|---|---|---|---|---|---|---|---|
| FA3 | 32 | Male | 63 | 527 | 1058 | 21 | 11 |
| FA4 | 48 | Female | 92.5 | 751 | 1027 | 20 | 27 |

Friedreich Ataxia Rating Scale. GAA1: smaller allele repeats; GAA2: longer allele repeats

hES cell-like colony morphology. The picked colonies were expanded and cultured in the presence of mitotically inactivated mouse embryonic fibroblasts (MEF) in iPS medium. The cells were incubated 37°C in an atmosphere of 5% $CO_2$ in air with 95% relative humidity. The culture medium was changed every second day.

*Reverse Transcription–Polymerase Chain Reaction Experiments* Total RNA was isolated from iPS colonies using the RNAeasy Micro kit (Qiagen) and cDNA synthesis was performed using Superscript III Reverse Transcriptase (Invitrogen). The cDNA samples were amplified by polymerase chain reaction (PCR) using Taq DNA polymerase Super Script III (Invitrogen) with sense and antisense primers (Geneworks, Hindmarsh, SA, Australia) designed for detection of human DNA target sequences (Table 2). Genomic DNA was isolated from iPS cell lines and fibroblasts using previously published protocols [15]. The amplified DNA fragments were analysed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide. Molecular sizes (bp) were calculated using 1 kb plus DNA ladder markers (M). The amplicons were purified, sequenced and corresponded to those of the expected human cDNA (data not shown).

Real-Time Quantitative RT-PCR

Total RNA was extracted from fibroblasts, iPS cells and differentiated iPS cells (differentiated EBs and neural crest progenitors) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration and purity of the RNA samples were assessed using the Nanodrop1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Prior to using these samples further, a minimum ratio of absorbance at 260 nm and 280 nm of two was considered as pure RNA. The total RNA fractions of each sample were then converted to cDNA using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA), and used as template for real-time quantitative polymerase chain reaction (qPCR) to compare gene expression. Q-PCRs were carried out using TaqMan Universal master mix (Applied Biosystems) and the 7900HT Fast Real-Time PCR system (Applied Biosystems). TaqMan gene expression assay for *FXN* (Hs00175940_m1) and for a range of cardiac markers: actin alpha cardiac muscle 1 (*ACTC1*; Hs01109515_m1), troponin T type 2 (*TNNT2*; Hs00165960_m1), troponin I (*TNNI3*; Hs00165957_m1) and NK2 transcription factor related, locus 5 (*NKX2.5*; Hs00231763_m1) were used

**Table 2** Sense and antisense primers

| Gene name | Forward primer (5' to 3') | Reverse primer (5' to 3') |
|---|---|---|
| For transgenes and endogenous genes PCR and RT-PCR | | |
| OCT3/4 | TATGCACAACGAGAGGATTTTG | TGTGTCCCAGGCTTCTTTATTT |
| SOX-2 | CACCTACAGCATGTCCTACTCG | GGTTTTCTCCATGCTGTTTCTT |
| KLF4 | TACAAAGAGTTCCCATCTCAAGG | TGGATTCCTCATTTTTCCTGAT |
| c-MYC | CTGAGACAGATCAGCAACAACC | TCTTTTATGCCCAAAGTCCAAT |
| NANOG | TCAATGATAGATTTCAGAGACAG | GGGTAGGTAGGTGCTGAGGC |
| REX1 | GGAAATAGCAGAGTGCTTCGC | CTCTTCTGTTCTGTTCACACAG |
| OCT3/4 (transgene) | CTAGTTAATTAAGAATCCCAGTG | CACTAGCCCCACTCCAACCT |
| SOX2 (transgene) | CTAGTTAATTAAGGATCCCAGG | TGTTGTGCATCTTGGGGTTCT |
| KLF4 (transgene) | ACAAAGAGTTCCCATCTCAAGGTG | TCCAAGCTAGCTTGCCAAACCTACAGG |
| c-MYC (transgene) | CTAGTTAATTAAGGATCCCAGTG | CAGCAGCTCGAATTTCTTCC |
| For bisulfite-sequencing PCR | | |
| OCT3/4 (1) | GGATGTTATTAAGATGAAGATAGTTGG | CCTAAACTCCCCTTCAAAATCTATT |
| OCT3/4 (2) | TAGTTGGGATGTGTAGAGTTTGAGA | TAAACCAAAACAATCCTTCTACTCC |

(Applied Biosystems). The relative quantitation was achieved by applying the comparative $C_T$ method ($\Delta\Delta C_T$) whereby the mRNA levels were normalized against the level of the hypoxanthine phosphoribosyltransferase 1 *(HPRT1)* mRNA (Human *HPRT1* Endogenous Control, Applied Biosystems, 4333768 F) and the control group was used as the calibrator. Data are expressed as mean±standard error of the mean (SEM). Significance of the differences was evaluated using t-tests and one way ANOVA followed by Newman Keuls. Statistical significance was established at $*p<0.05$, $**p<0.01$ and $***p<0.001$.

