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Lab Resource: Genetically-Modified Single Cell Line

Generation of LUMCi041-A-2: Equipping a PAX3 reporter iPSC line with doxycycline inducible H2B-mTurquoise2 for live cell imaging

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ABSTRACT

An induced pluripotent stem cell (iPSC) line, in which a H2B-fluorescent protein fusion is temporally expressed, is a valuable tool to track cells and study cell divisions and apoptosis. To this end we introduced a 3rd generation "all-in-one" doxycycline-inducible H2B-mTurquoise2 vector into the *AAVS1* locus of *PAX3-Venus* iPSCs via CRISPR/Cas9. H2B-mTurquoise2 expression is absent but readily induced by doxycycline allowing quantification of cell divisions and imaging of living cells. Besides being a universal reporter in iPSC-based differentiation and toxicity assays, the generated pluripotent and genomically normal LUMCi041-A-2 line is particularly suited to study PAX3-positive stages of development.

Unique stem cell line identifier	LUMCi041-A-2 Synonym of CRMi003-A-2 Subclone of CRMi003-A https://hpscreg.eu/cell-line/LUMCi041-A-2
Alternative name(s) of stem cell line	*DOXiH2BmTurq2*
Institution	Leiden University Medical Center
Contact information of the reported cell line distributor	Christiaan Arendzen, c.h.arendzen@lumc.nl
Type of cell line	iPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: fetal tissue (umbilical cord blood) Sex: male No disease known
Cell Source	Cord Blood CD34 + derived NCRM-1 with a *PAX3-Venus* targeted allele
Method of reprogramming	Episomal reprogramming with *SOX2, KLF4, MYC, Oct4, Lin28* and SV40 large T antigen.
Clonality	Clonal, puromycin selection followed by single cell purification through fluorescence-activated cell sorting (FACS)
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	Tissue culture treated multiwell plates (Greiner), hESC qualified Matrigel (Corning #354277), StemFlex medium (ThermoFisher Scientific Cat#A3349401)

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(*continued*)

Unique stem cell line identifier	LUMCi041-A-2 Synonym of CRMi003-A-2 Subclone of CRMi003-A https://hpscreg.eu/cell-line/LUMCi041-A-2
Type of Genetic Modification	Targeted insertion of an all-in-one cassette (containing rtTA3G, H2B-mTurquoise2, puromycin N-acetyl-transferase)
Associated disease	N/A
Gene/locus	*AAVS1/ PPP1R12C*
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Electroporation
All genetic material introduced into the cells	CRISPR/Cas9 plasmid, *AAVS1* guide RNA plasmid, 'all in one' 3rd generation TET-ON H2B-mTurquoise2 plasmid, BCL-XL expression plasmid
Analysis of the nuclease-targeted allele status	5′ PCR amplification and Sanger sequencing of the targeted region
Method of the off-target nuclease activity surveillance	N/A
Name of transgene	rtTA3G, H2B-mTurquoise2
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	Puromycin
Inducible/constitutive system details	Doxycycline inducible expression of H2B-mTurquoise2 via CAG promoter driven expression of rtTA3G
Date archived/stock date	N/A
Cell line repository/bank	N/A
Ethical/GMO work approvals	See parental line CRMi003-A at hpscreg.eu/cell-line/CRMi003-A LUMC GMO permit: IG06-02
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pCas9-GFP vector (Addgene #44719) *AAVS1* guide Vector: (Addgene #41818) BCL-XL vector: Li et al., Nucl. Acids Res. 2018

1. Resource table

Manuscript section expected contents clarification

2. Resource utility

We equipped a *PAX3-Venus* iPSC line with a doxycycline inducible Histone 2B (H2B)-mTurquoise2 reporter in the *AAVS1* locus. This reporter iPSC line is instrumental to track cells and to temporally assess cell divisions and apoptosis in iPSC-based differentiation and cell toxicity assays.

