Stem Cell Research 56 (2021) 102547

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Single Cell Line

An INSULIN-GFP/GLUCAGON-mCherry reporter line for the study of human pancreatic endocrine cell development

Tanya Labonne [a], Andrew G. Elefanty [a,b,c], Edouard G. Stanley [a,b,c,1], Jacqueline V. Schiesser [a,b,*,1]

[a] Murdoch Children's Research Institute, The Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia
[b] Department of Paediatrics, Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne, Parkville, Victoria 3052, Australia
[c] Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria 3800, Australia

A B S T R A C T

Insulin expressing beta cells and glucagon expressing alpha cells are the two most abundant endocrine cell types of the human pancreatic islet. Both alpha and beta cells can be generated in vitro via the differentiation of pluripotent stem cells (PSCs), affording the opportunity to study their ontogeny and to examine their developmental inter-relationship. To aid these analyses, we have generated a PSC line in which insulin expression is reported by GFP and glucagon expression is reported by mCherry. This cell line enables viable isolation of cells expressing each hormone and optimisation of methods that lead to their generation.

Resource Table

Unique stem cell line identifier	ESIBIe003-A-10 https://hpscreg.eu/cell-line/ESIBIe003-A-10
Alternative name(s) of stem cell line	MCRIe004-A-10 HES3 INS-GFP GCG-mCh
Institution	Murdoch Children's Research Institute, Melbourne, Australia
Contact information of distributor	Jacqueline Schiesser; jacqui.schiesser@mcri.edu.au
Type of cell line	ESC
Origin	Human
Additional origin info	Age: n/a Sex: Female Ethnicity if known: n/a
Cell Source	hES3 (ESIBIe003-A)
Clonality	Clonal
Method of reprogramming	n/a
Genetic Modification	YES
Type of Modification	Reporter knock-in
Associated disease	n/a
Gene/locus	Glucagon, 2q24.2
Method of modification	TALEN
Name of transgene or resistance	mCherry
Inducible/constitutive system	n/a
Date archived/stock date	25/6/2019
Cell line repository/bank	N/A
Ethical approval	RCH Human Research Ethics Committee 33001A

1. Resource utility

This reporter line expresses GFP and mCherry fluorescent proteins under the control of the endogenous INSULIN (INS) and GLUCAGON (GCG) loci respectively. This facilitates the identification of endocrine cells in real time and enables isolation of the two most common hormone expressing cell types in the pancreas by flow cytometry (Table 1).

2. Resource details

We previously generated $INS^{GFP/w}$ embryonic stem cell (ESC) reporter lines (Micallef et al., 2012) that enable the isolation of putative pancreatic beta cells from differentiating ESC cultures. In order to further characterise endocrine cells generated during the differentiation process, we inserted sequences encoding a mCherry reporter gene into the GLUCAGON (GCG) locus of $INS^{GFP/w}$ hESCs, using gene targeting by homologous recombination. The targeting vector comprised two homology arms flanking the mCherry gene and an adjacent cassette conferring resistance to the antibiotic, hygromycin (Fig. 1A). Following identification of correctly targeted clones, the PGKHygroR was subsequently removed by CRE-mediated recombination, a modification confirmed by PCR analysis of sequences spanning the junctions between the targeting vector and flanking genomic sequences (Fig. 1B). The resultant cell line, here designated as $INS^{GFP/w}GCG^{mCh/w}$ hESCs, appeared morphologically normal and expressed the pluripotency

* Corresponding author: Murdoch Children's Research Institute, The Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia.
E-mail address: jacqui.schiesser@mcri.edu.au (J.V. Schiesser).
[1] Edouard G. Stanley and Jacqueline V. Schiesser contributed equally to this study.

https://doi.org/10.1016/j.scr.2021.102547
Received 2 August 2021; Received in revised form 8 September 2021; Accepted 19 September 2021
Available online 28 September 2021