*Immunofluorescence* Cells were fixed with 4% paraformaldehyde (PFA) or ethanol (for OCT-4), blocked in 10% fetal calf serum-PBT, and immunostained using the following antibodies: mouse monoclonal anti-OCT3/4 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-nanog (eBioscience), mouse TG-30 reactive with CD9 (gift from Prof. M. Pera), mouse anti-Pax6 (Developmental Hybridoma Bank), mouse anti-S100β protein (Sigma-Aldrich), mouse anti-βIII-tubulin (Millipore, Billerica, MA, USA), rabbit anti-p75 (Promega), mouse anti-Peripherin (Sigma), mouse anti-HNK1 (gift from Dr D. Newgreen), and mouse anti-Neurofilament 200 (Sigma-Aldrich). Cells were then immunostained with the appropriate conjugated secondary antibodies (Alexa Fluor 568 or 488, Molecular probes-Invitrogen). Nuclei were counter-stained with Hoechst-33342 (Sigma-Aldrich). Specificity of the staining was verified by the absence of staining in negative controls consisting of the appropriate negative control immunoglobulin fraction (Dako). Assessment of long-term expression of stem cell markers was performed on FA3-iPS and FA4-iPS at different passages (p).

*GAA Expansion Analyses* Genomic DNA for GAA expansion analyses was extracted from fibroblast and iPS cells using the Gentra Puregene DNA extraction kit (QIAGEN Inc., Hilden, Germany) as per the manufacturer's instructions. The concentration and purity of the genomic DNA were assessed using the Nanodrop1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The size of the GAA expansion in intron 1 of the *FXN* gene was determined by PCR using the Expand Long Range dNTPack (Roche, Australia) with 50–100 ng template DNA and 0.4 μM of EXP-Bam-F 5′AAGGAAGTGGTA GAGGGTGTTTCACGAGGA3′ and EXP-Bam-R 5′ TTTGGATCCAACTCTGCTGACAACCCATGCTGTC CACA3′ primers. PCR products were electrophoresed on a 1% (*w*/*v*) agarose, 1xTAE gel alongside standard DNA markers designated M1 (1Kb Plus; Invitrogen, USA) and M2 (200 bp ladder, Promega, Australia). Size determination was performed using Eagle Sight, V3.0 (Stratagene, USA). The positive control (BAC clone RP11-265B8) and non-expanded alleles in the normal range yielded an 810 bp fragment.

*Bisulfite Genomic Sequencing* Genomic DNA (1 μg) from FA3- and FA4-iPS cells, their original fibroblasts and hES cell line MEL-1 were processed for bisulfite modification using CpGemone DNA modification kit (Millipore). One conserved CpG-enriched region in the Oct-4 promoter and one conserved CpG-enriched region in the Nanog promoter were selected to be amplified by PCR as described by Freburg *et al*. [16]. The PCR products were subcloned into pGEMT-easy vector (Promega). Ten clones of each sample were verified by sequencing with Sp6 universal primer and the methylation status was determined using BiQ Analyzer software [17]. All primer sequences are given in Table 2.

*Neural and Neural Crest Induction of FRDA Cell Lines* Neural induction by noggin (500 ng/mL, R&D) was performed as described [18]. Noggin-treated cells were harvested after 14 days by mechanical dissection and were further subcultured in suspension in low-attachment 96-well plates (Corning) containing neurobasal medium (NBM) with bFGF and EGF (20 ng/mL each, R&D) as neurospheres [19]. After 2 weeks of growth in suspension culture, the neurospheres were plated as previously described onto laminin- or fibronectin-coated glass chamber slides in NBM without growth factors for 5 days [19]. NBM was changed every second day. Differentiation towards neural crest-like cells was performed using a previously published protocol [20]. Briefly, iPS-derived neurospheres were plated onto a feeder layer of MEFs in NBM supplemented with bFGF and EGF (20 ng/mL each). After 24 h, cultures were treated with the small molecule Rho/ROCK inhibitor Y27632 at a final concentration of 25 μM to promote delamination of neural crest-like cells. For differentiation to sensory neurons, the same neural crest induction procedure was used and 24 h after treatment with Y27632, cultures were treated for a further 2 days in NBM supplemented with 10 ng/ml BMP2, 10 ng/ml BMP4 and 25 μM Y27632. Cultures were then maintained for 1 week in NBM supplemented with 10 ng/ml BMP2 and 10 ng/ml BMP4, followed by another week in NBM only. Cultures were then fixed and processed for immunohistochemistry analyses.

*Embryoid Body (EB) Formation* EB formation was performed as previously described [21]. Briefly, undifferentiated iPS cells were harvested after 6–13 days by mechanical dissociation and further subcultured in suspension in low-attachment plates (Corning) in medium (KSR medium) containing DMEM-F12, supplemented with 0.1 mM 2-mercaptoethanol, 1% NEAA, 2 mM glutamine, 25 U/mL

penicillin, 25 μg/mL streptomycin and supplemented with 20% KSR (all from Invitrogen). Medium was changed every second day. After 8 days in culture, EBs were cytospun onto glass chamber slides (4 min at 1000 rpm, Shandon Cytospin 4, ThermoFisher Scientific), air dried and fixed with 4% PFA. Cells were then blocked in 10% fetal calf serum-PBT, immunostained with markers of endoderm (rabbit anti-alpha-fetoprotein (AFP), Dako), mesoderm (mouse anti-CD31, Dako; rabbit anti-c-kit, Santa Cruz) or ectoderm (mouse anti-nestin, Millipore) and the appropriate conjugated secondary antibodies (Alexa Fluor 568 or 488, Molecular probes-Invitrogen). Slides were mounted and nuclei were counter-stained using Prolong Gold antifade reagent with DAPI (Invitrogen). Specificity of the staining was verified by the absence of staining in negative controls consisting of the appropriate negative control immunoglobulin fraction (Dako, data not shown).

