Stem Cell Research 49 (2020) 102016

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab resource: Stem Cell Line

A CRISPR-strategy for the generation of a detectable fluorescent hESC reporter line (WAe009-A-37) for the subpallial determinant GSX2

Dario Besusso, Andrea Cossu, Ayat Mohamed, Manuel Cernigoj, Paolo Codega, Maura Galimberti, Ilaria Campus, Paola Conforti, Elena Cattaneo*

Department of Biosciences, University of Milan, Milan 20122, Italy
Istituto Nazionale di Genetica Molecolare "Romeo ed Enrica Invernizzi", Milan 20133, Italy

A B S T R A C T

GSX2 is a homeobox transcription factor (TF) controlling the specification of the ventral lateral ganglionic eminence and its major derivative, the corpus striatum. Medium spiny neurons (MSNs) represent the largest cell component of the striatum and they are primarily affected in Huntington disease (HD). Here, we used CRISPR technology to generate a pluripotent GSX2-reporter human embryonic stem cell (hESC) line that can be leveraged to monitor striatal differentiation in real-time and to enrich for MSN-committed progenitors.

1. Resource Table:

Unique stem cell line identifier	WAe009-A-37
Alternative name(s) of stem cell line	H9-GSX2-tTA:GFP
Institution	University of Milan and INGM
Contact information of distributor	Dario Besusso, dario.besusso@unimi.it
Type of cell line	ESC
Origin	human
Additional origin info	*Age: N/A* Sex: female *Ethnicity if known: N/A*
Cell Source	H9 WA-09 hESC line
Method of reprogramming	N/A
Associated disease	N/A
Gene/locus	GSX2 (GS homeobox 2)/ 4q12
Method of modification	CRISPR/Cas9 and lentivirus
Gene correction	YES
Name of transgene or resistance	tTA-Advanced and EGFP
Inducible/constitutive system	TRE-EGFP (tetO-GFP)
Date archived/stock date	10/07/2010
Cell line repository/bank	N/A
Ethical approval	N/A

2. Resource utility

MSNs can be generated *in vitro* starting from hESCs with an efficiency currently not greater than 40% (Arber et al., 2015; Carri et al., 2013). In this context, a GSX2 reporter line could be leveraged to monitor and isolate MSN-committed progenitors to generate highly-pure MSNs culture for cell replacement therapy.

3. Resource details

HD is a neurodegenerative disorder caused by the loss of striatal medium spiny projection neurons (MSNs) of the corpus striatum (Bates et al., 2015). Cell replacement using hESC-derivatives represent a promising disease-modifying therapy, but current *in vitro* protocols are limited by poor efficiency. In this context, GSX2 expression could be leveraged to isolate subpallial-committed progenitors through the generation of a fluorescent reporter cell line. To this aim, we used Cas9-assisted targeting to modify the endogenous GSX2 locus of the hESC line RC17 by replacing the GSX2 stop codon with the sequence coding for the self-cleaving peptide GT2A followed by the fluorescent protein turboGFP (tGFP), to avoid protein fusion (Fig. 1A). First, the best performing gRNA was selected through a surveyor assay (Fig. 1B) and then gRNA3 (Fig. 1D) was used in the form of synthetic RNA in complex with recombinant w.t. Cas9 to achieve GSX2 specific targeting (RC17 GSX2-tGFP). After nucleofection and antibiotic selection, the surviving clones were screened using PCR primer sets spanning the insertion junctions (Fig. 1A, Table 2). For all positive clones, locus specific recombination was observed in only one of the two alleles (heterozygous). Since, GSX2 is a silent gene in self-renewal state and its expression can only be initiated with 20–30 days of *in vitro* differentiation, we designed a set of gRNAs for dCas9-mediated transcriptional activation of the endogenous locus (dCas9-VPR). After selecting optimal gRNAs for GSX2 activation (Fig. 1C), the RC17 GSX2-tGFP clone was nucleofected with dCas9-VPR together with selected gRNA plasmids to trigger GSX2 transcription and cells analysed by flow cytometry after 48 hrs. This confirmed GSX2-specific tGFP expression, therefore functional targeting of the cells (Fig. 1E). To asses GT2A cleavage efficiency, we performed western blot

* Corresponding author.

