Stem Cell Research 37 (2019) 101409

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Stem Cell Line

Human induced pluripotent stem cell line HMUi001-A derived from corneal stromal cells

Timur Bikkuzin[a,d,1], Yan Shi[a,1], Baoqi Sun[a], Yiyuan Guo[a], Xin Jin[a], Zhenbo Han[b,c], Valentin Pavlov[d], Hong Zhang[a,*]

[a] Eye Hospital, First Affiliated Hospital of Harbin Medical University, Harbin, China
[b] Department of Pharmacy, the Second Affiliated Hospital of Harbin Medical University (Institute of Clinical Pharmacy, the Heilongjiang Key Laboratory of Drug Research, Harbin Medical University), Harbin, China
[c] Department of Pharmacology (the State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), College of Pharmacy, Harbin Medical University, Harbin, China
[d] Central Laboratory of Scientific Research, Bashkir State Medical University, Ufa, Russian Federation

A B S T R A C T

A human corneal stroma induced pluripotent stem cell (HMUi001-A) line was created from primary cultured human corneal fibroblasts. Reprogramming was performed using episomal vector delivery of OCT4, SOX2, KLF4, L-MYC and LIN28. Further characterization of the HMUi001-A confirmed that the cell line was pluripotent, free from Epstein Barr viral genome, and retained normal karyotype.

Resource table

Unique stem cell line identifier	*HMUi001-A*
Alternative name(s) of stem cell line	*HCSiPSCs*
Institution	*Eye Hospital, First Affiliated Hospital of Harbin Medical University, Harbin, China*
Contact information of distributor	*Hong Zhang. Email:zelmykqnwyh@163.com*
Type of cell line	*iPSC*
Origin	*Human*
Additional origin info	*Age: 24* *Sex: male* *Ethnicity: Chinese*
Cell Source	*Human Corneal fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Non-integrating episomal vectors*
Genetic 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	*22.06.2017*
Cell line repository/bank	*https://hpscreg.eu/user/cellline/edit/HMUi001-A*
Ethical approval	*Ethics committee of the Harbin Medical University, approval number 44701*

Resource utility

In this study, we established an integration-free human iPSC line derived from human limbal corneal stromal cells (HLCSCs, corneal fibroblasts), a useful resource for specific differentiation into the corneal cells, which may be a more efficient method.

Resource details

Corneal fibroblasts which were obtained from corneal rim of a 24-year-old healthy male subject were reprogrammed by an integration-free Epi5™ Episomal iPSC Reprogramming kit containing OCT4, SOX2, KLF4, L-MYC and LIN28 (Takahashi et al., 2007). We established the hiPSC line (HMUi001-A) and further characterized for pluripotency. Immunocytochemistry analysis showed that the HMUi001-A line expressed pluripotent markers NANOG and TRA-1-60 (Fig. 1A). Western blot and real-time PCR results also demonstrated the expression of pluripotent markers were higher than the negative control HLCSCs, suggesting successful reprogramming of HMUi001-A. Purchased skin-derived AC-iPSCs (CELLAPY) were used for positive control (Fig. 1B, C). Level of episomal vector (EBNA) gene expression showed no statistical

* Corresponding author.
E-mail address: zelmykqnwyh@163.com (H. Zhang).
[1] Equal contributors.

https://doi.org/10.1016/j.scr.2019.101409
Received 20 November 2018; Received in revised form 10 February 2019; Accepted 14 February 2019
Available online 15 February 2019

