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Lab resource: Stem Cell Line

Induced pluripotent stem cell line (INSAi002-A) from a Fabry Disease patient hemizygote for the rare p.W287X mutation

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A B S T R A C T

Fabry Disease (FD) is a multisystemic X-linked disorder that belongs to the group of lysosomal storage disorders (LSDs). Causal mutations on alpha-galactosidase A (α-Gal A) commonly lead to abnormal protein and consequently to FD. Since it is an X-linked disease, males are primarily affected. This work describes the generation of induced Pluripotent Stem Cells (iPSCs) from skin fibroblasts from a FD patient, using non-integrative episomal vectors. Differentiation of iPSCs can be applied to generate a variety of cell types with high degree of genetic complexity that would otherwise be difficult to obtain.

Resource table

Unique stem cell line identifier	INSAi002-A
Alternative name(s) of stem cell line	FD-1
Institution	INSA
Contact information of distributor	Olga Amaral
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: Sex: male
Cell Source	Skin fibroblasts (Istituto “Giannina Gaslini” Biobank)
Clonality	Clonal
Method of reprogramming	Epi5 Episomal iPSC Reprogramming Kit
Genetic Modification	Yes
Type of Modification	Familial
Associated disease	Fabry Disease
Gene/locus	FD gene (Gene ID: 2717)/locus Xq22.1; Mutation in hemizygosity; p.W287X (rs104894839)
Method of modification	Not applicable
Name of transgene or resistance	Not applicable
Inducible/constitutive system	Not applicable
Date archived/stock date	01/05/2019
Cell line repository/bank	The cell line has not been deposited in a stem cell bank or repository but is physically cryopreserved at INSA's facilities in CSPGF in Porto, Portugal.
Ethical approval	Original fibroblast line was obtained from a non-commercial provider, Gaslini Istitute Biobank under Genetic Biobank Guidelines, Telethon - SIGU, Analysis 4/5, 2003. Italian Data Protection Authority (Garante Privacy), General Authorization for the processing of gene data n°8/2016. INSA project approval code 2015DGH1073.

1. Resource utility

In this work, we report the establishment of an iPSC line from a Fabry disease (FD) patient. The starting biological material was skin fibroblasts. Skin biopsies are not difficult to get and provide an easy to obtain material for generating iPSCs. The development of iPSCs and its application to the LSD field is contributing significantly to new strategies for pathogenesis modeling and drug testing.

This report presents the establishment of iPSCs derived from Fabry disease (FD, ORPHA324, OMIM:301,500) patient's fibroblasts. FD is one of the most common LSDs, it is X-linked and caused by mutations in the alpha-galactosidase A gene (*GLA*, Xq21.3-q22, OMIM: 300,644), which lead to the pathogenic deficient activity of the lysosomal hydrolase alpha-galactosidase A (α-Gal A, EC 3.2.1.22). This deficiency results in