https://doi.org/10.1016/j.scr.2020.102016
Received 6 August 2020; Received in revised form 9 September 2020; Accepted 26 September 2020
Available online 01 October 2020

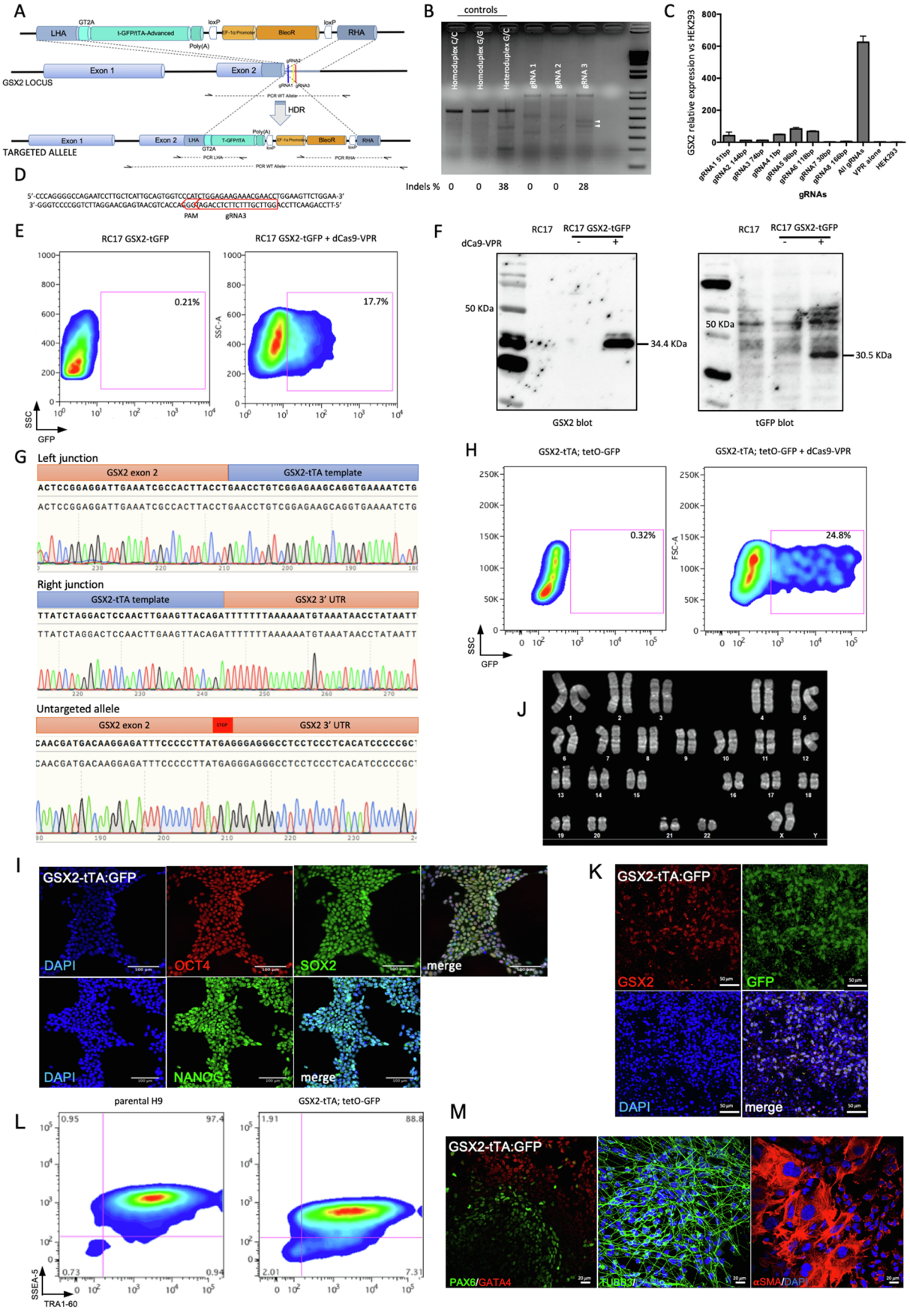

Fig. 1. Generation and characterisation of a detectable fluorescent hESC GSX2 reporter line.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Not shown but available with author
Phenotype	Immunocytochemisty	Assess staining/expression of pluripotency markers: Oct4, Nanog, Sox2	Fig. 1 panel I
	Flow cytometry	Assess pluripotency marker frequency: Tra 1–60: 96.1% SSEA-5: 90.7%	Fig. 1 panel L
Genotype	g-Banding	Normal 46, XX	Fig. 1 panel J
Identity	Microsatellite PCR (mPCR)	DNA Profiling not performed	N/A
	STR analysis	16 sites tested.	Not shown but available with author
Mutation analysis (IF APPLICABLE)	Sequencing	heterozygous type of mutation	Fig. 1 panel G
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	Negative	Fig. 1/supplementary
Differentiation potential	e.g. Embryoid body formation OR Teratoma formation OR Scorecard OR Directed differentiation	POU5F1 (OCT-4) expressed NANOG expressed SOX2 expressed TRA 1–60 expressed SSEA-5 expressed Differentiation potency: Endoderm: Expression of GATA4 by In vitro spontaneous differentiation. Ectoderm: Expression of TUBB3 and PAX6 by In vitro spontaneous differentiation. Mesoderm: Expression of ACTA2 (alphaSMA) by In vitro spontaneous differentiation.	Fig. 1 panel M
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	HIV 1: not tested HIV 2: not tested Hepatitis B: not tested Hepatitis C: not tested	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	not tested	N/A
	HLA tissue typing	not tested	N/A

analysis after GSX2 induction and found no evidence of protein fusion, but the presence of only the expected 30 (left panel) and 34 (right panel) KDa bands for GSX2 and tGFP, respectively (Fig. 1F). Despite correct targeting, when positive clones were subject to *in vitro* striatal differentiation, no reporter signal was detected by flow cytometry in the expected time window, even though GSX2 