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Lab resource: Stem cell line

Generation of a Nrf2 homozygous knockout human embryonic stem cell line using CRISPR/Cas9

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ABSTRACT

Nuclear factor erythroid 2-related factor 2 (NFE2L2 or Nrf2) is a well-known transcription factor that regulates the expression of a large number of anti-oxidant genes in mammalian cells (J.H. Kim et al., 2014). Here, we generated a homozygous Nrf2 knockout human embryonic stem cell (hESC) line, H9Nrf2KO-A13, using the CRISPR/Cas9 genome editing method. The Nrf2 homozygous knockout H9 cell line maintains pluripotency, differentiation potential into three germ layers, and a normal karyotype.

Resource table.

Name of stem cell line	H9Nrf2KO-A13
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 (Fig. 1, Table 1, Fig. S1, Tables S1,S2)
Link to related literature	N/A
Information in public databases	N/A
Ethics	Cell lines were used according to the institutional guidelines. The Institutional Review Board (IRB) of the Korea CDC authorized this study. IRB approval number: 2015-03-EXP-04-3C-A

1. Resource details

We generated a cell line (H9Nrf2KO-A13) that is a homozygous knockout of Nrf2 by targeting exon 4 of Nrf2 using the CRISPR/Cas9 gene editing system (Fig. 1A). H9 cells were electroporated with preassembled Cas9 protein/single-chain guide RNA (sgRNA) ribonucleoproteins (RNPs) targeting the transactivation domain of Nrf2 (Fig. 1B). Four days after electroporation, cells were reseeded as single cells to obtain single-cell-derived clones. To validate the Nrf2 knockout, we performed targeted deep sequencing. Among 106 clones, we found three homozygous mutant lines and a biallelic 14-nucleotide-deleted and 2-nucleotide-inserted line, H9Nrf2KO-A13 (Fig. 1C); these were expanded and stored for further characterization.

The H9Nrf2KO-A13 line was characterized both in terms of its molecular phenotype and differentiation potential. The H9Nrf2KO-A13 line is morphologically normal (Fig. 1D). ICC data revealed that the H9Nrf2KO-A13 line is OCT4-, SSEA4-, TRA-1-60-, and TRA-1-80-positive (Fig. 1E). We confirmed that the CRISPR/Cas9 gene editing process did not introduce any chromosomal abnormality by determining the cell line's karyotype (Fig. 1F). To confirm its differentiation potential, we subcutaneously injected the clone into immunodeficient mice to test for teratoma formation that resulted in cell types representing the three germ layers (Fig. 1G). Additionally, the mRNA expression levels of markers representing the three germ layers were examined in 14-day-cultured embryonic bodies (EBs) (Table 1). Finally, to confirm the safety of the cell line, we tested for mycoplasma (data not shown), viruses, and bacterial infections (Fig. S1).

In summary, the Nrf2 homozygous knockout hESC line described herein is karyotypically normal and exhibits pluripotency. This line is

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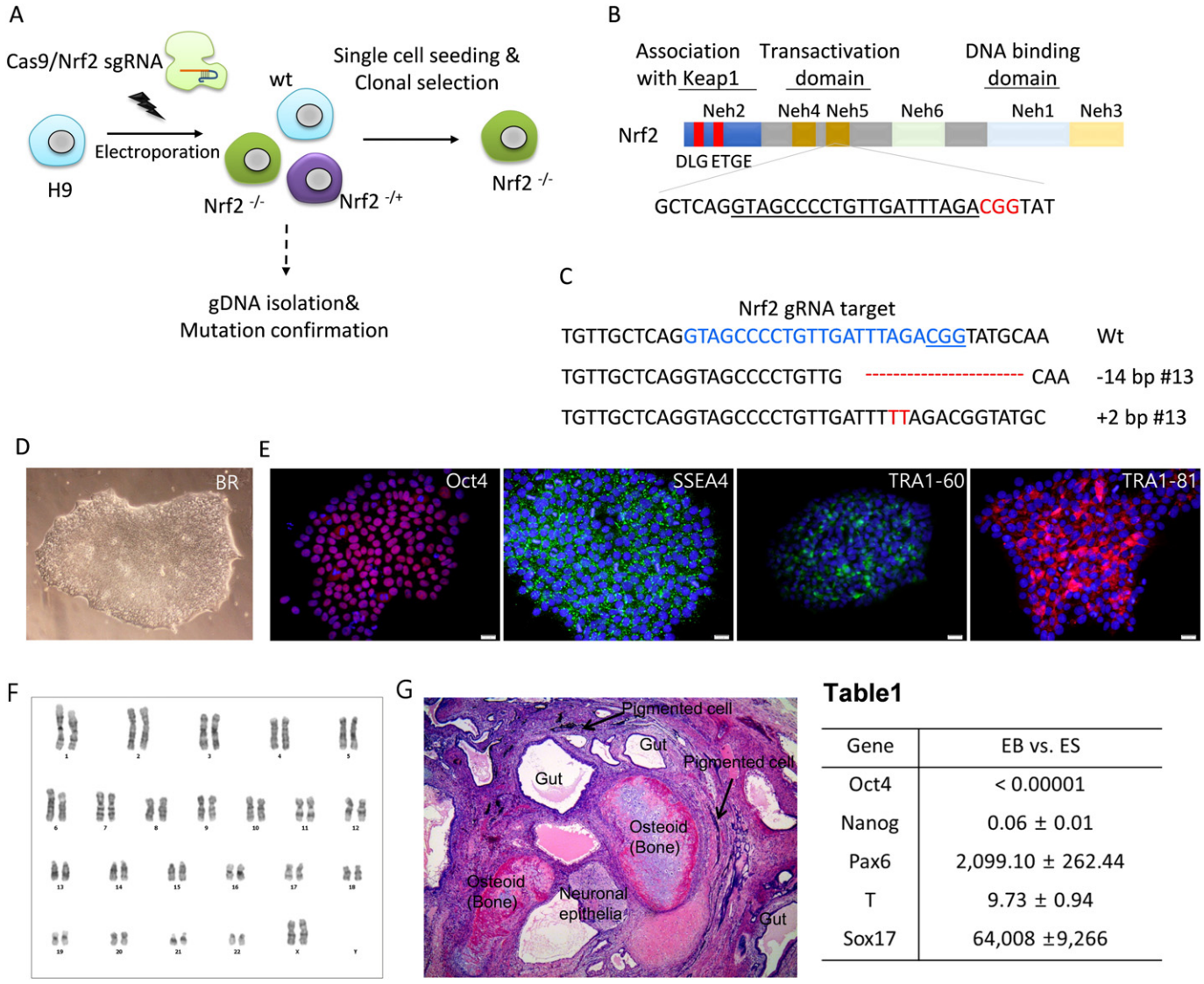

