

Lab Resource: Stem Cell Line

Establishment of a human iPSC line, IISHDOi004-A, from a patient with Usher syndrome associated with the mutation c.2276G > T; p.Cys759Phe in the *USH2A* gene



Francisco Zurita-Díaz^{a,b,c,d,1}, María del Carmen Ortuño-Costela^{a,b,c,d,1}, Ana Moreno-Izquierdo^{d,e}, Liliana Galbis^f, José María Millán^{c,g}, Carmen Ayuso^{c,f}, Rafael Garesse^{a,b,c,d}, M. Esther Gallardo^{a,b,c,d,*}

^a Departamento de Bioquímica, Facultad de Medicina, Universidad Autónoma de Madrid, Spain

^b Instituto de Investigaciones Biomédicas “Alberto Sols” (UAM-CSIC), Madrid, Spain

^c Centro de Investigación Biomédica en Red (CIBERER), Madrid, Spain

^d Instituto de Investigación Sanitaria Hospital 12 de Octubre (i+12), Madrid, Spain

^e Servicio de Genética, Hospital 12 de Octubre, Madrid, Spain

^f Departamento de Genética, Instituto de Investigación Sanitaria – Hospital Universitario Fundación Jiménez Díaz (IIS-FJD, UAM), Madrid, Spain

^g Instituto de Investigación Sanitaria Hospital La Fe, IIS-La Fe, Valencia, Spain

ABSTRACT

A human iPSC line, IISHDOi004-A, from fibroblasts obtained from a patient with Usher syndrome, harboring a homozygous mutation in the *USH2A* gene (c.2276G > T; p.Cys759Phe) has been generated. Reprogramming factors Oct3/4, Sox2, Klf4, and c-Myc were delivered using Sendai virus.

Resource table		Associated disease	Usher syndrome
		Gene/locus	Gene <i>USH2A</i> : c.2276G > T; p.Cys759Phe; Chromosome: 1q41
Unique stem cell line identifier	IISHDOi004-A	Method of modification	N/A
Alternative name(s) of stem cell line	RPAB16-FiPS4F3	Name of transgene or resistance	N/A
Institution	Instituto de Investigación Sanitaria Hospital 12 de Octubre, i + 12	Inducible/constitutive system	N/A
Contact information of distributor	Dr. M. Esther Gallardo egallardo.imas12@h12o.es	Date archived/stock date	December 2017
Type of cell line	iPSC	Cell line repository/bank	N/A
Origin	Human	Ethical approval	Patient informed consent was obtained. This study was reviewed and approved by the Institutional Ethical Committee of the “Fundación Jiménez Díaz”, 03/14; 404,327 1.
Additional origin info	Age: N/A Sex: Male Ethnicity if known: Spanish		
Cell Source	Skin fibroblasts		
Clonality	Clonal		
Method of reprogramming	Transgene free (Sendai virus)		
Genetic Modification	NO		
Type of Modification	N/A		

* Corresponding author at: Instituto de Investigación Sanitaria Hospital 12 de Octubre (i+12), Madrid, Spain
E-mail address: egallardo.imas12@h12o.es (M.E. Gallardo).

¹ These two authors contributed equally.

Resource utility

Usher syndrome is an autosomal recessive disease involving sensorineural hearing loss, retinitis pigmentosa, and, in some cases, vestibular dysfunction. There is no clinical treatment available for this disorder. The iPSC line reported here will be very useful for modelling this disease and for carrying out a high-throughput pharmacological screening.

Resource details

The human iPSC line, IISHDOI004-A, has been generated using a non-integrative methodology that involves the use of Sendai viruses containing the reprogramming factors Oct3/4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007). For this purpose, fibroblasts from a patient with Usher syndrome (Millán et al., 2011) have been obtained from a skin biopsy. These fibroblasts harboured a homozygous mutation in the *USH2A* gene (c.2276G > T; p.Cys759Phe). We have also confirmed the presence of this mutation in the iPSCs (Fig. 1A). IISHDOI004-A iPSC colonies displayed a typical ES-like colony morphology and growth behaviour (Fig. 1B) and they stained positive for alkaline phosphatase activity (Fig. 1C). The endogenous expression of the pluripotency associated transcription factors OCT4, SOX2, KLF4, NANOG, CRIPTO and REX1 was evaluated by quantitative real time polymerase chain reaction (qPCR) (Fig. 1H). Immunofluorescence analysis revealed expression of transcription factors OCT4, NANOG and SOX2, and typical ES cells surface markers SSEA3, SSEA4, TRA-1-60 and TRA-1-81 (Fig. 1E). We also confirmed the clearance of the vectors and the exogenous reprogramming factor genes by RT-PCR after eight culture passages (Fig. 1G). The iPSC line has been adapted to feeder-free culture conditions and a karyotype analysis after more than 20 culture passages has been performed (Fig. 1F). This analysis displayed a normal (46, XY) karyotype. We also verified that the line IISHDOI004-A was derived from the patient's fibroblasts by DNA fingerprinting analysis (archived at SCR journal). In addition, the line was confirmed by PCR analysis to be mycoplasma-negative (Fig. 1D). Finally, the capacity of the IISHDOI004-A iPSC line to differentiate into the three germ layers (endoderm, mesoderm and ectoderm) was evaluated *in vitro* using an embryoid body based assay (Fig. 1I).