and tGFP were detectable by qPCR (data not shown). Since, many TFs are characterized by low expression levels (Bao et al., 2019), we decided to adopt a molecular strategy that enables the amplification of the downstream signal. To this aim, we replaced the tGFP sequence of the targeting construct with the improved tetracycline-controlled transactivator (tTA-Advanced) and repeated the editing in the hESC line H9. The best positive clone (H9 GSX2-tTA) was selected by PCR/Sanger screening of targeted and w.t. alleles (Fig. 1G), infected with a lentivirus carrying a tetracycline responsive element followed by GFP (tetO-GFP), further subcloned and validated using the dCas9-VPR approach (H9 GSX2-tTA; tetO-GFP, Fig. 1H). This new clone was tested for mycoplasma contamination (Table 1), normal karyotype (Fig. 1J), expression of pluripotency markers (Fig. 1I and 1L), potency (Fig. 1M) and then was subjected to striatal *in vitro* differentiation to validate reporter expression after 27 days. Although, overall proportion of GFP expressing cells resulted lower than expected suggesting sporadic silencing, positive cells showed expected overlap between GFP and GSX2 by ICC (Fig. 1K), confirming the validity of the adopted strategy for the generation of detectable fluorescent reporter of weakly expressed TFs.

4. Materials and methods

4.1. Cell culture

The hESC RC17 p20 (Roslin CT) were maintained in StemPro medium (ThermoFisher) while H9 p37 (WiCell) were cultured using mTESR (StemCell Technology) and both passaged once a week using EDTA dissociation.

4.2. Immunocytochemistry

Cells were fixed in 4% (vol/vol) PFA for 15 min at RT, blocked and permeabilized using 5% normal goat serum (NGS; Vector), with 0.1% Triton X-100 in PBS for 1 h at room temperature. After washing, cells were incubated overnight at 4 °C with primary antibodies (see Table 2). Next, Alexa Fluor secondary antibodies (ThermoFisher scientific) were used 1:500 in PBS for 1 h RT, followed by 3X washes with PBST and 5 min incubation at RT with 1:10000 of DAPI (ThermoFisher scientific) in PBS.

