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Lab resource: Stem Cell Line

Induced pluripotent stem cell line heterozygous for p.R2447X mutation in filaggrin: KCLi002-A

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A B S T R A C T

We have generated an induced pluripotent stem cell (iPSC) line KCLi002-A (iOP107) from a female donor, heterozygous for the loss-of-function mutation p.R2447X in the filaggrin gene (*FLG*). Epidermal keratinocytes were reprogrammed using non-integrating Sendai virus vectors. The entire process of derivation and expansion of iPSCs were performed under xeno-free culture conditions. Characterization of KCLi002-A line included molecular karyotyping, mutation screening using restriction enzyme digestion Sanger sequencing and next generation sequencing (NGS), whereas pluripotency and differentiation potential were confirmed by expression of associated markers *in vitro* and *in vivo* teratoma assay.

Resource table.

Unique stem cell line identifier	KCLi002-A
Alternative name(s) of stem cell line	iOP107
Institution	King's College London, London UK
Contact information of distributor	Dusko ILIC, dusko.ilic@kcl.ac.uk
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: Unknown Sex: Female Ethnicity: Caucasian
Cell Source	Epidermal keratinocytes
Clonality	Clonal
Method of reprogramming	Non-integrating SeV-mediated delivery of OCT4, SOX2, c-MYC and KLF4
Genetic modification	NO
Type of modification	N/A
Associated disease	Atopic dermatitis (AD) or eczema (OMIM: 605803) Ichthyosis vulgaris (IV) (OMIM: 146700)
Gene/locus	Filaggrin gene (*FLG*), loss-of-function mutation NM_002016.1(*FLG*):c.7339C > T - nonsense (p.R2447X, p.Arg2447Ter)
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	April 2018
Cell line repository/bank	N/A
Ethical approval	Ethics Committee of the Medical University of Innsbruck, Austria (AN2016–0260)

Resource utility

Generation of a library of human iPSC lines with the most common variants in the *FLG* gene can be efficiently used to construct highly specific *in vitro* 3D skin models (Petrova et al., 2014) for drug discovery towards novel personalized therapies in AD.

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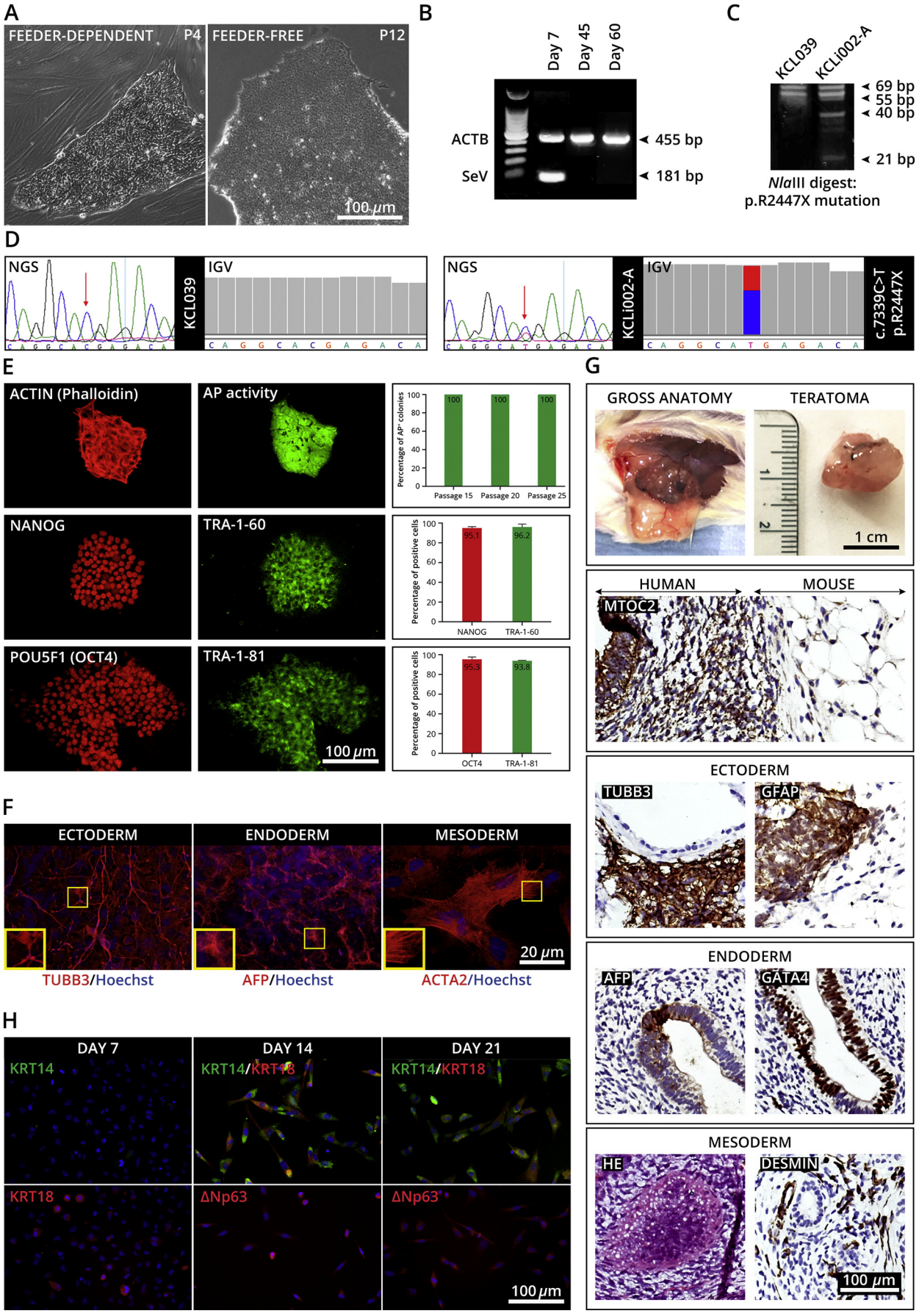

