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Lab resource: Stem Cell Line

Establishing a human embryonic stem cell clone with a heterozygous mutation in the DGCR8 gene

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A B S T R A C T

DiGeorge Syndrome (DGS) Critical Region 8 (DGCR8) is a primary candidate gene in they DGS. The DGCR8 microprocessor complex subunit is an essential cofactor in the canonical miRNA biogenesis which is involved in diverse cellular functions such as cell fate decisions, apoptosis and different signaling pathways. However, the role of DGCR8 in these processes or development of DGS is not fully understood. Here we present a heterozygous DGCR8 mutant human embryonic stem cell line (HuES9$^{DGCR8+/-}$) created by the CRISPR/Cas9 system. The generated HuES9$^{DGCR8+/-}$ cells maintain normal karyotype, morphology, pluripotency and differentiation capacity into all three germ layers.

Resource Table

Unique stem cell line identifier	*HVRDe009-A-1*
Alternative name(s) of stem cell line	*HuES9$^{DGCR8+/-}$*
Institution	*Research Centre for Natural Sciences, Eötvös Loránd Research Network*
Contact information of distributor	*Ágota Apáti, apati.agota@ttk.hu*
Type of cell line	*ESC*
Origin	*Human*
Additional origin info	*Sex: female*
Cell Source	*Blastocyst*
Clonality	*Single cell clone*
Method of reprogramming	*NA*
Genetic Modification	*YES*
Type of Modification	*Insertional mutagenesis, heterozygous*
Associated disease	*DiGeorge Syndrome*
Gene/locus	*DGCR8*
Method of modification	*CRISPR/Cas9*
Name of transgene or resistance	*GFP and Puromycin*
Inducible/constitutive system	*NA*
Date archived/stock date	*28.05.2020.*
Cell line repository/bank	*NA*

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(continued)

Unique stem cell line identifier	*HVRDe009-A-1*
Ethical approval	*HuES9 NIH Approval number: NIHhESC-09-0022 and Health Care Research Council, Human Reproduction Committee in Hungary (in Hungarian: Egészségügyi Tudományos Tanács, Humán Reprodukciós Bizottság (ETT HRB) Approval number: 6681/2012-EHR*

Resource utility

The generated HuES9$^{DGCR8+/-}$ cell line may help the better understanding of the molecular mechanisms underlying the complex symptoms of the DGS. Furthermore, this in vitro model system can be used for examinations of miRNA processing, pharmacological testing and drug screening.

Resource details

The DG syndrome is the most common micro-deletion syndrome associated with a broad range of developmental features affecting the cardiovascular, nervous, and immune systems. These abnormalities are caused by heterozygous deletions of chromosome 22q11.2 affecting about 40–50 protein-coding genes and about 40 non-protein coding genes. The exact cellular and biological phenotype of this chromosomal disease is difficult to understand due to the complex genetic background. The primary candidate gene in the deleted region is the DG syndrome critical region gene 8 (DGCR8), which is the essential cofactor