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1C
Phenotype	Qualitative analysis *(Immunofluorescence)*	Positive staining for the pluripotency marker OCT4	Fig. 1C
	Quantitative analysis *(Flow Cytometry)*	Positive staining for EPCAM, CD9 and SSEA4	Fig. 1C
Genotype	Karyotype (G-banding) and resolution	SNP array (resolution 0.50 Mb)	Submitted in archive with journal
Identity	Microsatellite PCR (mPCR) OR STR analysis	Not performed	N/A
		SNPduo compartitive analysis of SNP arrays of parental and gene edited clone. Identical genotypes (>99.9%) of entire genome indicating the lines are from the same individual	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	PCR	Correct Junction fragments generated by integration dependent primer pairs	Fig. 1B
Microbiology and virology	Mycoplasma	Negative for Mycoplasma as determined by PCR	Submitted in archive with journal
Differentiation potential	Directed differentiation to derivatives of ectoderm, mesoderm and endoderm	Differentiation to ectoderm marked by Beta-III Tubulin, mesoderm marked by CD34 and CD43, and endoderm marked by INSULIN and GLUCAGON	Fig. 1D (ectoderm), E (mesoderm) and F (endoderm)
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

marker OCT4 by immunofluorescence, as well as the stem cell markers CD9, EPCAM and SSEA-4 by flow cytometry (Fig. 1C). DNA fingerprinting by STR analysis confirmed the relationship between the GCG-mCherry-targeted line to the parental INS-GFP line (submitted in archive with journal). The $INS^{GFP/w}GCG^{mCh/w}$ hESC line had a normal molecular karyotype as determined by SNP analysis and tested negative for *Mycoplasma*.

We next tested whether $INS^{GFP/w}GCG^{mCh/w}$ hESCs retained the potential to differentiate into derivatives of the three primary germ layers, ectoderm, mesoderm and endoderm. $INS^{GFP/w}GCG^{mCh/w}$ hESCs differentiated toward the neural lineage, a derivative of ectoderm, formed cells that expressed the neural marker beta III-tubulin (Fig. 1D). To examine their capacity to form mesoderm lineages, $INS^{GFP/w}GCG^{mCh/w}$ hESCs were subjected to a differentiation protocol that generates endothelial and haematopoietic cells. Under these conditions, early mesoderm cells were identified by the expression of CD13 at differentiation day 4 whilst day 8 cultures contained CD34 + endothelial cells and a small fraction of CD43 + blood cells (Fig. 1E).

The ability of $INS^{GFP/w}GCG^{mCh/w}$ hESCs to form endoderm was examined by differentiating them toward pancreatic endocrine lineages, marked by the expression of INSULIN-GFP and GLUCAGON-mCherry. Consistent with previously observations using the parental $INS^{GFP/w}$ hESC line (Micallef et al., 2012), $INS^{GFP/w}GCG^{mCh/w}$ hESCs generated GFP+ (INS+) cells by differentiation day 28. Confocal microscopy and flow cytometry analysis revealed the presence of cells expressing either hormone alone or both INS and GCG (Fig. 1F, G). By differentiation day 38, most fluorescent cells expressed either GCG or INS but not both. At this stage, intracellular flow cytometry analysis was used to show that GCG expression was exclusively associated with mCherry + cells, confirming the fidelity of the reporter gene (Fig. 1H). When combined with the results of previous studies, these data indicate that $INS^{GFP/w}GCG^{mCh/w}$ hESCs enable the real time observation of cells expressing INS and GCG. As such, the $INS^{GFP/w}GCG^{mCh/w}$ hESCs dual reporter line will facilitate research aimed at optimising endocrine differentiation protocols and for studying the developmental relationship between beta cells and alpha cells.

