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Lab Resource: Multiple Cell Lines

Generation of integration-free induced pluripotent stem cell lines derived from two patients with X-linked Alport syndrome (XLAS)

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ABSTRACT

Skin biopsies were obtained from two male patients with X-linked Alport syndrome (XLAS) with hemizygous *COL4A5* mutations in exon 41 or exon 46. Dermal fibroblasts were extracted and reprogrammed by nucleofection with episomal plasmids carrying OCT3/4, SOX2, KLF4 LIN28, L-MYC and p53 shRNA. The generated induced Pluripotent Stem Cell (iPSC) lines AS-FiPS2-Ep6F-28 and AS-FiPS3-Ep6F-9 were free of genomically integrated reprogramming genes, had the specific mutations, a stable karyotype, expressed pluripotency markers and generated embryoid bodies which were differentiated towards the three germ layers *in vitro*. These iPSC lines offer a useful resource to study Alport syndrome pathomechanisms and drug testing.

Resource table.

Unique stem cell line identifier	ESi055-A ESi056-A
Alternative name of stem cell line	AS-FiPS2-Ep6F-28 (ESi055-A) AS-FiPS3-Ep6F-9 (ESi056-A)
Institution	Center of Regenerative Medicine in Barcelona
Contact information of distributor	Anna Veiga, aveiga@cmrb.eu
Type of cell lines	iPSC
Origin	human Fibroblasts from patients AS2 and AS3
Cell Source	Skin fibroblasts
Method of reprogramming	Nucleofection with non-integrating episomal plasmids carrying OCT3/4, SOX2, KLF4, LIN28, L-MYC, p53 shRNA
Multiline rationale	Same disease, two different patients
Gene modification	No modification
Type of modification	No modification
Associated disease	X-linked Alport syndrome (XLAS)
Gene/locus	AS-FiPS2-Ep6F-28: *COL4A5* (LRG_232t2, NM_033380.2) Genotype: c.3722G>A p.(G1241D), Exon41/Hemizygosis AS-FiPS3-Ep6F-9: *COL4A5* (LRG_232t2, NM_033380) Genotype: c.4052G>A p.(G1351D), Exon46/Hemizygosis
Method of modification	No modification
Name of transgene or resistance	No transgenes
Inducible/constitutive system	Not inducible
Date archived/stock date	February 2017
Cell line repository/bank	http://www.eng.isciii.es/ISCIII/es/contenidos/fd-el-instituto/fd-organizacion/fd-estructura-directiva/fd-subdireccion-general-investigacion-terapia-celular-medicina-regenerativa/fd-centros-unidades/fd-banco-nacional-lineas-celulares/fd-lineas-celulares-disponibles/lineas-de-celulas-iPS.shtml*direct URL.*
Ethical approval	Patient informed consent obtained/Ethics Review Board-competent authority approval obtained by Comité de Ética e Investigación Clínica-CEIC-CMRB (ADD01/2015, 14/2012) and by the Catalan Authority for Stem Cell Research (Approval number: 2231891)

Resource utility

X-linked Alport syndrome is a hereditary disorder causing chronic kidney disease progressing to end-stage renal disease. Alport syndrome is rare, but it accounts for around 1% of patients receiving renal replacement

