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Lab resource: Stem cell line

A homozygous Keap1-knockout human embryonic stem cell line generated using CRISPR/Cas9 mediates gene targeting

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ABSTRACT

Kelch-like ECH-associated protein 1 (keap1) is a cysteine-rich protein that interacts with transcription factor Nrf2 in a redox-sensitive manner, leading to the degradation of Nrf2 (Kim et al., 2014a). Disruption of Keap1 results in the induction of Nrf2-related signaling pathways involving the expression of a set of anti-oxidant and anti-inflammatory genes. We generated biallelic mutants of the *Keap1* gene using a CRISPR-Cas9 genome editing method in the H9 human embryonic stem cell (hESC). The Keap1 homozygous-knockout H9 cell line retained normal morphology, gene expression, and *in vivo* differentiation potential.

Resource table.

Name of stem cell line	H9Keap1KO-A31
Institution	Korea National Institute of Health
Person who created resource	So-Jung Kim[a, d], Omer Habbib[b, c], Jung-Hyun Kim[a]
Contact person and email	Jung-Hyun Kim, kjhcorea@korea.kr
Date archived/stock date	Nov 11, 2016
Origin	Human Embryonic Stem Cell Line WA09; NIH Registration Number 0062
Type of resource	Biological Reagent: Genetically modified human embryonic stem cell line
Sub-type	Cell line
Key transcription factors	N/A
Authentication	Cell line N/A Identity and purity of cell line confirmed (Figure 1, Table 1, Supplementary Figure 1, Supplementary Table 1)
Link to related literature	N/A
Information in public databases	N/A
Ethics	The cell line was used according to the institutional guidelines. The Institutional Review Board (IRB) of the Korea CDC approved the study. IRB approval number: 2015-03-EXP-04-3C-A

1. Resource details

To engineer a biallelic mutation in the *Keap1* gene, we introduced preassembled Cas9 protein/single-chain guide RNA (sgRNA) ribonucleoproteins (RNP) targeting the cysteine-rich N-terminal intervening region (IVR) domain of Keap1 (Fig. 1A). The sgRNA-induced indel frequency was measured 3 days post-transfection using T7 endonuclease I (T7E1), and the identity of the mutations was confirmed by deep sequencing (Fig. 1B, C). After single-cell seeding, individual clones were picked and expanded. We confirmed the targeted disruption of Keap1 by deep sequencing, which showed a high rate of mutation (66%), with homozygous deletions occurring in 13% of the clones.

For further characterization, we selected the H9Keap1KO-A31 line, with insertion of a biallelic single nucleotide (A) (Fig. 1D). The H9Keap1KO-A31 line grew well, showed normal morphology (Fig. 1E), and expressed the pluripotency markers OCT4, SSEA4, TRA-1-60, and TRA-1-80 (Fig. 1F). The cell line had a stable karyotype (46, XX) (Fig. 1G). It also showed differentiation potential which formed teratomas with tissues derived from all germlines, including gut (endoderm), osteoid (mesoderm), and neuronal epithelia and pigmented cells (ectoderm) (Fig. 1H). Additionally, mRNA expression of the three germ layer markers was detected in 14-day cultured embryonic bodies (EB) (Table 1). To confirm the safety of the cell line, we tested for mycoplasma (data not shown), virus, and bacterial infections (Fig. S1).

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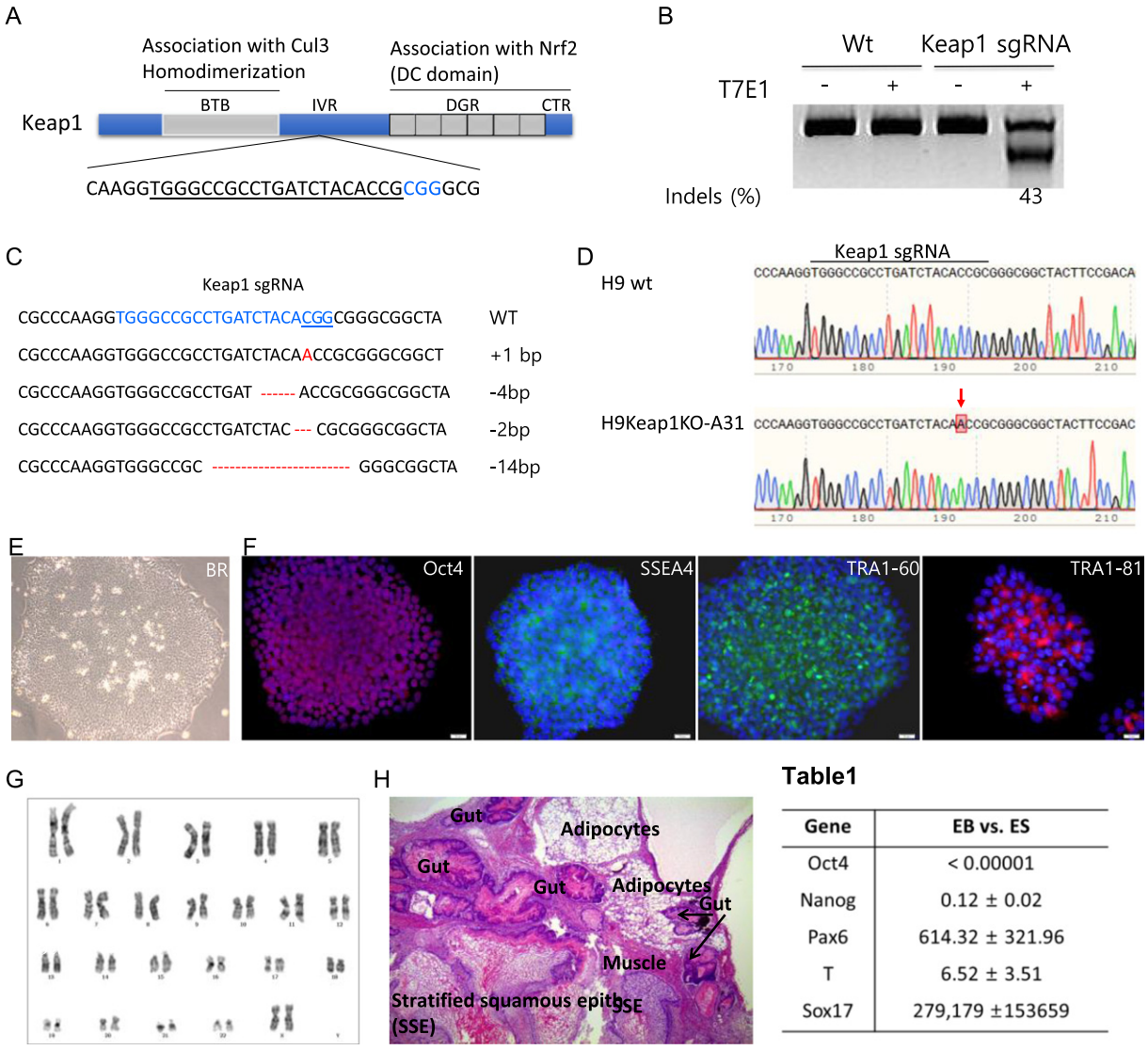

