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A human H1-HBB11-GFP reporter embryonic stem cell line (WAe001-A-2) generated using TALEN-based genome editing

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Abstract

Hemoglobin production during mammalian development is characterized by temporal switches of the genes coding for the α- and β-globin chains. Defects in this controlled process can lead to hemoglobinapathies such as sickle cell disease and β-thalassemia. The ability of human embryonic stem cells (hESC) to proceed through hematopoiesis could provide a clinically useful source of red blood cells. However, hESC-derived red cells exhibit an embryonic/fetal, but not adult, mode of hemoglobin expression. The resource described here is a hESC line engineered to express a reporter from its adult globin promoter, providing a screening platform for small molecules that lead to efficient induction of adult globin.

1. Resource utility

WAe001-A-2 also known as H1-HBB11-GFP has the stop codon of the HBB gene replaced by the 2A-GFP cassette. This cell line will monitor HBB expression via GFP expression. This line will be used to understand mechanisms governing HBB expression (Sankaran et al., 2015) as well as to screen for drugs that modulate HBB expression.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://doi.org/10.1016/j.scr.2020.101837.

2. Resource details

A WAe001-A-2 (H1-HBB11-GFP) reporter human embryonic stem cell line (clone 11) was generated using TALEN-based genome targeting of a 2A-eGFP cassette to replace the stop codon of endogenous *HBB*, thus creating a *HBB-GFP* allele, in WA01 (H1, NIH registration number 0043) hESCs (Table 1). Addgene plasmid #31938 was used to generate a donor plasmid HBB-2a-GFP-PGK-Puro (Fig. 1A). TALEN recognition sequences were designed using software found at www.taleffectors.com (Fig. 1B). Correct integration was confirmed by PCR of genomic DNA using HBB-F3 and GFP_R2 (Hockemeyer et al., 2012) primer pairs (data not shown) followed by sequencing across the integration site, revealing that the stop codon of HBB was correctly replaced by the 2A-GFP cassette (Fig. 1C). Southern blot analysis of HindIII-digested WAe001-A-2 and parental WA01 DNA samples using a DIG-labelled GFP probe confirmed there was only a single site at which the 2A-eGFP cassette was inserted into the genome (Fig. 1D). Further PCR screening indicated that the cell line was heterozygous and generated a wild type 279 bp band and a 497 bp band only seen in the H1-HBB11-GFP clone (Fig. 1E). The H1-HBB11-GFP clone was used in all further analysis and was named WAe001-A-2 as per https://hpscreg.eu. WAe001-A-2 exhibited a normal ESC morphology (Fig. 1F) and expressed the stem cell marker, Nanog (Fig. 1G). Three germ-layer differentiation ability was demonstrated by spontaneous in vitro differentiation of embryoid bodies with subsequent replating and immunocytochemical detection of smooth muscle actin (SMA) for mesoderm, alpha-feto protein (AFP) for endoderm, and beta-III tubulin (TUJI) for ectoderm (Fig. 1H). RTq-PCR analysis found comparable expression of Oct4, Nanog, Sox-2, and Tert in the unmodified parental line WA01 and in the newly created gene modified WAe001-A-2 cell line (Fig. 1I). Karyotype analysis was performed by WiCell at a 450–475 band resolution, which indicated the cell line was male and that there appeared to be an interstitial duplication in the long arm of chromosome 20 in thirteen of twenty cells examined (Fig. 1J). Unfortunately, the abnormality at this location is a recurrent acquired duplication in human pluripotent stem cell cultures. Identity of this line was confirmed by STR analysis that analyzed 28 allelic polymorphisms across the 15 STR loci (Supplementary Table 1) and confirmed that WA01 and WAe001-A-2 were identical. H1-HBB11-GFP (clone 11), H1-HBB1-GFP (clone 1), or H9 (negative control) cells were differentiated using the Kennedy et al. (2012) protocol. PCR showed βglobin expression from day 13 EBs that had been cocultured on OP9 cells for 21 days only in the H1-HBB11-GFP cells (Fig. 1K), and FACS analysis on the same population of cells detected GFP expression in 16% of the cells (Fig. 1L) whereas the negative control was < 2% (not shown).