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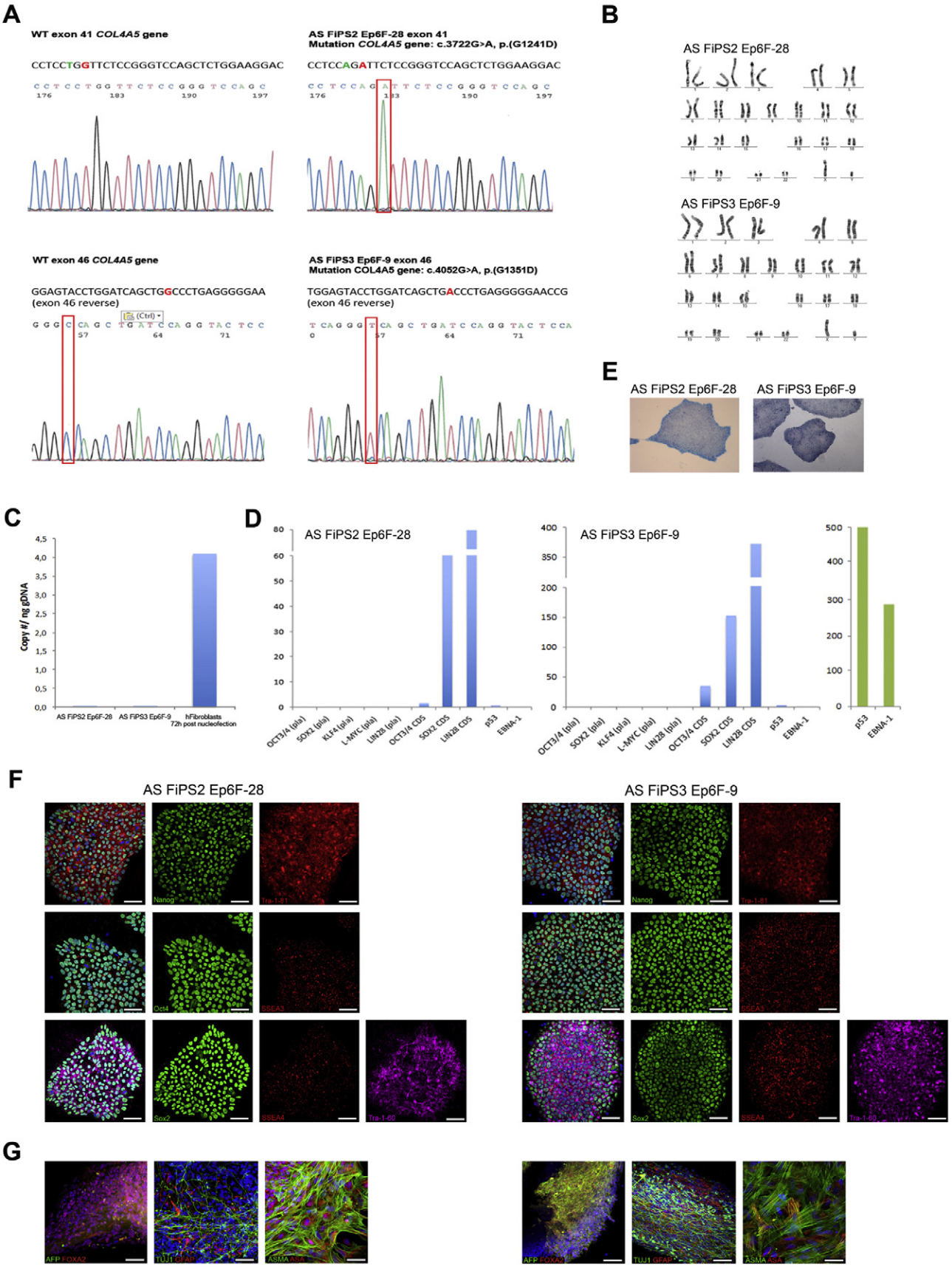
A
WT exon 41 COL4A5 gene
CCTCCTGGTTCTCCGGGTCCAGCTCTGGAAGGAC
AS FiPS2 Ep6F-28 exon 41
Mutation COL4A5 gene: c.3722G>A, p.(G1241D)
CCTCCAGATTCTCCGGGTCCAGCTCTGGAAGGAC
WT exon 46 COL4A5 gene
GGAGTACCTGGATCAGCTGGCCCTGAGGGGGAA
(exon 46 reverse)
AS FiPS3 Ep6F-9 exon 46
Mutation COL4A5 gene: c.4052G>A, p.(G1351D)
TGGAGTACCTGGATCAGCTGACCCTGAGGGGGAACCG
(exon 46 reverse)
B
AS FiPS2 Ep6F-28
AS FiPS3 Ep6F-9
E
AS FiPS2 Ep6F-28
AS FiPS3 Ep6F-9
C
Copy #/ ng gDNA
AS FiPS2 Ep6F-28
AS FiPS3 Ep6F-9
hFibroblasts 72h post nucleofection
D
AS FiPS2 Ep6F-28
AS FiPS3 Ep6F-9
OCT3/4 (pla)
SOX2 (pla)
KLF4 (pla)
L-MYC (pla)
LIN28 (pla)
OCT3/4 CD5
SOX2 CD5
LIN28 CD5
p53
EBNA-1
F
AS FiPS2 Ep6F-28
AS FiPS3 Ep6F-9
Nanog
Tra-1-81
Oct4
SSEA3
Sox2
SSEA4
Tra-1-60
G
AFP FOXA2
TUJ1 GFAP
ASMA ASA

