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Generation of four severe early-onset chronic obstructive pulmonary disease (COPD) patient-derived induced pluripotent stem cell lines from peripheral blood mononuclear cells

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A B S T R A C T

Evidence highlights the concept of multiple trajectories leading to COPD. Early-life events (i.e., in utero lung development) may influence the maximally attained lung function and increase the risk to develop COPD. Human pluripotent stem cells (hiPSC) represent a unique opportunity to model lung development. We generated hiPSC lines from four highly characterized COPD patients with early onset and severe phenotype. Peripheral blood mononuclear cells (PBMCs) were reprogrammed using integration-free Sendai Virus. The cell lines had normal karyotype, expressed pluripotency hallmarks, and differentiated into the three primary germ layers.

These lines offer a tool to study early-life origins of COPD.

1. Resource utility

Early onset of COPD is a poorly understood trajectory of the disease, actually considered to have developmental lung origins (Martinez, 2016). iPSC lines were generated from four severe COPD patients. These cell lines afford a unique model to investigate mechanisms relevant to pathologic lung development, and susceptibility to environmental exposure.

2. Resource details

In this study, peripheral blood mononuclear cells (PBMCs) were obtained from four early onset severe COPD patients. Cells were reprogrammed into iCOPD2 (UHOMi003-A), iCOPD6 (UHOMi006-A), iCOPD8 (UHOMi004-A), iCOPD9 (UHOMi005-A) lines respectively using non-integrating CytoTune-iPSC 2.0 Sendai Reprogramming kit.

PBMCs were isolated by Ficoll gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare)(Vissers et al., 1988) then washed with PBS plus foetal bovine serum (FBS) 10%. PBMCs were cultured in StemSpan with Erythroid Expansion Medium (SSEM, StemCell Technologies), according to the manufacturer's instructions. Erythroid

differentiation leads to erythroid progenitor cells expansion. The four reprogramming factors OCT3/4, SOX2, KLF4, and C-MYC were transiently expressed in the erythroid progenitor cells using the integration-free Sendai virus transduction method. The four cell lines had a normal morphology (Fig. 1, panel A, bright field microscopy; scale bar: 50 μ m) and karyotype analysis revealed normal number and structure of chromosomes in all four lines (Fig. 1, panel B). The generated iPSC lines were tested for the absence of exogenous reprogramming vector which was analysed by PCR using Sendai virus vector (SeV) specific primers after about 10 passages, (Fig. 1D). The viral sequence was also absent in the negative non transduced human embryonic stem cells (Fig. 1, panel D,) but present in positive control.

Expression of pluripotency genes was evidenced by q-RT-PCR, using previously published Hy03 healthy iPSC cell line (UHOMi002-A) as positive control, respectively for SOX2, NANOG and OCT4 (Fig. 1, panel C) (Fieldes et al., 2020).

Expression of pluripotency markers were assessed by immunocytochemistry staining, using antibodies against human OCT3/4 and NANOG (Fig. 1, E scale bar 50 μ m) and using surface marker TRA-1-60 (Fig. 1, panel F). The ability of COPD iPSC cell lines to differentiate into the three germ layers in vitro was confirmed by embryoid body (EB)

