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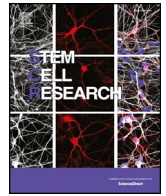
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Lab resource: Stem Cell Line

Generation of a human iPSC line, INMi004-A, with a point mutation in *CRX* associated with autosomal dominant Leber congenital amaurosis

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ABSTRACT

The human induced pluripotent stem cell (iPSC) line, INMi004-A, was generated using dermal fibroblasts from a 6 year-old patient with autosomal dominant Leber Congenital Amaurosis (LCA) caused by the point mutation c.695delC (p.Pro232Argfs*139) in the *CRX* gene. We used non-integrative Sendai virus vectors containing the human OSKM transcription factor cocktail to reprogram patient fibroblasts. The generated iPSC line contained the congenital deletion c.695delC in exon 4 of *CRX*, had a normal karyotype, and was capable of differentiation into all three germ layers. This cell line represents an important tool to study the pathophysiology of *CRX*-associated LCA.

Resource table

Unique stem cell line identifier	INMi004-A
Alternative name(s) of stem cell line	CRX-LCA-iPSC
Institution	Institute for Neurosciences of Montpellier, Montpellier, France
Contact information of distributor	Vasiliki Kalatzis vasiliki.kalatzis@inserm.fr
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 6 years old Sex: Male Ethnicity: Caucasian
Cell source	Dermal fibroblasts
Clonality	Clonal
Method of reprogramming	Non-integrative Sendai virus vectors
Genetic modification	Yes
Type of modification	Congenital mutation
Associated disease	Leber congenital amaurosis
Gene/locus	CRX 19q13.3
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	March 2018
Cell line repository/bank	https://hpscereg.eu/cell-line/INMi004-A
Ethical approval	Regional committee: CPP South-Mediterranean I (2014-A00549-38) National committee: ANSM (140549B-62)

1. Resource utility

The INMi004-A (*CRX*-LCA-iPSC) line described herein was derived from a patient presenting with autosomal dominant Leber Congenital Amaurosis (LCA) and carrying a point mutation, c.695delC, in the *CRX* gene. This iPSC line, which can be used to generate patient-specific photoreceptor cells, will permit modelling of the pathophysiology of *CRX*-associated LCA.

2. Resource details

CRX (cone-rod homeobox) is a transcription factor required for the activation of both cone and rod photoreceptor genes (Tran and Chen, 2014). *CRX* is essential for photoreceptor differentiation as well as maintenance (Furukawa et al., 1997), and mutations in this gene lead to inherited retinal dystrophies (IRDs) characterised by photoreceptor degeneration. Interestingly, three distinct IRD forms with a primarily autosomal dominant mode of transmission have been linked to *CRX* mutations: Leber Congenital Amaurosis (LCA), Retinitis Pigmentosa (RP) and Cone-Rod Dystrophy (CRD) (Berger et al., 2010). LCA is an early onset form of RP, which severely affects vision in the first decade of life. LCA (and RP) are characterised by degeneration of rods before cones leading to tunnel vision; by contrast, in CRD, cones degenerate first. Currently, there are 23 causative genes for LCA and *CRX* mutations account for ~3% of cases (<https://sph.uth.edu/retnet/>).

