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Generation of two iPS cell lines (FRIMOi003-A and FRIMOi004-A) derived from Stargardt patients carrying *ABCA4* compound heterozygous mutations

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Abstract

Recessive Stargardt disease (STGD1) is an autosomal recessive retinal dystrophy, caused by mutations in the retina-specific ATP-binding cassette transporter (*ABCA4*) gene, which plays a role as a retinaldehyde flippase in the photoreceptor outer segments. In this work, two human induced pluripotent stem cell (iPSC) lines were generated from STGD1 patients carrying compound heterozygous mutations in *ABCA4*. Skin fibroblasts were reprogrammed with the Yamanaka factors using a non-integrating, Sendai virus-based approach. Both iPSC lines displayed typical embryonic stem cell morphology, had normal karyotype, expressed several pluripotency markers and were able to differentiate into all three germ layers.

Resource utility

Mutations in *ABCA4* gene can cause a wide range of recessive retinal disorders, including recessive Stargardt disease (STGD1), the most common form of inherited juvenile macular degeneration (Allikmets et al., 1997; Michaelides et al., 2003). Several novel pharmacologic, gene, and stem-cell therapies are being developed as therapeutic strategies to protect or improve vision for patients with STGD1 (Lu et al., 2017). The generation of iPSC lines from patients carrying mutations in *ABCA4* will be valuable *in vitro* models for the screening of new treatments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101389.

Resource details

Two patients were diagnosed with recessive Stargardt disease at Institut de Microcirurgia Ocular (IMO). Analysis of the *ABCA4* gene identified one splicing mutation and one missense variant in the first patient (STGD_1), and three missense variants (in *cis* position) and one frameshift mutation in the second (STGD_2) (Riera et al., 2017). Detailed clinical and genetic data is shown in Table 1. Dermal fibroblasts were obtained from a skin biopsy and reprogrammed into iPSC using non-integrative Sendai virus containing the reprogramming factors OCT3/4, SOX2, KLF4 and c-MYC. Approximately three weeks after transduction, iPSC colonies were manually picked and expanded. One iPSC colony for each patient was fully characterized at the genetic and molecular level (STGD1_FiPS4F1.5 and STGD2_FiPS4F1.7). The results of these analyses are summarized in Table 2. The resulting iPSC lines showed typical human embryonic stem cell morphology, with large and well-defined polygonal colonies, high nuclear/cytoplasmic ratio and prominent nucleoli (Fig. 1A). Both iPSC lines had normal karyotype, 46XX and 46XY respectively (Fig. 1B), did not express Sendai virus and reprogramming genes and were *Mycoplasma* negative (Supplementary file). For the DNA fingerprinting analysis, we used 16 different markers, which confirmed that each iPSC line had the same genetic background as the donor fibroblasts (Table 2). Immunocytochemistry and flow cytometry assays showed that the iPSC lines were positive for the pluripotency transcription factors OCT4, NANOG and SOX2, and the surface marker SSEA4 (Fig. 1C and D). To verify the ability of these iPSC lines to differentiate into the three germ layers, an *in vitro* functional differentiation test was performed, confirming that both iPSC lines could generate endoderm, mesoderm and ectoderm (Fig. 1E). Finally, the presence of the *ABCA4* mutations in the iPSC lines was verified by Sanger sequencing (c.4253 + 4C > T and c.6089G > A variants in STGD1_FiPS4F1.5 cells, and c.514G > A, c.2023G > A, c.6148G > C and c.3211_3212insGT mutations in STGD2_FiPS4F1.7 cells) (Fig. 1F).

Materials and methods

Reprogramming of skin fibroblasts

Fibroblasts were expanded in DMEM (cat# 11960077, Life Technologies) supplemented with 10% FBS (cat# 10082147, Life Technologies) and 1× penicillin-streptomycin (cat# 15140122, Life Technologies), and reprogrammed following the manufacturer instructions (CytoTune™-iPS 2.0 Sendai Reprogramming Kit, cat# A16518, ThermoFisher Scientific). Six days after transduction, 2×10^4 cells were seeded on irradiated mouse embryonic fibroblasts (MEFs) (cat# A34181, ThermoFisher Scientific) in six wells of a 6-well plate, and DMEM media was replaced with hES media, containing DMEM/Ham's F-12 (cat# 11320–033, Life Technologies) supplemented with 20% KSR (cat # 10828028 Life Technologies), 1× non-essential amino acids (cat# 11140076, Life Technologies), 1× penicillin-streptomycin, 1× glutamine (cat# 25030081, Life Technologies), 1× β-mercaptoethanol (cat# 21985023, Life Technologies), and 10 ng/ml of FGF2 (cat# 233-FB, R&D Systems). Three weeks after reprogramming, 20 colonies were manually picked and placed onto a MEFs-coated 24-well plate. Selected iPSC colonies were expanded on MEFs for six passages, then cells were cultured and adapted to feeder-free conditions, onto