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Fig. 1. Characterization of KCLi002-A line. A, The colonies display typical morphology of pluripotent stem cells under feeder-dependent and feeder-free conditions. B, The absence of 181 bp positive SeV band at 45 and 60 days of culture indicates that the cells are SeV free. β-actin 455 bp band serves as an internal control. C, The line is heterozygous for p.R2447X mutation in *FLG* as indicated with restriction enzyme digestion. Mutation generates a site for *Nla*III that is not present in wildtype allele. KCL039 is used as a negative control. D, Amplicon array-based NGS spanning the mutation site. hESC line KCL039 is used as a negative control. E, Pluripotency-associated markers. Alkaline phosphatase activity (AP) was restricted to the iPSC colony (green) growing on AP-negative feeder cells. Both iPSC colony and feeders are positive for actin (phalloidin) staining (red). The cells in the iPSC colonies were positive > 90% for POU5F1 (red), TRA-1-81 (green), NANOG (red) and TRA-1-60 (green) pluripotency-associated markers. F, Spontaneous differentiation in vitro. The cells can differentiate into all three germ layers as demonstrated with markers specific for ectoderm (TUBB3), endoderm (AFP) and mesoderm (ACTA2). Nuclei are visualized with Hoechst 33342. G, Spontaneous differentiation in vivo. Gross anatomy and staining for human-specific MTCO2 marker indicated that the teratoma is incapsulated and did not invade surrounding tissues. Teratoma contained cells from all three germ layers as demonstrated with markers specific for ectoderm (TUBB3, GFAP), endoderm (AFP, GATA4) and mesoderm (AB-PAS, DESMIN). H, Directed differentiation into keratinocytes. The cells expressed keratinocyte-specific markers KRT14, KRT18, TP63 (ΔNp63) in time-dependent manner.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Light microscopy	hESC-like morphology (compact, dense, roundly shaped colonies with sharp edges, high nucleus to cytoplasm ratio)	Fig. 1 panel A
Phenotype	Qualitative analysis (Immunofluorescence staining and AP activity)	Expression of pluripotency- markers TRA-1-60, TRA-1-81, OCT4, NANOG; AP-positive	Fig. 1 panel E
	Quantitative analysis (immunofluorescence counting)	Percentage of cells positive for pluripotent markers: OCT4: 95%, NANOG: 95%, TRA-1-60: 96%, TRA-1-81: 94%	Fig. 1 panel E and Supplementary File 3
Genotype	Array CGH	46, XX	Supplementary File 1 (Specimen No. 17/34499 correlates with KCLi002-A)
Identity	STR analysis	DNA fingerprinting PCR, 17 specific markers tested	Supplementary file STR Analysis
Mutation analysis	Restriction enzyme digestion	Mutation p.R2447X creates a new *Nla*III site, which was used to screen short, highly specific PCR fragments for this variant. hESC line KCL039 is used as wild-type control.	Fig. 1 panel C
	FLG-specific PCR primer assays with NGS	Heterozygous, p.R2447X in *FLG* tandem repeat (TR) 7. hESC line KCL039 is used as wild-type control.	Fig. 1 panel D
Microbiology and virology	Sendai	RT-PCR analysis: negative	Fig. 1 panel B
	Mycoplasma	LookOut Mycoplasma PCR Detection Kit: negative	Supplementary File 2
Differentiation potential	Embryoid body formation	Expression of smooth muscle actin (ACTA2), α-feto protein (AFP) and β-III-tubulin (TUBB3)	Fig. 1 panel F
	Teratoma formation	Alcian blue/periodic acid Schiff (PAS)-stained cartilage and desmin for mesoderm, TUBB3 and glial fibrillary acidic protein (GFAP) for ectoderm, and GATA4 and AFP for endoderm, while human-specific mitochondrially encoded cytochrome C oxidase II (MTCO2) only immunostains human mitochondria in the cells of the teratoma	Fig. 1 panel G
	Directed differentiation into keratinocytes	The iPSC-derived keratinocytes expressed the epithelial cell markers: KRT14, KRT18, and isoform of TP63 (ΔNp63)	Fig. 1 panel H
Donor screening	HIV 1 + 2, Hepatitis B, Hepatitis C	Not tested	N/A
Genotype additional info	Blood group genotyping	Not tested	N/A
	HLA tissue typing	Not tested	N/A
	FLG polymorphism	TR8 heterozygote (8.1/8.2) TR10 homozygote (10.1)	N/A