4.3. GSX2 editing

pUC57-GSX2-tTA-BleoR donor plasmid was synthesized by Genscript and sgRNAs were designed and tested by Surveyor Assay

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers (IC)	*Mouse anti-OCT4*	*1:500*	*Santa Cruz Biotechnology, INC. Cat# sc-5279, RRID:AB_628051*
Pluripotency Markers (IC)	*Rabbit anti-Sox2*	*1:500*	*Millipore, Cat# AB5603, RRID:AB_2286686*
Pluripotency Markers (IC)	*Rabbit anti-Nanog*	*1:500*	*Abcam, Cat# ab21624, RRID:AB_446437*
Differentiation Marker	*Mouse anti-alpha SMA*	*1:500*	*Sigma-Aldrich, Cat# A2547, RRID:AB_476701*
Differentiation Marker	*Mouse anti-GATA 4*	*1:200*	*Santa Cruz Biotechnology, Cat# sc-25310, RRID:AB_627667*
Differentiation Marker	*Rabbit anti-PAX6*	*1:2500*	*BioLegend, Cat# 901302, RRID:AB_2749901*
Differentiation Marker	*Rabbit anti-beta III Tubulin (TUBB3)*	*1:1000*	*BioLegend, Cat# 802001, RRID:AB_2564645*
Secondary antibodies	*Alexa Fluor® 488 goat anti-rabbit IgG*	*1:500*	*Invitrogen, Cat# A11008, RRID:AB_143165*
Secondary antibodies	*Alexa Fluor® 568 goat anti-mouse IgG*	*1:500*	*Invitrogen, Cat# A11004, RRID:AB_2534072*
Pluripotency Markers (FACS)	*Anti-TRA-1-60-PE*	*1:11*	Miltenyi Biotec Cat# 130-100-347 *RRID:AB_2654227*
Pluripotency Markers (FACS)	*Anti-SSEA-5-VioBlue*	*1:11*	Miltenyi Biotec Cat# 130-106-657 *RRID:AB_2653536*
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Left Homology Arm-Template junction (PCR)	*GSX2-tTA edited locus*	*FW: 5′-*TGTCTCGACTCCGGAGGATT*-3′* *RV: 5′-*ACGTCCCCGCATGTTAGAAG*-3′*	
Right Homology Arm-Template junction (PCR)	*GSX2-tTA edited locus*	*FW: 5′-*GACGCCCGCCCTTAATATAACT*-3′* *RV: 5′-*TGAAAGAGAGTAGGGATGGGGA*-3′*	
W.T. Allele/Untargeted Allele (PCR)	*GSX2*	*FW: 5′-TGTCTCGACTCCGGAGGATT-3′* *RV: 5′-ACCAACCCTGTTTTTCCTCTGTT-3′*	
Sequencing WT Allele	GSX2	*5′-TGTCTCGACTCCGGAGGATT-3′*	
Sequencing left Homology Arm (LHA)	Targeted GSX2	*5′-*TGTCTCGACTCCGGAGGATT*-3′*	
Sequencing right Homology Arm (RHA)	Targeted GSX2	*5′-*TGAAAGAGAGTAGGGATGGGGA*-3′*	

using T7 endonuclease I (NEB). 2×10^6 cells were nucleofected with 1 μg of donor plasmid and 20 pmol of equimolar w.t. Cas9 and sgRNA RNP complex using Nucleofector kit (Lonza). After nucleofection, cells were plated in mTeSR (StemCell Technology) with 1:1000 of 10 μM RI and 1 μM of SCR7 for 24 h. Positive clones were selected using 100 μg/mL of Zeocin 3 days post nucleofection. Single clones were manually picked and expanded. Genomic DNA of each clones were extracted using NucleoSpin Tissue kit (Macherey Nagel). PCRs for genomic DNA were performed using Q5® High-Fidelity DNA Polymerase (NEB). Positive clones were then plated in low confluency in a 6-well plate and infected using 1 μl of lentivirus (1.9×10^4 TU/μl) carrying the sequence TRE-EGFP with 0.8 μg/ml Polybrene.

4.4. dCas9-VPR induction

GSX2 expression was induced in hESC through dCas9-VPR (Addgene plasmid #63798). 2×10^6 cells were nucleofected with 2 μg of dCas9-VPR plasmid and 4 plasmids carrying gRNA (each at 200 ng). Cells were dissociated with Accutase with 10 μM Rock kinase Inhibitor (RI), electroporated using Nucleofector kit (Lonza) and plated with mTSeR with RI in a 60 mm dish coated with Cultrex (Trevigen). Expression was evaluated 48hrs after nucleofection.