Matrigel-coated plates (cat# CB 40230, Corning) in mTeSR1 medium (cat# 5850, StemCell Technologies). Cells were weekly subcultured 1:10 using 50 mM EDTA in phosphate buffered saline (PBS) without calcium and magnesium and incubated at 37 °C in 5% CO_2 atmosphere.

Karyotype analysis

After six passages, karyotype was performed on twenty G-banded metaphase cells at 450–500 band resolution (Cell Line Genetics).

Mutation analysis

Genomic DNA was isolated from 10^6 cells using the DNeasy Blood & Tissue Kit (cat# 69504, Qiagen) following manufacturer's instructions. PCR amplification was performed using DNA AmpliTools Green Master Mix (cat# 4749, Biotools) and specific primers flanking the *ABCA4* mutations (Table 3) in a final volume of 50 µl. The PCR was performed using a SimpliAmp™ Thermal Cycler (Applied Biosystems) in a three-step process: denaturation for 2 min at 95 °C, followed by 35 cycles of 20 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The resulting PCR products were Sanger sequenced (Macrogen).

STR analysis

Identity analysis was performed by Cell Line Genetics on the fibroblast cells and the established iPSC line using the PowerPlex 16 System (cat# DC6531, Promega).

In vitro differentiation assay

In vitro functional differentiation was performed using the Human Pluripotent Stem Cell Functional Identification kit (cat# SC022, R&D Systems). Cells were fixed and stained to detect endoderm, mesoderm and ectoderm specific markers (SOX17, Brachyury and OTX2, respectively) (Table 3).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature (RT) and washed twice with 1× PBS before being permeabilized with 0.2% Triton X-100 in 1× PBS for 10 min. Cells were rinsed twice in 1% bovine serum albumin (BSA) in 1× PBS and blocked for 30 min at RT with DAKO blocking buffer (Agilent). Cells were incubated with primary antibodies in 1% BSA at 4 °C for O/N, and then washed twice and incubated with the appropriate secondary antibody for 1 h at RT (Table 3). Finally, cell nuclei were stained with DAPI (1:1000) at RT for 5 min, washed twice and visualized and captured using an Olympus IX73 inverted microscope connected to a XM10 monochrome camera (Olympus, Tokyo, Japan).

Flow cytometry

Single cell suspension was obtained using EDTA and cells were fixed in 4% PFA for 15 min. For nuclear staining, cells were also permeabilized with cold methanol for 20 min on ice. Cells were incubated for 15 min at RT with conjugated antibodies and isotype negative

controls diluted with DMEM supplemented with 10% FBS (Table 3), then FACS analyzed (Bio-Rad S3e (Bio-Rad)). FACS data were analyzed using FlowJo software.

RNA isolation and RT-PCR analysis

The clearance of Sendai virus and reprogramming factors was confirmed by RT-PCR after nine passages. Briefly, total RNA from 1×10^6 cells was obtained by using the RNeasy Protect Cell Mini Kit (cat# 74624, Qiagen), according to manufacturer's instructions. Then, cDNA was generated by reverse transcription using Transcriptor High Fidelity cDNA Synthesis Kit (cat# 05091284001, Roche Diagnostics). *GAPDH* was used as control for normalization. The PCR was performed in a SimpliAmp™ Thermal Cycler (Applied Biosystems) using the DNA AmpliTools Green Master Mix (cat# 4749, Biotools) in a final volume of 25 μl (primer sequences are given in Table 3). For amplification of *GAPDH*, a two-step PCR was performed as follows: first denaturation for 2 min at 95 °C, followed by 32 cycles of 20 s at 95 °C and 2 min at 63 °C. For amplification of Sendai virus genome and reprogramming factors, we performed a three-step PCR: denaturation for 2 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. The cDNA from cells collected one week after the virus infection was used as a positive control for the expression of Sendai virus genome and reprogramming factors.

