Lab Resource: Multiple Cell Lines

Generation of *PKD1* mono-allelic and bi-allelic knockout iPS cell lines using CRISPR-Cas9 system

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A B S T R A C T

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease, characterised by the development of multiple fluid-filled cysts in the kidneys and other organs. *PKD1* and *PKD2* are the two major causative genes encoding for polycystin-1 and polycystin-2, respectively. Here, we report the generation of two isogenic induced pluripotent stem cell (iPSC) lines with either heterozygous or compound heterozygous mutations in the *PKD1* gene using CRISPR-Cas9 technology. The $PKD1^{+/-}$ and $PKD1^{-/-}$ iPSCs maintain stem cell-like morphology, normal karyotype, pluripotency and differentiation capacity in the three germ layers.

Resource Table:

Unique stem cell lines identifier	IRFMNi003-A-3 IRFMNi003-A-4
Alternative names of stem cell lines	KO PKD1#16 ($PKD1^{+/-}$, heterozygous) KO PKD1#5 ($PKD1^{-/-}$, compound heterozygous)
Institution	Istituto di Ricerche Farmacologiche Mario Negri IRCCS
Contact information of distributor	Susanna Tomasoni susanna.tomasoni@marionegri.it
Type of cell lines	iPSC
Origin	Human
Cell Source	Peripheral mononuclear blood cells (PBMCs)
Clonality	Clonal
Method of reprogramming	Sendai Virus
Multiline rationale	Isogenic clones
Gene modification	YES
Type of modification	Deletion
Associated disease	Autosomal dominant polycystic kidney disease
Gene/locus	PKD1/16p13.3
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	November 2019
Cell line repository/bank	N/A
Ethical approval	Ethical Committee of Bergamo, Protocol number 58/19

1. Resource utility

Approximately 85% of all ADPKD cases are attributed to mutations in the *PKD1* gene. However, their pathological role remains a matter of debate. The generated *PKD1* mono-allelic and bi-allelic knockout iPSC lines are a useful resource for investigating pathogenic mechanisms of ADPKD, as well as for drug testing.

2. Resource details

ADPKD is typically transmitted as a dominant trait by loss-of-function mutations in *PKD1* and/or *PKD2* genes. Patients with ADPKD are typically heterozygous, with germline mutations in one allele (Watnick and Germino, 1999). ADPKD is characterised by great phenotypic variability, with *PKD1* mutations exhibiting a more severe disease form and onset at an earlier age (Hateboer et al., 1999). This is explained partially by a two-hit model, according to which a germline mutation (first hit) and a subsequent somatic mutation (second hit) are required for cyst formation. This model explains the focal nature of the disease. Moreover, several pieces of evidence indicate that cystogenesis is dependent on the gene dosage changes in *PKD1* (Ong and Harris, 2015). However, the relevance of these mechanisms in human ADPKD is not fully understood. Here, we generated two *PKD1* knockout iPSC lines (IRFMNi003-A-3 and IRFMNi003-A-4) by targeting the *PKD1* gene with the CRISPR-Cas9 system (Table 1). A single guide RNA targeting *PKD1* exon 36 (GTGGGTGCGAGCTTCCCCCC), previously designed and tested for its cutting efficiency by Freedman et al. (Freedman et al., 2015), was cloned into a plasmid containing Cas9 from S. pyogenes, fused with 2A-EGFP (pSpCAs9(BB)-2A-GFP, PX458; Addgene). The human iPSC line IRFMNi003-A, which had earlier been established using Sendai virus technology (Ciampi et al., 2016), was nucleofected with pSpCAs9(BB)-2A-GFP-sgPKD1 plasmid, followed by fluorescence-activated cell sorting (FACS) of GFP $^+$ cells, single cell seeding on a mouse embryonic fibroblast (MEF)-feeder layer and manual picking. Forty single cell-

