Title

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Lab resource: Stem cell line

Induced pluripotent stem cell line from an atopic dermatitis patient heterozygous for c.2282del4 mutation in filaggrin: KCLi001-A

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A B S T R A C T

We have generated an induced pluripotent stem cell (iPSC) line KCLi001-A (iOP118) from a female atopic dermatitis (AD) patient, heterozygous for the loss-of-function mutation c.2282del4 in the filaggrin gene (*FLG*). Epidermal keratinocytes were reprogrammed using non-integrating Sendai virus vectors. The entire process of derivation and expansion of AD-iPSCs were performed under xeno-free culture conditions. Characterization of KCLi001-A line included molecular karyotyping, mutation screening using restriction enzyme digestion and Sanger sequencing, while pluripotency and differentiation potential were confirmed by expression of associated markers in vitro and by in vivo teratoma assay.

Resource table

Unique stem cell line identifier	KCLi001-A
Alternative name(s) of stem cell line	iOP118
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	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	None
Type of modification	N/A
Associated disease	Atopic dermatitis (AD) or eczema, OMIM #605803
Gene/locus	Filaggrin gene (*FLG*), loss-of-function mutation NM_002016.1:c.2282del4
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	December 2017
Cell line repository/ bank	N/A
Ethical approval	Ethics Committee of the Medical University of Innsbruck, Austria (AN2016-0260)

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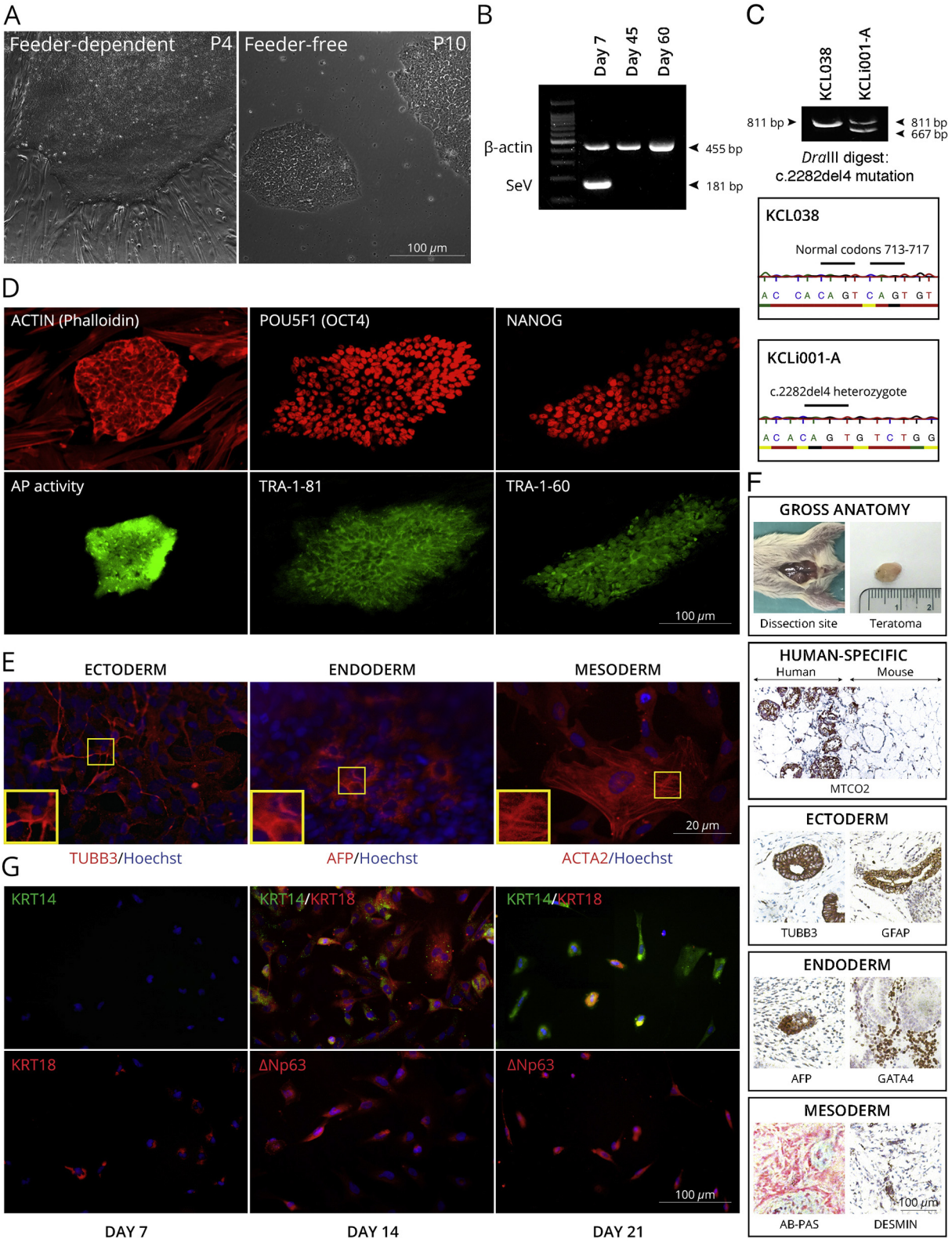

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Resource utility

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.