Mycoplasma detection

The presence of mycoplasma was regularly tested by using e-Myco™ plus Mycoplasma PCR Detection Kit (cat# 25234, Intron).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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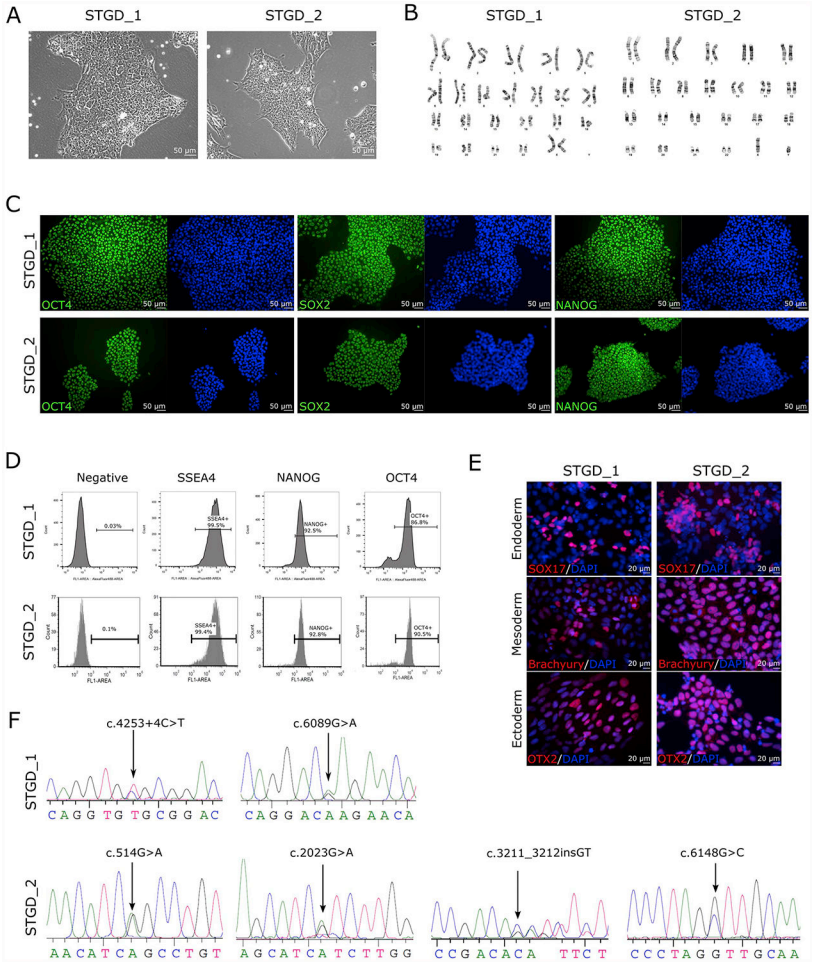

Fig. 1.
Characterization of the iPSC lines.

Table 1

Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
STGD1_FiPS4F1.5	STGD_1	Female	29	Caucasian	*ABCA4* compound heterozygous mutations Allele 1: c.4253 + 4C > T Allele 2: c.6089G > A (p.Arg2030Gln)	Stargardt disease
STGD2_FiPS4F1.7	STGD_2	Male	20	Caucasian	*ABCA4* compound heterozygous mutations: Allele 1: c.514G > A (p.Gly172Ser); c.2023G > A (p.Val675Ile); c.6148G > C (p.Val2050Leu) Allele 2: c.3211_3212insGT (p.Ser1071fs*14)	Stargardt disease

Table 2

Characterization and validation.