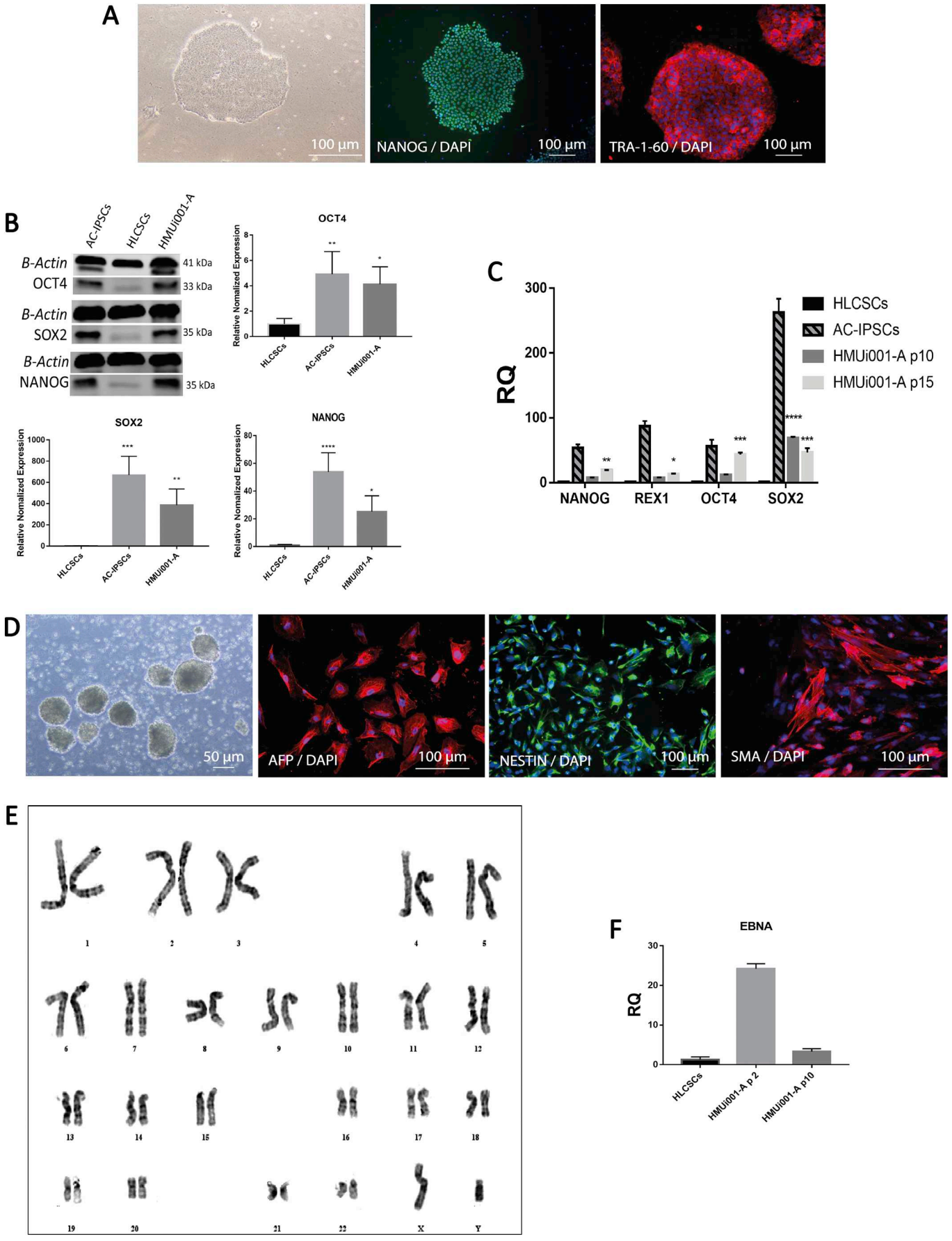

Fig. 1. Characterization of HMUi001-A cells.

difference between HLCSCs and HMUi001-A passage number 10 in comparison with the early passage number 2 (Fig. 1F). The potential of the generated iPSCs to differentiate into cells of all three germ layers was investigated by spontaneous differentiation into cells representative of ectoderm (NESTIN positive cells), mesoderm (SMA positive cells) and endoderm (AFP positive cells, Fig. 1D). Chromosome analysis results exhibited a normal diploid chromosomal content (46, XY) (Fig. 1E). Altogether, these results demonstrate that the HMUi001-A hiPSCs derived from the more efficient protocol are bona fide hiPS cells. Furthermore, STR analysis confirmed that the HMUi001-A hiPSCs originated from the parental corneal fibroblasts, and were free from mycoplasma as examined by MycoBlue Mycoplasma Detector (Vazyme, China) qualitative test (Supplementary Fig. 1).

Materials and methods

Cell culture

HLCSCs were isolated from donor corneal rims, cultured by the tissue explants adhered method. Briefly, the epithelial and endothelial layers were removed by mechanical scraping, and the remainder of the limbus stroma was cut into small pieces (1–2 mm). Then the small piece tissues were put into 24-wells plates, one piece per well. A small amount of fibroblasts culture medium: DMEM F12 Low Glucose (Biological industries), supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 0.5× penicillin/streptomycin (Invitrogen) was added into the plates, to maintain moist of tissue pieces. Then the plates were incubated for approximately 8 h at 37.0 °C, 5% CO2. After tissue adhered to the plate, the culture medium was added until 0.5 ml per well. The medium was changed every two days and cells were subcultured every 4–5 days at a split ratio of 1:5 using 0.25% trypsin (Sigma-Aldrich).

HiPSCs were feeder free cultured in TeSR-E8 basal medium (Stem Cell Technologies) on a Matrigel (Corning) -coated 6-well plate at 37° C and 5% CO2. Medium was replaced every day. The hiPSCs were subcultured every 3–4 days at a split ratio of 1:5 using ReLeSR™ enzyme (Stem Cell Technologies) Tables 1 and 2.