Materials and methods

Generation of iPSCs

Human fibroblasts from a patient with Usher syndrome harboring the mutation c.2276G > T; p.Cys759Phe in the *USH2A* gene were reprogrammed using the CytoTune-iPS 2.0 Sendai reprogramming kit following the instructions of the manufacturer. IISHDOI004-A line was maintained and expanded both on feeder and feeder-free conditions as described in Galera et al., 2016.

Phosphatase alkaline analysis

The iPSC line IISHDOI004-A was seeded on a feeder layer plate. After one week, direct phosphatase alkaline activity was determined using the phosphatase alkaline blue membrane substrate solution kit (Sigma, AB0300), (Table 1).

Mutation analysis

Total DNA from patient's fibroblasts and iPSCs was extracted using a commercial kit. Subsequently, a PCR was carried out with the primers shown in Table 2. Following PCR amplification, direct sequencing of amplicons was performed in an ABI 3730 sequencer (Applied Biosystems).

qPCR analysis

Total mRNA was isolated using TRIZOL and 1 µg was used to synthesize cDNA using the Quantitect RT cDNA synthesis kit. One µl of the reaction was used to quantify by qPCR the expression of the endogenous pluripotency associated genes (*OCT4*, *SOX2*, *KLF4*, *NANOG*, *CRIPTO* and *REX1*, Table 1). Primers are listed in Table 2 (Aasen et al., 2008). All the expression values were normalized to the *GAPDH* gene. Plots are representative of at least three independent experiments.

Karyotype analysis

Karyotype analyses were carried out using cells with more than twenty culture passages. Briefly, cells were treated with 10 µg/mL of Colcemid (Gibco) for 90 min at 37 °C, trypsinized, treated with hypotonic solution KCl 0.075 M, and fixed with Carnoy's fixative. Cells were then dropped on a microscope glass slide and dried. Metaphase cells were G banded using Wright staining. At least 20 metaphases were karyotyped.

Immunofluorescence analysis

Cells were grown on 0.1% gelatin-coated 35 mm culture plates (81,156, Ibidi), fixed with 4% paraformaldehyde for 30 min at RT and permeabilized using TBS + (0.1% Triton X-100 in Tris-buffered saline, TBS) for 45 min. Then the cells were incubated in TBS + + (3% donkey serum, 0.3% Triton X-100 in TBS) for two hours at RT. Primary antibodies were applied overnight at 4 °C. Secondary antibodies for two hours at RT. Nuclei were stained with DAPI (Sigma, 28718-90-3). The antibodies are listed in Table 2.

In vitro differentiation assay

The *in vitro* pluripotency capacity of the line IISHDOI004-A was tested by spontaneous embryoid body differentiation. The protocol used has been described in detail by Galera et al., 2016.

DNA fingerprinting analysis

For DNA fingerprinting analysis the markers D13S317, D7S820, VWA, D8S1179, D21S11, D19S433, D2S1338 and amelogenin for sex determination have been amplified by PCR and analyzed by ABI PRISM 3100 Genetic analyzer and Peak Scanner v3.5 (Applied Biosystems), (Table 2).

Mycoplasma detection

Mycoplasma detection was performed by PCR analysis using 1 mL of the cell culture supernatant (3 days culture, 90% confluence). Primers used are specified in Table 2. The 300 bp band represents a mycoplasma-positive sample (positive control, C+). The 570 bp band is an internal control to discard the inhibition of the polymerase.