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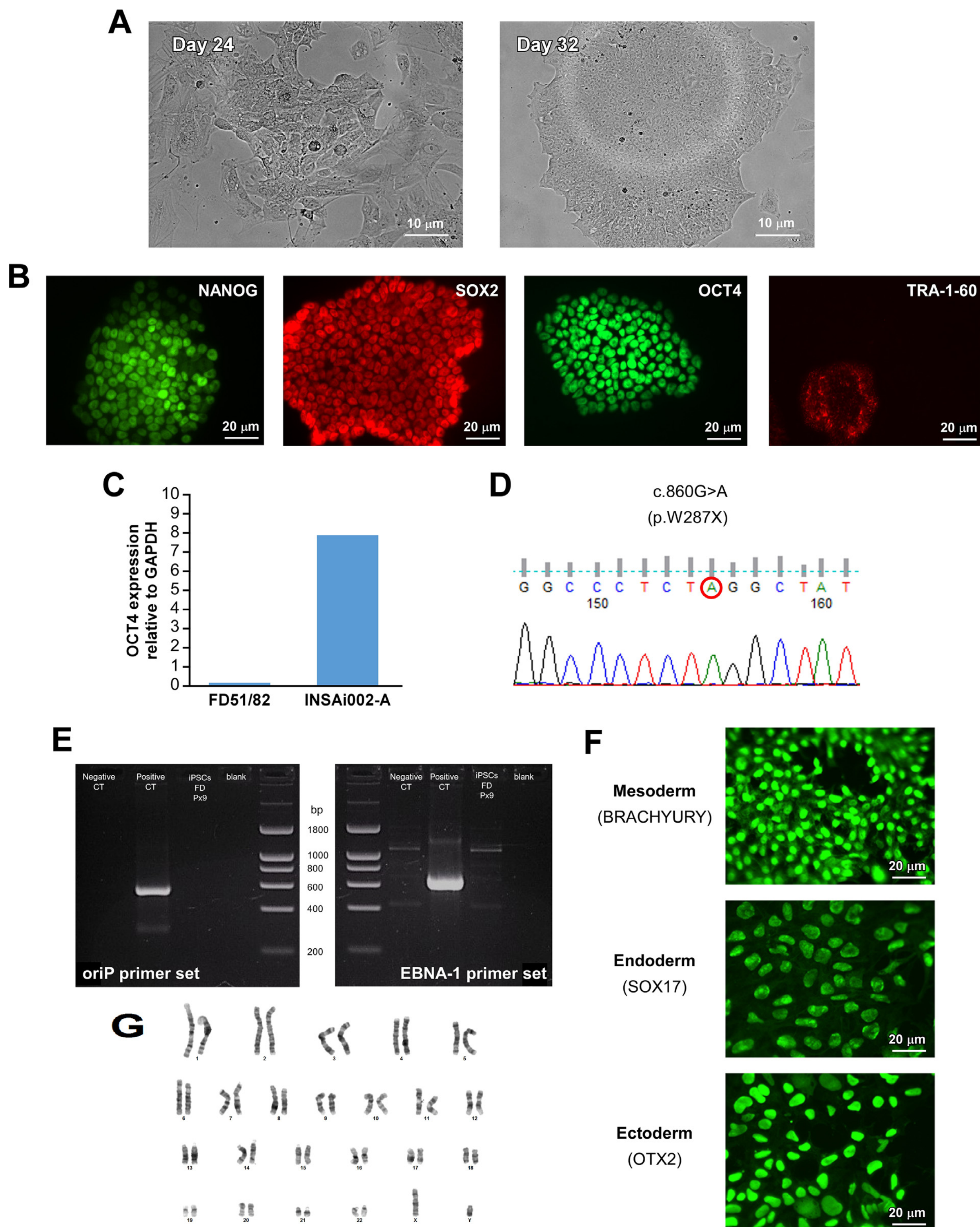

Fig. 1. Characterization of induced pluripotent stem cell line (INSAi002-A) from a Fabry Disease patient hemizygote for the rare pW287X mutation.

progressive accumulation of glycolipids and in the subsequent malfunction of the cells affected (for example, podocytes and cardiomyocytes). The iPSC line INSAi002-A was successfully generated from skin fibroblasts from a hemizygous FD patient with a rare nonsense mutation, p.W287X (Fig. 1). This mutation was first described in a patient with classic clinical manifestations (Davies et al., 1993). A nonsense point-mutation consisting of a TGG-to-TGA substitution in exon 6 of the *GLA* gene (NM_000169.2:c.861G > A) results in p.W287X harboring a stop codon predicting a premature termination of the α-Gal A and possibly a truncated protein. Furthermore, exon 6 mutations have been reported to be associated with cardiac variant phenotypes (Takata et al., al.,1997). This INSAi002-A can be used for reprogramming into specific cell types of interest and can be of particular importance for the derivation of cardiomyocytes and modeling for mechanistic studies of the readthrough response to inducer compounds (Lombardi et al., 2020).

Table 1.
Characterization and validation.

Classification	Test	Result	Data
Morphology	Light microscopy Photography	Normal	Fig. 1 panel A (morphology of a typical colony 24 (left) and 32 days (right) post-transduction with reprogramming vectors
Phenotype	Qualitative analysis Immunofluorescence (IF)	Positive for NANOG, SOX2, OCT4 and TRA-1-60	Fig. 1 panel B
	Quantitative analysis (RT-qPCR)	Positive for OCT4	Fig. 1 panel C
Genotype	Karyotype (G-banding) and resolution	46XY, Resolution 450–500	Fig. 1 panel G
Identity	STR analysis	DNA Profiling Performed	Supplementary file
Mutation analysis	Sequencing	Hemizygous p.W287X (rs104894839)	Fig. 1 panel D
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR: Negative	Not presented
	Sendai	RT-PCR analysis: Negative	Fig. 1 panel E
Differentiation potential	Embryoid body formation and Directed differentiation (IF)	Positive detection of 3 germ layers markers BRACHYURY, SOX17 and OTX2	Fig. 1 panel F