3. Materials and methods

3.1. Maintenance of human embryonic stem cells

H1(NIH code WA01), H9 (WA09) from the WiCell Research Institute (Madison, WI) and the HBB-GFP modified WAe001-A-2 cells were maintained as described (Kennedy et al., 2007).

3.2. Generation of a WAe001-A-2 reporter WA01 human embryonic stem cell line using TALEN-based genome engineering

Homologous arms were amplified using genomic DNA isolated from WA01 hESCs and primers to amplify the upstream homologous arm or the downstream homologous arm, respectively (see Table 2 for primer sequences). PCR products were run on a 1% agarose, gel, gel purified and digested with SbfI and NheI (upstream homologous arm), and AscI and NotI (downstream homologous arm), respectively, and ligated into the OCT4-2A-eGFP-PGK-Puro vector (Addgene; plasmid 31938) replacing the OCT4 homologous arms. TALENs targeting 5'-TGGCTAATGC CCTGGCC-3' upstream of the *HBB* stop codon, and 5'-TGGACAGCAA GAAAGCGAGC-3' downstream of the *HBB* stop codon, respectively, were assembled using the Joung Lab REAL Assembly TALEN kit (Addgene; Kit # 1000000017) according to protocol by Sander et al. (2011). To generate the reporter line, 10^7 WA01 hESCs were mixed with 40 μg of *HBB*-2A-eGFP-PGK-Puro donor plasmid and 5 μg of each TALEN encoding plasmid in 800 μL of PBS in a 0.4 cm cuvettes. Electroporation was performed at 250 V and 500 μF using a Gene Pulser Xcell system (BioRad). Cells were plated on irradiated DR4 MEFs and selected with 0.5 μg/mL puromycin starting on day 5 after electroporation. Resistant colonies were picked and expanded.

3.3. Southern blot analysis

10 μg of genomic DNA from potential *HBB*-eGFP reporter clones were digested with HindIII (NEB) overnight at 37 °C and run on a 1% agarose gel. The DNA was transferred from the gel to a positively charged nylon membrane using a standard protocol. The DIG DNA Labelling and Detection Kit (Roche) in combination with a GFP fragment (see Table 2 for primer sequences) was used for probe labelling, hybridization and signal detection according to the manufacturer's instruction.

3.4. PCR and quantitative real-time PCR

PCR using ~100 ng of genomic DNA was performed using Fusionflash High Fidelity PCR Master Mix (Finnzymes) at 98 °C for 1 min followed by 35 cycles at 98 °C for 1 min, 55 °C and 57 °C for 5 sec each, and 72 °C for 30 sec, followed by a final extension at 72 °C for 1 min in a Verity 96-well Thermal Cycler (Applied Biosystems). Quantitative real-time PCR on cDNA prepared from 500 ng RNA isolated from cells, using "PureLink RNA Mini Kit" Ambion Cat# 1517663, was performed using SyberGreen (Applied Biosystems) according to the manufacturer's instruction in a Lightcycler 480 (Roche) at 95 °C for 1 min (initial denaturing step), followed by 40 cycles of 95 °C for 3 sec and 60 °C for 30 s. Data were normalized to GADPH.