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

Fig. 1. Characterization of KCLi001-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 c.2282del4 mutation in FLG as indicated with restriction enzyme digestion and Sanger sequencing of PCR product spanning the mutation site. Mutation generates a site for DraIII that is not present in wildtype allele. The enzyme digestion cuts 811 bp product in mutated allele into 667 bp and 134 bp, whereas 811 bp remains intact in wildtype. hESC line KCL038 is used as a negative control. D, Pluripotency 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). iPSC colonies are positive for POU5F1 (red), TRA-1-81 (green), NANOG (red) and TRA-1-60 (green) pluripotency markers. E, 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. F, Spontaneous differentiation in vivo. Gross anatomy and staining for human-specific MTCO2 marker iPSC 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). G, 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 D
	Quantitative analysis (immunofluorescence counting)	Percentage of cells positive for pluripotent markers: OCT4–94%, NANOG – 95, TRA-1-60: 95%, TRA-1-81: 93%	Fig. 1 panel D
Genotype	Array CGH	46, XX	Submitted in archive with journal
Identity	STR analysis	DNA fingerprinting PCR, 17 specific markers tested	Submitted in archive with journal
Mutation analysis	Sequencing	Heterozygous, c.2282del4 in exon 3 of *FLG* hESC line KCL038 (Miere et al., 2016b) is used as wild-type control.	Fig. 1 panel C
	Restriction enzyme digestion	Mutation 2282del4 creates a new *Dra*III site, which was used to screen short, highly specific PCR fragments for this variant. hESC line KCL038 (Miere et al., 2016b) is used as wild-type control.	Fig. 1 panel C
Microbiology and virology	Mycoplasma	LookOut Mycoplasma PCR Detection Kit: negative (Supplementary file 1)	Supplementary Fig. 1
Differentiation potential	Embryoid body formation	Expression of smooth muscle actin (ACTA2), α-fetoprotein (AFP) and βIII-tubulin (TUBB3)	Fig. 1 panel E
	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 mitochondrially encoded cytochrome C oxidase II (MTCO2) only immunostains human mitochondria in the cells of the teratoma	Fig. 1 panel F
	Directed differentiation into keratinocytes	The iPSC-derived keratinocytes expressed the epithelial cell markers: KRT14, KRT18, and isoform of TP63 (ΔNp63)	Fig. 1 panel G
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

prevalence is continuously increasing, particularly in underdeveloped countries (Asher et al., 2006). Several loss-of-function mutations within *FLG* exon 3, including c.2282del4 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 AD patient who is heterozygous for c.2282del4, were reprogrammed into iPSCs following previously established protocol with genome non-integrating Sendai virus (SeV) vectors (Miere et al., 2016a). 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 ten passages, the elimination of the SeV vectors was confirmed in the KCLi001-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:c.2282del4) was retained in the iPSCs. This finding was also confirmed independently by Sanger sequencing (Fig. 1C). Endogenous expression of pluripotency-related molecular markers (TRA-1-60, TRA-1-81, OCT4, NANOG) in the iPSCs was assessed by double immunofluorescence technique (Fig. 1D). Furthermore, undifferentiated colonies were also positive for alkaline phosphatase (AP) (Fig. 1D). Differentiation capacity of the KCLi001-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. 1E), 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. 1F).

Since our aim is to use the line for modeling AD in vitro, we tested differentiation of the KCLi001-A iPSCs into epidermal keratinocytes (Petrova et al., 2014). The cells expressed keratinocyte-specific markers - keratins 14 and 18 (KRT14, KRT18), and isoform of TP63 known as ΔNp63 within three weeks in culture as expected (Fig. 1G).

Examination of the genomic integrity of our AD-iPSC line using array CGH after more than twenty passages showed a normal female karyotype (46, XX), whereas smaller imbalances have not been excluded (submitted in archive with journal). 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 atopic dermatitis. (Table 1).

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-cytokeratin, pan	1:300	Abcam Cat# ab7753, RRID:AB_306047
	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*	AATAGGTCTGGACACTCAGGT/-GGGAGGACTCAGACTGTTT	
Targeted mutation analysis/sequencing	*FLG*	CTCCAGTCAGCAGACAGCTC/-GTCTTACTCCAGTGCTGGGC	
Sendai Virus checking (RT-PCR)	*SeV*	GGATCACTAGGTGATATCGAGC/-ACCAGACAAGAGTTTAAGAGATATGTATC	
	KOS	ATGCACCGCTACGACGTGAGCGC/-ACCTTGACAATCCTGATGTGG	
	KLF-4	TTCCTGCATGCCAGAGGAGCCC/-AATGTATCGAAGGTGCTCAA	
	c-MYC	TAACTGACTAGCAGGCTTGTCG/-TCCACATACAGTCCTGGATGATGATG	

Materials and methods

Epidermal keratinocytes reprogramming

Patient keratinocytes of passage 3 were transduced with genome integration-free SeV virus kit (CytoTune 2.0, Life Technologies) as described (Miere et al., 2016a). 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 KCLi001-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 311 bp sequence in exon 3 of *FLG* was amplified and Eurofins Genomics provided DNA sequencing service. Mutation c. 2282del4 creates a new *Dra*III site, which was used to screen short, highly specific 811 bp *FLG* gene fragment, as described in more detail previously (Palmer et al., 2006).

Reverse transcription PCR analysis of SeV vectors

Total RNA was isolated from the cells 7, 45 and 60 days post-transduction (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 SeV transgenes was carried out using the SuperScript IV First-strand cDNA synthesis reaction kit (Invitrogen).

Pluripotency markers

The pluripotency status of KCLi001-A line was evaluated by immunostaining for three germ layer markers in spontaneously differentiated cells (Table 1) as previously described (Petrova et al., 2014).

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).

Spontaneuous 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

KCLi001-A were differentiated into keratinocytes following modified protocol Petrova et al. (2014). Briefly, the iPSC differentiation 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) and 10 ng/μl bone morphogenetic protein 4 (BMP4; R&D System). Differential expression of the lineage-specific markers was assessed at Day 7, 14 and 21 of the protocol with 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.2018.07.014.

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