Resource details

AD or eczema is an incurable, non-contagious, extensive inflammatory and extremely pruritic chronical cutaneous disorder. AD is one of the most common skin diseases which affects up to 20% of children and approximately 3% of adults worldwide, while its prevalence is continuously increasing, particularly in underdeveloped countries (Asher et al., 2006). Several loss-of-function mutations within *FLG* exon 3, including p.R2447X variant, are considered to be the most significant risk factors for atopic dermatitis in the European population (Palmer et al., 2006). The epidermal keratinocytes derived from a female donor who is heterozygous for p.R2447X, were reprogrammed into iPSCs following previously established protocol with genome non-integrating Sendai virus (SeV) vectors (Miere et al., 2016). Three weeks post-transduction colonies with a typical morphology of pluripotent stem cells appeared and were selected to establish feeder-free iPSC clones (Fig. 1A). After twelve passages, the elimination of the SeV vectors was confirmed in the KCLi002-A cell line by RT-PCR using specific primers (Fig. 1B). The clones were screened with restriction enzyme digestion and we have verified that the AD-related mutation (NM_002016.1: p.R2447X) was retained in the iPSCs (Fig. 1C). This finding was also confirmed independently by *FLG*-specific PCR primer assays with next generation sequencing (Fig. 1D). Endogenous expression of pluripotency-related molecular markers (TRA-1-60, TRA-1-81, OCT4, NANOG) in the iPSCs was assessed by double immunofluorescence (Fig. 1E). Furthermore, undifferentiated colonies were also positive for alkaline phosphatase (AP) activity (Fig. 1E). Differentiation capacity of the KCLi002-A cells into three germ layers was determined by specific immunofluorescence staining of AFP (liver, endoderm), ACTA2 (cardiac muscle, mesoderm), and TUBB3 (neurons, ectoderm) *in vitro* (Fig. 1F), as well as *in vivo* through a teratoma formation assay. All three germ layers, ectoderm, mesoderm, and endoderm, were present in the teratoma, as demonstrated by immunohistochemical analysis (Fig. 1G).

Since our aim is to use the line for modeling AD *in vitro*, we tested differentiation of the KCLi002-A iPSCs into epidermal keratinocytes (Petrova et al., 2014). The cells expressed early epithelial cell marker keratin 18 (KRT18) and keratinocyte-specific markers - keratin 14 (KRT14), and isoform of TP63 known as ΔNp63 within three weeks in culture as expected (Fig. 1H).