*Cardiac Differentiation* Eight day-EBs were plated onto 0.1% gelatine-coated dishes in KSR medium for at least 19 days. Medium was changed every second day. Cardiomyocytes were identified by the presence of beating areas.

*Karyotyping* G-banding of FA3-iPS (p11, 20 cells), FA4-iPS (p7, 20 cells) cell colonies was performed by Southern Cross Pathology Australia, Clayton, Australia using standard techniques.

*Teratoma Formation* FA3-iPS and FA4-iPS cell colonies (all at p4 and p5) with an undifferentiated morphology were mechanically harvested and injected into testis of severe combined immunodeficiency (SCID; NOD.CB17-prkdcscid/Jasmu) male mice [1]. Seven weeks later, the resulting teratomas were removed and fixed in HistoChoice (Amresco), and embedded in paraffin. Histological examination was performed on hematoxylin and eosin stained sections.

## Results

### Generation of iPS Cell Lines

Two FRDA patient skin fibroblast cell lines were successfully reprogrammed into iPS cells (FA3-iPS and FA4-iPS cells). Fibroblast cultures were grown from skin biopsies obtained from FRDA patients (Table 1). The fibroblast cell lines FA3 and FA4 were expanded to passage 5 prior to retroviral infection with Moloney-based retroviral vectors (pMXs) containing the human cDNA encoding *OCT-4, SOX2, c-MYC* and *KLF4* cDNAs. Nine days following infection, the fibroblasts displayed cell clumping (Fig. 1b), and by day 15 clumps of aggregated cells could be observed in the culture plates (Fig. 1c). Cells in the aggregated clumps exhibited a hES cell-like morphology with a high nucleus/cytoplasm ratio (Fig. 1d). Four weeks after infection, the colonies grew larger and were picked and expanded (Fig. 1e, f). The two FA-iPS cell lines grew robustly when maintained on mouse embryonic fibroblast (MEF) feeder layers.

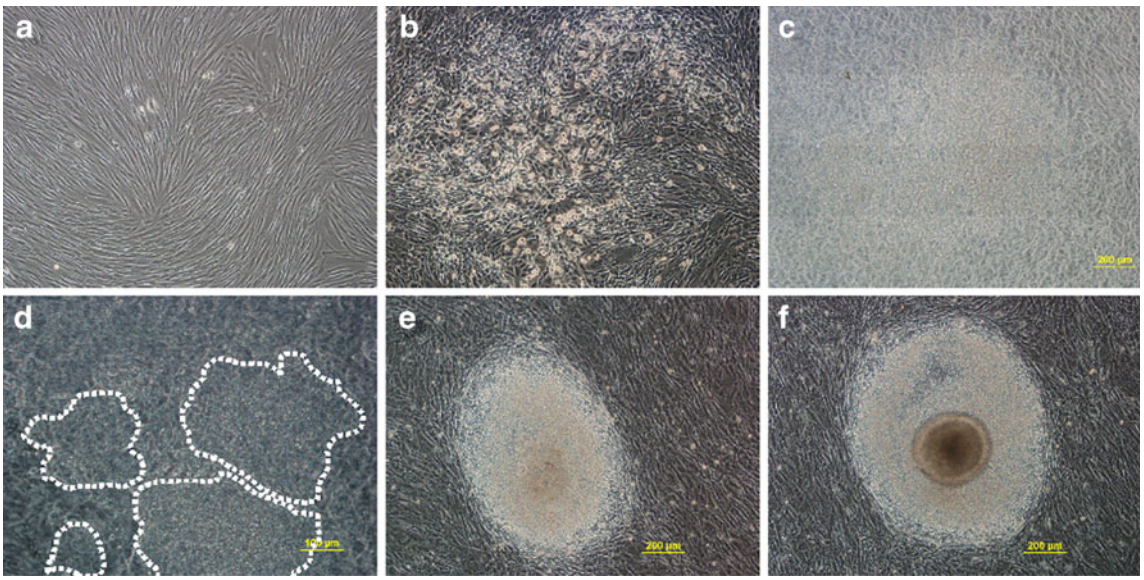


**Fig. 1** Generation of iPS cell lines from FRDA patients. Bright field photographs representative of iPS cell lines obtained from FRDA patients. **a** FA4 p5 fibroblasts following retroviral infection; **b** primary clumping of cells 8 days following infection; **c** aggregated cell clumps formed 15 days following infection; **d** hES cell-like cells observed in the colonies (dotted area) 21 days following infection; **e** FA-iPS cells following manual dissection of 4-week colonies (p1) and **f** FA4-iPS cells (p2)

## Characterization of iPS Cell Lines

Long-term culture and assessment of expression of stem cell markers confirmed successful maintenance of both FA-iPS cell lines, which have now been cultured for more than fifteen passages. Immunocytochemistry analysis demonstrated the continuing presence of pluripotent markers such as NANOG, OCT-4 and TG-30 in both cell lines (Fig. 2a–c) and no chromosomal abnormality was observed by G-banding (Fig. 2d).

