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Lab Resource: Single Cell Line

Generation of a homozygous knock-in human embryonic stem cell line expressing SNAP-tagged SOD1

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A B S T R A C T

Superoxide Dismutase 1 (SOD1) is an antioxidant enzyme that protects the cells from radical oxygen species. To study the behavior of endogenous SOD1 under a microscope, we genetically modified H1 human embryonic stem cells (hESCs) to express SOD1 fused with a SNAP-tag, a protein tag that can be covalently labeled with a variety of synthetic probes. The engineered homozygous clone expressing SOD1-SNAP fusion proteins has normal stem cell morphology and karyotype, expresses pluripotency markers, and can be differentiated into all three germ layers *in vitro*, providing a versatile platform for imaging-based studies of SOD1.

1. Resource Table:

Unique stem cell line identifier	WAe001-A-56
Alternative name(s) of stem cell line	H1_SOD1-SNAP / UHMCe002-A-56
Institution	Department of Chemistry, University of Houston, Houston
Contact information of distributor	Tai-Yen Chen (tchen37@central.uh.edu)
Type of cell line	Human embryonic stem cell
Origin	Human
Additional origin info	Age: N/A Sex: Male Ethnicity if known: N/A
Cell Source	H1 hESCs
Clonality	Clonal
Method of reprogramming	N/A
Genetic Modification	Yes
Type of Modification	Transgene expression (protein tag)
Associated disease	N/A
Gene/locus	21q22.11; SOD1 exon 5
Method of modification	CRISPR-Cas9
Name of transgene or resistance	SNAP
Inducible/constitutive system	N/A
Date archived/stock date	11/9/2020
Cell line repository/bank	https://hpscreg.eu/cell-line/WAe001-A-56
Ethical approval	Cell lines were used according to institutional guidelines. UTHealth approval number: SCRO-16–01

2. Resource utility

To study SOD1 at an endogenous level using imaging approaches, we genetically engineered H1 hESC to express SOD1-SNAP fusion protein that can be labeled with various probes. The line can be differentiated into three germ layers and provides a resource to visualize SOD1 under a microscope in different cell contexts.

3. Resource details

SOD1 is a ubiquitously expressed enzyme that converts superoxide radicals to oxygen and hydrogen peroxide. SOD1 malfunction is associated with familial amyotrophic lateral sclerosis (Bruijn et al., 2004). However, its molecular behaviors, such as subcellular distribution and interaction dynamics, are still largely unknown. To dissect the role of SOD1 at an endogenous level in different cell contexts using single-

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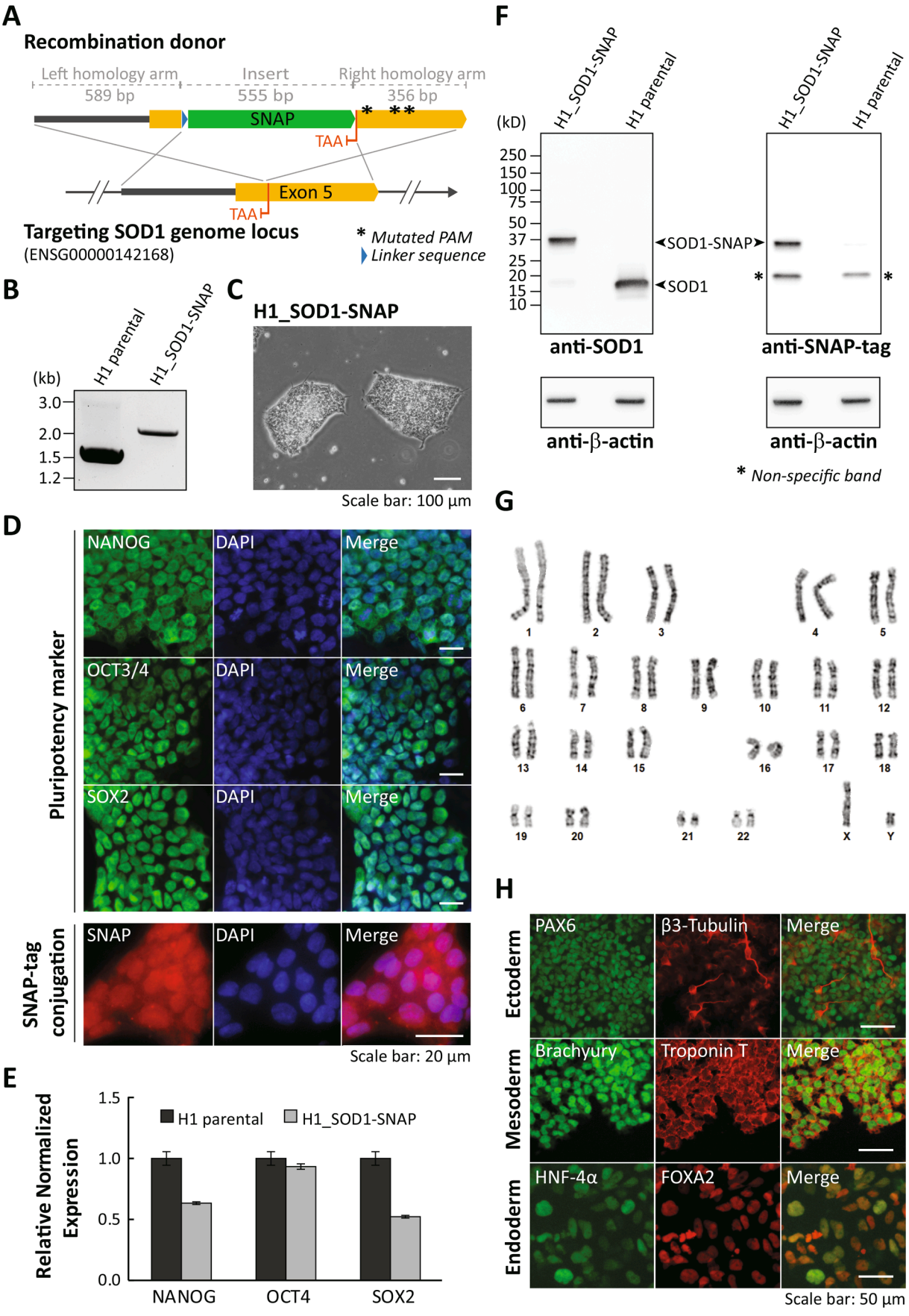