Examination of the genomic integrity of our iPSC line using array CGH after more than twenty passages showed a normal female karyotype (46, XX), whereas smaller imbalances have not been excluded (Supplementary File 1). In addition, the line has been verified to be

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

Class	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-TRA-1-60	1:100	Millipore Cat# MAB4360, RRID: AB_2119183
	Goat anti-NANOG	1:100	R&D Cat# AF1997, RRID: AB_355097
	Mouse anti-TRA-1-81	1:100	Millipore Cat# MAB4381, RRID: AB_177638
	Mouse anti-OCT4	1:100	SantaCruz Biotech.; Cat. No. SC-5279, RRID: AB_628051
Differentiation markers	Mouse anti-AFP	1:100	Sigma Cat# A8452, RRID: AB_258392
	Mouse anti-ACTA2	1:100	Sigma Cat# A5228, RRID: AB_262054
	Mouse anti-TUBB3	1:100	Sigma Cat# T5076, RRID: AB_532291
	Rabbit anti-KRT 14	1:1000	Abcam Cat# ab181595 RRID: N/A
	Mouse anti-KRT 18	1:1000	Sigma Cat# C8541, RRID: AB_476885
	Mouse anti-ΔNp63	1:100	Abcam Cat# ab172731 RRID: N/A
	Mouse anti-desmin	1:150	Dako Cat# M0760, RRID:AB_2335684
	Goat anti-GATA-4	1:10	R&D Systems Cat# AF2606, RRID:AB_2232177
	Rabbit anti-GFAP	1:200	Dako Cat# Z0334, RRID:AB_10013382
	Mouse anti-MTCO2	1:10	Abcam Cat# ab110258, RRID:AB_10887758
Secondary antibodies	Donkey anti-mouse Alexa Fluor 488-conjugated IgM	1:100	Jackson ImmunoResearch Labs Cat# 715–545-140, RRID:AB_2340845
	Donkey anti-goat Rhodamine X-conjugated IgG	1:100	Jackson ImmunoResearch Labs Cat# 705–295-147, RRID:AB_2340423
	Donkey anti-mouse Rhodamine-X-conjugated IgG	1:100	Jackson ImmunoResearch Labs Cat# 715–295-150, RRID:AB_2340831
	Donkey anti-rabbit FITC-conjugated IgG	1:100	Jackson ImmunoResearch Labs Cat# 711–095-152, RRID:AB_2315776

Primers

	Target	Forward/Reverse primer (5′-3′)
Genotyping	*FLG*	CCACACGTGGCCGGTCAGCA/-CTACCGAATGCTCGTGGTGGT
		CCACACGTGGCCGGTCAGCA/-GTCCTGACCCTCTTGGGACGT
Targeted mutation analysis	NM_002016.1(*FLG*):c.7339C > T (p.R2447X)	ACACACAGTCAGTGTCAGCC/-ACCAATTGCTCGTAGTGGGA
Sanger sequencing		CCACACGTGGCCGGTCAGCA/-GTCCTGACCCTCTTGGGACGT
Sendai Virus checking (RT-PCR)	*SeV*	GGATCACTAGGTGATATCGAGC/-ACCAGACAAGAGTTTAAGAGATATGTATC
	KOS	ATGCACCGCTACGACGTGAGCGC/-ACCTTGACAATCCTGATGTGG
	KLF-4	TTCCTGCATGCCAGAGGAGCCC/-AATGTATCGAAGGTGCTCAA
	c-MYC	TAACTGACTAGCAGGCTTGTCG/-TCCACATACAGTCCTGGATGATGATG
	ACTB	CAACCGCGAGAAGATGAC/-AGGAAGGCTGGAAGAGTG

unambiguously mycoplasma-negative in the PCR assay (Supplementary File 2). Taken together, these results prove that we have successfully produced a stable AD patient specific iPSC line which can provide a powerful tool for: 1) developing the first iPSC-derived 3D *in vitro* AD-human skin equivalents (HSE); 2) deciphering the molecular mechanisms of the disease; 3) innovative drug screening platform in AD. (Table 1).