We investigated the DNA methylation status of CpG dinucleotides in two CpG-rich regions in the *OCT-4* promoter [16]. Bisulfite genomic sequencing analysis showed that both *OCT-4* promoter regions were demethylated in the two FA-iPS cell lines, similar to hES cells (MEL-1), whereas the same regions were highly methylated in parental FA fibroblasts (FA3, FA4) (Fig. 2e). RT-PCR analyses showed that the FA3-iPS cells and FA4-iPS cells expressed endogenous *OCT-4, SOX2, NANOG* and *REX1* pluripotent marker genes as did the hES cells (MEL-1), while no transcript was detected in their parental fibroblasts (Suppl. Fig. 1). Endogenous *KLF4* and c-*MYC* transcripts were not found in FA4-iPS cells and MEL-1 cells. Similarly, no c-*MYC* expression and only weak expression of endogenous *KLF4* was detected in FA3-iPS cells. However, both *KLF4* and *c-MYC* were expressed in the parental fibroblasts (Suppl. Fig. 1). Exogenous *OCT-4* and *SOX2* transgenes were effectively silenced in both iPS cell lines, while *KLF4* (in FA4-iPS cells) and *c-MYC* (in both cell lines) transgenes remained detectable (Suppl. Fig. 1a). Genomic DNA PCR showed that both FA3- and FA4-iPS cells harboured integrated transgenes of the four transcription factors (Suppl. Fig. 1b).

## Assessment of Pluripotency of iPS Cell Lines

FA3-iPS cells and FA4-iPS cells gave rise to teratomas containing tissues representative of the three embryonic germ layers after inoculation in the testis of SCID mice (Suppl. Fig. 1). FA-iPS cells responded to noggin treatment and neural induction by forming neurospheres that could then be further differentiated to neuronal and glial cells