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
AS FiPS2 Ep6F-28 (ESi055-A)	*AS FiPS2 Ep6F-28*	*Male*	*25*	*Caucasian*	*COL4A5* (LRG_232t2, NM_033380.2) Genotype: c.3722G>A p.(G1241D), Exon41/Hemizygosis	*XLAS*
AS FiPS3 Ep6F-9 (ESi056-A)	*AS FiPS3 Ep6F-9*	*Male*	*41*	*Caucasian*	*COL4A5* (LRG_232t2, NM_033380) Genotype: c.4052G>A p.(G1351D), Exon46/Hemizygosis	*XLAS*

Table 2
Summary of AS FiPS2 Ep6F-28 and AS FiPS3 Ep6F-9 characterization.

Classification	Test	Result	Data
Morphology	Photography	Normal	Not shown but available with author
Phenotype	Immunocytochemistry	OCT4, SOX2, NANOG, TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4	Fig. 1 panel F
	Flow cytometry	N/A	
Genotype	Karyotype (G-banding) and resolution	46XX, resolution 500	Fig. 1 panel B
Identity	Microsatellite PCR (mPCR)	N/A	
	STR analysis	10 loci analyzed, all matching	Supplementary Fig. S1 panel A
Mutation analysis	Sequencing	Gene: *COL4A3* (LRG_230t1, NM_000091.4) Genotype: c.[345delG];[345delG], p.(P116Lfs*37), Exon6/Homozygosis	Fig. 1 panel A
	Southern Blot OR WGS	N/A, non-integrating reprogramming methodology	
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Fig. S1 panel B
Differentiation potential	Embryoid body formation	Proof of three germ layers formation from Embryoid bodies: α-fetoprotein (AFP) and forkhead box A2 (FOXA2), βIII-tubulin (TUJ1) and glial fibrillary acidic protein (GFAP), α-smooth muscle actin (ASMA) and α-sarcomeric actin (ASA)	Fig. 1 panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

therapy. The generated iPSC lines offer a useful resource to investigate pathogenic mechanisms in Alport syndrome, as well as for drug testing.

Resource details

Alport syndrome (AS) is a hereditary disorder causing chronic kidney disease progressing to end-stage renal disease, sensorineural hearing loss, and ocular abnormalities (Kashtan & Michael, 1996). AS is rare (1/5.000–10.000), but it accounts for around 1% of patients receiving renal replacement therapy. AS has three genetic modes of inheritance: X-linked AS (XLAS), autosomal recessive AS (ARAS), and autosomal dominant AS (ADAS) (Kashtan, 2015). XLAS is caused by mutations in the *COL4A5* gene encoding the type 4 collagen α5–chain and accounts for approximately 65% of patients with the disease. The patient AS2 presented in this study has a history of sensorineural deafness since early adolescence, haematuria since childhood as well as progressive proteinuria. He reached end-stage renal disease at 20 years of age and was transplanted one year later. He carries a hemizygous missense mutation in the *COL4A5* gene, involving a Glycine residue, causing XLAS. His mother as well as his aunts and cousins are affected by the disease. The patient AS3 has a history of haematuria and proteinuria since childhood without hypoacusia. He reached end-stage renal disease at 42 years of age and received a kidney graft from a living donor. He carries a hemizygous missense mutation in the *COL4A5* gene, involving a Glycine residue, causing XLAS. His mother, aunt and cousin are affected by the disease and they were all transplanted in their forties to sixties.