Materials and methods

Epidermal keratinocytes reprogramming

Patient keratinocytes of passage 2 were transduced with genome integration-free SeV virus kit (CytoTune 2.0, Life Technologies) as described (Miere et al., 2016). Clonal selection of fully reprogrammed cells was performed manually by picking individual clones with hESC-like appearance (Table 1). The iPSCs under feeder-free culture conditions were maintained on Matrigel (BD Biosciences) in TeSR2 medium (STEMCELL Technologies).

FLG mutation verification

Genomic DNA was extracted from KCLi002-A cells using DNeasy Blood & Tissue Kits (Qiagen) and samples were verified independently by restriction enzyme digestion and Sanger sequencing using the primers described in Table 2. A sequence in exon 3 of *FLG* was amplified and Eurofins Genomics provided DNA sequencing service. Mutation p.R2447X creates a new *Nla*III site, which was used to screen short, highly specific 185 bp *FLG* gene fragment. Furthermore, comprehensive *FLG* genotyping of AD-iPSCs was performed through amplicon array-based NGS sequencing, as described in more detail previously (Wong et al., 2018). The entire *FLG* coding region was analyzed for loss-of-function (LoF) mutations using a previously published tiled amplicon sequencing assay. iPSC cell line KCLi002 was found to have one *FLG* LoF mutation in the *FLG* tandem repeat 7 (NM_002016.1(*FLG*):c.7339C > T - nonsense (p.R2447X, p.Arg2447Ter)). No other LoF mutations were detected in the *FLG* coding region of this cell line, therefore KCLi002 was classified as a heterozygous carrier with the second allele a genetically wild type copy. WT hESC line, KCL039 was used as a control. This hESC line has no detectable mutation in the *FLG* gene.

Reverse transcription PCR analysis of SeV vectors

After twelve passages, total RNA was isolated from iPSC cells, while transduced cell pool at passage 2 was used as positive control (RNeasy mini Kit, Qiagen). SeV specific primers were used to assess the presence of remaining Sendai virus vectors (Table 2). RT-PCR for the detection of Sendai transgenes was carried out using the SuperScript IV First-strand cDNA synthesis reaction kit (Invitrogen).

Pluripotency markers

The pluripotency status of KCLi002-A line was evaluated by immunostaining for NANOG, OCT4, TRA-160, and TRA-1-81 (Table 1) as previously described (Petrova et al., 2012). Manual cell counting was used to quantify the expression of pluripotency markers. For each marker three random areas were selected for photography and analysis. Cell counts were performed in triplicate by one analyst (Supplementary File 3). The data from the three areas for each marker were averaged, and these values were used to generate line graphs of expression levels. Error bars were created using the standard error of the mean (SEM) for each triplicate group. Line graphs were generated using Microsoft Excel and translated into a vector format with Adobe Illustrator.

Alkaline phosphatase activity

Emerging iPSCs were analyzed for alkaline phosphatase activity by AP Live Stain (Thermo Fisher). After live staining, iPSCs were washed and fixed, and cytoskeletal actin filaments have been contrasted by labelling with rhodamine phalloidin (Molecular Probes).

Spontaneous differentiation into three germ layers

To test the differentiation capacity of our iPSC line, *in vitro* embryonic body formation, as well as *in vivo* conventional teratoma assay were assessed (Table 1), as previously described (Petrova et al., 2014).

Directed differentiation into keratinocytes

To derive keratinocytes from KCLi002-A cells, we modified an established protocol (Petrova et al., 2014). Briefly, the iPSC differentiation into keratinocytes was initiated on Vitronectin XF (STEMCELL Technologies)-coated surface in defined keratinocyte-serum-free medium (DKSFM, Gibco) supplemented with 1 μM all-trans retinoic acid (ATRA; Sigma-Aldrich), 10 ng/μl bone morphogenetic protein 4 (BMP4; R&D System). Differential expression of the lineage-specific markers after three weeks of culture was monitored by immunostaining (Table 1).

Molecular karyotyping

Array comparative genomic hybridization (aCGH) and short tandem repeat (STR) analysis of 17 STR loci were conducted at Viapath Genetics Centre.

Mycoplasma contamination detection

The absence of mycoplasma contamination was detected using LookOut® Mycoplasma PCR Detection Kit (Sigma-Aldrich).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101462.

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