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Lab resource: Stem Cell Line

Generation of a *TLE1* homozygous knockout human embryonic stem cell line using CRISPR-Cas9

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ABSTRACT

Here, we generated a biallelic mutation in the *TLE1* (Transducin Like Enhancer of Split 1) gene using CRISPR-Cas9 editing in the human embryonic stem cell (hESC) line WA01. The homozygous knockout cell line, TLE1-464-G04, displays loss of TLE1 protein expression while maintaining pluripotency, differentiation potential and genomic integrity.

Resource table.

Name of stem cell line	TLE1-464-G04
Institution	Broad Institute of MIT and Harvard, Cambridge, MA
Person who created resource	Amanda Herring, Anne M. Bara, Lindy E. Barrett
Contact person and email	Lindy Barrett, lbarrett@broadinstitute.org
Date archived/stock date	May 11, 2016
Origin	Human Embryonic Stem Cell Line WA01; NIH Registration Number 0043.
Type of resource	Biological reagent: Genetically modified human embryonic stem cell line
Sub-type	Cell line
Key transcription factors	N/A
Authentication	Identity and purity of cell line confirmed (Fig. 1)
Link to related literature	http://www.ncbi.nlm.nih.gov/pubmed/24157548 http://www.ncbi.nlm.nih.gov/pubmed/21076418 http://www.ncbi.nlm.nih.gov/pubmed/25186908 http://www.ncbi.nlm.nih.gov/pubmed/9804556
Information in public databases	N/A
Ethics	Cell lines were used according to institutional guidelines.

Resource details

Here, we targeted exon 2 of *TLE1* using CRISPR-Cas9 based genome engineering (Ran et al. 2013) to generate a *TLE1* homozygous knockout hESC line, TLE1-464-G04. This approach generated a 1 bp biallelic insertion in exon 2 of *TLE1* (Fig. 1A). This insertion was predicted to shift the *TLE1* full-length reading frame, resulting in loss of TLE1 expression. To this end, the hESC line WA01 was transfected with constructs expressing Cas9 nuclease coupled with puromycin resistance and an sgRNA targeting exon 2 of *TLE1*. Cells were then selected with puromycin and plated at clonal density to allow for targeted colonies to emerge. We manually isolated a total of 94 individual colonies, which were expanded in 96-well plates and then duplicated for cell freezing and extraction of genomic DNA. To detect the presence of frame-shift mutations, the region flanking the predicted cut site of exon 2 was PCR amplified, barcoded and deep-sequenced. The putative *TLE1* homozygous clone, TLE1-464-G04 ($TLE1^{-/-}$), and a paired wild-type *TLE1* clone, TLE1-464-A01 ($TLE1^{+/+}$), were expanded and banked for further analyses.

Western blot analysis revealed undetectable levels of full-length TLE1 protein in $TLE1^{-/-}$ cells relative to $TLE1^{+/+}$ cells (Fig. 1B). These results are consistent with the 1 bp insertion in exon 2 leading to disruption of TLE1 expression. Genomic integrity of $TLE1^{+/+}$ and $TLE1^{-/-}$ cell lines was confirmed by G-banded karyotype analysis (Fig. 1C). Stem cell colonies grown from both cell lines continued to express the pluripotency markers OCT4, SOX2, SSEA-4, and TRA-1-60 (Fig. 1D) and maintained three germ layer differentiation capacity, as evidenced by expression of AFP (endoderm), SMA (mesoderm) and β-III Tubulin (ectoderm) following *in vitro* embryoid body differentiation (Fig. 1E).

Taken together, the *TLE1* homozygous knockout hESC line described here is karyotypically normal and retains pluripotency characteristics. It has potential to gain insight into how *TLE1* functions in both normal development and disease.

Materials and methods

Cell culture

The XY hESC line WA01 was obtained from WiCell (Thomson et al. 1998) (www.wicell.org). Cells were grown in mTeSR1 medium (Stem Cell Technologies 05850) on plates pre-coated with Geltrex (Life Technologies A1413301) and maintained under standard conditions