3.5. Three germ layer differentiation

The HBB-Cl11-GFP line was treated with 0.5 mM EDTA in 1X PBS. Cells were gently scraped with a cell scraper to form small aggregates (10–20 cells). Aggregates were resuspended in hESC media in the absence of bFGF and in the presence of thiazovivin (2 μM). The EBs were cultured in this media for 5 days and the EBs allowed to settle in a falcon tube. The hESC media was aspirated off and resuspended in DMEM containing 10% FBS and replated on Matrigel coated plates and EB cells were allowed to attach. Media was

changed every 3 days and the culture was maintained for 14 days. Random differentiation was detected by simultaneous 3-color staining using three fluorochrome-conjugated antibodies provided with the 3 Germ Layer Immunocytochemistry Kit (Invitrogen; Catalog # A25538). Ectoderm derivatives were detected with TUJ1-rabbit (AF647), mesoderm differentiated cells with SMA-mouse (AF555), and endoderm derivatives with AFP-mouse IgG1 (AF488). The nuclei were counterstained with DAPI (blue).

3.6. Immunocytochemistry

Cultured or differentiated WAe001-A-2 hESCs were fixed with 4% Paraformaldehyde in PBS for 15 min at RT, washed with 1X PBS, permeabilized with 0.2% Triton-X 100 for 10 min at RT and rewashed with PBS. Cells were blocked using a 0.05% saponin solution (PBS, 1% Glucose, 0.02% sodium azide, 5 mM HEPES, 0.05% Saponin) containing 10% of the serum from the animal in which the secondary antibodies were generated in. For pluripotency marker detection, cells were incubated with a 1:200 dilution overnight at 4 °C of goat-polyclonal anti-Nanog (R&D Systems). Cells were washed three times in 0.05% saponin solution for 15 mins each. Cells were then incubated with donkey anti-goat Alexa Fluor 488 (Life Technologies) secondary antibody at a 1:500 concentration for 60 min. Cells were washed 3 times in 0.05% saponin solution to remove the unbound secondary antibody. DAPI at 1:1000 dilution was added for the last wash. For 3 germ layer identification, fixed cells were processed using the 3-Germ Layer Immunocytochemistry Kit (Life Technologies) according to the manufacturer's instruction. Briefly, cells were incubated with the blocking solution for 1 h at RT, then incubated with primary antibodies (rabbit anti-TUJI (1:250), mouse IgG1 anti-AFP (1:250), and mouse IgG2 anti-SMA (1:100)) overnight at 4 °C. The next day, cells were washed 3 times for 15 min in 0.05% saponin solution followed by incubation with secondary antibodies (Alexa Fluor 647 donkey anti-rabbit; Alexa Fluor 488 goat anti-mouse IgG1, and Alexa Fluor 555 goat anti-anti mouse IgG2a; all at 1:250) in 0.05% saponin in 1X PBS for 1 h at RT. After 3 washes with 1X PBS, nuclei were stained with DAPI (1:1000). Fluorescence was visualized using a Leica DMRA2 fluorescence microscope (Wetzlar, Germany) and images were recorded using a digital Hamamatsu CCD camera (Hamamatsu City-Japan).

3.7. Karyotype analysis and cell identity by short tandem repeat analysis

Chromosomal G-banding karyotype analysis (450–475 band resolution) as well as short tandem repeat analysis (28 allelic polymorphisms analyzed across the 15 STR loci) for parental cell WA01 and WAe001-A-2 authentication was performed by the WiCell Research Institute (Madison, WI).

3.8 Hematopoietic differentiation

For hematopoietic differentiation we followed a modified version of a published protocol (Kennedy et al., 2012). The control hESC line H9 and two of the TALEN clones (HBB-C11-GFP and HBB-Cl11-GFP) line were treated with 0.5 mM EDTA in 1X PBS. Cells were gently scraped with a cell scraper to form small aggregates (10–20 cells). Aggregates were resuspended in Stemline II hematopoietic stem cell expansion media (Sigma), supplemented with penicillin/streptomycin (10 ng/ml), L-glutamine (2 mM), ascorbic acid (1 mM), monothioglycerol (MTG, 4×10^{-4} M; Sigma-Aldrich), and transferrin (150 μg/ml)

