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Lab Resource: Single Cell Line

Generation of an iPSC line from congenital hypopituitarism patient with a nonsense heterozygous variant in *FOXA2*

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A B S T R A C T

Forkhead box A2 (FOXA2) is a pioneer transcription factor, necessary for human development. Mutations in *FOXA2* were recently associated with congenital hypopituitarism (CH); however, the pathogenic mechanism remains unknown. Induced pluripotent stem cells from a patient with CH carrying a heterozygous *FOXA2* variant (c.686C > A; p.S229*) were obtained by cellular reprogramming from the patient's peripheral blood mononuclear cells. The generated iPSCs exhibited all hallmarks of pluripotency, differentiated into the three germ layers, and presented a normal karyotype. This resource represents a valuable tool for investigating the role of FOXA2 in pituitary development and associated disorders.

Resource Table

Unique stem cell line identifier	INEUi005-A
Alternative name(s) of stem cell line	FOXA2^{S229*}-iPSCs R1 Clone
Institution	Instituto de Neurociencias, Fundación para la Lucha contra las Enfermedades Neurológicas de la Infancia (FLENI)
Contact information of distributor	Dr. Santiago Miriuka. smiriuka@fleni.org.ar / Dra. Lucia N. Moro lmoro@fleni.org.ar
Type of cell line	iPSC
Origin	Human
Additional origin info required for human ESC or iPSC	Age:14 Sex: Female Ethnicity if known: White Latin
Cell Source	PBMCs
Clonality	Clonal
Method of reprogramming	Lentiviral EF1a-hSTEMCCA-loxP vector expressing OCT-4, SOX-2, c-MYC, and KLF- 4

(*continued on next column*)

Resource Table (*continued*)

Unique stem cell line identifier	INEUi005-A
Genetic Modification	Yes
Type of Genetic Modification	Hereditary
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	Polymerase chain reaction (PCR) followed by agarose gel electrophoresis (Supplementary Fig. 1)
Associated disease	Congenital hypopituitarism
Gene/locus	FOXA2 NM_021784.4: c.686C > A
Date archived/stock date	23/12/2024
Cell line repository/bank	https://hpscreg.eu/cell-line/INEUi005-A
Ethical approval	The study was approved by a local Ethics Committee (Comité de ética en investigaciones biomédicas del Instituto FLENI) (code number: Protocol 32–24). Written informed consent was obtained from the patient's parents and the patient.

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1. Resource utility

The mechanism by which mutations in *FOXA2* lead to hypopituitarism has not yet been fully elucidated. Here, we generated an iPSCs line from a patient diagnosed with hypopituitarism and craniofacial malformations with a heterozygous *FOXA2* variant (c.686C > A;p.S229*). This new line represents a valuable tool for assessing the mechanism of a novel *FOXA2* variant, enlightening the molecular contribution of this gene during development.

2. Resource Details

Congenital hypopituitarism (CH) is a complex disorder characterized by the deficiency of one or more pituitary hormones, with an estimated incidence of 1 in 4000 to 1 in 10,000 live births (Bosch I Ara et al., 2020). More than 30 genes, mainly transcription factors, have been implicated in this disorder. *FOXA2* is one of these genes. Forkhead Box A2 (*FOXA2*, OMIM: *600288) is a pioneer transcription factor member of the forkhead DNA-binding class of proteins, involved in the development of multiple tissues including the floorplate, forebrain, liver, pancreas, and gastrointestinal tract (Ruiz i Altaba et al., 1993; Jin et al., 2001; Lee et al., 2005; Gao et al., 2008; Richmond and Breault, 2010; Ruiz i Altaba et al., 1993).

A role for Foxa2 in pituitary development has been proposed since it is highly expressed throughout the developing hypothalamic-pituitary axis in mice (Giri et al., 2017), and regulates sonic hedgehog (*SHH*) gene expression (Vajravelu et al., 2018), which is essential for pituitary stem cell differentiation (Treier et al., 2001).