A skin punch biopsy was taken from a 6 year-old patient with

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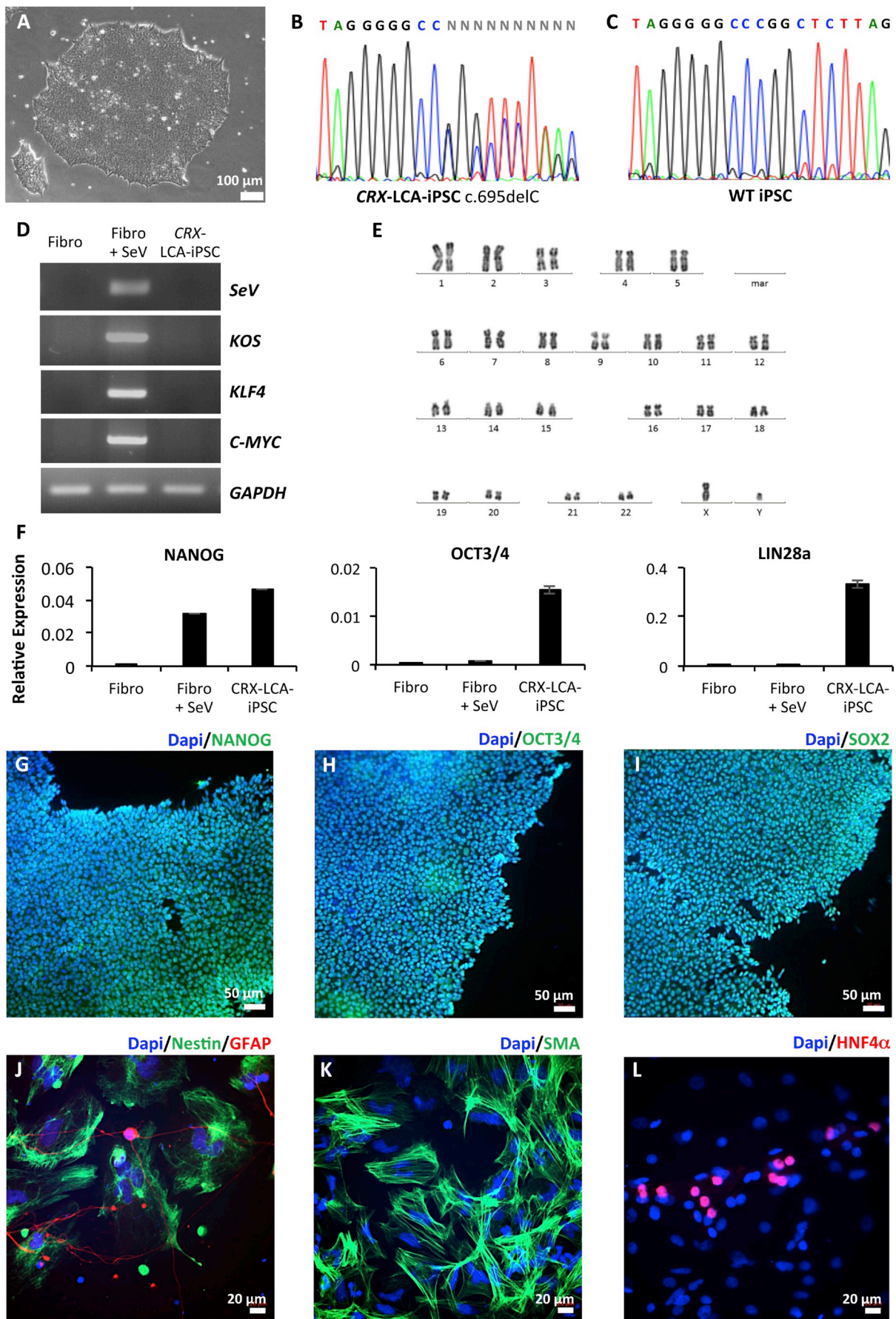


Fig. 1. Characterization of the INMi004-A (CRX-LCA-iPSC) line.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Microscopy	Normal morphology	Fig. 1A
Phenotype	Qualitative analysis	Positive for pluripotency markers: NANOG, OCT3/4 and SOX2	Fig. 1G-I
	Immunocytochemistry Quantitative analysis qPCR	Expression of pluripotency markers: NANOG, OCT3/4, LIN28a	Fig. 1F
Genotype	Karyotype (G-banding) and resolution	46,XY Resolution 400–500	Fig. 1E
Identity	Microsatellite PCR (mPCR) OR	DNA Profiling Performed	Available with authors
		D3S306 D7S640 D8S532 D17S787 D19S572	
Mutation analysis	STR analysis	Not performed	N/A
	Sequencing	Heterozygous, CRX c.695delC	Fig. 1B
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing luminescence. Negative	Supplementary Table 1
Differentiation potential	Embryoid body formation	Expression of Nestin and GFAP (ectoderm), SMA (mesoderm) and HNF4 α (endoderm) in iPSC derived EBs	Fig. 1J-L
Donor screening (optional)	Hepatitis B, Hepatitis C, HIV 1–2	Negative	Available with authors
Genotype additional info (optional)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

autosomal dominant LCA and harbouring the heterozygous c.695delC mutation in exon 4 of *CRX* (Supplementary Fig. 1). This mutation is predicted to cause a frameshift giving rise to a mutant protein, p.Pro232Argfs*139, with a long non-homologous C-terminal extension. Primary fibroblasts were isolated and reprogrammed into induced pluripotent stem cells (iPSCs) using non-integrative Sendai virus vectors carrying the *OCT3/4*, *SOX2*, *KLF4* and *cMYC* cocktail, (OSKM; Takahashi et al., 2007). The generated *CRX*-LCA-iPSC colonies displayed a typical round morphology with refractive edges and consisted of small tightly packed cells with a high nuclear/cytoplasmic ratio (Fig. 1A). We confirmed the presence of the c.695delC mutation in the *CRX*-LCA-iPSC line (Fig. 1B), as compared to wild-type (WT) iPSC (Fig. 1C), by Sanger sequencing. After twelve cell culture passages, we could not detect the Sendai vectors by reverse-transcription (RT)-PCR amplification using primers specific to the reprogramming cassettes (Fig. 1D). By contrast, we detected the Sendai vectors in transduced patient fibroblasts (Fibro + SeV; positive control) as compared to non-transduced cells (Fibro; negative control). The *CRX*-LCA-iPSC line displayed a normal karyotype (46,XY) after ten passages (Fig. 1E), excluding the appearance of large chromosomal rearrangements during reprogramming.