3. Materials and methods

3.1. Generation of targeted GCG-$^{mCherry/w}$ hESCs

The GLUCAGON-mCherry targeting construct comprised a 2 kb 5′ homology arm, mCherry coding sequences, a loxP flanked phosphoglycerol kinase (PGK)-promoter-hygromycin resistance cassette and a 2.9 kb 3′ homology arm. $INSULIN^{GFP/w}$ HES3 ESCs (Micallef et al., 2012) were electroporated with GLUCAGON specific TALENs and the targeting construct as described previously (Kao et al., 2016). Hygromycin resistant clones were screened for the presence of gene targeting events using primer P1, corresponding to genomic sequences 5′ of the 5′ end of the targeting vector, in conjunction with P2, a reverse primer located in the mCherry coding sequence. Correctly targeted clones were expanded and the PGK-hygromycin resistance cassette removed as previously described (Micallef et al., 2012), verified by PCR using primers P3 and P4. Single-cell cloning was performed using single-cell deposition as previously described (Micallef et al., 2012). A single clone, designated $INS^{GFP/w}GCG^{mCh/w}$, was used for subsequent analyses.

3.2. hESC culture and in vitro differentiation

Cells were cultured and passaged enzymatically essentially as described previously (Costa et al., 2007). To assess ectodermal differentiation potential, hESCs were differentiated as described (Goulburn et al., 2011) and neural progenitors identified using immunofluorescence analysis. For mesoderm differentiation, hESCs were differentiated to CD34 + cells according to Ng et al. (2008) and analysed using flow cytometry. Endodermal differentiations were performed as described by Micallef et al. (2012).

3.3. Extracellular and intracellular staining by flow cytometry

Cells were dissociated using TrypLE™ select (ThermoFisher) and analysed using a LSR Fortessa (Becton Dickinson, Bioscience). Expression of extracellular markers was evaluated in cells that were incubated with conjugated antibodies (Table 2) diluted in PBS containing 2% FCS for 20 min on ice. Dead cells were identified by propidium iodide (Sigma) staining. Expression of intracellular markers was evaluated in cells following fixation and permeablisation in Cytofix/Cytoperm (Becton Dickinson, Bioscience) as specified by the manufacturer.

3.4. Live cell sorting

Single cell cloning was performed by dissociating cultures using TrypLE™ select (ThermoFisher) and single cells deposited into individuals wells of a 96 well plate using a BD Aria (Becton Dickinson, Bioscience).

