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Lab Resource: Multiple Cell Lines

GMP-compatible manufacturing of three iPS cell lines from human peripheral blood

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A B S T R A C T

The utilization of human induced pluripotent stem cells (hiPSCs) for disease modeling and drug discovery is already reality, and several first-in-man-applications as cellular therapeutics have been initiated. Implementation of good manufacturing practice (GMP)-compliant protocols for the generation of hiPSC lines is crucial to increase the application safety as well as to fulfil the legal requirements for clinical trials approval. Here we describe the development of a GMP-compatible protocol for the reprogramming of $CD34^+$ hematopoietic stem cells from peripheral blood ($CD34^+$ PBHSC) into hiPSCs using Sendai virus-based reprogramming vectors. Three GMP-compatible hiPSC (GMP-hiPSC) lines were manufactured and characterized under these conditions.

Resource table

Unique stem cell lines identifier	MHHi008-A MHHi008-B MHHi008-C
Alternative names of stem cell lines	$CD34^+$hPBHSC_GMPDU_SeV-iPS8 $CD34^+$hPBHSC_GMPDU_SeV-iPS16 $CD34^+$hPBHSC_GMPDU_SeV-iPS18
Institution	Hannover Medical School
Contact information of distributor	Alexandra Haase haase.alexandra@mh-hannover.de Ulrich Martin martin.ulrich@mh-hannover.de
Type of cell lines	iPSC
Origin	Human
Cell source	CD34 positive hematopoietic stem cells from peripheral blood
Clonality	Clonal
Method of reprogramming	Sendai Virus, Transgene-free
Multiline rationale	Isogenic clones
Gene modification	NO
Type of modification	N/A
Associated disease	N/A
Gene/locus	N/A
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	July 2018
Cell line repository/bank	N/A
Ethical approval	The Local Ethics Committee approved the study and informed consent was obtained from the donor.

Resource utility

The development of a GMP-compliant protocol for production of patient-specific, clinical-grade hiPSCs including the isolation and cultivation of the source $CD34^+$PBHSCs and the complete reprogramming process enabling the manufacturing of GMP-grade hiPSC lines is shown.

Resource details

We have recently described the generation of human iPSCs from $CD34^+$ cord blood hematopoietic stem cells under complete animal-derived component free conditions (Haase et al., 2017). To further develop our protocols towards production of clinical-grade, patient-specific hiPSC, we have now evaluated and compiled various GMP-compliant components and process steps for the generation of transgene-free hiPS cells. $CD34^+$PBHSCs were isolated from blood donations by using the complete closed and automated cell manufacturing platform CliniMACS™ Plus (Miltenyi Biotec) and reprogramming was performed using CTS™ CytoTune™-iPS 2.1 Sendai Reprogramming Kit

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
CD34 $^{+}$ hPBHSC_GMPDU_SeV-iPS8 (MHHi008-A)	MHHi008-A	male	32	Caucasian	N/A	N/A
CD34 $^{+}$ hPBHSC_GMPDU_SeV-iPS16 (MHHi008-B)	MHHi008-B	male	32	Caucasian	N/A	N/A
CD34 $^{+}$ hPBHSC_GMPDU_SeV-iPS18 (MHHi008-C)	MHHi008-C	male	32	Caucasian	N/A	N/A

(Thermo Fisher Scientific) delivering the reprogramming factors Oct3/4, Sox2, Klf-4 and L-Myc. All media, material and compounds needed for the isolation and cultivation of the source cells as well as the reprogramming process and the establishment of hiPSC lines were either GMP-compliant or part of a special product line (CTS™, Cell Therapy Systems, Thermo Fisher Scientific) approved for manufacturing of cell-, gene- and tissue-based products. Three of the resulting clonal GMP-conform hiPSC lines (Table 1) were selected for further detailed characterization post-reprogramming as described below. Immunofluorescence staining revealed that the tested GMP-hiPSC lines strongly expressed the pluripotency markers Oct3/4, SOX2, TRA-1-60 and SSEA-4 (Fig. 1, Panel A, Scale bars represent 100 µm). A normal human karyotype 46, XY was detected by fluorescence R-banding karyotyping (Fig. 1, Panel B) and genetic identity of the generated GMP-hiPSC lines was confirmed by STR analysis (Supplementary Table S1). Quantitative analysis of pluripotency markers by flow cytometry assessed over 99% TRA-1-60 positive cells and a positive cell population of > 84% for Oct4 (Fig. 1, Panel C). The complete lack of residual SeV genomes and exogenous reprogramming factor transcripts in all clones were tested by qRT-PCR (Fig. 1, Panel D) with primers shown in Table 3/additional table. Three germ layer differentiation potential was confirmed by positive staining against mesodermal (sarcomeric α-actinin (ACTN2); cardiac Troponin T (cTnT)), endodermal (α-fetoprotein (AFP); SOX17) and ectodermal markers (β-3-tubulin (TUB3); desmin) after EB-based spontaneous differentiation (d21 of differentiation (Fig. 1, Panel E, Scale bars represent 100 µm)). Details of antibodies used for immunofluorescence staining are shown in Table 3. All characteristics of the three GMP-hiPSC lines are summarized in Table 2.