3. Resource details

H2B proteins form together with H2A, H3 and H4 the core of the nucleosome. Once H2B has been incorporated during the S-phase of the cell cycle degradation is negligible and H2B loss over time is the result of H2B re-distribution over mother and daughter cells after each cell division. The features of H2B are maintained when H2B is fused to fluorescent proteins (FP). Consequently, permanent H2B-FP expression has been successfully used to study mitosis (Sivakumar et al., 2014), chromosomal architectural dynamics (Kanda et al., 1998) and apoptosis-induced chromosomal degradation (Nolin et al., 2016), whereas temporal expression has been applied to study the proliferation rate of hematopoietic stem cells (HSCs) and the consequences thereof in mouse models (Bernitz et al., 2016). We reasoned that a H2B-FP iPSC line, in which the H2B fusion protein is inducibly expressed, is a sought-after tool to study and trace cell divisions and apoptosis in iPSC-based differentiation and toxicity assays. To this end we introduced a 3rd generation 'all-in-one' doxycycline (DOX) inducible vector containing promoterless puromycin N-acetyl transferase, a CAG promoter driving transcription of rtTA3G, and H2B-mTurquoise2 (H2B-mTurq2) controlled by six 3rd generation TetO elements (Fig. 1A) into one of the *AAVS1* alleles of *PAX3-Venus* iPSCs. Upon culturing of the iPSCs in maintenance medium in the presence of 1 μg/ml Dox, puromycin selected colonies homogeneously expressed H2B-mTurq2 (Fig. 1B). To ensure the single cell origin of the clones, mTurquoise2 positive single cells were further purified by fluorescence activated cell sorting (FACS) (Fig. 1C). Correct insertion of the donor DNA into the *AAVS1* locus was verified by PCR and Sanger sequencing of the remaining wild-type allele (Fig. 1D, E). To establish the utility of this iPSC line for quantification of cell divisions we directly compared H2B-mTurq2 labelling to the results obtained with CFSE labelling, which is often used for visualization of distinct cell generations. Analysis of the cells by flow cytometry after a 48 hrs pulse of DOX followed by a chase initiated 36 hrs after withdrawal of DOX, a time period required for complete degradation of non-incorporated H2B-mTurq2, did not reveal substantial differences between the two labelling methods. Both methods demonstrated two-fold dilutions of the label upon each cell division (Fig. 1F). Additional characterization confirmed the pluripotent nature and genome integrity of the generated, mycoplasma negative, DOX inducible H2B-mTurq2 iPSC line (Fig. 1G, H, Supl Fig. 1E).

4. Materials and methods

4.1. Cell culture

Human iPSCs were cultured in StemFlex (ThermoFisher Scientific) on Matrigel (Corning) coated plates at 37 °C and 5% CO_2. Once a week cells were passaged as aggregates using GCDR (Stemcell Technologies).

4.2. Donor plasmid generation

We removed mCherry from pUCM-AAVS1-TO-hNGN2 (Addgene #105840) using PmeI and Acc65I restriction. Subsequently, hNGN2 was exchanged for a synthesized FRT-H2B/mTurquoise2-FRT fragment (SynthesisGene, China) using PacI and NotI resulting in pUCM-AAVS1-TO-H2B/mTurq2.