HiPSC generation

500,000 corneal fibroblasts were transfected using Lipofectamine® 3000 Reagent Protocol (Invitrogen) with Epi5 reprogramming vectors mixes (pCE-OCT3/4, pCE-hSK, pCE-hUL, all from Invitrogen). The transfected cells were plated down onto the plates covered with Matrigel in OptiMEM medium (Gibco) supplemented with 10% FBS at 37 °C and 5% CO2. On day 2 post reprogramming, we replaced it with complete TeSR-E8 media. On day 30, hiPSC colonies were isolated by manual dissection and transplanted by ReLeSR onto a new plate coated with Matrigel. The established HMUi001-A hiPSCs were further expanded and characterized for pluripotency.

Spontaneous differentiation of hiPS Cells

To determine the differentiation ability of HMUi001-A in vitro, we harvested hiPSCs using ReLeSR for 5 min at 37 °C until colonies were detached. The clumps were washed with serum-free media: DMEM/F12 + GlutaMAX-I supplemented with 20% KnockOut serum replacement, 1% MEM non-essential amino acids (NEAA), 0.1 mM b-mercaptoethanol, 100 U/ml penicillin/streptomycin (all from Thermo Fisher Scientific) and transferred onto 6-well ultra-low attachment plate (Corning) to form embryoid bodies (EBs). After 10 days culturing EBs were plated onto 0.1% gelatin-coated plates using the same medium for additional 5 days. The medium was changed every other day. Anti-AFP, Anti-SMA and Anti-NESTIN markers were analysed by immunofluorescence staining.

Immunofluorescence staining

The cell colonies were fixed in 4% paraformaldehyde (Thermo Fisher Scientific) for 20 min at room temperature. After rinsing with PBS, 0.1% Triton X-100 (Thermo Fisher Scientific) was added for 15 min. Then the cells were washed with PBS again and blocked with 5% BSA for 30 min. Next, the colonies were incubated under conditions with following antibodies diluted in PBS with 1% BSA, as appropriate: TRA-1-60 1:100 (Millipore), NANOG 1:100 (Novus), a-SMA 1:100 (R&D System.), AFP 1:100 (Adnova), NESTIN 1:100 (Abcam) at 4 °C overnight. After rinsing three times with PBS, cells were incubated with the corresponding Alexa-Fluor 594-conjugated secondary antibody (Thermo Fisher Scientific) for 1 h at room temperature. The nuclei were stained with DAPI (Abcam). Fluorescent images were taken using an Axiovert200M (Carl Zeiss) microscope.

Western blot analysis

Total protein of hiPS cells was extracted using RIPA buffer (Thermo Fisher Scientific) in presence of 2% protease inhibitor (Sigma-Aldrich). Protein (30 μg per sample) was separated using 10% SDS–PAGE and then transferred onto nitrocellulose membranes (Thermo Fisher Scientific). The membrane was blocked with 5% non-fat milk (BD Biosciences) and 0,1% Tween 20 (Sigma-Aldrich) in Tris-buffered saline (TBST), then immunoblotted overnight using OCT4 (Santa Cruz Biotechnology), NANOG (Novus) and SOX2 (R&D Systems) primary antibodies in TBST at 4 °C with gentle shaking. Fluorochrome-labelled secondary antibody (Alexa Fluor 800, Thermo Fisher Scientific) was used to identify the appropriate primary antibody. Immunoreactivity was detected with the Odyssey fluorescent scanning system (LI-COR)

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel A
Phenotype	Qualitative analysis (Immunofluorescence)	Positive staining for TRA-1-60 and NANOG	Fig. 1 panel A
	Quantitative analysis (Western Blot, RT-PCR)	Expression of pluripotency markers OCT4, SOX2, NANOG, REX1.	Fig. 1 panel B and C
Genotype	Karyotype (G-banding) and resolution	46XY, Resolution 450–500	Fig. 1 panel E
Identity	Microsatellite PCR (mPCR)	Not performed	N/A
	STR analysis	20 sites tested, all sites are matched	Available with authors
Mutation analysis (IF APPLICABLE)	Sequencing	Not performed	N/A
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	MycoBlue Mycoplasma Detector qualitative test. Negative	Supplementary Fig. 1
Differentiation potential	Embryoid body formation and directed differentiation	Positive staining for endodermal (AFP), mesodermal (SMA) and ectodermal (NESTIN)	Fig. 1 panel D
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

Table 2
Reagents details.

Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotent markers	Mouse anti-TRA-1-60	1:100	Millipore Cat# MAB4360, RRID:AB_2119183
	Mouse anti-NANOG	1:100	Novus Cat# NBP1–47427, RRID:AB_10010380
Differentiation Markers	Mouse anti-AFP	1:100	Abnova Corporation Cat# H00000174-M01, RRID:AB_464229
	Mouse anti-SMA	1:100	R and D Systems Cat# MAB1420, RRID:AB_262054
	Mouse anti-NESTIN	1:100	Abcam Cat# ab6320, RRID:AB_308832
Secondary antibodies	Alexa Fluor 594 donkey anti-mouse IgG (H?+?L)	1:500	Thermo Fisher Scientific Cat# A-21203, RRID:AB_2535789

Antibodies used for Western blot			
	Antibody	Dilution	Company Cat # and RRID
Pluripotent markers	Mouse anti-OCT4	1:1000	Santa Cruz Biotechnology, Cat # sc-5279, RRID: AB_628051
	Mouse anti-NANOG	1:1000	Novus Cat# NBP1–47427, RRID:AB_10010380
	Mouse anti-Sox2	1:1000	R and D Systems Cat# MAB2018, RRID:AB_358009
Loading control	Mouse anti-ß actin	1:2000	ZSGB-Bio Cat# TA-09, RRID:AB_2636897
Secondary antibodies	Alexa Fluor Plus 800 goat anti-mouse IgG (H?+?L)	1:1000	Thermo Fisher Scientific Cat# A32730, RRID:AB_2633279

Primers		
	Target	Forward/Reverse primer (5'-3')
Pluripotency markers	OCT4/408?bp	AGCCCTCATTTCACCAGGCC/CAAAACCCGGAGGAGTCCCA
	SOX2/522?bp	CATTTGCTGTGGGTGATGGG/TCTTGGCTCCATGGGTTCG
	NANOG/356?bp	CAGCCCCGATTCTTCCACCAGTCCC/ CGGAAGATTCCCAGTCGGGTTCACC
	REX1/472?bp	CAGATCCTAAACAGCTCGCAGAAT/ GCGTACGCAAATTAAAGTCCAGA
Episomal vector	EBNA/582?bp	ATCAGGGCCAAGACATAGAGATG/GCCAATGCAACTTGGACGTT
Housekeeping gene	ß-ACTIN/436?bp	CGAGCACAGAGCCTCGCCTTT/TGCCGTGCTCGATGGGGTAC

and analysed by Image Studio software. β-actin (ZSGB-Bio, China) was used as a loading control.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen). PCR primers were purchased from Invitrogen. The concentration of RNA was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). For detection of REX1, NANOG, EBNA, endogenous SOX2 and OCT4 expression level, cDNA was synthesized from 5 ng of total RNA using the ReverTraAce® qPCR RT Kit (TOYOBO, Japan). Quantitative real-time PCR was performed using SYBR Green Real-Time PCR MasterMix (TOYOBO) according to the manufacturer's protocols using an ABI 7500 Sequence Detection System (Life Technologies). PCR amplification was performed in a 20-μl reaction volume containing 2 μl cDNA, 6 μl DEPC, 10 μl SYBR Master Mix, 1 μl forward primer and 1 μl reverse primer. The amplification consisted of holding 95 °C for 60 s, 40 cycles of 15 s at 95 °C, 15 s at 60 °C, and 45 s at 72 °C, followed by a 5 min extension at 72 °C after the last cycle. β-actin was used as an internal control.

Karyotyping

Cells from passage 9 were used for karyotypic analysis. At least 20 metaphase chromosomes were evaluated for the standard G-banded karyotyping using 500 resolution. The karyotyping analysis was carried out by CELLAPY, Beijing.

Short tandem repeat (STR) analysis

STR analysis was carried out by Cobioer Biosciences, Nanjing. Genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. Samples were amplified using GenePrint System (Promega) and processed using the ABI3730x1 Genetic Analyzer. Loci detected were AMEL, D1S1656, D5S818, D12S391, FGA, D13S317, D7S820, D16S539, VWA, TH01, TPOX, CSF1PO, D2S1338, D21S11, D18S51, D8S1179, D3S1358, D6S1043, PENTAE, D19S433 and PENTAD. Data was analysed by GeneMapper4.0 software.

Mycoplasma test

Mycoplasma analysis was performed using qualitative MycoBlue Mycoplasma Detector (D101–02, Vazyme, China). Briefly, after adding 1 μl of the iPSCs culture supernatant to the reaction system and incubated at 60 °C for 1 h, the results were determined by visual observation according to manufacturer's protocol.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101409.

Acknowledgments

The authors thank Prof. Cai from the Second Affiliated Hospital of Harbin Medical University for kindly providing the AC-iPSC line. As well, as we thank Bao Fengyang (Harbin Medical University) for technical assistance.

References

Takahashi, K., et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872.