4.5. Striatal differentiation protocol

Cells were differentiated into striatal projection medium spiny neuron (MSNs) using a protocol previously described by Delli Carri et al. (2013). Briefly 6×10^5 cell were plated in each well of 6-well plate 2 days before neuronal induction. At day 0, the medium was replaced with DMEM/F12 plus N2 (1X), B27 (without Retinoic Acid 0.5X, Life Technology), with 10 μM SB431542 and 500 nM LDN. At day 3, cells were split 1:2 and at day 5, 200 ng/ml SHH and 100 ng/ml DKK-1 (Preprotech) were added to the medium. At day 11, cells were detached and re-plated in 1 drop of medium containing $4x10^5$ cells in a 12-well plate. From day 11, the medium was composed by DMEM/F12 plus N2, B27, 200 ng/ml SHH, 100 ng/ml DKK-1 and 30 ng/ml BDNF until day 26. From day 27 to day 50, the medium was replaced with DMEM/F12 plus N2, B27 and 30 ng/ml BDNF.

4.6. Spontaneous in vitro differentiation

For spontaneous *in vitro* differentiation, embryoid bodies (EBs) were formed using 10^3 cells/well in a V-bottom 96 wells plate (STAR LAB) with 150 μl/well of mTESR/RI and centrifugated at 800rmp for 1′ at RT. Three days later, the medium was changed with DMEM/F12 (Gibco) with Penicillin (100 U/ml) and Streptomycin (100 μg/ml) (Euroclone), L-Glutamine (2 mM, Gibco), 20% KSR, (Gibco), NEAA (1:100, Gibco), 2-Mercaptoethanol (100 μM, Gibco). A week later, EBs were allowed to attach onto Matrigel-coated 24wells optical plates and left to spontaneously differentiate for further 7 days.

4.7. Karyotype

The karyotyping by g-banding analysis were performed by ISENET Biobanking service unit in Milan, Italy (www.isenetbiobanking.com).

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 work was conducted under the sponsorship of CHDI Foundation (United States, grant ID A-558 11103) and was also supported by the European Union funded projects NeurostemcellRepair (European Union, FP7, 559 GA no. 602278, 2013-17) and NSC-Reconstruct (European Union, H2020, GA no 874758, 2020-23).

References

Arber, C., Precious, S.V., Cambray, S., Risner-Janiczek, J.R., Kelly, C., Noakes, Z., Fjodorova, M., Heuer, A., Ungless, M.A., Rodriguez, T.A., Rosser, A.E., Dunnett, S.B., Li, M., 2015. Activin A directs striatal projection neuron differentiation of human pluripotent stem cells. Development 142 (7), 1375–1386.

Bao, X., Adil, M.M., Muckom, R., Zimmermann, J.A., Tran, A., Suhy, N., Xu, Y., Sampayo, R.G., Clark, D.S., Schaffer, D.V., 2019. Gene editing to generate versatile human

pluripotent stem cell reporter lines for analysis of differentiation and lineage tracing: tracing cell lineages in hPSCs with cre reporter. Stem Cells 37 (12), 1556–1566.

Bates, G.P., Dorsey, R., Gusella, J.F., Hayden, M.R., Kay, C., Leavitt, B.R., Nance, M., Ross, C.A., Scahill, R.I., Wetzel, R., Wild, E.J., Tabrizi, S.J., 2015. Huntington disease. Nat Rev Dis Primers 1 (1). https://doi.org/10.1038/nrdp.2015.5.

Carri, A.D., Onorati, M., Lelos, M.J., Castiglioni, V., Faedo, A., Menon, R., Camnasio, S., Vuono, R., Spaiardi, P., Talpo, F., Toselli, M., Martino, G., Barker, R.A., Dunnett, S.B., Biella, G., Cattaneo, E., 2013. Developmentally coordinated extrinsic signals drive human pluripotent stem cell differentiation toward authentic DARPP-32^{+} medium-sized spiny neurons. Development 140 (2), 301–312.

Delli Carri, A., Onorati, M., Castiglioni, V., Faedo, A., Camnasio, S., Toselli, M., Biella, G., Cattaneo, E., 2013. Human Pluripotent Stem Cell Differentiation into Authentic Striatal Projection Neurons. Stem Cell Rev and Rep 9 (4), 461–474. https://doi.org/10.1007/s12015-013-9441-8.