4.3. Reporter insertion and clone selection

Plasmids (pUCM-AAVS1-TO-H2B/mTurquoise2 (210 ng) and pCas9-

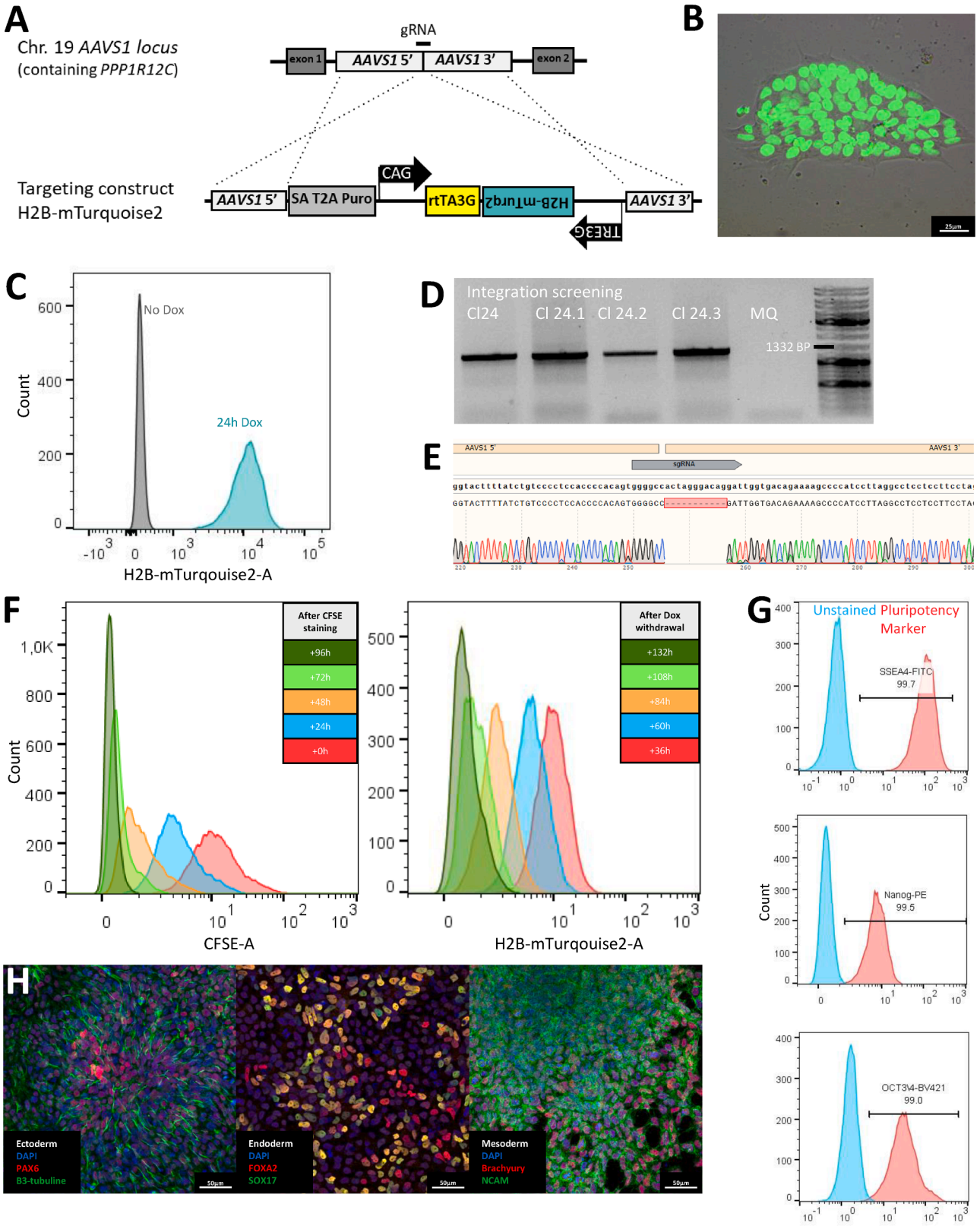

Fig. 1. Characterization and functional validation of LUMCi041-A-2.

Table 1
Characterization and validation.