Dermal fibroblasts were derived from skin biopsies of patients AS2 and AS3 carrying the hemizygous *COL4A5* mutations c.3722G>A, p.(G1241D) on exon 41 or c.4052G>A p.(G1351D) on exon 46, respectively, confirmed by Sanger sequencing (Fig. 1A). Fibroblasts were reprogrammed by nucleofection of non-integrating episomal plasmids encoding six human factors (OCT3/4, SOX2, KLF4, LIN28, L-Myc and a p53 knock down shRNA). The resulting iPSC lines were named AS-FiPS2-Ep6F-28 and AS-FiPS3-Ep6F-9 (Tables 1 and 2) and were karyotypically normal (Fig. 1 B). Absence of episomal plasmids was shown by determining episomal plasmid copy number in genomic DNA (gDNA) from the iPSC line by absolute quantitative real time PCR (aqRT-PCR). As positive control, gDNA from human fibroblasts 72 h post nucleofection was used (Fig. 1C). mRNA expression levels of episomal plasmid derived genes and endogenous pluripotency markers (Fig. 1D, pla or CDS, respectively, in blue), and p53 and EBNA-1 expression of control fibroblasts 72 h post nucleofection (Fig. 1D, in green) were analyzed by quantitative reverse transcriptase PCR (qPCR) using specific primers (Table 3). Expression of pluripotency markers was confirmed by immunocytochemistry with antibodies against endogenous human OCT4, SOX2, NANOG, TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4 (Fig. 1F, scale bars 50 μm) and alkaline phosphatase (AP) activity (Fig. 1E). The differentiation capacity of the lines was tested by embryoid body (EB) formation and differentiation *in vitro* towards the three germ layers, as shown by immunofluorescence analyses demonstrating the expression of definitive endoderm (AFP and FOXA2), ectoderm (TUJ1 and GFAP) and mesoderm (ASMA and ASA) markers (Fig. 1G,

Fig. 1. Characterization of AS FiPS2 Ep6F-28 and AS FiPS3 Ep6F-9 lines. A. WT DNA sequences and mutations in the same region of the iPSC lines. B. Karyotype of representative metaphase showing normal 46 chromosomes (XY). C. Absolute quantitative real time PCR showing absence of episomal plasmids in iPSCs (left and middle column) and presence of plasmids in GFP nucleofected control fibroblasts (right column). D. mRNA expression levels of transgenes (pla) and endogenous pluripotency markers (CDS) (in blue), and p53 and EBNA-1 expression of GFP nucleofected control fibroblasts (in green). E. Positive alkaline phosphatase activity in iPSC colonies growing under feeder-free conditions. F. Confocal images showing immunodetection of pluripotency-associated markers of AS FiPS lines. Scale bar: 50 μm. G. Immunofluorescence analyses of *in vitro* differentiation of EBs using specific antibodies against the endodermal markers α-fetoprotein (AFP) and forkhead box A2 (FOXA2), ectodermal markers βIII-tubulin (TUJ1) and glial fibrillary acidic protein (GFAP), and mesodermal markers α-smooth muscle actin (ASMA) and a-sarcomeric actin (ASA). Nuclei were counterstained with DAPI. Scale bar: 50 μm.

Table 3
List of antibodies and primers used.