Deletion and/or mutations of *FOXA2* have been linked to CH (Boda et al., 2019; Dayem-Quere et al., 2013; Garcia-Heras et al., 2005; Williams et al., 2011) in association with biliary atresia and heterotaxy (Tsai et al., 2015), congenital hyperinsulinism (Giri et al., 2017), or gastrointestinal malformations (Boda et al., 2019; Elsayed et al., 2020); however, the molecular contribution of *FOXA2* to the pathogenesis of CH and its role during pituitary development remains unknown.

Here, Induced pluripotent stem cells (iPSCs) were generated from a female patient diagnosed with growth hormone deficiency at the age of 1.7 years, anterior pituitary hypoplasia, and craniofacial midline defects, carrying a heterozygous *nonsense* variant in *FOXA2* (NM_021784.4: c.686C > A; p.S229* (previously reported (Vishnopolska et al., 2021)). Peripheral blood mononuclear cells (PBMCs) were isolated from the patient's blood, amplified for 10 days in expansion media with factors, and transduced with an EF1a-hSTEMCCA-loxP lentiviral vector expressing OCT-4, KLF4, SOX-2, and c-MYC, as described in (Sommer et al., 2009). A single clone of FOXA2^{S229*}-iPSCs, termed as INEUi005-A, was isolated and selected for characterization. INEUi005-A showed typical multicellular iPSC colonies with distinct borders, an elevated nuclei-to-cytoplasm ratio, and high alkaline phosphatase (ALP) activity (Fig. 1A). Authentication of iPSCs against donor PBMCs was confirmed through short tandem repeat (STR) profiling (Table 1), and karyotype analysis revealed a normal 46, XX karyotype screened by 30 metaphases at a 440-band resolution (Fig. 1B). *FOXA2* c.686C > A;p.S229* heterozygous mutation was confirmed in INEUi005-A by Sanger sequencing (Fig. 1C). The mRNA expression levels of pluripotency-associated genes, *OCT4*, *NANOG*, and *SOX2* were similar to the control iPSC line, INEUi002-A (Questa et al., 2016), and upregulated versus human fibroblasts (HF) (One-Way ANOVA, followed by Dunnett's − test; *p < 0,05, **p < 0,01; ***p < 0,001) (Fig. 1D). At the protein level, the assessment of 15 stem-specific and cell-commitment markers in FOXA2S229*-iPSCs (INEUi005-A)cell lysates confirmed their pluripotent status, as no differences were observed versus Control-iPSCs (INEUi002-A) (Fig E). Moreover, immunofluorescence studies demonstrated positive staining of nuclear SOX2, NANOG, and OCT-4, and the stem cell surface marker, TRA1-60 (Fig. 1F). The spontaneous *in vitro* capacity of differentiation was evaluated by the embryoid body assay showing positive expression of specific markers for each lineage: TUJ1 (ectoderm), TBX6 (mesoderm), and SOX17 (endoderm), showing that INEUi005-A iPSCs are able to differentiate into the three embryonic lineages (Fig. 1G).

3. Materials and Methods

3.1. Cellular Reprogramming and iPSCs Culture

Peripheral blood mononuclear cells (PBMCs) were obtained from 3 ml peripheral blood and separated by density gradient centrifugation using Ficoll-Paque media (Sigma). PBMCs were cultured in expansion media containing QBSF-60 Serum-Free Medium (Quality Biological, Cat#: 160–204–101), 100 µg/mL Penicillin/streptomycin (Gibco), 100 µg/mL Glutamin (Gibco) and 50 µg/mL ascorbic acid, enriched with 50 ng/mL SCF, 10 ng/mL IL-3, 2U/mL EPO, 40 ng/mL IGF-1 and 1 µM Dexamethasone, as in (Park and Mostoslavsky, 2018). After 9 days of expansion, 150,000 PBMCs were lentivirally transduced with the EF1a-hSTEMCCA-loxP vector expressing OCT-4, SOX-2, c-MYC, and KLF- 4 (MOI = 1), and transferred to Geltrex (Gibco) coated dishes with ReproTeSR media (Stem Cell Technologies). Single iPSCs colonies were picked manually and expanded in culture till passages 10–13. iPSCs were maintained in StemFlex media in a cell incubator at 37 °C in a 5 % CO_2 atmosphere and passed when cells reached 70 % confluence using Versene, for maintenance, or TrypLE when single cell dissociation was needed, with 10 uM Y-27632 Rock inhibitor (Tocris) on Geltrex-coated plates.