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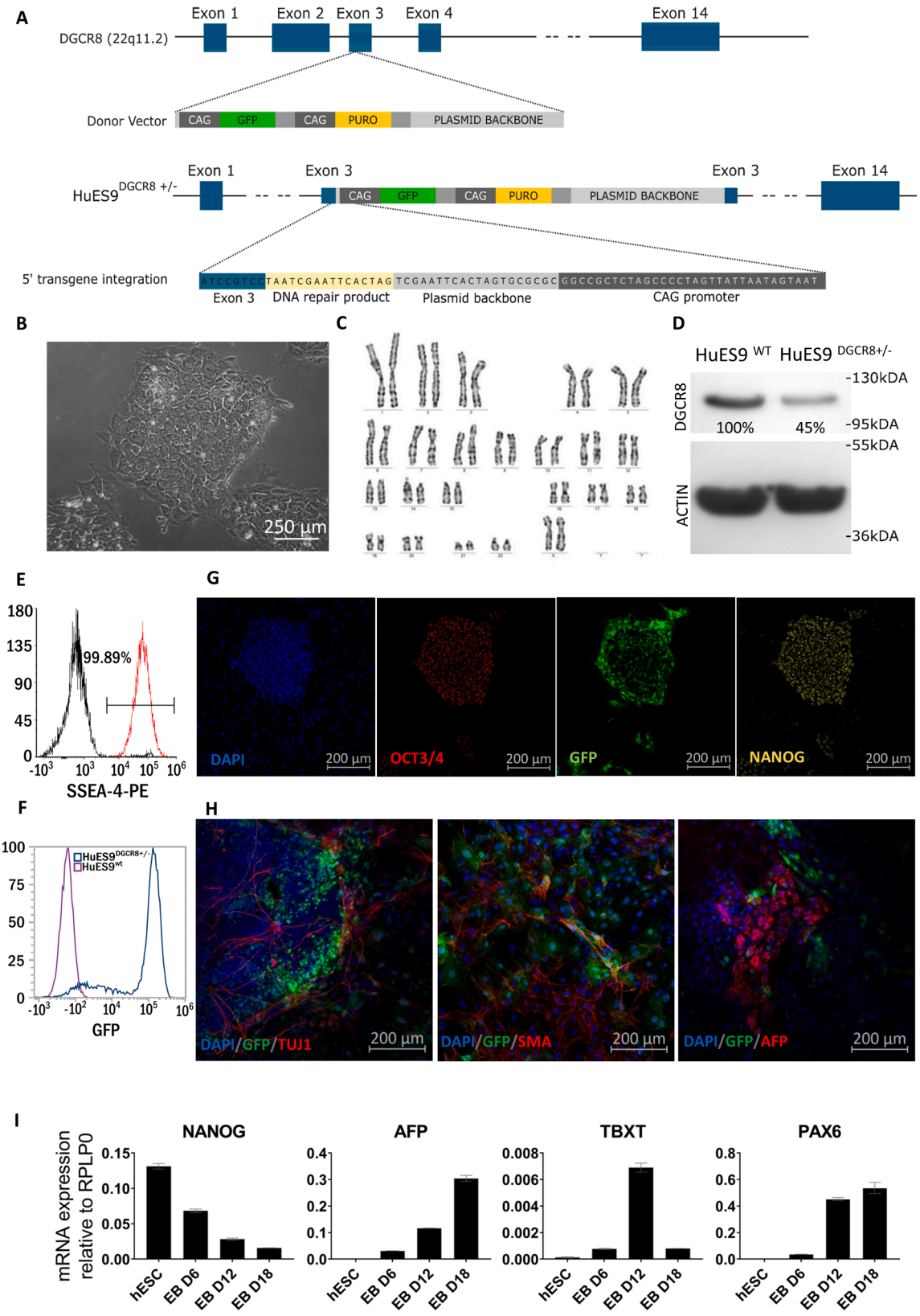

Fig. 1. Generation and characterization of a DGCR8 mutant human embryonic stem cell line HVRDe009-A-1.

for Drosha in primary miRNA processing (Tomari and Zamore, 2005). We aim to study the effect of DGCR8 on cell differentiation and the function of mature cell types, to understand its role in the disease. For this purpose, we generated a heterozygous mutant human embryonic stem cell line by a CRISPR/Cas9-based knock-out/knock-in method (Fig. 1A). Unique sequences in Exon 3, which were applied to knock out DGCR8 expression in mouse embryonic stem cells (Yeom et al., 2006.), were used to design an appropriate sgRNA for targeting DGCR8

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	normal	Fig. 1 panel B
Phenotype	Immunocytochemistry	OCT3/4, NANOG positive	Fig. 1 panel G
	RT-qPCR	NANOG positive	Fig. 1 panel I
	Flow cytometry	SSEA-4: <99%	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	46XX, Resolution 450–500 bands per haploid chromosome set	Fig. 1 panel C
Identity		DNA Profiling Performed	Supplementary file panel F
	STR analysis	17 sites tested, all matching between HuES9^{wt} and HuES9$^{DGCR8+/-}$	
Mutation analysis (IF APPLICABLE)	Sanger sequencing	Heterozygous mutation, off-target validation	Fig. 1 panel A and Supplementary file panel B, G
	Copy number determination	One insertion in genome	Supplementary file panel C
Microbiology and virology	Mycoplasma	Mycoplasma testing /Negative	Supplementary file panel D
Differentiation potential	Embryoid body formation	Immunocytochemistry: AFP, SMA and B-III-TUBULIN mRNA expression: AFP, BRY and PAX6	Fig. 1 panels H and I
Donor screening (OPTIONAL)	NA		
Genotype additional info (OPTIONAL)	NA		