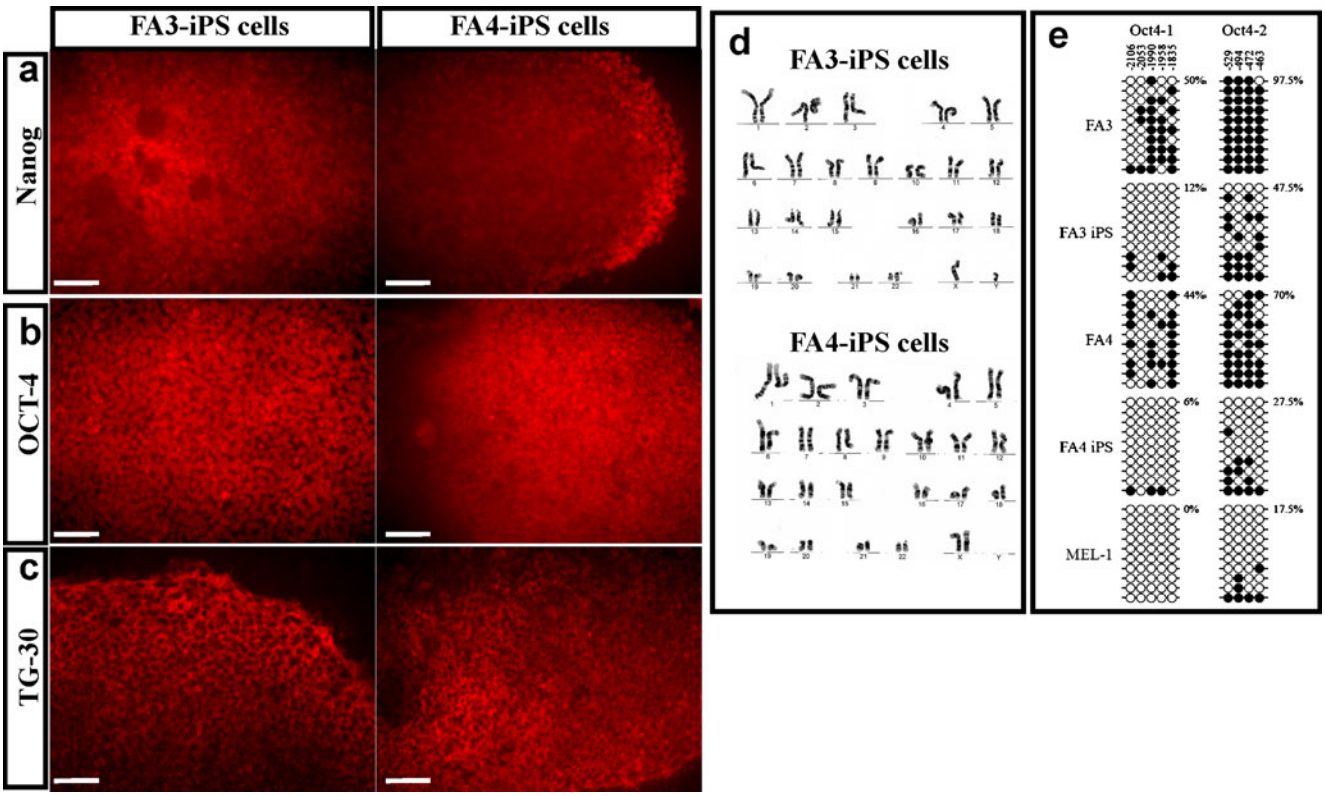


**Fig. 2** Characterization of human iPS cell lines FA3 and FA4. Immunostaining of FA3-iPS (p5) and FA4-iPS (p5) cells with **a** NANOG, **b** OCT-4, **c** TG-30. Scale bars: 100 μm. **d** Karyotyping of FA3-iPS (p11, 20 cells) and FA4-iPS cells (p7, 20 cells). **e** Methylation analyses by bisulfite sequencing analysis of *OCT-4* methylation in FA3- and FA4-fibroblasts and corresponding iPS cells. The hES cell line MEL-1 was analysed as a control. Numbers at the top indicate the CpG position relative to the transcription start site. Global percentages of methylated cytosines (% Me) are shown. Each row of circles for a given amplicon represents the methylation status of each CpG in one bacterial clone for the region. Ten clones are shown. Open and filled circles indicate unmethylated and methylated CpG dinucleotides, respectively

(Fig. 3). Both of the FA-iPS cell lines formed embryoid bodies (EBs) containing cells representative of the three germ layers (Suppl. Fig. 3).

### Assessment of GAA Expansion and *FXN* Level in Fibroblasts and iPS Cell Lines

The GAA expansions for each allele within the *FXN* gene were detected to be about 532 and 1026 triple repeats for the FA3 fibroblasts and 226 and 718 triple repeats for the FA4 fibroblasts. The generation of iPS cells from FA3 and FA4 fibroblasts showed both an expansion and a contraction of the GAA triple repeats after reprogramming (Fig 4a, Suppl. Fig. 4). There was also variation of GAA repeat length across different colonies for FA3-iPS cells, highlighting GAA repeat instability across cells (Fig. 4a, Suppl. Fig. 1). Importantly, quantitative RT-PCR (qPCR) for *FXN* mRNA revealed that both fibroblasts and iPS cells generated from FRDA patients have statistically significant lower levels of FXN mRNA than control fibroblasts and iPS cells (Fig. 4b, c). These data demonstrate that iPS cell lines generated from FRDA patients maintain the GAA expansion and low *FXN* mRNA levels.

### FA-iPS Cells Differentiate into Cell Types Affected in FRDA

Sensory neurons of the dorsal root ganglia (DRG) are severely affected and die in FRDA [22]. Using a protocol we recently described for the differentiation of hES cells into neural crest-like cells [20], which are progenitors to peripheral DRG sensory neurons, we differentiated the two FA-iPS cell lines into migrating p75+ and HNK+cells, properties characteristic of neural crest (Fig. 5). Neural crest progenitors derived from FA3-iPS cells were further differentiated towards peripheral sensory neurons with BMP2 and BMP4 treatment. Differentiated cultures showed bipolar, peripherin-expressing neurons (Fig. 5), which are morphological and phenotypic characteristics of sensory and peripheral neurons, respectively.

Cardiomyocytes are also affected in FRDA [22]. Q-PCR analysis performed on plated EBs from both FA-iPS cell lines revealed the expression and upregulation of various cardiac lineage markers such as *NKX2.5, ACTC1* and *TNNT2* when compared to their respective undifferentiated state (Fig. 6a, b). *TNNI3* was found to be expressed in both cell line-derived EBs and upregulated in FA4 (Fig. 6a, b). Furthermore, plated EBs from both FA-iPS cell lines generated spontaneously beating cells, representative of functional cardiomyocytes (Suppl. Movies 1, 2). Lastly, assessment of *FXN* levels in FA3 iPS cells revealed that both differentiated EBs and neural crest progenitors from FA-iPS cells retained low levels of *FXN* mRNA, confirming the ability of the iPS cell lines to differentiate into cells representative of the cardiac and neural lineages that maintain low *FXN* mRNA levels (Fig. 6c).

## Discussion

Pluripotent stem cell lines from patients with a clinically complex inherited disease such as FRDA are of interest for two reasons. First, they represent a possible reagent to offer stem cell therapy, where they will be less hazardous than