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
IRFMNi003-A-3	KO PKD1#16	Female	32	Caucasian	PKD1 mono-allelic knockout	Autosomal dominant polycystic kidney disease
IRFMNi003-A-4	KO PKD1#5	Female	32	Caucasian	PKD1 bi-allelic knockout	Autosomal dominant polycystic kidney disease
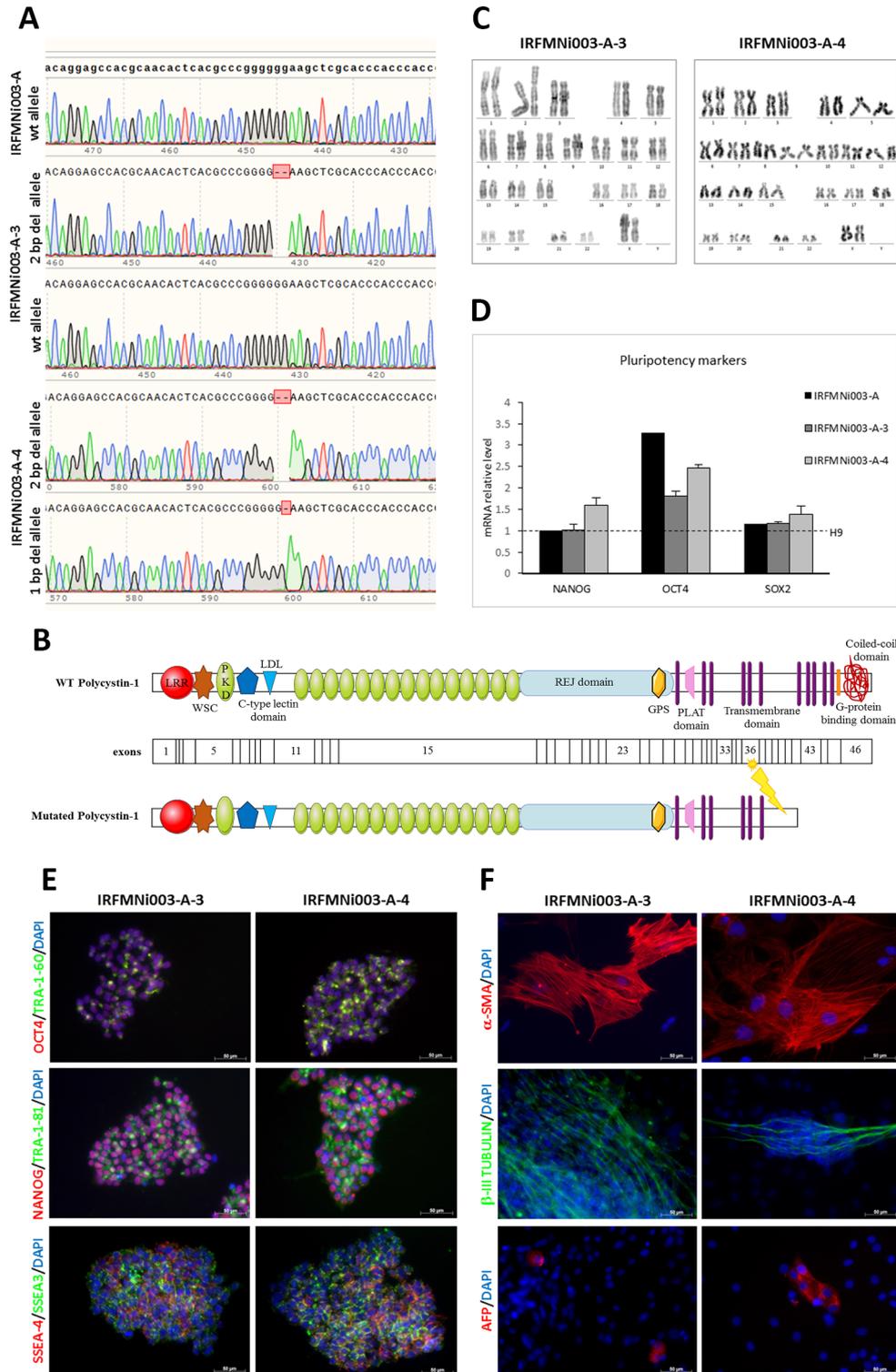
Fig. 1. Characterisation of IRFMNi003-A-3 and IRFMNi003-A-4 iPS cell lines.

Table 2
Characterisation and validation.

Classification	Test	Result	Data
Morphology	Photography	*Normal*	*Supplementary Fig. 2*
Phenotype	Qualitative analysis *by Immunocytochemistry*	*Both cell lines express the pluripotency markers OCT4, NANOG, TRA-1–60, TRA-1–81, SSEA-3, SSEA-4*	Fig. 1 *panel E Supplementary Fig. 4*
	Quantitative analysis *by RT-qPCR*	*Both cell lines express the pluripotency markers OCT4, NANOG and SOX2 at levels comparable to the parental iPSC line*	Fig. 1 *panel* D
Genotype	Karyotype (G-banding) and resolution	*46XX, Resolution 400–550*	Fig. 1 *panel* C
Identity	Microsatellite PCR (mPCR) OR	*N/A*	
	STR analysis	*18 sites tested/Matched*	*Submitted in archive with journal*
Mutation analysis (IF APPLICABLE)	Sequencing	*Heterozygous and compound heterozygous indel in PKD1 exon 36*	Fig. 1 *panel A and Supplementary Fig. 1*
	Southern Blot OR WGS	N/A	*N/A*
Microbiology and virology	Mycoplasma	*Mycoplasma testing by PCR. Negative*	*Supplementary Fig. 3*
Differentiation potential	Embryoid body formation	*Mesoderm: smooth muscle actin Ectoderm: β-III-tubulin Endoderm α-feto protein*	Fig. 1 *panel* F
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*