3.2. Karyotyping, STR analysis, and Genotyping

Chromosomal G-band analysis (passage 11) were performed by Kromos Cytogenetic Laboratory (Buenos Aires, Argentina) (Supplementary Fig. 3). Short tandem repeats (STR) were analyzed by The Servicio de Huellas Digitales Genéticas (Facultad de Farmacia y Bioquímica, UBA, Buenos Aires, Argentina). Genomic DNA obtained from the iPSC line was PCR-amplified with specific primers to detect *FOXA2* mutation c.686C > A;p.S229* and Sanger sequenced in Macrogen.

3.3. Phosphatase alkaline assay

Alkaline phosphatase activity was assessed using the Sigma Kit 86R-1KT, following the manufacturer's instructions with slight modifications.

3.4. Real-time quantitative reverse transcription PCR (qRT-PCR)

Cellular extracts (passages 11, 12, and 13) were resuspended in TRIzol™ reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. Reverse transcription was performed on 1ug of RNA using the MMLV reverse transcriptase (Promega), random hexamers, and dNTPs for cDNA synthesis. Real-time qPCR was done using FastStart Universal SYBR Green Master Mix (Roche) and specific primers (Table 2) and amplified in a StepOnePlus Real-Time PCR (95 °C/15 s, 60 °C/1 min, 40 cycles). LinRegPCR software was used for mRNA values analysis, normalizing the gene values against the geometrical mean of two housekeeping genes, *GAPDH* and *RPL7*. Results were transformed with the log2 function and relativized to HF mean values.

3.5. Immunofluorescence

iPSCs (passage 13) were fixed with paraformaldehyde 4 % for 10 min, followed by washes with PBS 1X. Permeabilization and blocking were done in PBS-Triton X100, 3 % normal goat serum for 30 min, followed by incubation with primary antibody at 4 °C overnight. The next day, cells were washed and incubated with a secondary antibody

