Supplementary Material

KCNJ13 gene deletion impairs cell alignment and phagocytosis in retinal pigment epithelium derived from human induced pluripotent stem cells

Kir7.1 regulates RPE alignment and phagocytosis

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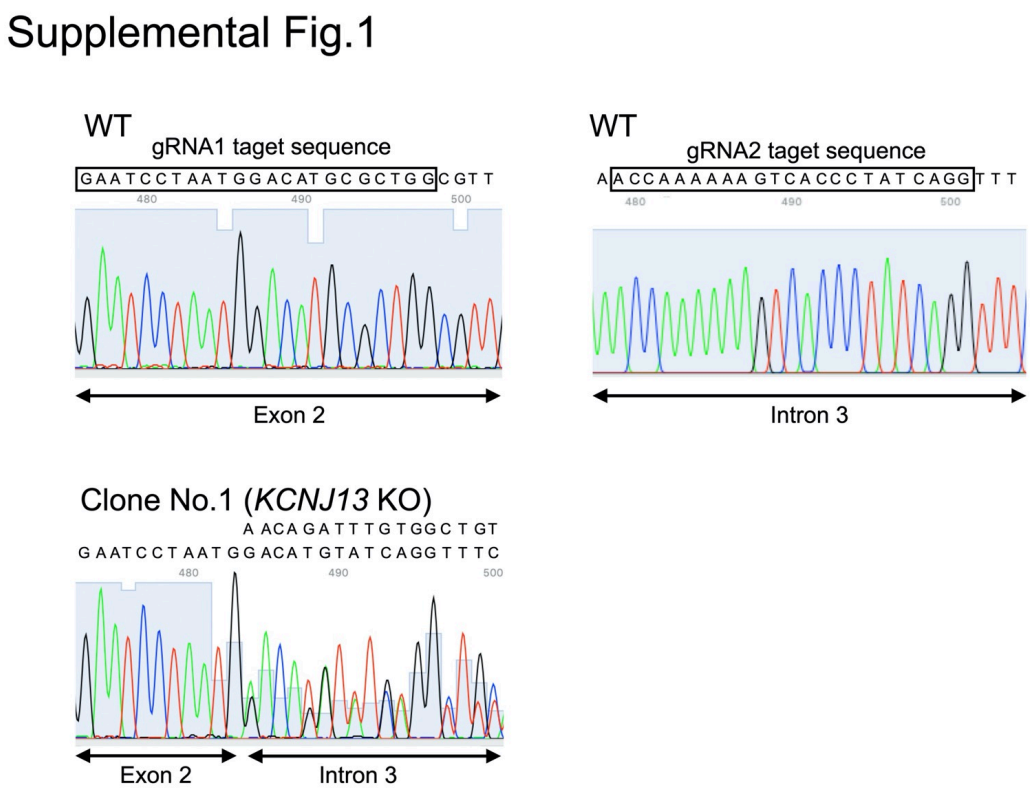
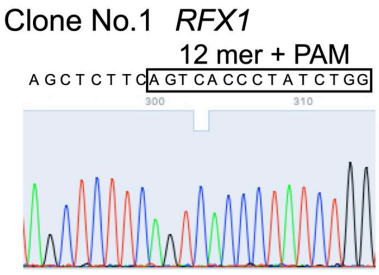
Supplementary Figures



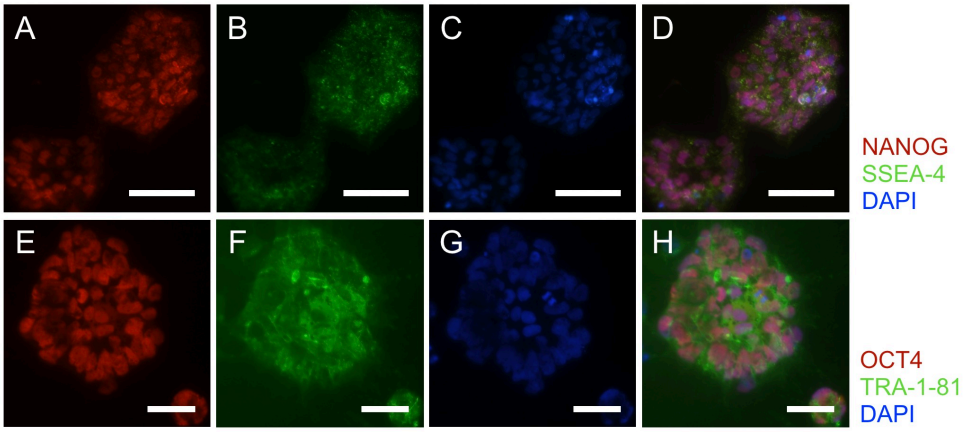
Supplementary Figure S1. Genomic DNA was extracted from WT and clone No.1 iPSCs and subjected to PCR. Sequence analysis of the obtained PCR products was performed. We confirmed that the *KCNJ13* gene was knocked out in the clone No. 1 iPSCs.