Fig. 1. The generation and characterization of H1_SOD1-SNAP.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel C
Phenotype	Qualitative analysis: *Immunocytochemistry*	Positive staining for pluripotency markers NANOG, OCT3/4, and SOX2	Fig. 1 panel D
	Quantitative analysis: *RT-qPCR*	NANOG: 63 ± 1%; OCT4: 93 ± 2%; SOX2: 52 ± 1% (Mean ± SEM)	Fig. 1 panel E
Genotype	Karyotype (G-banding) and resolution	46XYResolution: 672 × 672	Fig. 1 panel G
Identity	Microsatellite PCR (mPCR) OR	N/A	N/A
	STR analysis	14 sites tested; all match parental H1	Data available with authors and submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Match recombination donor	Supplementary Fig. 1
	Southern Blot	Homozygous insertion	Supplementary Figure 2
Microbiology and virology	Mycoplasma: *PCR*	Negative	Supplementary Figure 3
Differentiation potential	*In vitro* differentiation: *Immunocytochemistry*	Positive staining for ectoderm markers: PAX6 and β3-Tubulin; mesoderm markers: Brachyury and Troponin T; endoderm markers: FOXA2 and HNF-4α	Fig. 1 panel H
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

molecule super-resolution microscopy (Chen et al., 2015), we genetically tagged the SOD1 with SNAP-tag, a mutated form of human O^6-alkylguanine-DNA-alkyltransferase, that can be covalently labeled with various synthetic probes (Keppler et al., 2003) in H1 hESCs. The genetically modified H1 hESC homozygous clone (H1_SOD1-SNAP) was obtained by two rounds of CRISPR-Cas9 mediated homologous recombination. The SNAP gene was in-frame inserted at the C-terminus of the SOD1 coding region following a three-amino-acid-linker (Fig. 1A). H1 hESCs were co-transfected with single-guide RNA (sgRNA)-Cas9 plasmids and recombination donor containing plasmids. The emerged individual clones were picked and screened for SNAP insertion. A heterozygous clone consisting of one SNAP knock-in allele was first obtained and subjected to a second-round CRISPR-Cas9 induced SNAP knock-in. We then successfully generated a homozygous SNAP knock-in clone confirmed by genotyping PCR, Sanger sequencing, and Southern blot analysis (Fig. 1B, Suppl. Fig. 1 and Suppl. Fig. 2, respectively). The expression of the SOD1-SNAP fusion protein in the knock-in cell lysate is confirmed by immunoblotting using anti-SNAP and anti-SOD1 antibodies (Fig. 1F and Suppl. Fig. 4). The majority of SOD1-SNAP protein remains intact in the knock-in cell lysate with an only barely detectable level of un-tagged SOD1 signal, likely originating from the cleavage (Fig. 1F). The overall SOD1-SNAP protein level is reduced compared to the endogenous SOD1 level in H1 parental cells, suggesting the tag appendix may affect the expression of fusion proteins but not compromise SOD1 function (Suppl. Fig. 5). SOD1-SNAP fusion protein expression with intact conjugation activity of SNAP-tag is also confirmed by the positive signal after labeled with fluorescent SNAP-ligand in H1_SOD1-SNAP hESCs (Fig. 1D, bottom panel). The knock-in line exhibits normal hESC morphology with a high nuclear-cytoplasmic ratio, prominent nucleoli, and tightly packed colonies with a well-define edge (Fig. 1C). Immunostaining demonstrated the expression of pluripotency transcription factors NANOG, OCT3/4, and SOX2 in the knock-in cells (Fig. 1D), despite their transcript levels slightly dropped as compared to the H1 parental quantified using RT-qPCR (Fig. 1E). *In vitro* differentiation showed that the knock-in clone still possesses the ability to be differentiated into ectoderm, mesoderm, and endoderm, demonstrated by positive immunostaining of the differentiated cells using germ layer-specific markers (Fig. 1H). The knock-in clone has a normal karyotype (Fig. 1G) and is free of mycoplasma contamination (Suppl. Fig. 3). Its short tandem repeat (STR) profile confirmed a matched identity to the parental H1 (data available upon request). The characterization and validation of the H1_SOD1-SNAP were summarized in Table 1. In short, H1_SOD1-SNAP cells are pluripotent H1 hESCs with normal karyotype and express SOD1-SNAP fusion proteins that can be labeled with SNAP-tag fluorophores providing a versatile platform for imaging-based studies of SOD1.