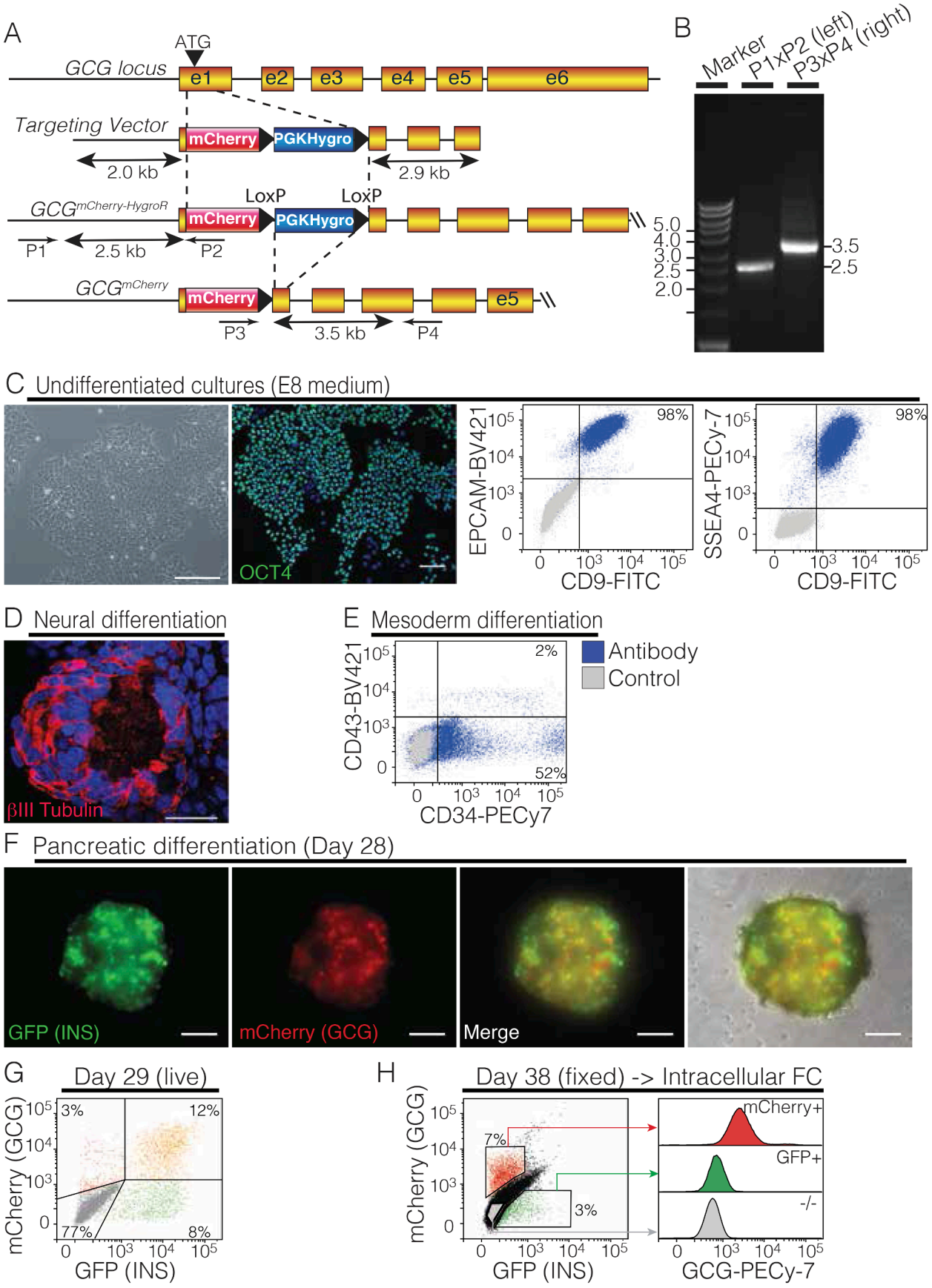

Fig. 1. Characterization and validation of an INSULIN$^{GFP/w}$ GLUCAGON$^{mCh/w}$ human embryonic stem cell line.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Marker	Rabbit anti-OCT4	1:200	Cell Signaling Technology Cat# 2840, RRID:AB_2167691
Pluripotency Marker	FITC Mouse anti-human CD9	1:10	BD Biosciences Cat#555371, RRID: AB_395773
Pluripotency Marker	Brilliant Violet 421 Mouse anti-human CD326 (EpCAM)	1:50	Biolegend Cat#324220, RRID:AB_2563847
Pluripotency Marker	PE/Cyanine 7 Mouse anti-human SSEA-4	1:50	Biolegend Cat#330420, RRID:AB_2629631
Differentiation Marker	PE/Cyanine 7 Mouse anti-human CD34	1:100	Biolegend Cat#343516, RRID:AB_187725
Differentiation Marker	Brilliant Violet Mouse anti-human CD43	1:25	BD Biosciences Cat#562916, RRID: AB_2737890
Differentiation Marker	Mouse anti-Beta III Tubulin	1:200	Promega Cat#G712A, RRID:AB_430874
Differentiation Marker	Mouse anti-human Glucagon	1:100	Sigma-Aldrich Cat#G2654, RRID: AB_259852
Secondary Antibody	Alexa Fluor 594 Donkey anti-mouse IgG (H + L)	1:1000	Invitrogen Cat#A-21203, RRID: AB_141633
Secondary Antibody	Alexa Fluor 647 Donkey anti-mouse IgG (H + L)	1:1000	Invitrogen Cat#A-21244, RRID: AB_2535812
Secondary Antibody	PE/Cyanine 7 Goat anti-mouse IgG	1:100	Biolegend Cat#405315, RRID:AB_10662421