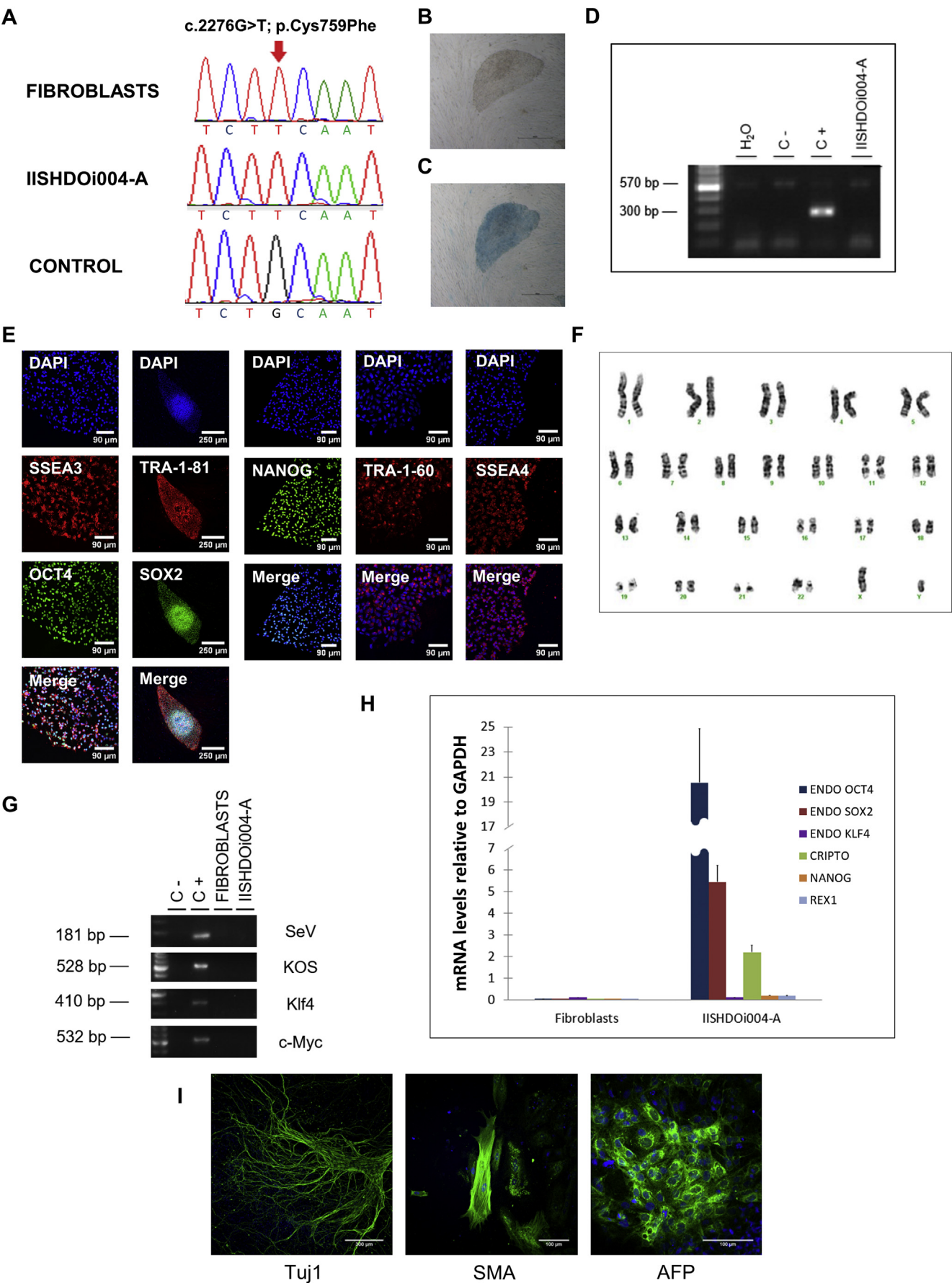


Fig. 1. Molecular and functional characterization of the IISHDOi004-A iPSC line.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology Phenotype	Photography	Normal	Fig. 1 panel B
	Qualitative analysis: immunocytochemistry	Positive for the pluripotency markers: SSEA3, SSEA4, TRA-1-81, TRA-1-60, OCT4, NANOG, SOX2	Fig. 1 panel E
	Qualitative analysis: alkaline phosphatase activity	Positive	Fig. 1 panel C
	Quantitative analysis: gene expression (qPCR)	Positive for the pluripotency markers OCT4, KLF4, SOX2, CRIPTO, NANOG, REX1	Fig. 1 panel H
Genotype Identity	Karyotype (G-banding) and resolution	46, XY Resolution 450–500	Fig. 1 panel F
	STR analysis	DNA profiling performed	Submitted to SCR journal for archiving
Mutation analysis (IF APPLICABLE)	Sequencing	8 loci, all matched (D2S1338, D7S820, D8S1179, D13S317, D19S433, D21S11, VWA, amelogenin) Confirmation of the mutation: <i>USH2A</i> c. 2276G > T; p.Cys759Phe	Submitted to SCR journal for archiving Fig. 1 panel A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology Differentiation potential	Mycoplasma	Mycoplasma testing by PCR: negative	Fig. 1 panel D
	Embryoid body formation and directed differentiation	Positive for: smooth muscle actin (SMA), β -tubulin (Tuj1) and alpha-fetoprotein (AFP)	Fig. 1 panel I
Donor screening (OPTIONAL) Genotype additional info (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse anti-TRA-1-81	1:150	Millipore Cat# MAB4381, RRID: AB_177638
	Mouse anti-TRA-1-60	1:150	Millipore Cat# MAB4360, RRID: AB_11211864
	Rabbit anti-SOX2	1:100	Thermo Fisher Scientific Cat# PA1-16968, RRID: AB_2195781
	Mouse anti-SSEA4	1:10	Millipore Cat# MAB4304, RRID: AB_177629
	Rat anti-SSEA3	1:20	Abcam Cat# ab16286, RRID: AB_882700
	Goat anti-NANOG	1:25	R and D Systems Cat# AF1997, RRID: AB_355097
	Mouse anti-OCT4	1:100	Santa Cruz Biotechnology Cat# sc-5279, RRID: AB_628051
Differentiation markers	Mouse anti- β tubulin isotype III	1:300	Sigma-Aldrich Cat# T8660, RRID: AB_528427
	Mouse anti- AFP	1:300	Sigma-Aldrich Cat# WH0000174M1, RRID: AB_1839587
	Mouse anti- SMA	1:400	Sigma-Aldrich Cat# A2547, RRID: AB_476701
Secondary antibodies	Cy [™] 2-conjugated AffiniPure Donkey Anti-Goat IgG (H + L)	1:50	Jackson ImmunoResearch Labs Cat# 705-225-147, RRID: AB_2307341