Classification (optional *italicized*)	**Test**	**Result**	**Data**
Morphology	Photography	Normal	Fig. 1B
Pluripotency status evidence for the described cell line	Quantitative analysis (flow cytometry)	OCT3/4: 99.0%	Fig. 1G
	Quantitative analysis (fluorescence microscopy; flow cytometry)	Nanog:99.5% SSEA4:99.7%	Fig. 1C
		H2B-mTurquoise2 expression induced by 24 h treatment with 1 μg/ml doxycycline	
	Qualitative analysis		Fig. 1B
Karyotype	G-banding	Normal, 46XY	Supplementary Fig. 1A
	CNV analysis KaryoStat	Normal	Supplementary Fig. 1C
Genotyping for the desired genomic alteration/allelic status of the gene of interest	Genomic DNA PCR specific for homology directed insertion (3′ donor arm)	Specific Integration into one of the *AAVS1* alleles was verified by PCR screening	Fig. 1D & 1E
	Non targeted allele-specific PCR	Sanger sequencing of the non-targeted *AAVS1* allele	
Verification of the absence of random plasmid integration events	PCR/Southern *[mandatory]*	N/A	
Parental and modified cell line genetic identity evidence	STR analysis	24 markers analyzed	Results available on hPSCreg.eu
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR)	Heterozygous	See Fig. 1E
	PCR-based analyses	Detection of correctly-targeted and randomly-integrated selectable targeting construct status	See Fig. 1D
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	Not done	Not done
Off-target nuclease analysis-	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	Not done	
Specific pathogen-free status	Mycoplasma	Mycoplasma testing using Lonza's mycoplasma detection kit, negative	Supplementary Fig. 1D
Multilineage differentiation potential	Directed Trilineage differentiation analysed with immunofluorescence.	Positive immunostaining for germ layer specific markers: ectoderm (PAX6, TUBB3), endoderm (FOXA2, SOX17), mesoderm (T, NCAM)	Fig. 1H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not done	Parental line is negative
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	Not done	
	HLA tissue typing	Not done	

GFP (Addgene #44719) (210 ng), pgRNA_AAVS1-T2 (Addgene #41818) (70 ng) and pEF1_BCL-XL (50 ng) were electroporated into *PAX3-Venus* hiPSCs (Neon system 10 μl, ThermoFisher Scientific, 1000v, 50 ms, 2 pulses). After electroporation cells were seeded onto Matrigel coated plates in StemFlex with 10 μM Fasudil (LC Laboratories). Cells were selected with puromycin (0.25 μg/ml) for 5 days. Genomic DNA was isolated from surviving colonies. 5′ homologous recombination specific flanks were amplified using forward AAVS1 primer and donor specific reverse primer (AAVS1-HRrev) PCR conditions. Positive clones were purified by FACS (Aria II sorter (BD)) following 48 hrs of 1 μg/ml doxycycline (Sigma) treatment. Single cells were seeded in StemFlex containing Fasudil onto Matrigel coated 96-well plates. Surviving clones were expanded and one clone was selected for extensive characterization.

4.4. Trilineage differentiation

To determine pluripotency hiPSCs were differentiated into meso-, endo- and ectoderm using the Trilineage differentiation kit (StemCell Technologies) on Matrigel coated coverslips. Cells were fixed with 2% PFA at RT for 20 min, permabilized with 0.05% Triton-X100/1x PBS and incubated with primairy antibodies in PBS-4%NSS at 4 °C O/N. After three washes with 1x PBS, cells were incubated with secondary antibodies for 1 hr at RT (Table 2). Nuclei were stained using DAPI (1 μg/ml). Coverslips were mounted in ProLong Gold (ThermoFisher) and images were captured on a SP8 confocol microscope (Leica)(Table 1).

4.5. Expression of pluripotency markers

hiPSCs were harvested as single cells with GCDR for 8 min at 37 °C. Fixation and permabilzation was done with the Fix & Perm kit (ThermoFisher). During permeabilization cells were stained by incubating with conjugated antibodies against OCT3/4, Nanog and SSEA4 (Table 1) for 1 hr at RT . Cells were analysed using a VYB flowcytometer (Miltenyi).

4.6. Reporter validation

One day after passaging doxycycline (1 μg/ml, Sigma) was added to culture medium for 48 hrs. Cells were stained, while in culture, with Cell Trace CFSE (ThermoFisher) 36 h after removal of doxycycline. Cells were analyzed at indicated time points following dissociation with GCDR and fixation with Fix & Perm Medium A (ThermoFisher). Samples were measured on a Miltenyi VYB flowcytometer.