Primers

	Target	**Forward/Reverse primer (5′-3′)**
Screening primer P1	GLUCAGON	ggttttctttggatgcatctgagt
Screening primer P2	mCherry	tcgccctcgatctcgaactcgtg
Screening primer P3	mCherry	cagtctcaatggatacaggctatc
Screening primer P4	GLUCAGON	catcgtggaacagtacgaacgcg

3.5. Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature. Following this, cells were placed in blocking buffer (10% FCS + 0.1% Triton-X in 1x PBS) for 1 h at room temperature. Primary antibodies were diluted in blocking buffer and incubated overnight at 4 °C. Secondary antibodies were diluted in blocking buffer and incubated at room temperature for 1 h (antibodies listed in Table 2). Nuclei were stained with DAPI at 1 μg/ml (Sigma-Aldrich) and cells visualised using a LSM 780 confocal microscope running Zen Black software (Carl-Zeiss). Image analysis was performed using ImageJ software.

3.6. SNP analysis

Karyotypes were analysed using Infinium CoreExome-24 SNP arrays. Data was compared to the human reference sequence hg19/CRCh37 (Feb 2009). The parental hES3 INSULIN-$^{GFP/w}$ was analysed using the SNP array and this data compared to the corresponding HES3 INSULIN-GFP/w GLUCAGON-mCh/w line using SNPduo comparative analysis (http://pevsnerlab.kennedykrieger.org/SNPduo). No differences were detected between the parental single-knock in and the derivative double knock-in line.

3.7. Mycoplasma screening

hESCs were tested for Mycoplasma by PCR by the commercial service provider, Cerberus Sciences (Adelaide, Australia).

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.

Acknowledgements

This study was funded in part by Australian National Health & Medical Research Council research fellowships awarded to A.G.E. (GNT1117596) and E.G.S (GNT1079004) and a project grant awarded to A.G.E and E.G.S (GNT1062268) Additional infrastructure funding to the Murdoch Children's Research Institute was provided by the Australian Government National Health and Medical Research Council Independent Research Institute Infrastructure Support Scheme and the Victorian Government's Operational Infrastructure Support Program.

References

Costa, M., Sourris, K., Hatzistavrou, T., Elefanty, A.G., Stanley, E.G., 2008. Expansion of human embryonic stem cells in vitro. Curr. Protocols Stem Cell Biol. Chapter 1 Unit 1C.1.1-1C.1.7.

Goulburn, A.L., Alden, D., Davis, R.P., Micallef, S.J., Ng, E.S., Yu, Q.C., Lim, S.M., Soh, C.-L., Elliott, D.A., Hatzistavrou, T., Bourke, J., Watmuff, B., Lang, R.J., Haynes, J.M., Pouton, C.W., Giudice, A., Trounson, A.O., Anderson, S.A., Stanley, E. G., Elefanty, A.G., 2011. A targeted NKX2.1 human embryonic stem cell reporter line enables identification of human basal forebrain derivatives. Stem Cells 29, 462–473.

Kao, T., Labonne, T., Niclis, J., Chaurasia, R., Lokmic, Z., Qian, E., Bruveris, F., Howden, S., Motazedian, A., Schiesser, J., Costa, M., Sourris, K., Ng, E., Anderson, D., Giudice, A., Farlie, P., Cheung, M., Lamande, S., Penington, A., Parish, C., Thomson, L., Rafii, A., Elliott, D., Elefanty, A., Stanley, E., 2016. GAPTrap: a simple expression system for pluripotent stem cells and their derivatives. Stem Cell Rep. 7, 518–526.

Micallef, S.J., Li, X., Schiesser, J.V., Hirst, C.E., Yu, Q.C., Lim, S.M., Nostro, M.C., Elliott, D.A., Sarangi, F., Harrison, L.C., Keller, G., Elefanty, A.G., Stanley, E.G., 2012. INS(GFP/w) human embryonic stem cells facilitate isolation of in vitro derived insulin-producing cells. Diabetologia 55 (3), 694–706.

Ng, E.S., Davis, R., Stanley, E.G., Elefanty, A.G., 2008. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nature Protocols 3, 768–776.