	Cy [™] 2-conjugated AffiniPure Goat Anti-Mouse IgG, Fc γ Subclass 2b specific	1:50	Jackson ImmunoResearch Labs Cat# 115-225-207, RRID: AB_2338749
	Cy [™] 2-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L)	1:50	Jackson ImmunoResearch Labs Cat# 111-225-144, RRID: AB_2338021
	Cy [™] 3-conjugated AffiniPure Goat Anti-Rat IgM, μ chain specific	1:250	Jackson ImmunoResearch Labs Cat# 112-165-075, RRID: AB_2338249
	Cy [™] 3-conjugated AffiniPure Goat Anti-Mouse IgG, Fc γ Subclass 3 specific	1:250	Jackson ImmunoResearch Labs Cat# 115-165-209, RRID: AB_2338698
	Cy [™] 3-conjugated AffiniPure Donkey Anti-Mouse IgM, μ chain specific	1:250	Jackson ImmunoResearch Labs Cat# 715-165-020, RRID: AB_2340811
	Goat anti-mouse IgG (H + L), Alexa Fluor 488	1:500	Thermo Fisher Scientific Cat# A-11029, RRID: AB_2534088
Primers	Target	Forward/reverse primer (5'-3')	
Pluripotency markers (qPCR)	<i>Endo-KLF4</i>	AGCCTAAATGATGGTGCTTGGT / TTGAAAACCTTTGGCTTCCTTGTT	
	<i>Endo-OCT4</i>	GGGTTTTTTGGGATTAAGTCTCTCA / GCCCCACCCCTTTGTGTT	
	<i>Endo-SOX2</i>	CAAAAATGGCCATGCAGGTT / AGTTGGGATCGAACAAAAGCTATT	
	<i>REX1</i>	CCTGCAGGCGGAAATAGAAC / GCACACATAGCCATCACAAGG	
	<i>CRIPTO</i>	CGGAACCTGTGAGCAGCATGT / GGGCAGCCAGGTGTCATG	
House-Keeping Genes (qPCR)	<i>NANOG</i>	ACAACTGGCCGAAGAATAGCA / GGTTCCCACTCGGGTTTCAC	
	<i>GAPDH</i>	GCACCGTCAAGGCTGAGAAC / AGGGATCTCGCTCCTGGAA	
	<i>Targeted mutation analysis/sequencing</i>	AGTAAGATTGGCCCCCTATGGC / CTCCTTCAACATTTGGGCTTGC	
	<i>SeV</i>	GGATCACTAGGTGATATCGAGC / ACCAGACAAGAGTTTAAGAGATATGTATC	
	<i>KOS</i>	ATGCACCGCTACGACGTGAGCGC / ACCTTGACAATCCTGATGTGG	
Virus silencing	<i>Klf4</i>	TTCCTGCATGCCAGAGGAGCCC / AATGTATCGAAGGTGCTCAA	
	<i>c-Myc</i>	TAACCTAGCTAGCAGGCTTGTGC / TCCACATACAGTCTGGATGATGATG	
	<i>D2S1338</i>	[6-FAM] CCAGTGGGATTTGGAAACAGA / ACCTAGCATGGTACCTGCAG	
	<i>D7S820</i>	[6-FAM] TGTGATAGTTTGAAGCAAGCACTAACG / CTGAGGTATCAAAAACCTCAGAGG	
	<i>D8S1179</i>	[6-FAM] TTTTGTGATTTTCATGTGTACATTTCG / CGTAGCTATAATTAGTTTCATTTC	
STR analysis	<i>D13S317</i>	[6-FAM] ACAGAAGTCTGGGATGTGGA / GCCCAAAAAGACAGACAGAA	
	<i>D19S433</i>	[6-FAM] CCTGGGCAACAGAATAAGAT / TAGGTTTTTAAGGAACAGGTGG	
	<i>D21S11</i>	[6-FAM] GTGAGTCAATTCCCAAG / GTTGTAATTAGTCAATGTTTCCC	
	<i>VWA</i>	[6-FAM] CCCTAGTGGATGATAAGAATAATC / GGACAGATGATAAATACATAGGATGGATGG	
	<i>Amelogenin</i>	[6-FAM] CCCTGGGCTCTGTAAAGAAATAGT / ATCAGAGCTTAACTGGGAAGCTG	
Mycoplasma detection	<i>GPO-3 / MGSO</i>	GGGAGCAAACAGGATTAGATACCCT / TGCACCATCTGTCTACTCTGTTAACCTC	