All results are summarized in Fig. 1. The fibroblasts were reprogrammed using non-integrative episomal vectors co-expressing the reprogramming genes Oct4, Sox2, Klf4, L-Myc, and Lin28, on a feeder-free system with vitronectin. Formation of iPSC-like colonies were clearly observable 24 days after transfection of the reprogramming vectors into FD fibroblasts (Fig. 1A). A typical human pluripotent stem cell-like morphology was detected by 30 days after transfection (Fig. 1A). A clone of FD-derived iPSC chosen to be better characterized, termed INSAi002-A cell line, showed the expression of endogenous pluripotency markers such as NANOG, SOX2, OCT4 and TRA-1–60 detected by immunofluorescence (Fig. 1B). A quantitative analysis by RT-qPCR of OCT4 expression showed an elevated expression in INSAi002-A cells in comparison to control fibroblasts (Fig. 1C), indicating further that INSAi002-A cells were reprogrammed. The INSAi002-A iPSCs retained the FD-causing *GLA* mutation (Fig. 1D) and displayed a normal 46XY karyotype (Fig. 1G) . In addition, these cells were devoid of any of the episomal vectors integration, as confirmed by RT-qPCR using primers specific for the vectors (Fig. 1E). In addition, The INSAi002-A iPSCs differentiated into all three germ layers as indicated by the positive immunofluorescence detection of mesoderm (BRACHYURY), endoderm (SOX17) and ectoderm (OTX2) markers, revealing their pluripotent nature (Fig. 1F). Altogether, the observations mentioned above along with STR analysis, indicated an efficient iPSCs production preserving the original genetic characteristics of the donor fibroblasts, using episomal vectors under feeder free conditions.

2. Materials and methods

2.1. Biological sample

The skin fibroblasts cell line from FD patient was obtained from Istituto Giannina Gaslini Bioresource Bank (Genova, Italy) in compliance with the ethical recommendations issued by the Ovideo agreement of 1997 and the International Declaration on Human Genetics Data of 2003 (Filocamo et al., 2014). The FD cell line was from a hemizygous male patient bearing the nonsense mutation p.W287X (rs104894839).

2.2. Fibroblasts culture

Cells were maintained in DMEM (1X) + GlutaMAX medium (Gibco) supplemented with 10% fetal bovine serum and 1% PEN-STREP (Lonza BioWhittaker) 5000 U Penicilin/mL in an incubator.

2.3. Reprogramming of fibroblasts to iPSCs

Cellular reprogramming fibroblasts to iPSCs was carried out using a non-integrative approach using episomal vectors. Fibroblasts were plated in 6-well plates coated with vitronectin, once confluences around 75% were reached transfection was performed. We used the Epi5 Episomal iPSC Reprogramming Kit (Invitrogen) on skin fibroblasts. The Epi5 iPSC kit contains an optimized mixture of three episomal vectors with an oriP/EBNA-1 (Epstein-Barr nuclear antigen-1) backbone for delivering the reprogramming genes Oct4, Sox2, Klf4, L-Myc, and Lin28. Additionally, it uses an optimized mixture of two vectors expressing mp53DD (a dominant negative mutation of p53) and EBNA1, which together improve the reprogramming efficiency of the system. The silencing of the viral promoter driving EBNA-1 expression, the loss of the episomes at a rate of approximately 5% per cell cycle due to defects in vector synthesis, and partitioning allows the removal of episomal vectors from the iPSCs without any additional manipulation The transfection of cells was done with Lipofectamine 3000 (Invitrogen) in Opti-MEM medium (Gibco).. Around 24 days after transfection, iPSC-like colonies were observed and were manually picked for further expansion and characterization. The cell culture matrix used for iPSCs growth was Vitronectin (VTNN from Gibco).

2.4. Cell culture and passage

Cells were cultured in 60 mm plates coated with 1/100 VTN—N diluted in 1X DPBS, and the incubator was at 37 °C with 5% CO_2. Once high confluence was reached, the medium was removed, cells were washed with 1X DPBS and treated with 1 mL of 0,5 mM EDTA solution for 4 min at room temperature for iPSCs detachment. After iPSCs detachment, EDTA is removed and iPSCs passage is done through resuspension of the cells with supplemented Gibco StemFlex Medium and 1% PEN-STREP (Lonza BioWhittaker) 5000 U Penicilin/mL. To the new matrix treated dishes is added 50 μL of cell suspension in a 2 mL volume medium.

2.5. Detection of episomal vectors by PCR in iPSCs

DNA samples were extracted from cell pellets using QIAamp® DNA Blood Mini Kit (Qiagen). Detection of the presence of Episomal iPSC Reprogramming Vectors in reprogrammed iPSC colonies was done by PCR using the PCR primers listed on Table 1. The EBNA-1 primer set can detect all five episomal plasmids in the kit. The oriP primer set can detect all episomal plasmids in the kit except pCXB-EBNA1, which lacks the OriP gene. After 9 passages, no episomal vectors were detected on FD iPSCs using PCR, indicating that our iPSCs were free of vectors footprint. Characterization and validation is shown in Fig. 1 and Table 1.