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency markers (flow cytometry)	anti-OCT3/4 BV421	1:25	BDbiosciences Cat#565644
	anti-Nanog PE	1:5	RRID: AB_2739320
	anti-SSEA4 FITC	1:25	BDbiosciences Cat#560483
			RRID: AB_1645522
			Miltenyi Cat#130-098-371
			RRID: AB_2653517
Differentiation markers	Rabbit anti-PAX6	1:200	Cell signalling Technology
ectoderm	Mouse anti-TUBB3	1:3000	Cat#60433
endoderm	Rabbit anti-FOXA2	1:100	RRID: AB_2797599
mesoderm	Goat anti- SOX17	1:100	Biolegend Cat#MMS-435p
	Rabbit anti-T	1:1600	RRID: AB_2313773
	Mouse anti-NCAM	1:400	Millipore Cat#07-633
			RRID: AB_390153
			R&D Cat#AF1924
			RRID: AB_355060
			Cell signalling Technology
			Cat#81694
			RRID: AB_2799983
			Cell signalling Technology
			Cat#3576
			RRID: AB_2149540
Secondary antibodies	Donkey-anti-mouse Alexa488	1:250	Invitrogen Cat#A-21202
Differentation markers	Donkey-anti-goat Alexa568	1:250	RRID: AB_141607
	Donkey anti-rabbit Alexa647	1:250	Invitrogen Cat#A-21432
			RRID: AB_141788
			Invitrogen Cat#A-31573
			RRID: AB_2536183
Other staining	DAPI	1 μg/ml	ThermoFisher Cat#D3571
	CFSE labeling	5 μM	RRID: AB_2307445
			ThermoFisher Cat#C34570
Site-specific nuclease			
Nuclease information	Nuclease type/nomenclature	spCas9	
Delivery method	Transfection	Vectors delivered using electroporation. The Neon electroporation system (ThermoFisher) was used.	
Selection/enrichment strategy	Puromycin selection and FACS	Antibiotic resistance and selection for mTurquoise2 positive cells after doxycycline treatment	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
e.g. Genotyping (desired allele/transgene presence detection)	AAVS1 - pUCM (5′ flank)	#1047: 5′-GACCTGCCTGGAGAAGGAT-3′	
	AAVS1	AAVS1DOXrev: 5′-GGTGGAGATATCAGCTTTACTAG-3’	
e.g. Targeted mutation analysis/sequencing	Sequencing data from both alleles	See Fig. 1D and 1E	
gRNA oligonucleotide/crRNA sequence		$gRNA^{T2}$: 5′-GGGGCCACTAGGGACAGGAT-3′	
Genomic target sequence(s)	Including PAM and other sequences likely to affect UCN activity	GGGGCCACTAGGGACAGGAT*TGG*	
ODNs/plasmids/RNA templates used as templates for HDR-mediated site-directed mutagenesis. Backbone modifications in utilized ODNS have to be noted using standard nomenclature.	plasmid	pUCM AAVS1-TO-H2BmTurq2	

4.7. Live cell imaging

Two days before passaging cells were treated with doxycycline (1 μg/ml, Sigma). Cells were dissociated with GCDR at 37 °C for 8 min. 1-5x10^3 cells were plated onto a Matrigel coated well of a 12-well plate in Stemflex containing ClonR(1:20, Stem Cell Technologies) and doxycycline (1 μg/ml). One day after passaging plates were transferred to the incubator of an AF6000 imaging system (Leica). Pictures were taken every 30 min for a period of 24 hrs.

4.8. Genome analysis

Karyotype was determined by standard G-banding and via KaryoStat (ThermoFisher).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: H.M.M. Mikkers reports financial support was provided by Interreg Vlaanderen-Nederland (project#0433: Biomat on microfluidic chip).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102592.

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