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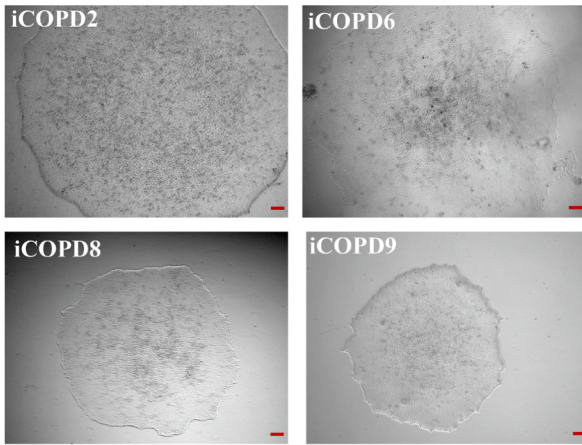
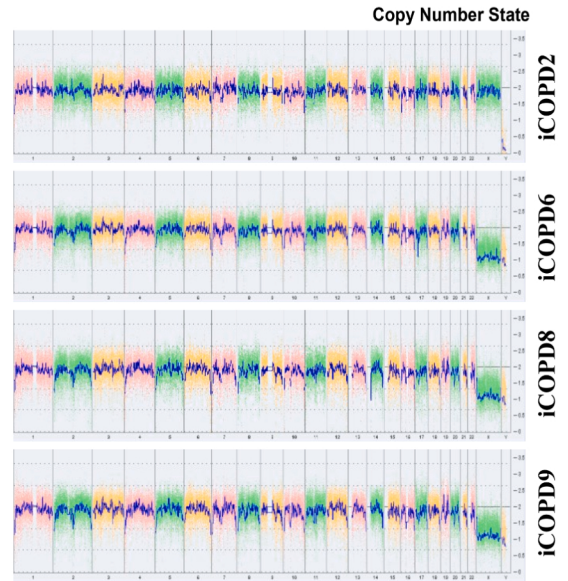
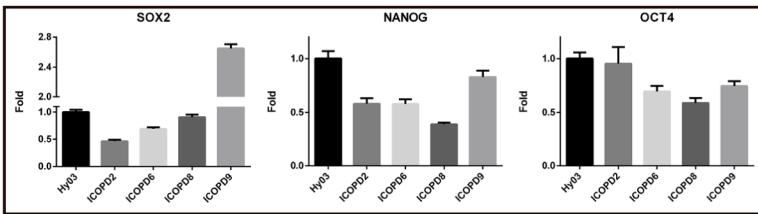
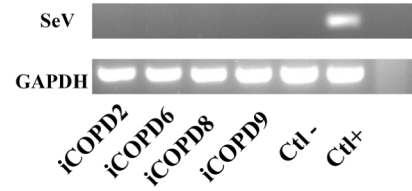
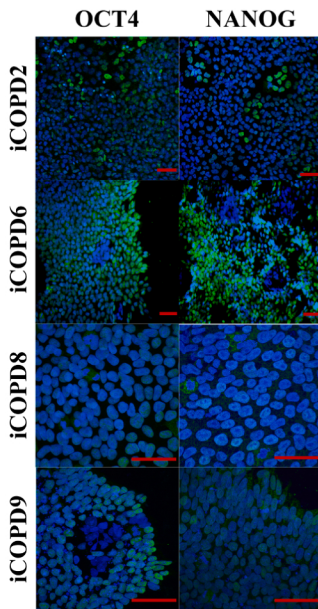
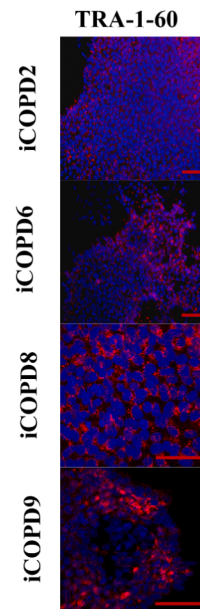
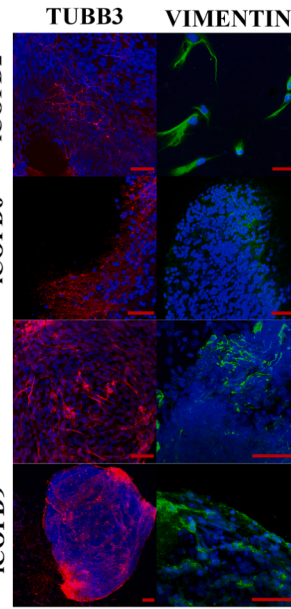
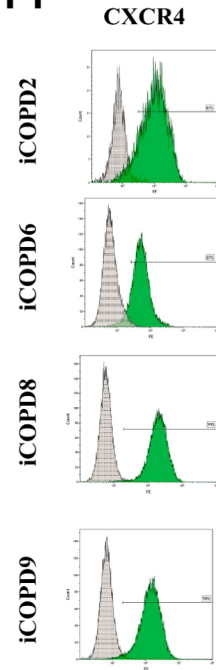
A**B****C****D****E****F****G****H***(caption on next page)*