2.6. Pluripotency characterization

To characterize the iPSCs obtained, we have used the Fluorescent

Table 2.
Reagent details.

Antibodies used for immunocytochemistry	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-Nanog, clone 7F7.1, Alexa Fluor® 488 conjugate	1:100	Millipore Cat# MABD24, RRID:AB_11203826
	Mouse anti-Sox-2, clone 10H9.1, Cy3 conjugate	1:100	Millipore Cat# MAB4423, RRID:AB_11205572
	Mouse anti-Oct-4 (POU5f1), clone 7F9.2, Alexa Fluor® 488 conjugate	1:100	Millipore Cat# MAB4419, RRID:AB_1977399
	Mouse anti-TRA-1-60, clone TRA-1-60, Cy3 conjugate	1:100	Millipore Cat# MAB4360, RRID:AB_2119183
Germ Layer Markers	Goat Anti-Human Brachyury Polyclonal Antibody, unconjugated (Mesoderm)	1:10	R and D Systems Cat# AF2085, RRID:AB_2200235
	Goat Anti-Human Sox17 Polyclonal Antibody, unconjugated (Endoderm)	1:10	R and D Systems Cat# AF1924, RRID:AB_355060
	Goat Anti-Human Otx2 Polyclonal Antibody, unconjugated (Ectoderm)	1:10	R and D Systems Cat# AF1979, RRID:AB_2157172
Secondary antibodies	Donkey anti-Goat IgG (*H* + *L*) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:200	Thermo Fisher Scientific Cat# A-11055, RRID:AB_2534102

Primers	Target	TaqMan® assay
Pluripotency Marker (qPCR)	OCT-4	hs00999632_g1
House-Keeping Gene (qPCR)	GAPDH	hs02786624_g1
	Target	Forward/Reverse primer (5′ – 3′)
Targeted mutation sequencing	GLA exon 6	CCTGCGGTAGGCTTGTT/AGGCCCAAGACAAAGTT
Epissomal Vectors	OriP	TTCCACGAGGGTAGTGAACC/TCGGGGGTGTTAGAGACAAC
	EBNA-1	ATCGTCAAAGCTGCACACAG/CCCAGGAGTCCCAGTAGTCA

Human ES/iPS Cell Characterization Kit (Merk Milipore). This kit includes conjugated antibodies to study pluripotent transcription factors: Oct-4, Sox-2 and Nanog; and cell surface epitopes, TRA-1–60. The qualitative analysis by immunofluorescence showed positive staining for all pluripotency markers confirming the pluripotent status.

2.7. Genetic characterization

Genotype was established in fibroblasts and in iPSCs and the mutations confirmed (Fig. 1D). See Table 2 for primers used. PCR products were purified with the ExoSAP-IT (Applied Biosystems), for Sanger sequencing BigDye®Terminator v3.1 (Applied Biosystems) was use. STR analysis used the AmpFeSTR® Identifier® (Applied Biosystems).

Karyotyping was performed on G-banded metaphase chromosomes using standard procedures and at least four metaphases were examined per sample. The result of the karyotype analysis was a normal 46XY (Fig. 1G).

2.8. iPSCs differentiation into the three germ layers

To determine if a cell is truly a pluripotent stem cell, it is important to verify its ability to differentiate into each of the three germ layers. We used the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems®) to differentiate into endoderm and ectoderm. In addition, we used the Differentiation Base Media Supplement (50X), that comes with this kit, with CHIR99021 supplement (Stemcell Technologies), a WNT pathway activator that promotes direct mesoderm differentiation. We used antibodies against OTX2, BRACHYURY, and SOX17 (ectoderm, mesoderm and endoderm markers, respectively), for the immunocytochemical studies (Fig. 1F). In brief, seeded iPSCs were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10 min, incubated with PBST with 1% BSA (Sigma-Aldrich) for 30 min and stained by standard immunofluorescence procedures. Cells were analyzed on DM400 M fluorescence microscope (Leica). Antibodies are listed in Table 2.

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CRediT authorship contribution statement

Ana Joana Duarte: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Diogo Ribeiro:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **Renato Santos:** Methodology, Visualization. **Luciana Moreira:** Methodology, Visualization, Writing - original draft. **José Bragança:** Conceptualization, Writing - review & editing. **Olga Amaral:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing - original draft, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101794.

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