Antibodies used for immunocytochemistry				
	Antibody	Specie	Dilution	Company Cat # and RRID
Pluripotency markers	OCT4	Mouse	1:25	Santa Cruz, sc-5279
	NANOG	Goat	1:25	R&D Systems, AF1997
	SOX2	Rabbit	1:100	ABR, PA1–16968
	SSEA3	Rat	1:2	Hybridoma Bank, MC-631
	SSEA4	Mouse	1:2	Hybridoma Bank, MC-813-70
	TRA-1-60	Mouse	1:100	Millipore, MAB4360
	TRA-1-81	Mouse	1:100	Millipore, MAB4381
Differentiation markers	TUJ1	Mouse	1:40	Covance, MMS-435P
	GFAP	Rabbit	1:1000	Dako, Z0334
	ASMA	Mouse	1:400	Sigma, A5228
	ASA	Mouse	1:400	Sigma, A2172
	AFP	Rabbit	1:200	Dako, A0008
	FOXA2	Goat	1:50	R&D Systems, AF2400
Secondary antibodies	A488-mouse		1:500	Jackson, 715-545-151 and 115-546-071
	Cy3-rat		1:500	Jackson, 112-165-020
	A488-rabbit		1:500	Jackson, 711-545-152
	Cy3-mouse IgG		1:500	Jackson, 115-165-071
	DL649-mouse IgM		1:500	Jackson, 115-495-075
	A488-goat		1:500	Jackson, 705-545-147
	Cy3-mouse		1:500	Jackson, 715-165-140
	Cy3-goat		1:500	Jackson, 705-165-147
	Cy3-rabbit		1:500	Jackson, 711-165-152
	Cy3-mouse IgM		1:500	Jackson, 115-165-075

Primers		
	Target	Forward/Reverse primer (5′-3′)
episomal plasmids (aqRT-PCR)	EBNA-1	ATCAGGGCCAAGACATAGAGATG/GCCAATGCAACTTGGACGTT
episomal plasmids (qPCR)	pCXLE-Oct3/4 (pla)	CATTCAAACTGAGGTAAGGG/TAGCGTAAAAGGAGCAACATAG
	pCXLE-SOX2 (pla)	TTCACATGTCCCAGCACTACCAGA/TTTGTTTGACAGGAGCGACAAT
	pCXLE-KLF4 (pla)	CCACCTCGCCTTACACATGAAGA/TAGCGTAAAAGGAGCAACATAG
	pCXLE-LIN28 (pla)	AGCCATATGGTAGCCTCATGTCCGC/TAGCGTAAAAGGAGCAACATAG
Endogenous pluripotency genes (qPCR) and controls (qPCR)	Endogenous Oct3/4 (cds)	CCCCAGGGCCCCATTTTGGTACC/ACCTCAGTTTGAATGCATGGGAGAGC
	Endogenous SOX2 (cds)	TTCACATGTCCCAGCACTACCAGA/TCACATGTGTGAGAGGGGCAGTGTGC
	Endogenous LIN28 (cds)	AGCCATATGGTAGCCTCATGTCCGC/TCAATTCTGTGCCTCCGGGAGCAGGGTAGG
	p53	TCTGTCCCTTCCCAGAAAACC/CAAGAAGCCCAGACGGAAAC
	EBNA-1	ATCAGGGCCAAGACATAGAGATG/GCCAATGCAACTTGGACGTT
House-keeping gene (qPCR)	GAPDH	GTAACCCGTTGAACCCCATTC/CCATCCAATCGGTAGTAGCG
Genotyping	*COL4A5*-exon 41	ATTGCCCTAATGTATGTGAATAGC/CAAGCTATTTACTTGTAAGAAGTTAT
	COL4A5-exon 46	AGTTTGACTCTAGAAATAGTGC/GCATCTAAGTATCAGGTATAAC

scale bars 50 μm). The iPSC identity was confirmed by short tandem repeat analysis and compared with the patient's fibroblasts (Supplementary Fig. S1A).

Materials and methods

Reprogramming of fibroblasts

Fibroblasts were cultured in IMDM supplemented with 10% FBS and 1% penicillin–streptomycin (all Gibco) at 37 °C and 5% CO_2. 1×10^6 fibroblasts were reprogrammed at passage 6 by nucleofection with three pCXLE episomal plasmids carrying OCT3/4, SOX2, KLF4, LIN28, L-Myc and a p53 knock down shRNA (Addgene). Five days after nucleofection fibroblasts were trypsinized and seeded onto γ-irradiated human foreskin fibroblasts (iHFF) in human embryonic stem cell medium [Knockout DMEM supplemented with 20% Knockout serum replacement, 2 mM Glutamax, 1% penicillin–streptomycin, 0,1 mM β-mercaptoethanol, 1% non-essential amino acids (NEAA), (all Gibco), and 10 ng/ml bFGF (Millipore)]. In parallel, fibroblasts were nucleofected with a pCXLE-GFP plasmid in the same way. Three days after nucleofection, efficiency was calculated by FACS analysis of GFP positive cells. Cell pellets were prepared to extract gDNA and mRNA and used as positive control for determining episomal plasmid copy numbers and silencing of transgenes. Approximately 15 days after seeding nucleofected fibroblasts, the first iPSC colonies appeared. Colonies were picked manually and passaged for expansion. From passage 5 on, colonies were adapted to feeder-free conditions.