derived clones were then expanded on matrigel-coated plates and analysed through Sanger sequencing. From the edited clones we selected a heterozygous clone (IRFMNi003-A-3) with a 2 bp deletion on one allele (c.10744_10745delCC, P3582Gfs*44) and a compound heterozygous clone (IRFMNi003-A-4) in which indels (insertion-deletions) occurred on both alleles, carrying a 1 bp deletion (c.10745_10745delC, P3582Rfs*3) on one allele and a 2 bp deletion (c.10744_10745delCC, P3582Gfs*44) on the other (Fig. 1A; Supplementary Fig. 1). These mutations were predicted to be disease-causing by Mutation Taster (http://www.mutationtaster.org/) and to lead to severely truncated proteins, no longer functional because they lack part of the transmembrane domains and all the coiled-coil domain (Ong and Harris, 2015) (Fig. 1B). The PKD1 knockout clones maintained normal pluripotent morphology (Supplementary Fig. 2) and had a normal karyotype (Fig. 1C). Quantitative real-time PCR (qRT-PCR) and immunofluorescence analyses showed that IRFMNi003-A-3 and IRFMNi003-A-4 expressed the key pluripotency markers (Fig. 1D and E, Supplementary Fig. 4). Moreover, the cells had the ability to spontaneously differentiate *in vitro* into the three germ layers (Fig. 1F). Sanger sequencing analysis of 13 potential off-target sites of the sgRNA (Supplementary Table 1) was performed and no off-target mutations were detected (data not shown). The Short Tandem Repeat (STR) analysis showed a perfect match on 17 different loci between the 2 cell lines and the parental iPSC (Table 2, data not shown but available from authors). Finally, PCR analysis confirmed that iPS cells were negative for mycoplasma contamination (Supplementary Fig. 3).

These *PKD1*-modified iPSC lines will offer a useful resource for the *in vitro* study of ADPKD pathogenic mechanisms and drug testing.

3. Materials and methods

3.1. Cell culture

Cell cultures were maintained under standard conditions (humidified chamber at 37 °C and 5% CO_2). The iPSCs were cultured on hES-qualified Matrigel-coated plates (Corning) in mTeSR1 medium (StemCell Technologies) while MEFs in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco) and 0.1 mM non-essential amino acids (NEAA; Gibco). MEFs were mitotically inactivated through mitomycin-c treatment (Sigma-Aldrich) 48 h before iPSC seeding. Mycoplasma contamination was detected using the N-GARDE Mycoplasma Detection PCR Kit (Euroclone).

3.2. CRISPR/Cas9-mediated PKD1 modified iPSC

18×10^6 iPSCs were nucleofected with 72 μg pSpCas9(BB)-2A-GFP-sg*PKD1* and plated on matrigel-coated 6-well plates in mTeSR1 with 10 μM Y-27632 (Sigma-Aldrich). After 24 h, GFP$^+$ single cells were isolated through cell sorting (FACSAria IIu; BD Bioscience), plated on MEF feeder-coated plates with a density of 500 cells/cm^2 and then the single cell-derived colonies were expanded for another 8 days. Forty emergent clones were picked manually and immediately plated on matrigel-coated wells in mTeSR1 medium.

3.3. Genotyping and sequencing

Genomic DNA was extracted from cells using a DNeasy Blood and Tissue kit (Qiagen). The *PKD1* gene was amplified with Long Range DNA Polymerase (biotechrabbit) using the primers listed in Table 3. PCR amplicons were Sanger sequenced using a BigDye® Terminator v3.1 sequencing kit on the 3730 DNA Analyzer (Applied Biosystems). Hypothesised deletions were disentangled and verified using the TIDE (Tracking of Indels by DEcomposition) online software tool. Mutations were further confirmed by TOPO TA cloning (Invitrogen) and Sanger sequencing. The results were aligned using SnapGene software.