Fig. 1. Characterization of the iPSC cell lines. A. The four cell lines display a normal morphology (bright field microscopy; scale bar: 50 μ m). B. KaryoStat analysis (ThermoFisher) revealed normal number and structure of chromosomes in all four lines. C. Expression of pluripotency genes was evidenced by q-RT-PCR, using previously published Hy03 healthy iPSC cell line (UHOMi002-A) as positive control, respectively for SOX2, NANOG and OCT4. D. The iPSC lines were tested for the absence of exogenous reprogramming vector by PCR using Sendai virus vector (SeV) specific primers after about 10 passages. The viral sequence was also absent in the negative non transduced human embryonic stem cells (Ctl-) but present in positive control (Ctl +). E. Expression of pluripotency markers assessed by immunofluorescence, using antibodies against human OCT3/4 and NANOG (scale bar 50 μ m). F. Expression of pluripotency markers assessed by immunofluorescence, using antibodies against surface marker TRA-1-6). G. The ability of COPD iPSC cell lines to differentiate into the three germ layers in vitro was confirmed by embryoid body (EB) formation. EB expressed ectodermal (β -III-tubulin) and mesodermal (vimentin) markers as observed by immunofluorescence. H. EB expressed endodermal protein CXCR4 as shown by flow cytometry method.

formation. EB formation was performed by transferring iPSC clumps to low-adhesion dishes (Falcon) in E8 medium. After 24 h, EB were suspended in differentiation medium composed of RPMI-1640 medium (Gibco) supplemented with 10% FBS (Gibco) and 1% Penicillin-streptomycin. The medium was changed every two days for 30 days. After this period, the EB were plated on Geltrex-coated dishes and cultured for about a week. Cells were then fixed in 4% paraformaldehyde for immunofluorescence analysis. They expressed ectodermal (β -III-tubulin), mesodermal (vimentin) (Fig. 1, panel G) and endodermal protein CXCR4 by flow cytometry method (Fig. 1, panel H).

Microsatellite analysis showed matching profiles of short tandem repeats (STR) amplified from genomic DNA UHOMi00 3,4, 5 & 6-A iPSC cells and patient-derived PBMCs (matching of 16/16 loci: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, AMEL, D5S818, FGA, D19S433, vWA, TPOX and D18S51; available with authors). The four iPSC cell lines were also confirmed to be free of mycoplasma. Of note, ratio < 0.9 means that the sample was negative for mycoplasma (Supplemental Table 1).

3. Materials and methods

3.1. Reprogramming of PBMCs to iPSCs

Erythroid progenitor cells (EPC) were transduced by Sendai-virus with the CytoTune[®]-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific), following manufacturer's instruction. The calculated volumes of each of the three CytoTune[™] 2.0 Sendai-virus (SeV) were added to the EPC (KOS MOI = 5, hc-Myc MOI = 5, hKlf4 MOI = 3). The

colonies with an embryonic stem cells (ES)-like appearance were manually isolated based on morphologic features.

3.2. Cell culture of iPSC cell lines

COPD cell lines were cultured on Geltrex matrix (Thermo Fisher Scientific) in E8 medium (Thermo Fisher Scientific), according to the manufacturer's instruction. The cells were cultivated at 37 °C in normoxic conditions/ 5% CO₂ and passaged manually. They were then adapted by passaging them using EDTA dissociation (Versene Solution, Thermo Fisher Scientific) every week into clumps, using 1:10 split ratio.

3.3. Karyotyping

Karyotyping was performed at mechanic passage 20. Karyotyping was performed using a service provider. The KaryoStat[™] assay allows for digital visualization of chromosome aberrations with a resolution similar to g-banding karyotyping. The size of structural aberration that can be detected is > 2 Mb for chromosomal gains and > 1 Mb for chromosomal losses. Genomic DNA purification Cells were prepared according to the Genomic DNA Purification Kit (Catalog #: K0512) and quantified using the Qubit[™] dsDNA BR Assay Kit (Catalog #: Q32850). GeneChip[®] Preparation 250 ng total gDNA was used to prepare the GeneChip[®] for KaryoStat[™] according to the manual and is an array that looks for copy number variants and single nucleotide polymorphisms across the genome.