(Supplementary file A). The synthesized oligo was cloned into a px330 vector (Addgene #42230), carrying the Cas9 nuclease, meanwhile, a specific, self-cleaving NHEJ donor vector (Tálas et al., 2017) containing CAG promoter-driven GFP and Puromycin resistance genes (Kolacsek et al., 2011) was constructed to disrupt DGCR8 expression. HuES9 cells were electroporated with the 2 plasmids, and after puromycin selection, GFP expressing single-cell clones were established and expanded in puromycin containing mTeSR medium on Matrigel coated plates. Clones were genotyped using the inverse PCR method and one out of 20 single-cell clones showed the targeted integration in a heterozygous manner. Sanger sequencing of the integrated transgene showed a 16 bp long NHEJ repair product upstream of the donor DNA (Fig. 1A and Supplementary file B). To exclude possible off-target insertions, predicted sites were sequenced, moreover, one copy of GFP was demonstrated by quantitative real-time PCR (Supplementary file G and C) (Kolacsek et al., 2011) providing uniform GFP expression (Fig. 1F). The cells maintained stem cell-like morphology and normal karyotype after transfection, antibiotic selection, and single-cell cloning (Fig. 1B and C). The cell line was also free of mycoplasma (Supplementary file D). Western blot analysis of DGCR8 in the HuES9$^{DGCR8+/-}$ hESCs showed decreased DGCR8 expression compared to the wild-type HuES9 cells (Fig. 1D and Supplementary file E). HuES9 $^{DGCR8+/-}$ cells constantly expressed pluripotency markers OCT4 and NANOG (Fig. 1G and I left panel). Moreover, flow cytometry analysis showed over 99% expression of SSEA4 (Fig. 1E). We further investigated the differentiation capacity of the HuES9$^{DGCR8+/-}$ cells by in vitro embryoid body (EB) formation. Immunostaining and real-time qPCR analysis confirmed continuous decline of pluripotency marker NANOG and the trilineage differentiation potential of HuES9$^{DGCR8+/-}$ by the expression of ectoderm (TUJ1, PAX6) mesoderm (TBXT, SMA) and endoderm (AFP) markers (Fig. 1H and I). Short tandem repeat (STR) analysis confirmed the identity of HuES9$^{DGCR8+/-}$ cells (Supplementary file F). Here we describe a single cell derived, heterozygous DGCR8 knock-out/ GFP knock-in, human embryonic stem cell line (Table 1 and 2).

1. Materials and methods

1.1. Cell culturing

HuES9 and the generated HuES9$^{DGCR8+/-}$ cell lines were maintained on Matrigel®-coated plates (Corning) in mTeSR1 (Stem Cell Technologies) media. HuES9$^{DGCR8+/-}$ culture media was supplemented with 0.8 μM puromycin (Thermofisher Scientific). Cells were passaged with Accutase (Thermofisher Scientific) and were replaced in mTeSR1-Y (mTeSR1 supplemented with 10 μM Y27632-2HCl (Selleckchem)).

1.2. CRISPR/Cas9 genome editing

The sgRNA for the precise genome editing was designed using the Zhang lab's guide design tool (http://crispr.mit.edu/). The sgRNA oligonucleotides were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene #42230) plasmid. The GFP and Puromycin resistance genes were cloned into the donor plasmid (Tálas et al., 2017). 4×10^6 HuES9 cells were electroporated in Amaxa Nucleofector using the Human Stem Cell Nucleofector™ Kit (Lonza) with the A-23 program, then plated onto Matrigel®-coated plates, in mTeSR1-Y. For cloning, GFP expressing cells were plated in Matrigel®-coated 96-well plates, in mTeSR1-Y supplemented with 30% MEF-CM (DMEM, supplemented with 15% FBS), harvested from mouse embryonic fibroblast (MEF) culture (Thermofisher Scientific). Single-cell clones were expanded and screened by PCR and Sanger sequencing for determination of integration site and potential off-target modifications.