Table1

Gene	EB vs. ES
Oct4	< 0.00001
Nanog	0.12 ± 0.02
Pax6	614.32 ± 321.96
T	6.52 ± 3.51
Sox17	279,179 ±153659

Fig. 1. A) Schematic overview of the *Keap1* gene and the Cas9/gRNA target site. B) Keap1-specific sgRNA RNP-mediated mutations measured with the T7E1 assay. C) Mutant DNA sequences at the Keap1 locus. D) Sanger-sequencing of the H9Keap1KO-A31 line. E) Cell morphology. F) Image of immunostaining and G) karyotype. H) Image of an H&E-stained teratoma (×40). Note the endoderm (gut), mesoderm (muscle, adipocytes), and ectoderm (stratified squamous epithelia) tissues. Table 1. The relative fold changes of gene expression between EB and ES.

In summary, the Keap1 homozygous-knockout hESC line described here is karyotypically normal and retains pluripotency. It has the potential to offer insight into the roles of the Nrf2-Keap1 signaling pathway in development.

2. Materials and methods

2.1. Cell culture

The human embryonic stem cell H9 was obtained from the WiCell Research Institute (WA91; NIH Registration Number 0062) (Thomson et al., 1998). Cells were grown in E8 medium (Gibco) on plates pre-coated with Laminin 521 (Corning). The cells were passaged using ReLeSR (Stem Cell Technologies).

2.2. CRISPR-Cas9 gene editing

First, 40 μg of *in-vitro* transcribed sgRNA and 30 μg of recombinant Cas9 protein were electroporated using the P3 Primary Cell 4D-Nucleofector® X Kit (Lonza) (Kim et al., 2014b). After confirming mutation with the T7E1 assay, genomic DNA was isolated using a QuickExtract genome isolation kit (Epicentre QE09050). The target region was amplified and subjected to paired-end read sequencing using Illumina MiSeq at LAS (Table S1).

2.3. EB formation

The hESCs were detached from a Vitronectin (Gibco)-coated plate using a cell scraper and transferred into a 15-mL conical tube. After a gentle spin-down, the cells were resuspended in EB medium, which is composed of Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco), 20% knockout serum replacement (Gibco), 1% nonessential amino acids (Gibco), and 0.1 mM β-mercaptoethanol (Invitrogen) and cultured for 14 days.

2.4. Teratoma assay

First, 10^6 cells were injected into NOD Scid Gamma (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice (Jackson Laboratory). After teratomas formed, the mice were sacrificed, and the formaldehyde-fixed tumor tissues were stained with hematoxylin and eosin (H&E).

2.5. Real-time quantitative PCR (qRT-PCR) analysis

Total RNA was extracted from the cells using the RNeasy Mini Kit (QIAGEN 74106), according to the manufacturer's instructions. cDNA was synthesized using RNA-to-cDNA EcoDry Premix (Clontech). The qRT-PCR assays were performed using TaqMan™ Master Mix (ABI) (Kim et al., 2014a).

2.6. Immunostaining

The cells were fixed and permeabilized. The fixed samples were incubated for 24 h at 4 °C with anti-Oct4 (Santa Cruz) or anti-SSEA-4 (Millipore) and with TRA-1-60 (Millipore), or TRA-1-81 (Millipore) primary antibodies. Then, the samples were washed and incubated in fluorescein-conjugated secondary antibodies with DAPI (Thermo). Finally, the slides were photographed using an Olympus DP72 camera and imaged with cellSens software.

2.7. Karyotyping

The G-banding karyotype was analyzed in 20 single clones, and the band resolution was 500.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2016.12.028.

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