The activation of endogenous pluripotency genes, *NANOG*, *OCT3/4* and *LIN28a*, was determined by quantitative PCR (qPCR) analysis of the *CRX*-LCA-iPSC line (Fig. 1F) using the same controls as for the RT-PCR study. All three pluripotency genes were expressed in *CRX*-CRD-iPSC but not in the non-transduced fibroblasts (Fig. 1F); an intermediate level of *NANOG* expression was already detectable in fibroblasts 7 days post-transduction. Immunofluorescence analysis confirmed the expression of the endogenous pluripotency markers NANOG (Fig. 1G), OCT3/4 (Fig. 1H) and SOX2 (Fig. 1I). Moreover, an *in vitro* embryoid body (EB) formation assay was used to test the capacity of the *CRX*-LCA-iPSC line to undergo tri-lineage differentiation, as determined by immunofluorescence staining using antibodies against Nestin and Glial Fibrillary Acidic Protein (GFAP) (Fig. 1J) for the ectoderm layer, Smooth Muscle Actin (SMA; Fig. 1K) for the mesoderm layer, and Hepatocyte Nuclear Factor 4 alpha (HNF4 α) (Fig. 1L) for the endoderm layer. In addition, PCR analysis of five microsatellite markers showed a perfect match between the *CRX*-LCA-iPSC line and fibroblasts of the same individual, in comparison to WT iPSC (available with the authors). Lastly, we routinely monitored the *CRX*-LCA-iPSC line for mycoplasma contamination (Supplementary Table 1).

3. Materials and methods

3.1. Fibroblast culture

Dermal fibroblasts were derived from a skin biopsy and cultured in AmnioMAX C100 basal media with GlutaMAX (Gibco) containing 10% decomplemented foetal calf serum (FCS; Lonza), 1% penicillin-streptomycin-amphotericin B (Lonza) and 2% AmnioMax-C100 supplement (Gibco).

3.2. Cell reprogramming

Fibroblasts were seeded in high-glucose DMEM with GlutaMAX (Gibco) containing 10% FCS, 1% non-essential amino acids (NEAA; Gibco) and 55 mM β -mercaptoethanol (Gibco), and reprogrammed using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher). At day 7, cells were passaged onto plates coated with Matrigel hESC-Qualified Matrix (Corning). At day 8, the medium was changed to TeSR-E7 Basal Medium (Stemcell Technologies). Emerging iPSC colonies were mechanically passaged into Essential 8 (E8) medium (Gibco) and cultured under 5% CO₂ at 37 °C. Subsequent passages (1:10 dilution; twice weekly) were performed using Versene solution (Gibco).

3.3. Mutation and microsatellite analysis

Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen). For mutation analysis, the DNA was PCR-amplified using the AmpliTaq Gold 360 Master Mix (Applied Biosystems) and *CRX*-specific primers (Table 2). The amplicon was cleaned with the ExoSAP-IT PCR Clean-up kit (GE Healthcare) and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit V3.1. For microsatellite analysis, DNA was amplified using primers for informative markers (Table 1) and PCR products were mixed with Genescan 400HD ROX size standard. All reactions were analysed on an Applied Biosystems 3130xL genetic analyser.

3.4. RT-PCR and qPCR analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and cDNA synthesis performed using the SuperScript® III First-Strand Synthesis System with random hexamers (Life Technologies), according