Materials and methods

Isolation and reprogramming of CD34^{+}PBHSC

All materials, media and components used for isolation and cultivation of source cells as well as for reprogramming and establishment of GMP-hiPSCs were either exclusively labelled as GMP-grade or were part of the Cell Therapy System (CTS) from Thermo Fisher Scientific. CD34 $^{+}$ PBHSC were isolated by the closed and automated cell manufacturing platform CliniMACS™ Plus (Huenecke et al., 2016 and www.miltenyibiotec.com) and were further cultivated in HSC-Brew GMP Medium (all Miltenyi Biotec).Cryopreservation was performed in CTS™Synth-a-Freeze (Thermo Fisher Scientific). For reprogramming CD34^{+}PBHSCs were thawed three days before reprogramming and 200,000 cells were transduced with CTS™ CytoTune™-iPS 2.1 Sendai Reprogramming Kit according to the manufactures protocol with slight modifications to comply with GMP-compliant conditions. Transduction was performed on day 0 using the CTSTMCytotuneTM2.1 Sendai reprogramming vectors (according to the manufacture's protocol with KOS MOI = 5, hl-Myc MOI = 5, hKlf4 MOI = 3) in HSC-Brew GMP Medium without polybrene. On day 1 and 3 we performed medium replacement with fresh HSC-Brew GMP Medium. Transduced cells were carefully centrifuged to remove the residual HSC medium and seeded onto CTS ™ Recombinant Human Vitronectin (Thermo Fisher Scientific)-coated plates in iPS-Brew GMP Medium (Miltenyi Biotec) on day 4. We performed half medium exchanges with iPS-Brew GMP Medium until first adherent cell spots appeared (day 5–8). On day 8–16 the medium was exchanged completely every second day. Cell colonies with appropriate iPSC morphology were picked and transferred onto fresh CTS ™ Recombinant Human Vitronectin-coated wells in iPS-Brew GMP Medium on day 16. iPSC clones were further cultivated for 10 passages (as described below) before temperature shift was performed for 6 days at 39 °C to enhance the loss of reprogramming vectors. All clones were tested for the removal of SeV genomes and transgenes by quantitative realtime PCR analysis.

Culture conditions

Cultivation and establishment of GMP-hiPSCs was performed in iPS-Brew GMP Medium (Miltenyi Biotec). In all steps CTS ™ Recombinant Human Vitronectin (Thermo Fisher Scientific) was used as cell culture surface according to the manufacturer's protocol. Cell passaging was performed by detaching hiPSC colonies with Versene solution (Thermo Fisher Scientific).

Quantitative realtime PCR analysis

Total RNA was isolated with RNA-isolation mini kit according to manufacturer's protocol (Qiagen, Hilden, Germany). The cDNA was synthesized from 2 µg total RNA using 1 µl (200 U) Superscript III RT, 4 µl 5 × first strand buffer, 1 µl of random primer, 1 µl of DTT (0.1 mM), 1 µl of dNTP mix (10 mM) in 20 µl reaction volume. Reaction conditions were 25 °C 10 min, 42 °C 50 min and 85 °C 5 min. Two microliters of cDNA were subjected to qRT-PCR amplification in a StepOnePlus Thermocycler (Applied Biosystems, Darmstadt, Germany) in 10 µl TaqMan Fast Advanced Master Mix, 1 µl Primer, 7 µl Nuclease-free water (Ambion, USA). qRT-PCR conditions were 95 °C 20 s initial step following 40 cycles with 95 °C 1 s, 60 °C 20 s. TaqMan Assay Details are listed in additional table.