Acknowledgments

We are very grateful to Virginia Rufo for her help with fibroblasts culturing, Victoria Cerrada for her help in the iPS cell characterization and José Luís Díaz Recuero for performing the skin biopsy. This work was supported by grants from: 1) the “Fondo de Investigación Sanitaria, Instituto de Salud Carlos III cofunded by FEDER Funds”: PI10/0703, PI13/00556 and PI16/00789 to RG, PI15/00484 to MEG and PI16/0425 to CA. 2) “Centro de Investigación Biomédica en Red en Enfermedades Raras” (CIBERER): grants 13-717/132.05 and ER16P3AC717 to RG. 3) “Comunidad Autónoma de Madrid” (grant number S2010/BMD-2402 to RG) and 4) FUNDALUCE 2015 to CA. MdC.O-C and F.Z-D receive grant support from the Ministerio de Educación, Cultura y Deporte (FPU13/00544 and FPU16/03895). MEG is supported by a “Miguel Servet” contract (CP16/00046) from Instituto de Salud Carlos III and FEDER Funds. LG is supported by a “Rio

Hortega” contract (16/00126) from Instituto de Salud Carlos III and FEDER Funds.

References

- Aasen, T., Raya, A., Barrero, M.J., Garreta, E., Consiglio, A., Gonzalez, F., Vassena, R., Bilić, J., Pekarik, V., Tiscornia, G., Edel, M., Boué, S., Izpisua Belmonte, J.C., 2008. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat. Biotechnol.* 26 (11), 1276–1284.
- Galera, T., Zurita, F., González-Páramos, C., Moreno-Izquierdo, A., Fraga, M.F., Fernández, A.F., Garesse, R., Gallardo, M.E., 2016. Generation of a human iPSC line from a patient with Leigh syndrome. *Stem Cell Res.* 16 (1), 63–66.
- Millán, J.M., Aller, E., Jaijo, T., Blanco-Kelly, F., Gimenez-Pardo, A., Ayuso, C., 2011. An update on the genetics of Usher syndrome. *J. Ophthalmol.* 2011, 417217.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131 (5), 861–872.

Marker	Fibroblasts		IISHDOi004-A	
D2S1338	191	195	191	195
D7S820	212	220	212	220
D8S1179	175	179	175	179
D13S317	186	190	186	190
D19S433	194	202	194	202
D21S11	225	239	225	239
VWA	141	154	141	154
Amelogenin	X	Y	X	Y