1.3. Western blot

After briefly sonicated, samples were run on 8% acrylamide gels, then electroblotted onto PVDF membranes (BioRad). Membranes were blocked by 5% milk/TBS-Tween, and incubated with Anti-DGCR8 antibody (Table 2) overnight at 4 °C. Membranes were then incubated with Anti-Rabbit IgG secondary antibody (Table 2) for 60 min at room temperature (RT). For signal detection ECL reagent (Thermofisher Scientific) was used, and the membranes were exposed to Agfa films. Anti-beta Actin antibody (Table 2) was used to normalize DGCR8 expression. DGCR8 protein level in HuES9$^{DGCR8+/-}$ cell line relative to parental cell line and normalized to beta-actin endogenous control is indicated.

1.4. In vitro spontaneous differentiation

hESC colonies were first dissociated with Collagenase (Thermofisher Scientific) and cultured in suspension on low attachment plates in EB medium (KO-DMEM supplemented with 20% FBS, 1 mM L-GLU, 1% non-essential amino acids, and 0,1 mM ß-mercaptoethanol) (Thermofisher Scientific) for 6 days. Then embryoid bodies (EBs) were transmitted onto 0.1% gelatin (Thermofisher Scientific) coated 24 well tissue culture plates or confocal chamber slides (Nalgene) and allowed to differentiate for another 12 days in DMEM supplemented with 10% FBS. The derivatives of EBs were characterized by immunocytochemical staining and by qPCR.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Targeted marker (ICC/WB)	Rabbit anti-DGCR8	1:1000	Abcam, Cat# ab191875, RRID: n/a
Loading control (WB)	Anti-beta Actin	1:10000	Abcam Cat# ab20272, RRID:AB_445482
Pluripotency marker (ICC)	Mouse anti-Oct3/4	1:50	Santa Cruz Biotechnology Cat# sc-5279, RRID:AB_628051
Pluripotency marker (ICC)	Goat anti-Nanog	1:100	R and D Systems Cat# AF1997, RRID: AB_355097
Pluripotency marker (Flow)	Mouse anti-SSEA-4-APC Isotype control: IgG3-APC	1:100 1:100	R and D Systems Cat# FAB1435A, RRID: AB_494994 R and D Systems Cat# IC007A, RRID: AB_952035
Endoderm marker (ICC)	Mouse anti-AFP	1:500	Sigma-Aldrich Cat# A8452, RRID: AB_258392
Mesoderm marker (ICC)	Mouse anti-SMA	1:500	Abcam Cat# ab7817, RRID:AB_262054
Ectoderm marker (ICC)	Mouse anti-ß-III-Tubulin	1:2000	R and D Systems Cat# MAB1195, RRID: AB_357520
Secondary antibody (ICC)	Goat-Anti-Mouse (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:250	Thermo Fisher Scientific Cat# A-11029, RRID: AB_2534088
Secondary antibody (ICC)	Donkey-Anti-Goat (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1:250	Thermo Fisher Scientific Cat# A-11055, RRID: AB_2534102
Secondary antibody (WB)	Anti-Rabbit IgG	1:5000	Thermo Fisher Scientific, Cat# G-21234, RRID: AB_2536530

Primers		
	Target	Forward/Reverse primer (5′-3′)
Pluripotency Marker(qPCR)	NANOG	Hs02387400_g1 (Thermo Fisher Scientific)
Endoderm differentiation marker (qPCR)	AFP	Hs00173490_m1 (Thermo Fisher Scientific)
Mesoderm differentiation marker (qPCR)	TBXT	Hs00610080_m1 (Thermo Fisher Scientific)
Ectoderm differentiation markers (qPCR)	PAX6	Hs00240871_m1 (Thermo Fisher Scientific)
House-Keeping Gene (qPCR)	RPLP0	Hs99999902_m1 (Thermo Fisher Scientific)
Genotyping and sequencing	DGCR8	Fwd: AGTTTGGCCCATGGGTAGG/Rev: GGAACACCCACTGCTTCTGAC
Inverse PCR	CRISPR-targeted gDNA	Fwd: GCGACTCTAGAGTCGTGGCCTTGGC / Rev: AGGCGGGCCATTTACCGTAAG
Off-target validation	predicted off-target sites	#1 CREB5-For/ TTACCACACACCAAACCATAGGC #1 CREB5-Rev/ CTCTGCACTGCCTTTCATTCAC #2 ANAPC11-For/ GGCCCATTTGAGATCTTTGAAG #2 ANAPC11-Rev CTACGGGAGAACAGCAAGCC #3 DYNC-For/ TGACAATGTGCATGAATTTGCC #3 DYNC-Rev/ CAATGTGCCACGGAAAGTTTG #4 ZMAT3-For/ GTACCCTAATGGACACATGGACG #4 ZMAT3-Rev/ CAGATAAGGGTGGACTGCTGTACTC #5 OR2L13-For/ TTTATCATCTTCCGCACCTGC #5 OR2L13-Rev/ CTGACAGTCGGGAGCAAGAAG