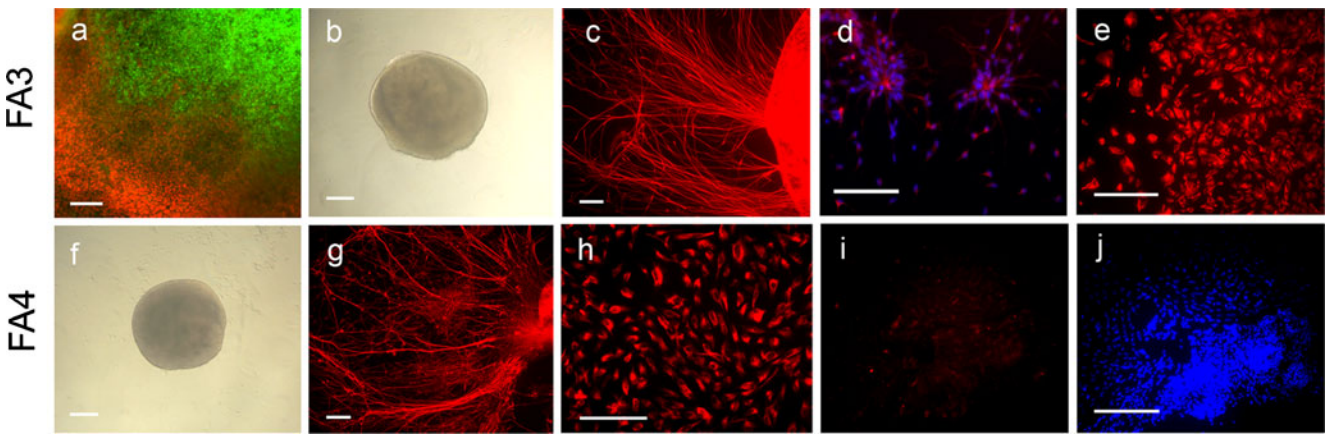


**Fig. 3** Neural differentiation of FRDA iPS cell lines. a FA3-iPS cells treated with noggin for 14 days are induced to differentiate to neural lineage as shown by Pax6 (green) expression. Undifferentiated cells retain OCT-4 expression (red). **b** Neurosphere derived from noggin-treated FA3-iPS cells. **c** FA3 iPS-derived neurospheres differentiated to neurons show expression of βIII tubulin and neurofilament 200 **(d)**. **e** Glial differentiation of FA3-iPS-derived neurospheres, show expression of S100β. **f** Neurosphere derived from noggin-treated FA4 iPS cells. **g** FA4-iPS-derived neurospheres differentiated to neurons and glia show expression of βIII tubulin **(g)** and S100β **(h),** respectively. **i** Negative control mouse IgG immunostaining and corresponding Dapi counterstain **(j)** of differentiated neurospheres derived from FA4-iPS cells. **(a, c–e, g–j)** scale bar: 100 μm, **(b, f)** scale bar: 200 μm

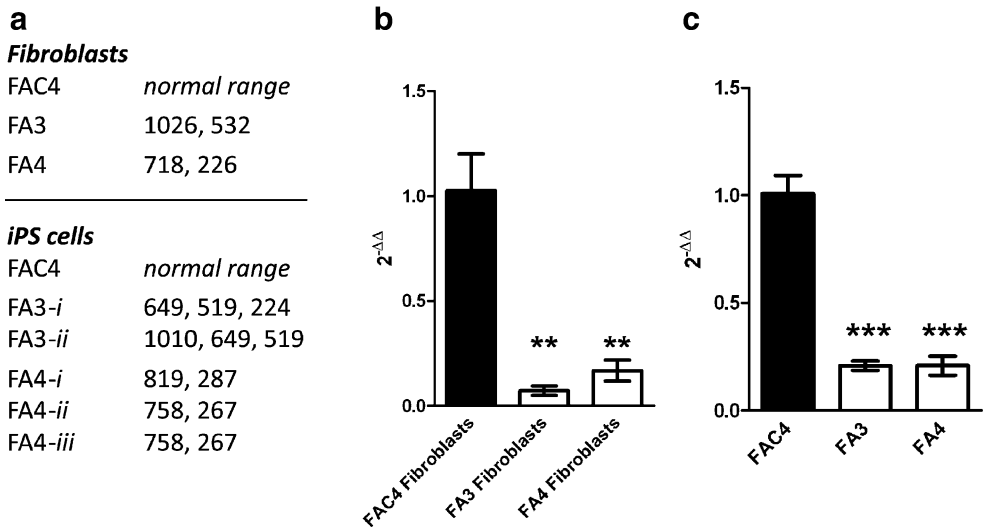


**Fig. 4** Assessment of GAA expansion and *FXN* level in fibroblasts and iPS cell lines. a Number of GAA triplet repeats in control (FAC4) and FRDA (FA3, FA4) fibroblast and iPS cells. Multiple repeats are observed in each sample since multiple sub-clones were pooled. **b, c** *FXN* qPCR for FAC4, FA3 and FA4 fibroblasts **(b)** and iPS cells **(c)** showing reduced levels of *FXN* mRNA expression in the FRDA samples. Data are representative of three independent experiments and are expressed as mean±SEM of $2^{-\Delta\Delta ct}$ (*FXN*) with data normalized to expression levels of HPRT1 and relative to FAC4. Statistical significance was established at *$p<0.05$, **$p<0.01$ and ***$p<0.001$ by *t*-test

cells derived from an unrelated embryo or donor since they share all immunological determinants with the patient from whom they are derived. Second, they may provide a cellular model that accurately reflects the molecular and cellular nature of the disease, both for basic scientific understanding of disease mechanisms and for development of drug screening assays. The value of stem cells as an *in vitro* model is enhanced for diseases such as for FRDA, where the variability in clinical severity is still poorly understood.