Immunocytological staining

Cells were fixed with 4% paraformaldehyde and stained by standard protocols using primary and secondary antibodies, as listed in Table 3. Corresponding isotype antibodies were used for negative control staining and cells were counterstained with DAPI (Sigma) and analysed with an AxioObserver A1 fluorescence microscope and AxioVision software (Zeiss).

Flow cytometry analysis

Cells were stained for flow cytometric analysis against OCT 3/4 or TRA-1-60 with antibodies listed in Table 3 according to standard protocols for either intracellular or surface antigens. Samples were analysed using an Accuri™ C6 Plus (BD Bioscience). Flow cytometric data evaluation was done with FlowJo 7.6.5 software (Celeza).

In vitro differentiation

HiPSC colonies were detached using a cell scraper, transferred into ultra-low attachment plates (Corning) and cultivated in differentiation medium for 7 days. Formed embryoid bodies were plated onto gelatin-coated cell culture plates for another 14 days before analysis as described previously (Haase et al., 2009).

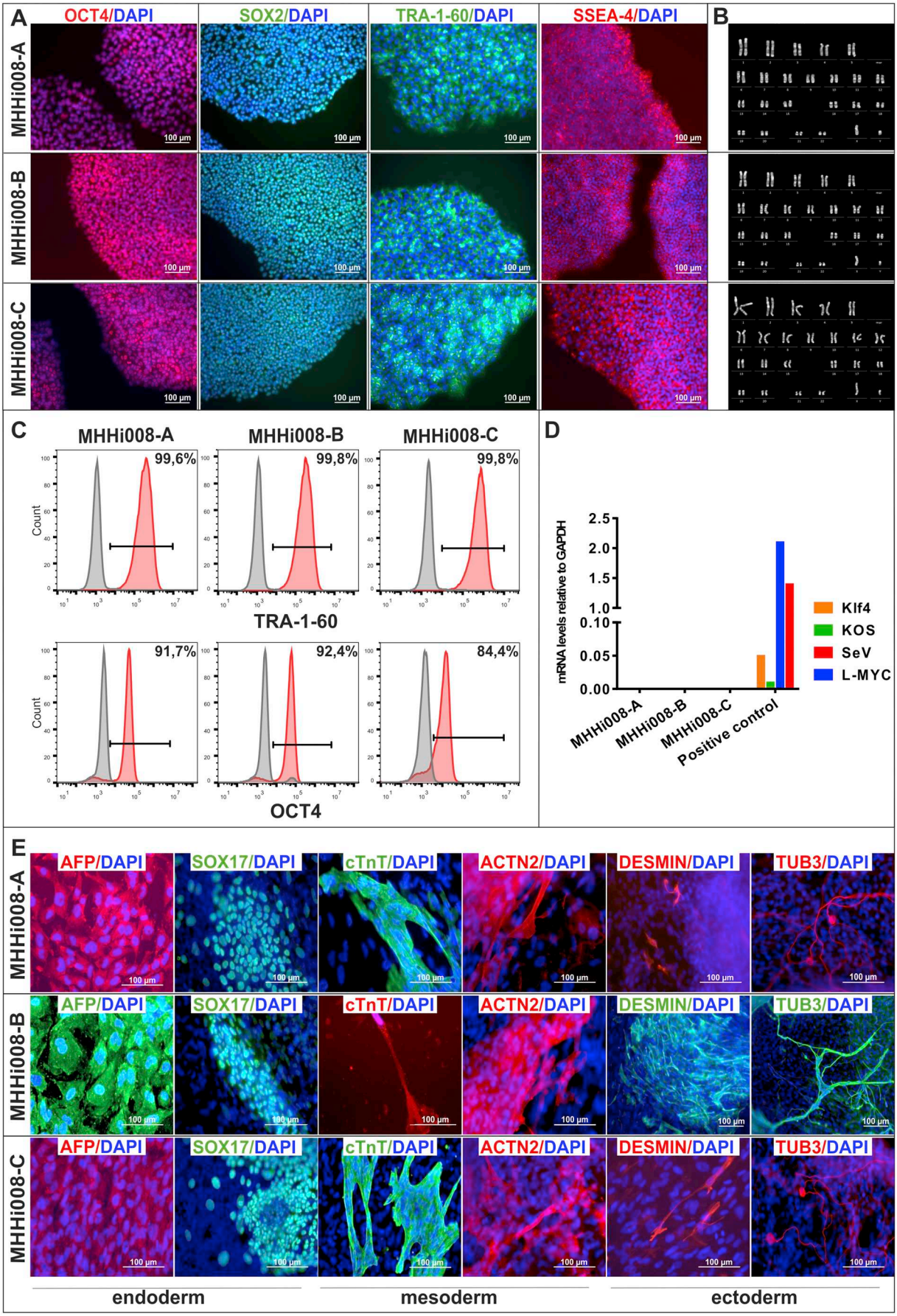

Fig. 1. Characterization of GMP-compatible iPS cell lines MHHi008-A, MHHi008-B and MHHi008-C.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Not shown but available with author
Phenotype	Qualitative analysis	Expression of pluripotency markers: Oct4, Sox2, TRA-1-60, SSEA-4	Fig. 1 panel A
	Quantitative analysis	All tested lines expressed high levels of pluripotency markers. OCT4: > 84%; TRA-1-60: > 99%	Fig. 1 panel C
Genotype	Karyotype (R-banding) and resolution	46XY Resolution: min 300 bands	Fig. 1 panel B
Identity	STR analysis	DNA Profiling not performed	N/A
		STR profile for 16 specific sites tested, all matched	Submitted in archive with journal
Mutation analysis	Sequencing	N/A	N/A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence(MycoAlert Detection Kit, Lonza) Negative (ratio reading B/A 0.42–0.46)	Not shown but available with author
Differentiation potential	Embryoid body formation	Genes of three germ layers expressed: card. troponinT (cTnT), sarc. α-actinin (ACTN2), α-fetoprotein (AFP), SOX17, Desmin, β-3-tubulin (TUB3)	Fig. 1 panel E
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	Negative	Not shown but available with author
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 3