Table 1
Characterization and validation.

	Test	Result	Data
Morphology	Photography Bright field	Normal for the four iPSC cell lines	Fig. 1 panel A
Phenotype	Qualitative analysis	Expression of pluripotency markers: OCT4, NANOG, TRA-1-60	Fig. 1 panel E & F
	Immunocytochemistry	Expression of SOX2, NANOG and OCT4	Fig. 1 panel C
	Quantitative analysis RT-qPCR analysis		
Genotype Identity	Karyotype (G-banding) and resolution	Normal for the 4 cell lines not performed	Fig. 1 panel B N. A
	Microsatellite PCR (mPCR)	Specific 16 loci tested, 16/16 matched between iPSC cell line and patient derived PBMCs	submitted in archive with journal
	STR analysis	Mycoplasma testing by luminescence. Negative	Supplemental Table 1
Microbiology and virology	Mycoplasma		
Differentiation potential	Embryoid body formation	Embryoid bodies show the three germ layers formation: Immunocytochemistry	Fig. 1 panel G & H
		ectoderm: β III-tubulin (TUBB3); mesoderm: vimentin marker.	
		Flow cytometry endoderm: C-X-C Motif Chemokine Receptor (CXCR4), respectively UHOMi003-A: 87 % UHOMi004-A: 99 % UHOMi005-A: 98 % UHOMi006-A: 87 %	
List of recommended germ layer markers	Expression of these markers has to be demonstrated at mRNA (RT PCR) or protein (IF) levels, at least 2 markers need to be shown per germ layer	Ectoderm: TUBB3/TUJ1, Endoderm: CXCR4 Mesoderm: VIMENTIN	Fig. 1 panel G & H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	not shown but available with the authors
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	N.A.	N. A
		N. A	N. A

3.4. Immunofluorescence labelling

Cells were tested after passage 20 and fixed in 4% PFA for 15 min at room temperature (RT) and washed 3 times in phosphate-buffered saline (PBS, Gibco). The cells were permeabilized with 0.5% Triton X-100 (Sigma Aldrich) for 15 min, blocked with 10% normal donkey serum (Sigma Aldrich) for 1 h at RT. Cells were incubated with the primary antibodies in PBS BSA 1% 0.1% Triton X-100 overnight at 4 °C. (listed in Table 2). Secondary antibodies were used at the dilution of 1:2000 during one hour at RT. Cell nuclei were stained with DAPI (Sigma Aldrich). Images were captured with Zeiss Axioimager Z1 Apotome and Confocal Microscopy Zeiss LMS700.

3.5. Flow cytometry analysis

Cells were tested after passage 20 and harvested using cell dissociation reagent (Versene Solution) and washed twice in PBS. Cells were then incubated 15 min at room temperature with a Live Cell/Dead Cell discriminator staining (Violet Zombie, Biolegend). IPS cells were incubated with a conjugated primary or the control isotype antibody for 30 min at 4 °C. After staining, cells were washed twice and analysed by flow cytometry using Beckman Coulter Gallios. Analysis was performed with Kaluza software.

3.6. Testing for Sendai virus clearance

Total RNA was isolated from iPSC cells within the first week after transfection with Sendai virus and iPSCs after passage 15. RNA extraction and RT were performed as previously described (qRT-PCR section). PCR was performed using Q5 High-Fidelity DNA Polymerase with the primers listed in Table 2. The PCR mix was composed of cDNA (50 ng), the forward and reverse primers 1.5 µl at 10 µM for each, dNTPs (10 nM), Q5 buffer and Q5 DNA Pol5 (New England BioLabs). DNA was melted for 3 min at 95 °C, then 35 cycles (95 °C) for 15 s, 55 °C for 15 s and 72 °C for 30 s), and final elongation was performed at 72 °C for 2 min using C100 Thermal Cycler (Bio-Rad). Products were analysed on 2.5 % agarose gel, ran at 130 V electric field. Finally, the picture was acquired using ChemiDoc™MP Imaging System (Bio-Rad).