henceforth referred to as Base Medium. BMP-4 (10 ng/ml) was added to Base Medium and aggregates along with 2 μM Thiazovivin (Selleck) to promote survival/ EB formation and incubated overnight at 37°. Subsequently the culture was fed with Base Medium supplemented with BMP-4 (10 μg/ml) and bFGF (5 μg/ml) for the next 24 h. At Day 2, EBs were allowed to settle and media was aspirated and replaced with Base Medium containing BMP-4 (10 μg/ml), bFGF (5 ng/ml), and Activin A (5 ng/ml) for the following 48 h (days 2–4). At Day 4, EBs were allowed to settle and media was aspirated and replaced with Base Medium containing bFGF (5 ng/ml), VEGF (15 ng/ml), IL-6, 10 ng/ml) IL-11 (5 ng/ml) and IGF-1 (25 ng/ml) and and DKK1 (150 ng/ml) referred to henceforth as Base Medium 1. On day 6, cultures we fed with Base Medium 1 supplemented with SCF (50 ng/ml) and EPO (2 U/ml final) referred henceforth as Base Medium 2. At Day 8, cultures were fed with Base Medium 2 supplemented with TPO (30 ng/ml), Flt-3 (10 ng/ml) and IL-3 (30 ng/ml). At Day 8, EBs / floating cells were spun down and media aspirated and cells resuspended in Base Medium containing VEGF (15 ng/ml), IL-6 (10 ng/ml) IL-11 (5 ng/ml), IGF-1(25 ng/ml), SCF (50 ng/ml), EPO (2 U/ml final), TPO (30 ng/ml), Flt-3 (10 ng/ml) and IL-3 (30 ng/ml) referred to as Base Medium 3. At Day 11, an aliquot of the culture was harvested, trypsinized and 20,000 cells were plated onto irradiated OP9 feeders in the presence of Base Medium 3 whereas the other aliquot was allowed to differentiate for another two more days (Day 11–13) and fed with 1 ml of Base Medium 3/one well of a 6-well plate. At day 13, EBs were harvested, trypsinized and 20,000 single cells were transferred to OP9 cells and cultured with Base Medium 3. Culture was fed every 3 days and cells were prepared for FACS analysis and RT-PCR after 11 days of EB culture or after 11 days of EB culture or 13 days or EB culture followed by either 21 or 23 days of culture on OP9 cells. Cells were then harvested and analyzed for GFP expression by FACS analysis or for βglobin expression by PCR analysis (Fig. 1K and L).