Reagent details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Mouse anti-OCT4	1:100	Santa Cruz Cat# sc-5279, RRID:AB_629051
	Mouse anti-SOX2	1:200	Santa Cruz Cat# sc-17,320 RRID:AB_2286684
	Mouse anti-TRA-1-60	1:100	Abcam Cat# 16288 RRID:AB_778563
	Mouse anti-SSEA-4	1:100	DSHB Cat# MC-813-70 RRID:AB_528477
Differentiation markers	Mouse anti-TroponinT	1:100	Thermo Scientific Cat# MA5–12960 RRID:AB_11000742
	Mouse anti-α-Actinin, Sarcomeric	1:800	Sigma Aldrich Cat# A7811 RRID:AB_476766
	Mouse anti-α-Fetoprotein	1:300	R&D Cat# MAB1368 RRID:AB_357658
	Goat anti-SOX17	1:200	R&D Cat# AF1924 RRID:AB_355060
	Mouse anti-Desmin	1:20	Progen Cat# 10519 RRID: unknown
	Mouse anti-β-3-Tubulin	1:400	Millipore Cat #05–559 RRID:AB_309804
Secondary antibodies	Cy2 Donkey Anti-Mouse IgM	1:200	Jackson Immunoresearch Cat# 715–225-020 RRID:AB_2340824
	488AF Donkey Anti-Goat IgG	1:200	Jackson Immunoresearch Cat# 705–545-147 RRID:AB_2336933
	Cy2 Donkey Anti-Mouse IgG	1:200	Jackson Immunoresearch Cat#715–225-150 RRID:AB_2340826
	Cy3 Donkey Anti-Mouse IgG	1:200	Jackson Immunoresearch Cat# 715–165-150 RRID:AB_2340820

Primers	
TaqMan assay ID	Target
Mr04269880_mr	Sendai
Mr04421257_mr	Sendai-KOS
Mr04421256_mr	Sendai-Klf4
Mr04944276_mr	Sendai-L-Myc
HS02758991_g1	GAPDH

Karyotype analysis

After treatment of adherent hiPSCs with colcemid (Invitrogen) for 30 min, cells were detached with trypsin and metaphases were prepared according to standard procedures. Fluorescence R-banding using chromomycin A3 and methyl green was performed as previously described (Schlegelberger et al., 1999). At least 20 metaphases were analysed per clone at a minimum of 300 bands. Karyotypes were described according

to the international System for Human Cytogenetic Nomenclature (ISCN).

STR analysis

Cells were analysed and compared with source cells by STR profiling of 16 specific sites by Microsynth AG (www.microsynth.ch).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101394.

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Statement on documentation

Reprogramming experiments were documented as per institutional policies on approved paper-based instructions, protocols, and records. All records were independently reviewed and validated.

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