3.4. Pluripotency gene expression analysis

Total RNA was isolated through the TRIzol Reagent-based procedure (Invitrogen) and treated with DNAse (Promega). cDNA was synthetised from 2 μg of total RNA with the Vilo Superscript kit (Invitrogen). qRT-PCR reactions were performed using TaqMan gene expression assays (Applied Biosystems), using predesigned TaqMan probes for the genes of interest according to the supplier's recommendations (Table 3). Human embryonic stem cell line H9 was taken as reference sample.

3.5. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (Società Italiana Chimici) and permeabilised with 0.3% Triton X-100 (Sigma-Aldrich) for 10 min at room temperature (RT). After blocking with 5% bovine serum albumin (Sigma-Aldrich) for 1 h, cells were incubated with primary antibodies overnight at 4 °C followed by the corresponding Alexa 594- or Alexa 488-conjugated secondary antibodies for 1 h at RT. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-OCT4	1:100	Santa Cruz Biotechnology Cat# sc-5279; RRID: AB_628051
	Rabbit anti-NANOG	1:100	Santa Cruz Biotechnology Cat# sc-33759; RRID: AB_2150401
	Mouse anti-TRA-1–60	1:200	Millipore Cat# MAB4360, RRID: AB_2119183
	Mouse anti-TRA-1–81	1:200	Millipore Cat# MAB4381, RRID: AB_177638
	Mouse anti-SSEA-4	1:100	Santa Cruz Biotechnology Cat# sc-21704, RRID: AB_628289
	Rat anti-SSEA-3	1:100	Santa Cruz Biotechnology Cat# sc-21703, RRID: AB_628288
Differentiation Markers	Mouse anti-Tubulin beta-III, clone TU-20, Alexa Fluor 488 Conjugated	1:100	Millipore Cat# CBL412X, RRID: AB_1977541
	Mouse anti-Actin, alpha-Smooth Muscle - Cy3	1:100	Sigma-Aldrich Cat# C6198, RRID: AB_476856
	AFP (AFP-11) antibody	1:100	Santa Cruz Biotechnology Cat# sc-51506, RRID: AB_626514
Secondary antibodies	Donkey anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	1:300	Thermo Fisher Scientific Cat# A10040, RRID: AB_2534016
	Goat anti-Mouse IgM Heavy Chain Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:300	Thermo Fisher Scientific Cat# A-21042, RRID: AB_2535711
	Donkey anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	1:300	Thermo Fisher Scientific Cat# A10036, RRID: AB_2534012
	Goat anti-Rat IgM (Heavy chain) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:300	Thermo Fisher Scientific Cat# A-21212, RRID: AB_2535798

Primers

	Target	Forward/Reverse primer (5′-3′)
Pluripotency Markers (qPCR)	NANOG	Hs02387400_g1 (Thermo Fisher Scientific)
	OCT4	Hs00742896_s1 (Thermo Fisher Scientific)
	SOX2	Hs00602736_s1 (Thermo Fisher Scientific)
House-Keeping Genes (qPCR)	HPRT1	Hs99999909_m1 (Thermo Fisher Scientific)
Genotyping	PKD1	Fwd: GGATGCCCGTACCGCGTGAT/ Rev: GTGGCACAGCCTCGGTGGTC
Targeted mutation sequencing	PKD1	Fwd: CCCGGAACCCCACCT/Rev: GTTTGGGGTAGGGTCTTCC

Aldrich). Images were taken using the Axio Observer Z1 fluorescence microscope (Zeiss). The antibodies used are listed in Table 3.

3.6. Embryoid body (EB) formation

The iPSCs were detached using dispase (StemCell Technologies) and cultured in ultra-low adhesion six well plates in DMEM/F-12 medium, supplemented with 20% KO serum (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 0.1 mM NEAA plus 10 μM Y-27632. After 24 h, Y-27632 was removed and EBs were cultured for another 6 days with medium change performed every other day. Thereafter, EBs were transferred onto a gelatine-coated plate and cultured for another 8 days.

3.7. Karyotyping and cell authentication

Karyotype analysis was performed in collaboration with the Genetic Medicine Laboratory of the Azienda Socio Sanitaria Territoriale Papa Giovanni XXIII, Bergamo (Italy). Cells were treated with Colcemid (Roche) for 3 h, then trypsinised and processed for karyotype analysis. 20 metaphase chromosome spreads were analysed with a G-band resolution of 400–550. Cell line authentication was performed using the ATCC Human Short Tandem Repeat (STR) Profiling Cell Authentication Service.

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.2020.101881.

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