4. Materials and methods

4.1. Cell culture

H1 hESCs were cultured as previously described (Wen et al., 2019).

4.2. CRISPR-Cas9

We adopted the protocol from Koch et al. (Koch et al., 2018) and designed sgRNAs using Benchling. The sgRNAs were cloned in pSpCas9 (BB)-2A-Puro (PX459) plasmid, a gift from Feng Zhang (Addgene plasmid # 48139; http://n2t.net/addgene:48139; RRID: Addgene_48139). The reported homozygous clone was obtained by two rounds of editing using CRISPR sgRNA-1 and 2 (Table 2). H1 hESCs were co-electrotransfected (300 V/ 500 μF, Bio-Rad Gene Pulser Xcell) with sgRNA-PX459 and recombination donor sequence (Genewiz) carried by pMiniT 2.0 (New England Biolabs, NEB). Transfected cells were plated on irradiated B6-Puro Mouse Embryonic Fibroblast feeders (Thermo Fisher) followed by puromycin selection for 3 days. Clones were manually picked and expanded under a feeder-free system.

4.3. PCR and sequencing

Genomic DNA was extracted using MasterPure Complete DNA and RNA Purification Kits (Epicentre). PCR screening was performed using AccuStart II GelTrack SuperMix (QuantaBio) with annealing temperature (T_a) at 64 °C. The product sizes are 1542 bp for H1 parental and 2097 bp for H1_SOD1-SNAP. Sample for Sanger sequencing (Eton Bioscience) was prepared using Q5 Hot Start High-Fidelity DNA Polymerase (NEB) with the same primer set at T_a 66 °C and extension for 1 min each cycle.

4.4. Southern blotting

Genomic DNA was digested with HindIII-HF (NEB) overnight. SOD1 probes were synthesized using PCR DIG Probe Synthesis Kit (Roche) at T_a 62 °C and extension for 40 sec each cycle. Southern blot was performed according to the Roche DIG Manual for Filter Hybridization with the hybridization temperature at 37 °C. Expected fragment sizes are 810 bp for SOD1 and 1365 bp for SOD1-SNAP.

Table 2
Reagents details.