3.7. RT-qPCR analysis

Reverse transcription (RT) was performed on cells around passage 20 using SuperScript™ First-Strand Synthesis System (ref 11904-018, Invitrogen) as recommended by the manufacturer in 20 µl reaction volume that included 1 µg RNA (extracted with RNeasy Mini Kit, ref 74106, Qiagen), Superscript II RT, oligo-dT primer, dNTP mixture, MgCl₂, 0.1 M DTT and RNase inhibitor. Quantitative PCR (qPCR) was then performed using the LightCycler® 480 SYBR Green I Master (04707516001, Roche) with 2 µl of the RT reaction product (1:20 dilution) and 0.5µl of each primer diluted at 10 µM (Integrated DNA Technologies IDT) in a total volume of 10 µl. PCR amplifications were carried out using a LightCycler 480 apparatus and the following programme: 1 cycle of 95 °C for 10 s; 40 cycles of 95 °C for 10 s, 60 °C for 15 s; 72 °C for 15 s, 1 cycle 95 °C for 5 s, 65 °C for 1 min, 97 °C forever. Gene expression levels were normalised to the expression of the housekeeping gene GAPDH, using the following formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ unknown - ΔCt positive control.

3.8. Short tandem repeat analysis (STR)

DNA extraction was performed as described below on cells after passage 10. STR analysis of generated iPSCs and parental PBMCs was performed to detect 16 loci. STR was performed using a service provider (Eurofins). Briefly, DNA isolation was carried out from cell pellet. Genetic characteristics were determined by PCR-single-locus-technology.

Table 2

Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit Polyclonal anti OCT4	1:400	Santa Cruz Technology Cat# RRID AB_2167703
	Rabbit polyclonal anti NANOG	1:250	Abcam Cat# ab80892 RRID AB_2150114
	Mouse monoclonal [TRA-1-60]	1:100	Abcam Anti-TRA-1-60 (R) antibody [TRA-1-60] RRID (ab16288)
			BD BiosciencesRRID 557145
Differentiation Markers	Mouse anti-CXCR4 (PE)	1:200	Santa Cruz Biotechnology Cat# sc-7557, RRID: AB_793998
	Polyclonal goat anti VIMENTIN	1:1000	Covance Research Products Inc. Cat# MMS-435P, RRID: AB_2313773
	Mouse anti βIII-TUBULIN		
Secondary antibodies	donkey anti mouse	1:2000	Thermo Fisher Scientific Cat# A-31570, RRID: AB_2536180
	Alexa fluor 555	1:2000	Thermo Fisher Scientific Cat# A-21206, RRID: AB_2535862
	donkey anti goat	1:200	BD Biosciences RRID556653
	Alexa fluor 647		
	Mouse IgG2a, (PE)		
Primers			
	Target	Forward/Reverse primer (5'-3')	
Virus clearance (qPCR)	SeV	GGATCACTAGGTGATATCGAGC	
	product size: 181 bp	ACCAGACAAGAG TTTAAGAGATATGTATC	
House-Keeping Genes	GAPDH (PCR)	CTG GCG TCT TCA CCA CCA TGG CAT CAC GCC ACA GTT TCC CGG	
	product size: 300 bp		
Pluripotency genes (qRT-PCR)	OCT4	GGG CTC TCC CAT GCA TTC AAA C CAC CTT CCC TCC AAC CAG TTG C	
	SOX2	GGCCATTAAACGGCACACTGCC TTACTCTCCTCTTTTGACCCCTCC	
	NANOG	TGA TTT GTG GGC CTG AAG AAA GAG GCA TCT CAG CAG AAG ACA	
House -Keeping Genes	GAPDH (qRT-PCR)	GAC CTG ACC TGC CGT CTA GAA A CCT GCT TCA CCA CCT TCT TGA	

16 independent PCR-systems D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, AMEL, D5S818, FGA, D19S433, vWA, TPOX and D18S51 were investigated. In parallel, positive, and negative controls were carried out yielding correct results.

3.9. Mycoplasma testing

Cell culture supernatants were tested around passage 20 using bioluminescence MycoAlert® Detection Kit (Lonza) according to manufacturer's instructions.

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