3.9. Mycoplasma test

The culture was screened for mycoplasma using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Cat # LT07–218) according to manufactures instructions (Supplementary Table 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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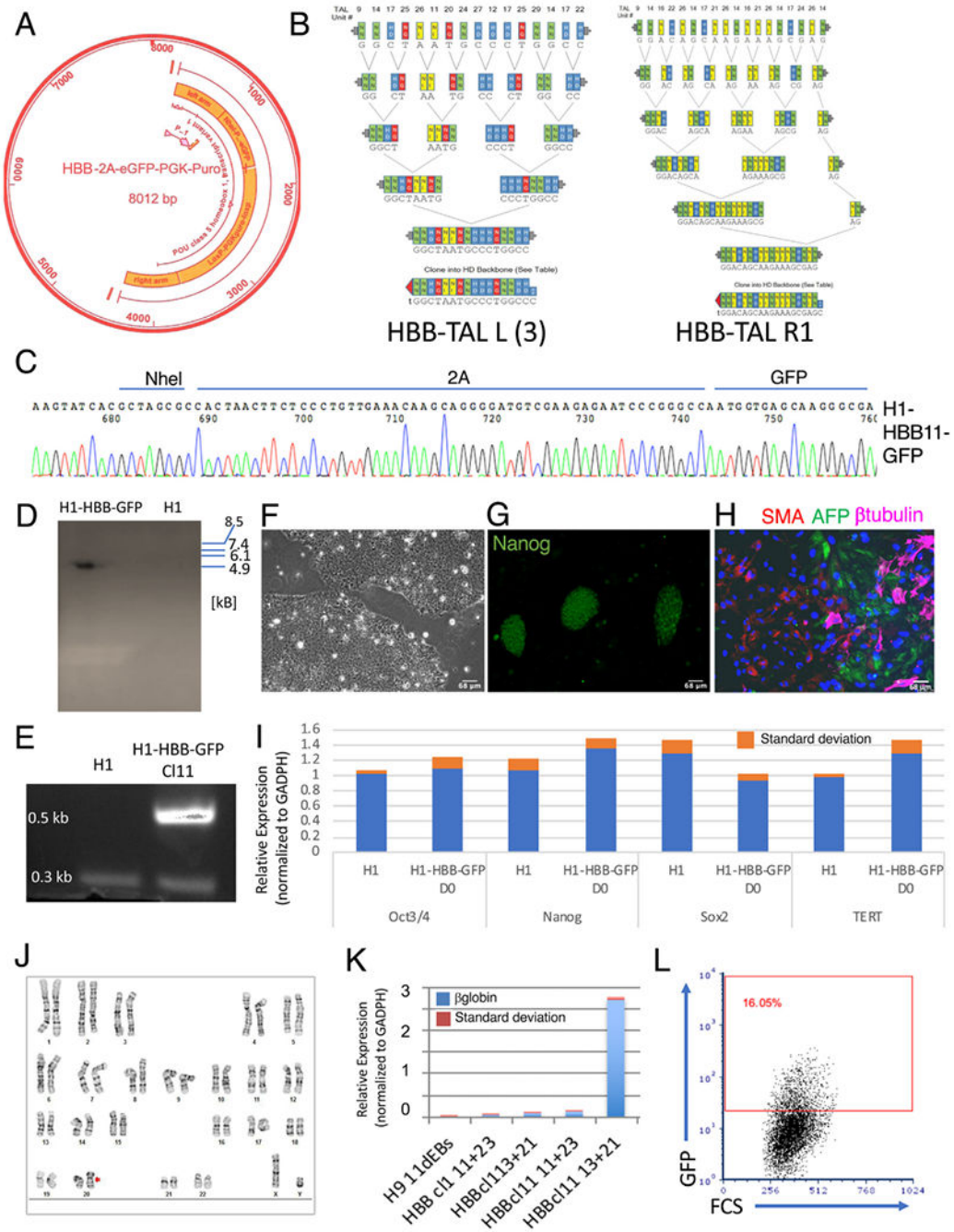

Fig. 1.

Generation and characterization of the H1-HBB11-GFP human embryonic stem cell line.

Table 1

Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal ESC morphology	Fig. 1F
Phenotype	Qualitative analysis Immunocytochemistry	Positive staining for NANOG	Fig. 1G
	Quantitative analysis RT-qPCR	RT-qPCR	Fig. 1I
Genotype	Karyotype (G-banding) and resolution	46 XY, (WiCell) Resolution 450–475	Fig. 1J
Identity	Microsatellite PCR	Not performed	NA
	STR analysis	Performed. 28 allelic polymorphisms analyzed across the 15 STR. All alleles matched the parental WA01 line.	Supplementary Table 1
Mutation analysis	Sequencing	Heterozygous reporter line	Fig. 1C and Fig. 1E
	Southern Blot OR WGS	One insertion, no off target insertions	Fig. 1D
Microbiology and virology	Mycoplasma	Mycoplasma testing luminescence : Negative	Supplementary Table 2
Differentiation potential	Embryoid body formation	Positive for smooth muscle actin (SMA), β-tubulin (TUJI) and α-feto protein (AFP).	Fig. 1F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not done	Not done
Genotype additional info (OPTIONAL)	Blood group genotyping	Not done	Not done
	HLA tissue typing	Not done	Not done