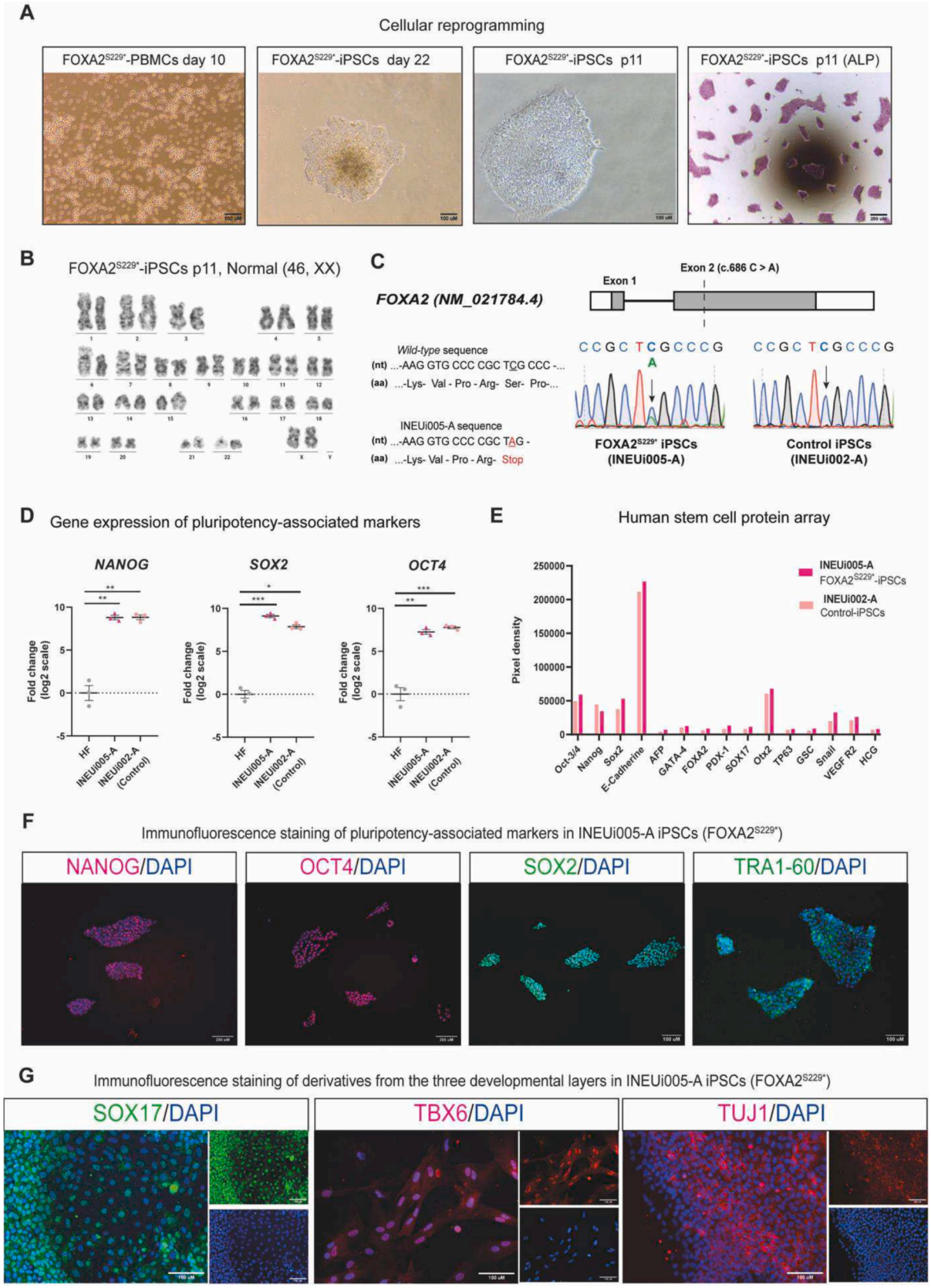

Fig. 1. Generation of FOXA2^{S229}*-iPSCs (INEUi005-A).