Table1

Gene	EB vs. ES
Oct4	< 0.00001
Nanog	0.06 ± 0.01
Pax6	2,099.10 ± 262.44
T	9.73 ± 0.94
Sox17	64,008 ±9,266

Fig. 1. A) Schematic diagram of the generation of Nrf2 homozygous knockout clones. B) Schematic of the Nrf2 gene and the Cas9/gRNA target site. C) Mutation information for the targeted region of the H9Nrf2KO-A13 clone. Images represent cell morphological (D), immunocytochemistry staining with the indicated markers of pluripotency (E), and karyotype analysis (F). G) H&E staining of a teratoma, indicating differentiation into the three germ layers (×40). Note the tissues from the endoderm (gut), mesoderm (osteoid), and ectoderm (neuronal epithelium, pigmented cells). **Table 1.** The relative fold changes of gene expression between EB and ES.

a valuable laboratory resource to offer insight into the roles of the Nrf2 pathway in development.

2. Materials and methods

2.1. Cell culture

The human embryonic stem cell H9 line was obtained from Wicell (WA91; NIH Registration Number 0062). Cells were grown in Essential 8 Medium (Gibco) on laminin 521 (Corning)-coated plates (Thomson et al., 1998). Cells were passaged using ReLeSR (Stem Cell Technologies).

2.2. Guide RNA

RNA was *in vitro*-transcribed using the MEGAshortscript T7 Kit (Ambion) according to the manufacturer's manual. sgRNA template was generated by the annealing and extension of two complementary oligonuceotides (Table S1). Transcribed RNA was purified using the MEGAclear Transcription Clean-Up Kit (Ambion). Purified RNA was quantified spectrophotometrically.

2.3. Electroporation and targeted deep sequencing

Human ESCs were dissociated into single cells using ReLeSR. Cells were resuspended in Nucleofection solution and electroporated with 30 μg Cas9 and 40 μg of *in vitro*-transcribed sgRNA using the Amaxa P3 Primary Cell 4D–Nucleofector Kit (Lonza) (S. Kim et al., 2014). After 4 days, cells were replated as single cells at a very low density on laminin 521-coated plates in Essential 8 Medium supplemented with a Rho kinase (ROCK) inhibitor (Stemgent). Individual colonies were picked and expanded. Genomic DNA was then extracted using QuickExtract (Epicentre). The target region was amplified and subjected to paired-end read sequencing using Illumina MiSeq at LAS (Table S2).

2.4. EB formation

hESCs on vitronectin (Gibco)-coated plates were detached using a cell scraper. Cells were gently spin-downed and re-suspended in EB medium comprised of DMEM/F12 (Gibco), 20% knockout serum replacement (Gibco), 1% nonessential amino acids (Gibco), and 0.1 mM β-mercaptoethanol (Invitrogen) for 14 days.

2.5. Teratoma assay

The cells (10^6) were injected into NOD Scid Gamma (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice (The Jackson Laboratory). The formed teratomas were fixed in formaldehyde, paraffin-embedded, and sectioned. To analyze the three germ layers, the sectioned slides were histologically examined by hematoxylin and eosin (H&E) staining.

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

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

2.7. Immunostaining

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

2.8. Karyotyping

The G-banding karyotype analysis was performed on 20 single clones at a band resolution of 500.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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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.2016.12.027.

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