Classification	Test	Result	Data
Morphology Phenotype	Photography	Normal	Fig. 1 panel A
	Qualitative analysis Immunocytochemistry	Assess staining of pluripotency markers: OCT4, SOX2, NANOG	Fig. 1 panel C
	Quantitative analysis Flow cytometry	STGD_1: SSEA, 99.5%; NANOG, 92.5%; OCT4, 86.8%	Fig. 1 panel D
		STGD_2: SSEA, 99.4%; NANOG, 92.8%; OCT4, 90.5%	
Genotype	Karyotype (G-banding) and resolution	STGD_1: 46, XX	Fig. 1 panel B
		STGD_2: 46, XY	
		Resolution 450–500	
Identity	STR analysis	16 *loci* analyzed, all matching	Available with the authors
Mutation analysis (IF APPLICABLE)	Sequencing	STGD_1: compound heterozygous, splicing and missense mutations	Fig. 1 panel F
		STGD_2: compound heterozygous, missense and insertion mutations	
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary file
Differentiation potential	Directed differentiation	Positive OTX2 ectodermal staining, positive Brachyury mesodermal staining and positive SOX17 endodermal staining	Fig. 1 panel E
Donor screening (OPTIONAL)	N/A	N/A	N/A
Genotype additional info (OPTIONAL)	N/A	N/A	N/A

Table 3

Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Marker	Rabbit anti-OCT4 (Alexa Fluor 488 Conjugate)	1:50	Cell Signaling Technology Cat# 5177S, RRID: AB_10693303
Pluripotency Marker	Mouse anti-SOX2 (Alexa Fluor 488 Conjugate)	1:50	Santa Cruz Biotechnology Cat# sc-365823 AF488, RRID: AB_10842165
Pluripotency Marker	Rabbit anti-NANOG	1:400	Cell Signaling Technology Cat# 4903P, RRID: AB_10559205
Pluripotency Marker	Mouse anti-SSEA-4 (Alexa Fluor 488 Conjugate)	1:20	BD Pharmingen Cat#560308, RRID: AB_1645371
Pluripotency Marker	Mouse anti-NANOG (Alexa Fluor 488 Conjugate)	1:10	BD Pharmingen Cat#560791, RRID: AB_1937305
Differentiation Marker	Goat anti-OTX2	1:20	R&D Systems Cat# AF1979, RRID: AB 2157172
Differentiation Marker	Goat anti-Brachyury	1:20	R&D Systems Cat# AF2085, RRID: AB_2200235
Differentiation Marker	Goat anti-SOX17 (NL557 Conjugate)	1:10	R&D Systems Cat# NL1924R, RRID: AB_2195645
Secondary antibody	Rabbit anti-Goat IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A27012, RRID: AB_2536077
Secondary antibody	Goat anti-Rabbit IgG Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A11008, RRID: AB_143165

Primers		
	Target	**Forward/Reverse primer (5'−3')**
Sendai virus detection	*SeV,* 181 bp	GGATCACTAGGTGATATCGAGC
		ACCAGACAAGAGTTTAAGAGATATGTATC
Transgene detection	*KOS,* 528 bp	ATGCACCGCTACGACGTGAGCGC
		ACCTTGACAATCCTGATGTGG
Transgene detection	*KLF4,* 410 bp	TTCCTGCATGCCAGAGGAGCCC
		AATGTATCGAAGGTGCTCAA
Transgene detection	*c-MYC,* 532 bp	TAACTGACTAGCAGGCTTGTCG
		TCCACATACAGTCCTGGATGATGATG
House-keeping gene	*GAPDH,* 983 bp	TGAAGGTCGGAGTCAACGGATTTGG
		CATGTAGGCCATGAGGTCCACCAC
Mutation sequencing	*ABCA4* (c.514G > A), 423 bp	GGCAAGAGCCTCACCTCTAG
		TAAATACAAGGCATTAAGTGATGG
Mutation sequencing	*ABCA4* (c.2023G > A), 511 bp	AGCCCATTAAATGTCCAGGGGAGG
		GTTGGCTAAAAGGAAGGGCTG
Mutation sequencing	*ABCA4* (c.3211_3212insGT), 409 bp	TAAACATCTAAGAGGCAGCACC

Primers

	Target	Forward/Reverse primer (5'–3')
		TCACCAAGTCACTGATAAACCC
Mutation sequencing	*ABCA4* (c.4253 + 4C > T), 473 bp	AGAAGCCCCAACCCTCTCCAC
		CTTCCCAAGCAGTCCTGCCT
Mutation sequencing	*ABCA4* (c.6089G > A), 415 bp	ATGCATTTCTGAAGCCAAATAGG
		AGACACATAGTAAACATTTGTTGG
Mutation sequencing	*ABCA4* (c.6148G > C), 435 bp	GCCTGTTTCAGCCTAGCTGG
		GCTGTGTGAACCAAACACTGG