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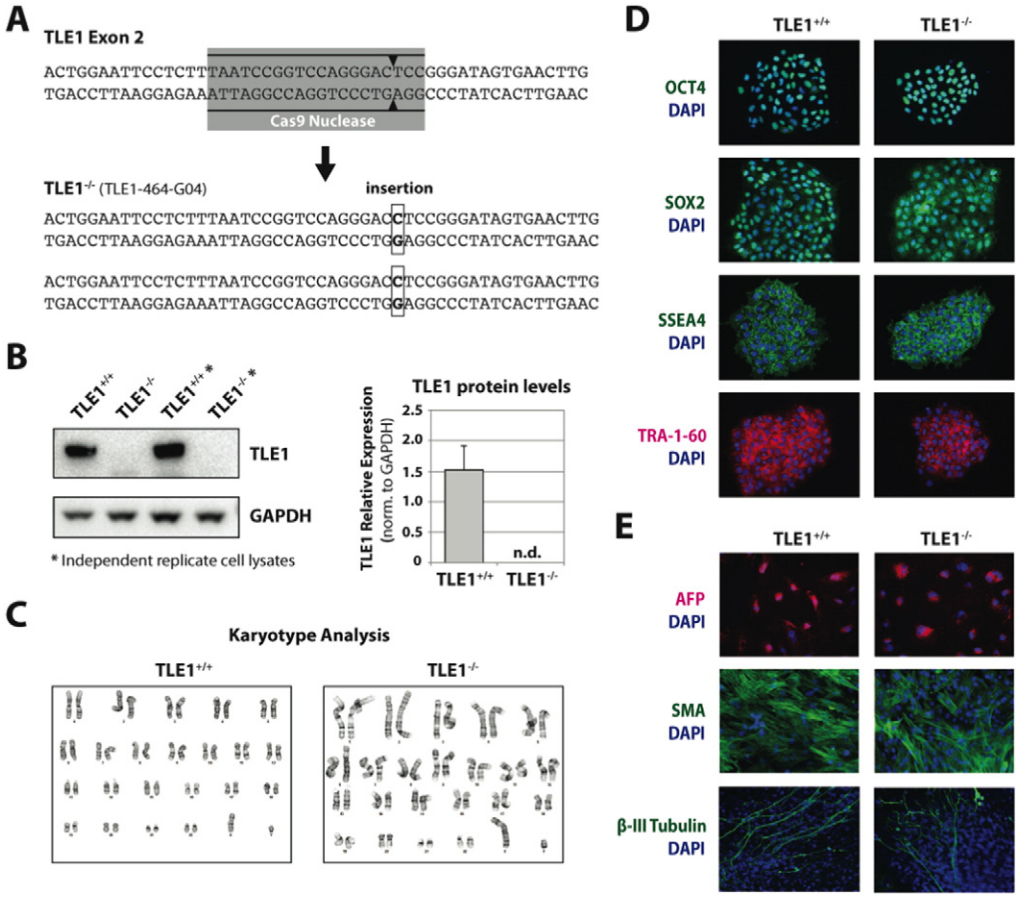

Fig. 1. Generation of the *TLE1* homozygous knockout hESC line TLE1-464-G04 by CRISPR-Cas9 editing. (A) Cas9 nuclease approach to create a 1 bp insertion in exon 2 of *TLE1*. Grey box denotes the sgRNA target site and arrows show the location of Cas9 cleavage. (B) Western blot analysis reveals non-detectable (n.d.) levels of TLE1 protein in TLE1-464-G04 ($TLE1^{-/-}$) cells relative to TLE1-464-A01 ($TLE1^{+/+}$) cells. Relative expression of TLE1 protein was quantified by calculating pixel density of TLE1 signal relative to GAPDH signal from the blot. Errors bars represent standard error of the TLE1/GAPDH ratios from the two independent $TLE1^{+/+}$ sample lysates. (C) Chromosome analyses of $TLE1^{+/+}$ and $TLE1^{-/-}$ lines show a normal karyotype (46, XY). (D) Immunostaining of $TLE1^{+/+}$ and $TLE1^{-/-}$ cells with pluripotency markers OCT4, SOX2, SSEA-4, and TRA-1-60. (E) Immunostaining of embryoid bodies derived from $TLE1^{+/+}$ and $TLE1^{-/-}$ cells reveals differentiation to all three germ layers: AFP, endoderm; SMA, mesoderm; β-III Tubulin, ectoderm.

(37 °C, 5% CO_2). Cells were passaged using 1 mM EDTA (Fluka 03690) in PBS or TrypLE Express (Life Technologies 12604021).

CRISPR guide selection and cloning

A CRISPR guide targeting exon 2 of *TLE1* was designed using http://crispr.mit.edu. Oligonucleotides (IDT) corresponding to the sgRNA (TAATCCGGTCCAGGGACTCC) were cloned into a pU6-sgRNA vector (a gift from Feng Zhang, Broad Institute) to generate the guide plasmid.

Gene targeting and clonal selection

For electroporation, hESCs were pre-incubated with "1:1 medium" composed of a 1:1 mixture of mTeSR1 medium and "hESC medium" [hESC medium: KO DMEM (Gibco 10829-018) with 20% KOSR (Gibco 10828-028), 1% Glutamax (Gibco 35050-061), 1% MEM NEAA (Corning 25-025-Cl), 0.1% 2-mercaptoethanol (Gibco 21985-023) and 20 ng/ml bFGF (EMD Millipore GF003AF)] supplemented with 10 μM ROCK inhibitor (ϒ-27632). For electroporation, 2.5×10^6 cells were re-suspended in R Buffer, mixed with DNA and electroporated at 1050 V, 30 ms, 2 pulses using 100 μl tips (NEON, Life Technologies MPK10096) with 1.4 μg guide plasmid and 7 μg Cas9 nuclease-puromycin plasmid pX459 (Addgene Plasmid # 48139).