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1A
Phenotype	RT-qPCR	Positive for pluripotency markers: NANOG, OCT3/4, and SOX2	Fig. 1D
	Protein array	Expression of pluripotency markers: NANOG, OCT3/4, SOX2, and epithelial cadherin (E-Cadherin). Low expression of cell commitment marker proteins	Fig. 1E
	Immunocytochemistry	Positive for pluripotency markers: NANOG, OCT3/4, SOX2, and TRA 1–60	Fig. 1F
Genotype	Karyotype (G-banding) and resolution	46, XX 30 metaphases were analyzed at 440-band resolution.	Fig. 1B
Identity	STR analysis	27 sites tested, matched	Available with the authors
Mutation analysis (IF APPLICABLE)	Sequencing	Amplified product by PCR and Sanger sequencing. Heterozygous	Fig. 1C
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR. Negative	Supplementary Fig. 2.
Differentiation potential	Embryoid body formation	Expression of βIII-tubulin (TUJ1), SRY-box 17 (SOX17), and T-Box Transcription Factor 6 (TBX6) were shown by immunofluorescence as proof of three germ layers formation	Fig. 1G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	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 #**	**RRID**
Pluripotency Markers	Mouse anti-OCT4 IgG	1:200	Santa Cruz Biotechnology Cat# sc-5279	RRID:AB_628051
	Rabbit anti-SOX2	1:100	Cell Signaling Technology Cat#3579	RRID:AB_2195767
	Mouse anti-NANOG IgG	1:100	Santa Cruz Biotechnology Cat# sc-293121	RRID:AB_2665475
	Mouse anti-TRA1-60 IgM	1:200	Santa Cruz Biotechnology Cat# sc-21705	RRID:AB_628385
Differentiation Markers	Mouse anti-TUJ1 IgG	1:400	Covance Cat# MMS-435P	RRID:AB_2313773
	Rabbit anti-TBX6	1:100	Invitrogen Cat# PA5-35102	RRID:AB_2552412
	Rabbit anti-SOX17	1:100	Invitrogen PA5-72815	RRID: AB_2718669
Secondary antibodies	Alexa Fluor 488 goat anti-rabbit IgG	1:400	Invitrogen Cat#A11034	RRID:AB_24766217
	Alexa Fluor 594 goat anti-rabbit IgG	1:400	Invitrogen Cat#A11032	RRID:AB_2534091
	Primers			
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**	
Pluripotency Markers (qPCR)	*SOX2*	110 bp	AGCATGGAGAAAACCCGGTACGC/CGTGAGTGTGGATGGGATTGGTGT	
	NANOG	110 bp	AAAGGATCTTCACCTATGCC/GAAGGAAGAGGAGAGACAGT	
	OCT4	128 bp	CTGGGTTGATCCTCGGACCT/CACAGAACTCATACGGCGGG	
House-Keeping Genes (qPCR)	*RPL7*	138 bp	AATGGCGAGGATGGCAAG/TGACGAAGGCGAAGAAGC	
	GAPDH	98 bp	ACAGCCTCAAGATCATCAG/GAGTCCTTCCACGATACC	
Genotyping	*FOXA2*	225 bp	CAGCAGAGCCCCAACAAGAT/GCAGCCGTTCTCGAACAT	
STEMCCA expression	STEMCCA – Oct4/Klf4	561 bp	CAACGAGAGGATTTTGAGGC/ATCGTTGAACTCCTCGGTCTCTCT	
	STEMCCA – Sox2/C-Myc	550 bp	TTGGCTCCATGGGTTCGGTG/AAGGGTGTGACCGCAACGTAGG	
Mycoplasma	*Mycoplasma* sp.	500 bp	ACACCATGGGAGYTGGTAAT/CTTCWTCGACTTYCAGACCCAAGGCAT	

(1:400, Molecular probes) and nuclei were stained with DAPI. Cells were visualized in an Evos XL Core inverted microscope.

3.6. Embryoid Body Assay

Spontaneous differentiation of iPSCs (passage 13) into the three developmental layers, endoderm, mesoderm, and ectoderm, was evaluated using an embryoid body (EBs)-based method developed in our laboratory and described in (Isaja et al., 2022) using KSR-EB medium (DMEM/F12, 20 % Knockout™ Serum Replacement, 1 % Gibco™ GlutaMAX supplement, 1 % Gibco™ MEM nonessential amino acids solution, 0.1 mM 2-mercaptoethanol) until day 4. Aggregates were transferred to gelatin-coated culture dishes in 20 % Fetal Bovine Serum (FBS) KSR-EB medium and cultured for 21 days to promote the differentiation.

3.7. Stem Cell Array Kit

Analysis of fifteen stem cell markers were analyzed using the Proteome Profiler Human Pluripotent Stem Cell Antibody Array (Catalog # ARY010) following the manufacturer's instructions. Cell lysates (passage 13) were collected in RIPA buffer with protease and phosphatase inhibitors for protein extraction.

CRediT authorship contribution statement

María Andrea Camilletti: Writing – original draft, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Chirino Felker Gonzalo Tomás:** Methodology, Investigation. **Castañeda Sheila:** Methodology. **Zabalegui Federico:** Methodology. **Amin Guadalupe:** Methodology. **Belgorosky Alicia:** Investigation. **Ciaccio Marta:** Investigation. **Di Palma Maria Isabel:** Investigation. **Vaiani Elisa:** Investigation. **Waisman Ariel:** Resources, Investigation. **Miriuka Santiago:** Resources, Funding acquisition. **Pérez Millán María Inés:** Investigation, Funding acquisition, Conceptualization. **Moro Lucia Natalia:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2025.103777.

Data availability

Data will be made available on request.

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