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-NANOG	1/200	Abcam Cat# ab21624, RRID:AB_446437
Pluripotency markers	Mouse anti-OCT3/4	1/200	Santa Cruz Biotechnology Cat# sc-5279, RRID:AB_628051
Pluripotency markers	Rabbit anti-SOX2	1/200	Thermo Fisher Scientific Cat# 48-1400, RRID:AB_2533841
Differentiation markers	Mouse anti-Nestin	1/200	Novus Biologicals Cat# NBP1-92717, RRID:AB_11020601
Differentiation markers	Rabbit anti-GFAP	1/200	Dako Cat# Z0334, RRID:AB_10013382
Differentiation markers	Mouse anti-SMA	1/200	Dako Cat# M0851, RRID:AB_2223500
Differentiation markers	Rabbit anti-HNF4 α	1/200	Santa Cruz Cat# sc-8987, RRID:AB_2116913
Secondary antibodies	Alexa Fluor [®] 488	1/500	Jackson ImmunoResearch, Cat# 711-545-152, RRID: AB_2313584
Secondary antibodies	AffiniPure Donkey Anti-Rabbit IgG (H + L)		
Secondary antibodies	Alexa Fluor [®] 488	1/500	Jackson ImmunoResearch, Cat# 715-545-150, RRID:AB_2340846
Secondary antibodies	AffiniPure Donkey Anti-Mouse IgG (H + L)		
Secondary antibodies	Alexa Fluor [®] 647	1/500	Jackson ImmunoResearch, Cat# 711-606-152, RRID:AB_2340625
Secondary antibodies	AffiniPure Donkey Anti-Rabbit IgG (H + L)		
Primers			
	Target	Forward/Reverse primer (5'-3')	
Exogenous reprogramming transgene (RT-PCR)	<i>SeV</i> (181 bp)	GGATCACTAGGTGATATCGAGC ACCAGACAAGAGTTAAGAGATATGTATC	
Exogenous reprogramming transgene (RT-PCR)	<i>KOS</i> (528 bp)	ATGCACCGCTACGACGTGAGCGC ACCTTGACAATCCTGATGTGG	
Exogenous reprogramming transgene (RT-PCR)	<i>KLF4</i> (410 bp)	TTCCTGCATGCCAGAGGAGCC AATGTATCGAAGGTGCTCAA	
Exogenous reprogramming transgene (RT-PCR)	<i>C-MYC</i> (532 bp)	TAACTGACTAGCAGGCTTGTCG TCCACATACAGTCTGGATGATGATG	
Pluripotency marker (qPCR)	<i>NANOG</i> (158 bp)	CAAAGGCAAACAACCCACTT TCTGCTGGAGGCTGAGGTAT	
Pluripotency marker (qPCR)	<i>OCT3/4</i> (168 bp)	GTACTCCTCGGTCCCTTTCC CAAAAACCTGGCACAACCT	
Pluripotency marker (qPCR)	<i>LIN28a</i> (166 bp)	GGGGAATCACCTTACAACT CTTGGCTCCATGAATCTGGT	
Housekeeping gene (RT-PCR and qPCR)	<i>GAPDH</i> (112 bp)	AACCATGAGAAGTATGACAAC CTTCCACGATACCAAAGTT	
Targeted mutation analysis	<i>CRX</i> (787 bp)	ATGTGAACCCAGCACCTCTC CCGATGGAGAGAGATGGAGA	

to the manufacturer's protocols. RT-PCR was performed as follows: denaturation (95 °C, 10 min), amplification (95 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min for 35 cycles), and a final extension (72 °C, 10 min). For qPCR analysis, the cDNA was diluted to 2.5 ng/ μ l and 2 μ l was used for each reaction with the FastStart SYBR Green I Master mix on a LightCycler 480 II thermal cycler (Roche). Gene expression was normalized to *GAPDH* expression. Primers sequences are listed in Table 2.

3.5. Karyotype analysis

Preparation of the iPSCs was performed as previously described without modification (Torriano et al., 2017). Twenty metaphase spreads were counted and karyotype analysis was performed by the Chromostem facility (CHU Montpellier, France).

3.6. Immunofluorescence staining

Cells were fixed using 4% PFA (20 min, RT), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich; 15 min, RT) and blocked (45 min, RT) in 1% BSA (Sigma-Aldrich)/10% donkey serum (Millipore). Primary antibodies were incubated overnight, 4 °C (no primary antibody for negative control), and fluorescence-conjugated secondary antibodies 1 h, RT (Jackson ImmunoResearch). All antibodies were diluted in 1% BSA/1% donkey serum. Nuclei were stained with 0.2 μ g/ml bisBenzimide (Sigma-Aldrich). Cells were observed using a Zeiss ApoTome 2 Upright wide-field microscope.

3.7. In vitro differentiation assay

iPSCs were dissociated with Accutase (Stemcell Technologies) and seeded on ultra-low attachment dishes for 2 days in E8 containing Y27632 StemMACS. At day 3, the medium was changed to DMEM/F12 (Gibco) with 20% knockout serum replacement (Gibco), 1% penicillin-streptomycin (Gibco), 1% GlutaMax, 55 mM β -mercaptoethanol and 1% NEAA. At day 7, the embryoid bodies were seeded onto Matrigel-coated wells in the same medium and cultured for 10 days before immunofluorescence staining.

3.8. Mycoplasma analysis

Mycoplasma detection was performed on cell culture supernatant using the MycoAlert Mycoplasma Detection Kit (Lonza) and a CLARIOstar microplate reader (BMG Labtech).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2019.101476>.

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