Table 2

Details of reagents and primers.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	*Goat anti -hNanog*	*1:200*	R&D Cat#AF1997. RRID:AB_355097
Differentiation Markers	Rabbit anti TUJ1	*1:500*	Molecular probes Cat# A25532 “3-Germ layer Immunocytochemistry Kit”. RRID: AB_2651003
	Mouse IgG1 antiAFP	*1:200*	Molecular probes Cat# A25530 “3-Germ layer Immunocytochemistry Kit”. RRID: AB_2651004
	Mouse IgG2 anti-SMA	*1:500*	Molecular probes Cat# A25531 “3-Germ layer Immunocytochemistry Kit”. RRID: AB_2651005
Secondary antibodies	Alexa Fluor™ 647 donkey anti-rabbit; for use with anti-TUJ1	1:250	Thermo Fisher Scientific Cat# A-21206, RRID:AB_2535792
	Alexa Fluor™ 488 goat anti-mouse IgG1; for use with anti-AFP	1:250	Thermo Fisher Scientific Cat# A25536, RRID:AB_2651011)
	Alexa Fluor™ 555 goat anti-mouse IgG2a; for use with anti-SMA	1:250	Thermo Fisher Scientific Cat# A25533 RRID:AB_2651012
Primers	Target	*Forward/Reverse primer (5’-3’)*	
Cloning primers	HBB upstream	*GACCTGCAGGTTGGAATATATGTGTGCTTATTTGC/GAGCTAGCGTGATACTTGTGGGCCAG*	
	HBB downstream	*GAGGCGCGCCTAAGCTCGCTTTCTTGCTG/GAGCGGCCGCTCAAACCATGACCCCTGTT*	
Targeted mutation analysis/ sequencing	Homo/Hetero Reverse GFP	*AGTCCAAGCTAGGCCCTTTT (forward)/TGCTCAAGGCCCTTCATAAT (reverse1) AAGTCGTGCTTCATGTG (reverse2)*	
Pluripotency (qPCR)	hOCT3/4	*CATTCAAACTGAGGTAAGGG/TAGCGTAAAAGGAGCAACATAG*	
	hNanog	*CCTGAAGACGTGTGAAGATGAG/GCTGATTAGGCTCCAACCATAC*	
	hSox2	*AGAAGAGGAGAGAGAAAGAAAGGGAGAGA/GAGAGAGGCAAACTGGAATCAGGATCAAA*	
	hTERT	*TGAAAGCCAAGAACGCAGGGATG/TGTCGAGTCAGCTTGAGCAGGAATG*	
House-Keeping	GAPDH	*ACATCAAGAAGGTGGTGAAGCAGG/TCAAAGGTGGAGGAGTGGGTGT*	
Differentiation markers	βglobin	*TGTCCACTCCTGATGCTGTTATGG/AGCTTAGTGATACTTGTGGGGCAG*	

Resource Table

Unique stem cell line identifier	WAe001-A-2.
Alternative name of stem cell line	H1-HBB11-GFP
Institution	Icahn school of Medicine at Mount Sinai
Contact information of distributor	James Bieker/james.bieker@mssm.edu
Type of cell line	ESC
Origin	Human
Additional origin info	Same as that as WA01. Age: unknown Sex: male Ethnicity: unknown
Cell Source	WA01 cells
Clonality	Clonal
Method of reprogramming	NA
Genetic Modification	YES
Type of Modification	Stop codon of HBB gene replaced with a 2A-GFP-PGK-puro cassette.
Associated disease	NA
Gene/locus	HBB/11p15.4
Method of modification	TALENs
Gene correction	NO
Name of transgene or resistance	GFP
Inducible/constitutive system	Reporter cell line
Date archived/stock date	Not done
Cell line repository/bank	Not done
Ethical approval	In vitro ESC and iPSC work have received IRB exemption from the Embryonic Stem Cell Research Oversight (ESCRO) committee at Icahn School of Medicine at Mount Sinai.