aqRT-PCR and qPCR analysis

To verify the absence of episomal plasmids in generated iPSCs, gDNA was extracted and aqRT-PCR with specific primers (Table 3) against plasmid derived EBNA1 was performed. As positive control gDNA from fibroblasts extracted 72 h post nucleofection was used. For qPCR mRNA was isolated by Trizol-based procedure and 1 μg of mRNA was reverse transcribed with Cloned AMV First-strand cDNA kit (Life technologies). For aqRT-PCR and qPCR reactions SYBR green (Life technologies) was used. Primer sequences are listed in Table 3. Ct values were normalized by means of the housekeeping gene GAPDH.

Karyotyping

Genomic integrity of iPSCs was evaluated by G banded metaphase karyotype analysis (Ambar, Barcelona). 70% confluent iPSC colonies were treated with colcemid (Gibco), trypsinized, incubated with hypotonic solution, fixed in Carnoy fixative (75% methanol, 25% acetic acid) and karyotypes performed following standard procedures.

Alkaline phosphatase, immunocytochemistry for pluripotency markers and in vitro differentiation

To detect AP activity, iPSCs were fixed with 4% paraformaldehyde for 1 min, washed with PBS and incubated with AP staining solution (Sigma) following the manufacturers' directions. To detect pluripotency

markers, immunofluorescence analyses were performed. iPSCs were fixed with 4% paraformaldehyde, blocked and permeabilized with TBS + 0.5% Triton X-100 + 6% donkey serum. Primary antibodies (Table 3) were incubated overnight in TBS + 0.1% Triton X-100 + 6% donkey serum. Secondary antibodies (Table 3) were incubated for 2 h at 37 °C. Nuclei were stained with DAPI. *in vitro* differentiation towards the three germ layers was promoted by EB formation. iPSCs colonies were lifted manually and incubated in ultra-low attachment plates in mTeSR1 medium. After 24 h medium was changed to differentiation medium for additional 24–48 h (Ectoderm medium: 50% Neurobasal, 50% DMEM/F12, 1% N2, 1% B27, 1% Glutamax and 1% Penicillin-Streptomycin; Endoderm medium: Knockout-DMEM, 10% FBS, 1% NEAA, 0.1% β-mercaptoethanol, 1% Glutamax and 1% Penicillin-Streptomycin; Mesoderm medium: Endoderm medium supplemented with 0,5 mM ascorbic acid). EBs were seeded on matrigel-coated slide flasks, cultured in differentiation media for 15–20 days and analyzed by immunofluorescence. Confocal images were taken using Leica TSC SPE or SP5 microscopes.

Confirmation of mutation

Exon 41 or exon 46 of *COL4A5* gene was amplified by PCR using exon flanking primers (Table 3). The PCR product was Sanger sequenced using the BigDye DNA Sequencing kit v1.1 on an ABI 3130 Genetic Analyzer (Applied Biosystems).

Authentication and mycoplasma testing

To confirm line identity genomic DNA was extracted from iPSCs and patients fibroblasts and used for STR analysis (Supplement Fig. S1 panel A). Routinely media samples were tested for absence of mycoplasma contaminations by PCR (Supplement Fig. S1 panel B).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2017.08.019.

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Author contributions

A.V, M. M and A. R designed the study. B. K, L.MS, B. A, Y.M, E. A, and G. B performed the experiments. B·K wrote the paper. R.T and M.F coordinated with patients and arranged for patient samples.

Conflict of interest

The authors declare no conflict of interest.

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