While animal models for some Mendelian-inherited diseases, such as thalassaemia, have led to significant improvements in our understanding of the disorder, others (such as FRDA) pose difficulties due to problems working

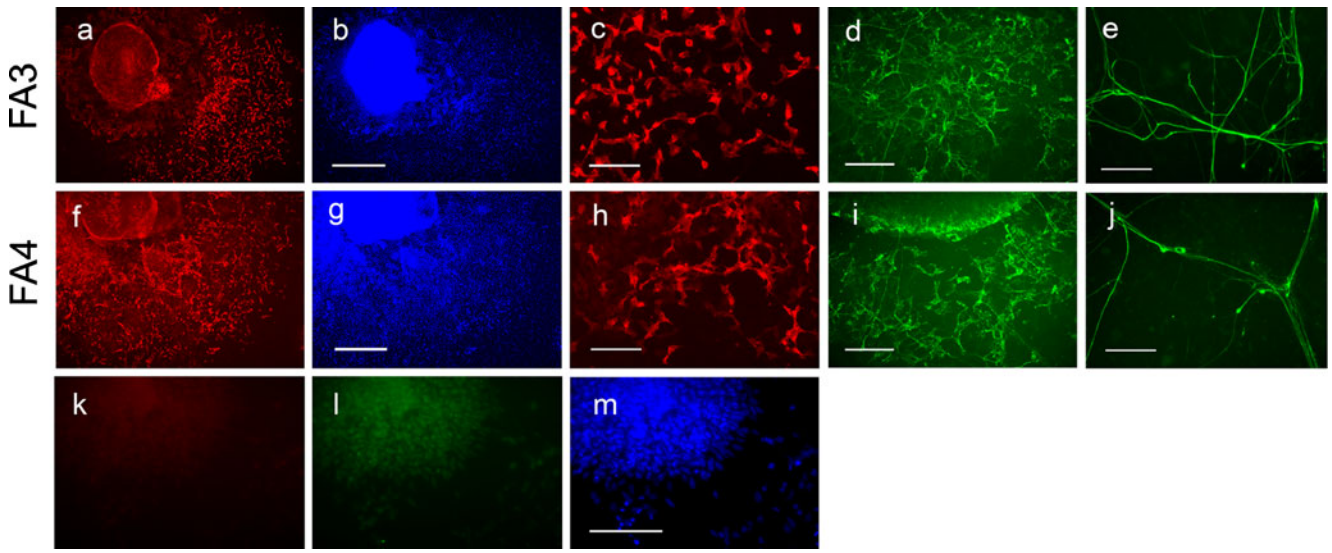


**Fig. 5** Generation of neural crest-like cells and peripheral neurons from FRDA iPS cells. FA3-iPS (**a–e**) and FA4-iPS (**f–j**) cells were differentiated to neural crest-like cells, as shown by expression of p75 (**a, c, f, h**) and HNK1 **(d, i)**. **b, g** Dapi counterstains of 'a' and 'f' images, respectively. **(e, j)** Neural crest-like progenitors derived from FA3- (**e**) and FA4- (**j**) iPS cells were differentiated towards sensory neurons by BMP treatment, resulting in bi-polar neurons that express the peripheral neuronal marker, peripherin. Negative control mouse IgG (**k**) and rabbit IgG (**l**) immunostaining images of FA3 neural crest-like progenitors. **m** Dapi counterstain of 'k' and 'l'. (**a–b, f–g)** Scale bars: 200 μm; (**c–e, h–m**): 100 μm

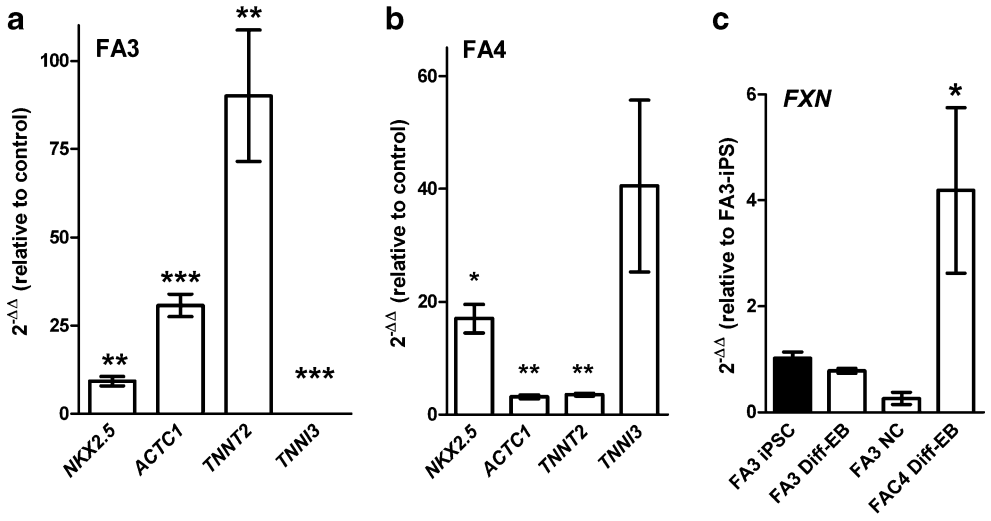


**Fig. 6** Generation of cardiac cells from FRDA iPS cells and *FXN* levels in differentiated EBs and neural crest progenitors. **a, b** Q-PCR analysis performed on plated EBs from FA3- (**a**) and FA4- (**b**) iPS cell lines for *Nkx2.5, ACTC1*, *TNNT2* and *TNNI3*. Data are representative of independent experiments performed in triplicate and are expressed as ± SEM of $2^{-\Delta\Delta ct}$ with data normalized to expression levels of *HPRT1* and relative to their respective undifferentiated iPS cells. Statistical significance was established at $*p<0.05$, $**p<0.01$ and $***p<0.001$ by t-test. **c** *FXN* qPCR performed on undifferentiated FA3-iPS cells (FA3 iPSC), plated EBs from FA3-iPS cells (FA Diff-EB), neural crest progenitors derived from FA3-iPS cells (FA3 NC) and plated EBs from FAC4 iPS cells (FAC4 Diff-EB). Data are representative of independent experiments performed in triplicate and are expressed as mean±SEM of $2^{-\Delta\Delta ct}$ with data normalized to expression levels of *HPRT1* and relative to FA3 iPSC. Statistical significance was established at $*p<0.05$, $**p<0.01$ and $***p<0.001$ by one-way ANOVA followed by Newman Keuls test (**c**)