Following electroporation, cells were dispensed into 10 cm Geltrex coated plates in 1:1 medium plus ROCK inhibitor. After 24 h, cells were treated with 1 μM puromycin (Sigma Aldrich P8833) for 24 h. Cells were then maintained in 1:1 medium for 10 days to allow for colony formation.

A total of 94 individual hESC colonies were manually picked and seeded into Geltrex-coated 96-well plates in 1:1 medium plus ROCK inhibitor. Colonies were allowed to expand for 10 days and clones in 96-well plates were then duplicated for cell freezing and genomic DNA extraction. Clones were frozen in 96-well plates using 50% 1:1 medium with ROCK inhibitor, 40% FBS (VWR SH30070.03) and 10% DMSO (Sigma D2650). Genomic DNA was extracted overnight at 55 °C in DirectPCR Lysis Reagent (Viagen 102-T) with Proteinase K (Roche 03115828001) followed by a 1 h 90 °C incubation.

Screening and sequence analysis

Primers containing Illumina multiplexing adapters (Ilumina) and specific to exon 2 of *TLE1* (F: ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACGCCCGCGACCAC and R: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCCAGACGCCGCACC, underlined = adaptor sequence) were used to PCR amplify a 146 bp TLE1 sequencing amplicon with Q5 Hot Start High-Fidelity Master Mix (NEB M04945). A second round of PCR amplification was performed on the 146 bp TLE1 amplicon used to incorporate well-specific barcode IDs (Broad Institute). Barcoded PCR products were pooled and gel-purified (Zymo Research D4008), run on a 2100 BioAnalyzer (Agilent Technologies) for quality assessment and submitted for MiSeq paired-end deep sequencing (Broad Institute). Sequencing results were analysed using the OutKnocker indel-calling program (www.OutKnocker.org) (Schmid-Burgk et al. 2014).

Embryoid body differentiation

Embryoid bodies (EBs) were generated as described (Klim et al. 2010). Briefly, hESCs were grown in ultra low-attachment flasks in "EB medium" [composed of IMDM (Life Technologies 12440079), 15% FBS, 1% NEAA and 0.1 mM 2-mercaptoethanol] for 2.5 weeks. EBs were then seeded into Geltrex-coated 12-well plates and allowed to differentiate under adherent conditions in EB medium for one additional week prior to fixation and immunostaining. For neuron generation, EBs were treated with dual SMAD inhibition (10 μM SB431542 and 100 nM LDN193189).

Western blot analysis

Two independently grown replicates of TLE1-464-G04 and TLE1-464-A01 were lysed using Pierce IP lysis buffer (Life Technologies 87787) with protease inhibitors (Sigma Aldrich 11836153001). 20 μg protein as determined by Pierce BCA Protein Assay kit (Thermo Scientific 23227) was loaded onto Bolt 4–12% Bis-Tris Plus gels (Invitrogen). Gels were dry blotted to nitrocellulose membranes (iBlot 2 NC Ministacks, Invitrogen) by the iBlot 2 transfer system (Invitrogen). The following antibodies were used: TLE1 (Abcam ab183742; 1:2000), GAPDH (EMD MAB374; 1:2000), α-rabbit HRP-linked F(ab')2 (GE Life Sciences NA9340; 1:5000) and α-mouse HRP-linked F(ab')2 (GE Life Sciences NA9310; 1:5000). Blots were visualized on a ChemiDoc MP Imaging System (BioRad) using a SuperSignal West Femto kit (Pierce). Pixel density quantification of TLE1 and GAPDH was performed with ImageJ software (https://imagej.nih.gov/ij/).

Immunostaining

hESC colonies and EBs were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, blocked and permeabilized with 0.1% TritonX-100 and 4% serum in PBS for 1 h and incubated with the appropriate primary antibody. Following primary antibody incubation, cells were washed with PBS and incubated with the appropriate secondary antibody (Alexa Fluor 488 or 594, 1:500, Invitrogen) for 1 h. Cells were then washed with PBS and incubated with DAPI before imaging at 20× magnification. The following primary antibodies were used: OCT4 (R&D Systems AF1759; 1:250), SSEA-4 (SCBT SC21704; 1:250), TRA-1-60 (SCBT SC21705; 1:200), SOX2 (R&D Systems MAB2018; 1:200), AFP (Sigma A8452; 1:250), SMA (Sigma A2547; 1:2000), β-III Tubulin (R&D Systems MAB1195; 1:3000).

Analysis of genomic integrity

Cytogenetic analysis was performed by Cell Line Genetics (www.clgenetics.com) on twenty G-banded metaphase cells from each cell line.

Acknowledgments

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