1.5. Immunofluorescent staining and flow cytometry

Cells were fixed, blocked, and permeabilized as described previously (Erdei et al., 2014.). Next, cells were incubated separately with primary antibodies (Table 2) for 60 min at RT. Cells were then incubated with corresponding secondary antibodies for 60 min at RT. Cell nuclei were stained with DAPI. SSEA4 Flow Cytometry analysis was performed as described previously (Erdei et al., 2014.).

1.6. Real-time PCR analysis (RT-PCR)

Total RNA was isolated using TRIzol Reagent (Thermofisher Scientific). cDNA samples were synthesized from 400 ng of total RNA using the Promega Reverse Transcription System. RT-PCR analyses were performed using TaqMan® assays (Table 2) and analyzed by the StepOne™ Real-Time PCR System (Thermofisher Scientific). All quantitative gene expression data were normalized to the expression level of RPLP0.

1.7. Karyotype and STR analysis

Karyotyping and STR analysis were performed by UD-GenoMed Medical Genomic Technologies Ltd.

1.8. Mycoplasma test

MycoAlert™ Mycoplasma Detection Kit was used according to the manufacturer's instructions (Lonza).

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.102134.

References

Erdei, Z., Lőrincz, R., Szebényi, K., Péntek, A., Varga, N., Likó, I., Várady, G., Szakács, G., Orbán, T.I., Sarkadi, B., Apáti, Á., 2014. Expression pattern of the human ABC transporters in pluripotent embryonic stem cells and in their derivatives: ABC Transporters in Pluripotent hESC. Cytometry 86 (5), 299–310. https://doi.org/10.1002/cyto.b.21168. Epub 2014 Apr 11. PMID: 24729538.

Kolacsek, O., Krízsik, V., Schamberger, A., Erdei, Z., Apáti, A., Várady, G., Mátés, L., Izsvák, Z., Ivics, Z., Sarkadi, B., Orbán, T.I., 2011. Reliable transgene-independent method for determining Sleeping Beauty transposon copy numbers. Mob. DNA 2 (1), 5. https://doi.org/10.1186/1759-8753-2-5. PMID: 21371313.

Tálas, A., Kulcsár, P.I., Weinhardt, N., Borsy, A., Tóth, E., Szebényi, K., Krausz, S.L., Huszár, K., Vida, I., Sturm, Á., Gordos, B., Hoffmann, O.I., Bencsura, P., Nyeste, A., Ligeti, Z., Fodor, E., Welker, E., 2017. A convenient method to pre-screen candidate guide RNAs for CRISPR/Cas9 gene editing by NHEJ-mediated integration of a 'self-cleaving' GFP-expression plasmid. DNA Res. 24 (6), 609–621. https://doi.org/10.1093/dnares/dsx029. PMID: 28679166.

Tomari, Y., Zamore, P.D., 2005. MicroRNA biogenesis: drosha can't cut it without a partner. Curr. Biol. 15 (2), R61–R64. https://doi.org/10.1016/j.cub.2004.12.057. PMID: 15668159.

Yeom, K.H., Lee, Y., Han, J., Suh, M.R., Kim, V.N., 2006. Characterization of DGCR8/Pasha, the essential cofactor for Drosha in primary miRNA processing. Nucleic Acids Res. 34 (16), 4622–4629. https://doi.org/10.1093/nar/gkl458. Epub 2006 Sep 8 PMID: 16963499.