with unstable trinucleotide repeats in genomic DNA. The transgenic and knock-out animal models available to date for FRDA do not accurately reflect the disease: they are either mildly symptomatic [23, 24] or engineered to be tissue-specific *Fxn* knock-outs only [25]. FRDA cell lines from patients have been generated previously, but these have been from cell types that are not primarily affected, such as lymphoblast and fibroblast cells [26]. Affected cell types such as cardiomyocytes or dorsal root ganglion neurons represent a much better cell type to screen candidate drugs and other potential therapies. In this report we demonstrate that pluripotent stem cells can be derived from FRDA patients using iPS cell technology, and that these iPS cells can be differentiated to cell type lineages primarily affected in the disease, cardiomyocytes and sensory neural progenitors. Furthermore, *FXN l*evels were consistently reduced in FRDA iPS cells and their derivatives. This data demonstrates that FRDA iPS cell lines can be utilized to study degenerative mechanisms occurring in specific cell types. Such studies would involve extensive characterization of specific cells derived from FRDA iPS cells, such as cardiomyocytes and DRG sensory neurons, to examine function, survival, mitochondrial activity and epigenetic modifications associated with the disease. Derivation of a human cellular system that accurately models the effects of the genetic defect causing FRDA is highly valuable for studying disease progression as well as providing an infinite source of diseased cell types for establishing high-throughput drug screening assays.

The potential therapeutic application of FRDA iPS cells requires correction of the *FXN* mutations. We have previously shown that human pluripotent stem cells from cord blood can be genetically modified by stable transfection with BAC clones containing a copy of the normal *FXN* gene [27], which is expressed when the cells are differentiated into neurons. It is therefore feasible that a similar approach will allow correction of the FRDA gene defect in the iPS cells.

For clinical therapies, non-viral approaches would need to be used to generate FA iPS cell lines. The FA iPS cell lines described in our study were developed using murine molony leukaemia virus (MMLV)-based retroviral expression of *OCT-4*, *SOX2*, *c-MYC* and *KLF4*. Ideally, retroviral-mediated expression of these four factors should be silenced during iPS cell derivation and complemented by reactivated expression from the endogenous gene loci [3, 5]. We analysed the expression of the endogenous loci and retroviral transgenes, and found that expression of endogenous *OCT-4* and *SOX2* loci were reactivated to levels similar to those found in hESC. This was accompanied by complete silencing of exogenous viral factors in the FA3- and FA4-iPS cells. However, there was variable but persistent expression of the retroviral *c-MYC* and *KLF4* transgenes in the iPS cell lines, a trend previously reported for other human iPS cells [7, 10, 28–31] and pig iPS cells

[32, 33]. Myc genes (including c-Myc) are key regulators of cell proliferation. It has been demonstrated that it is expressed in fibroblasts [34]. KLFs regulate cell-fate decisions, including development and differentiation of specific tissues. KLF4 is highly expressed in epithelial tissues [35]. Our RT-PCR analysis showed that MEL-1 cells, a human ES cell line, expressed low level endogenous KLF4 and c-MYC in our culture conditions. The generated iPS cells in this study expressed a similar low level of endogenous KLF and c-MYC expressions, indicating a down-regulation of the both genes comparing with the expressions in their parental fibroblasts. Nevertheless, the consequence of this persistent expression in iPS cells might indicate a potential for cell transformation. Therefore, new approaches to deliver reprogramming factors to the target cells, especially non-integrated methods, need to be investigated and optimized.

Interestingly, reprogramming of FRDA fibroblasts to iPS cells resulted in both expansions and contractions of the GAA triplet repeats within the *FXN* locus. Differences between expansion size at diagnosis (done on a blood sample DNA; Table 1) and new iPS cell analyses is not surprising. Indeed, many factors can cause such changes, including the fact that these are different cells types (blood versus fibroblast versus iPS cells), viral transduction/pluripotency process, and culturing factors in between. Furthermore, variation of GAA repeat lengths have been found in different cell types within the same FRDA patient or FRDA mouse model, with higher expansions observed in heart, brain and dorsal root ganglia tissue [36–39]. It has also been shown that the GAA repeat can progressively lengthen with age, especially within degenerating tissues such as cerebellum and DRG [38, 40].

The instability of GAA repeat length found within the FA-iPS cell lines may be a reflection of underlying mechanisms occurring in patients with FRDA rather than a cell culture artefact. This reinforces the usefulness of having FA-iPS cell lines to study epigenetic changes that take place within the *FXN* locus during disease progression. In summary, FA-iPS cell lines would provide a useful human cellular model system to study FRDA, as well as for drug discovery and the development of cell replacement therapies.

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