Antibodies used for immunocytochemistry (ICC)/immunoblotting (I.B.)			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Marker	Goat anti-NANOG	10 μg/ml (ICC)	R&D Systems Cat# AF1997, RRID: AB_355097
	Mouse anti-OCT3/4	1:250 (ICC)	Santa Cruz Biotechnology Cat# sc-5279, RRID: AB_628051
	Mouse anti-SOX2	1:250 (ICC)	Santa Cruz Biotechnology Cat# sc-365823, RRID: AB_10842165
Differentiation Marker	Mouse anti-PAX6	1:50 (ICC)	DSHB Cat# pax6, RRID: AB_528427
	Rabbit anti-β3-Tubulin	1:300 (ICC)	Cell Signaling Technology Cat# 5568, RRID: AB_10694505
	Goat anti-Brachyury	10 μg/ml (ICC)	R&D Systems Cat# AF2085, RRID: AB_2200235
	Mouse anti-Troponin T	10 μg/ml (ICC)	R&D Systems Cat# MAB1874, RRID: AB_2206731
	Rabbit anti-FOXA2	1:400 (ICC)	Cell Signaling Technology Cat# 8186, RRID: AB_10891055
	Mouse anti-HNF-4α	1: 200 (ICC)	Santa Cruz Biotechnology Cat# sc-374229, RRID: AB_10989766
Characterization Marker	Rabbit anti-SOD1	1:500 (IB)	Sigma-Aldrich Cat# HPA001401, RRID: AB_1080132
	Rabbit anti-SNAP-tag	1:1000 (IB)	New England Biolabs Cat# P9310S, RRID: AB_10631145
	Mouse anti-β-Actin	1:5000 (IB)	Sigma-Aldrich Cat# A5441, RRID: AB_476744
Secondary Antibody	Donkey anti-Mouse IgG (Alexa Fluor 488 conjugated)	1:1000 (ICC)	Thermo Fisher Scientific Cat# A-21202, RRID: AB_141607
	Donkey anti-Goat IgG (Alexa Fluor 488 conjugated)	1:1000 (ICC)	Thermo Fisher Scientific Cat# A-11055, RRID: AB_2534102
	Donkey anti-Mouse IgG (Alexa Fluor 594 conjugated)	1:1000 (ICC)	Thermo Fisher Scientific Cat# A-21203, RRID: AB_141633
	Donkey anti-Rabbit IgG (Alexa Fluor 594 conjugated)	1:1000 (ICC)	Thermo Fisher Scientific Cat# A-21207, RRID: AB_141637
	Goat anti-Rabbit IgG (HRP-linked)	1:5000 (IB)	Cell Signaling Technology Cat# 7074, RRID: AB_2099233
	Peroxidase-AffiniPure Goat anti-Mouse IgG	1:10000 (IB)	Jackson ImmunoResearch Labs Cat# 115–035-062, RRID: AB_2338504

Primers		
	Target	**Forward/Reverse primer (5′-3′)**
CRISPR sgRNA-1	SOD1 exon 5	CACCGTCGCCCAATAAACATTCCCT/ AAACAGGGAATGTTTATTGGGCGAC
CRISPR sgRNA-2	SOD1 exon 5	CACCGAGGATAACAGATGAGTTAAG/ AAACCTTAACTCATCTGTTATCCTC
Genotyping (insertion screening)	SOD1	ACAAAGATGGTGTGGCCGAT/ ACCCATCTGTGATTTAAGTCTGG
Southern Blot Probe Synthesis	SOD1	ACAGCTTCAGTGGAACAGATTTAG/ AAGGACAGCCTATTTGTCTAAGCAG
Pluripotency Gene (qPCR)	NANOG	TTTGTGGGCCTGAAGAAAACT/ AGGGCTGTCCTGAATAAGCAG
	OCT4	AACCTGGAGTTTGTGCCAGGGTTT/ TGAACTTCACCTTCCCTCCAACCA
	SOX2	AGAAGAGGAGAGAGAAAGAAAGGGAGAGA/ GAGAGAGGCAAACTGGAATCAGGATCAAA
House-Keeping Gene (qPCR)	GAPDH	CCACTCCTCCACCTTTGAC/ ACCCTGTTGCTGTAGCCA

4.5. RT-qPCR

Total RNA was extracted the same way as DNA and converted to cDNA using iScript R.T. Supermix (Bio-Rad). Quantitative PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with the following program: 50 °C for 10 min, 95 °C for 5 min, 40 cycles of 95 °C for 10 sec and 60 °C for 30 sec, 95 °C for 10 sec, and Melt Curve 65 to 95 °C, increment 0.5 °C for 5 sec. Data were analyzed in triplicate and normalized to GAPDH expression.

4.6. Immunoblotting, immunostaining, and SNAP-tag conjugation

Immunoblotting and immunostaining were performed as previously described (Wen et al., 2019). For SNAP-tag conjugation, live cells were incubated with 3 μM SNAP-Cell TMR-Star (NEB) for 30 min, washed and recovered, then fixed and permeabilized before imaging.

4.7. Karyotyping and STR analysis

The University of Texas M.D. Anderson Cancer Center Cytogenetics and Cell Authentication Core Facility conducted the G-band karyotyping (ten metaphase chromosome spreads) and STR analysis. The number of STRs at 14 loci was tested and compared to the H1 STR profile.

4.8. In vitro differentiation to three germ layers

In vitro differentiation was performed using STEMdiff Trilineage Differentiation Kit (STEMCELL Technologies).

4.9. Mycoplasma testing

Cells were cultured for 48 h before screening and more than 80% confluence. Spent media was collected from culture and subjected to mycoplasma detection using Mycoplasma PCR Detection Kit (Applied Biological Materials).

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 https://doi.org/10.1016/j.scr.2021.102415.

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