Supplemental Fig.2



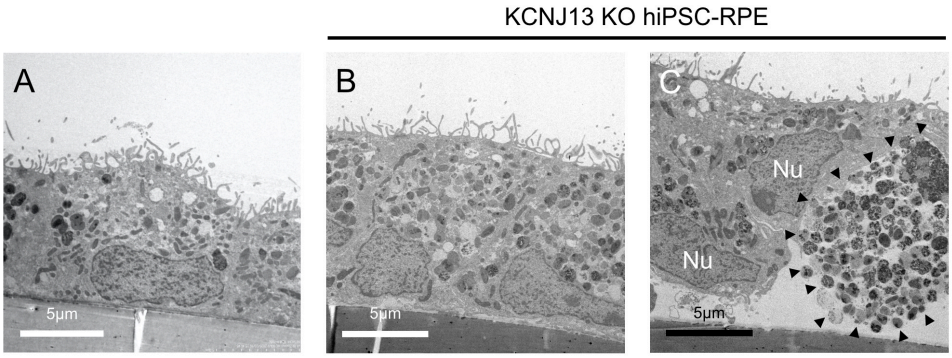
Supplementary Figure S2. The absence of off-target mutations by *KCNJ13* gRNAs was confirmed by DNA sequencing. Candidate off-target sites include the *RFX1* gene, which has a sequence in accordance with 12 mer + PAM of *KCNJ13* gRNA1 target sequence. We confirmed that no gene editing occurred in the *RFX1* gene in all the *KCNJ13*-edited iPSCs.

Supplemental Fig.3



Supplementary Figure S3. Expression of undifferentiation markers in the *KCNJ13*-KO iPSCs. Immunofluorescence images for NANOG (**A**), SSEA-4 (**B**), OCT4 (**E**), and TRA-1-81 (**F**) are shown. (**C, G**) Nuclei are stained with DAPI. (**D, H**) Merged views. Scale bar = 100 μm (**A-D**), 50 μm (**E-H**).

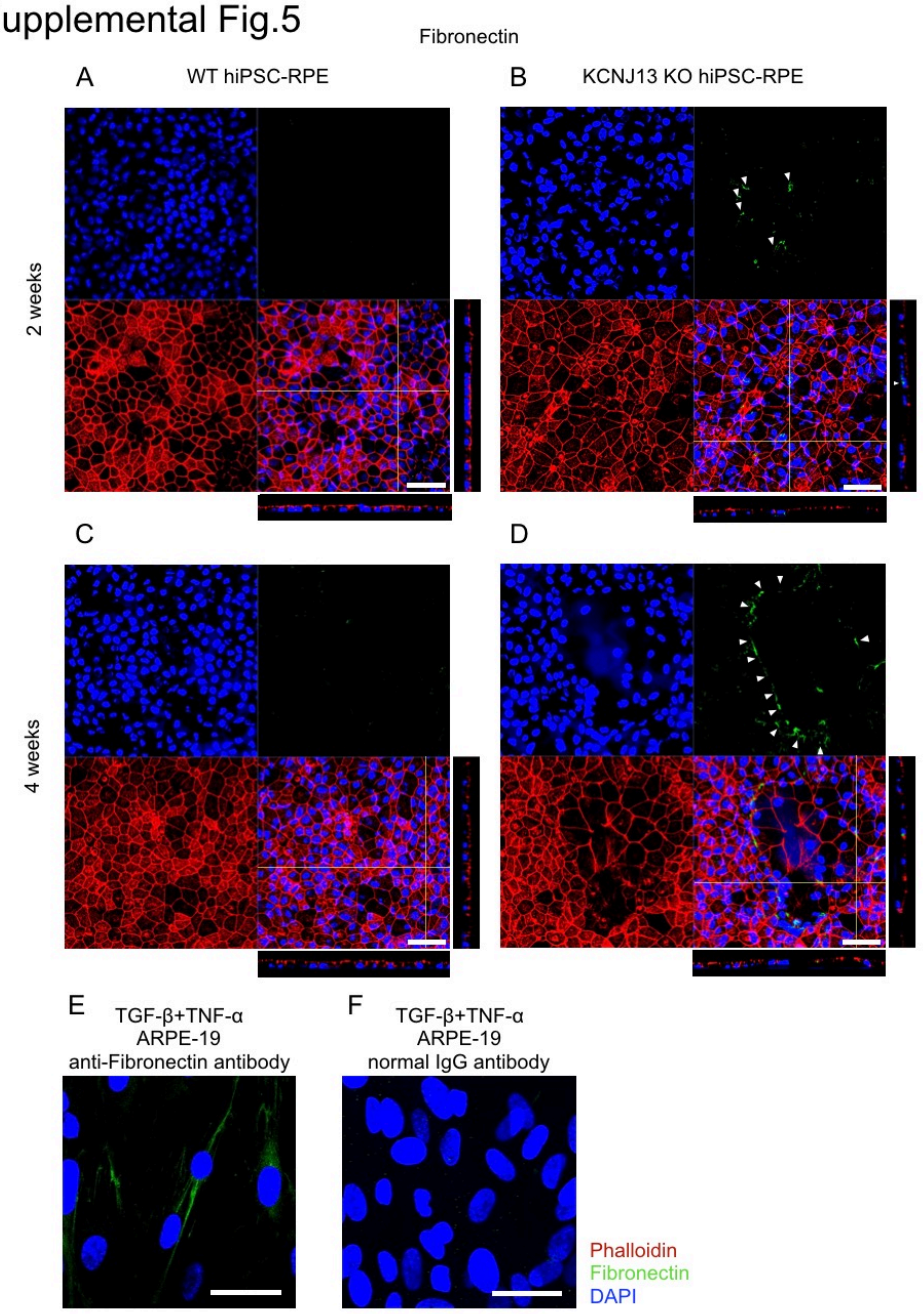
Supplemental Fig.4



Supplementary Figure S4.

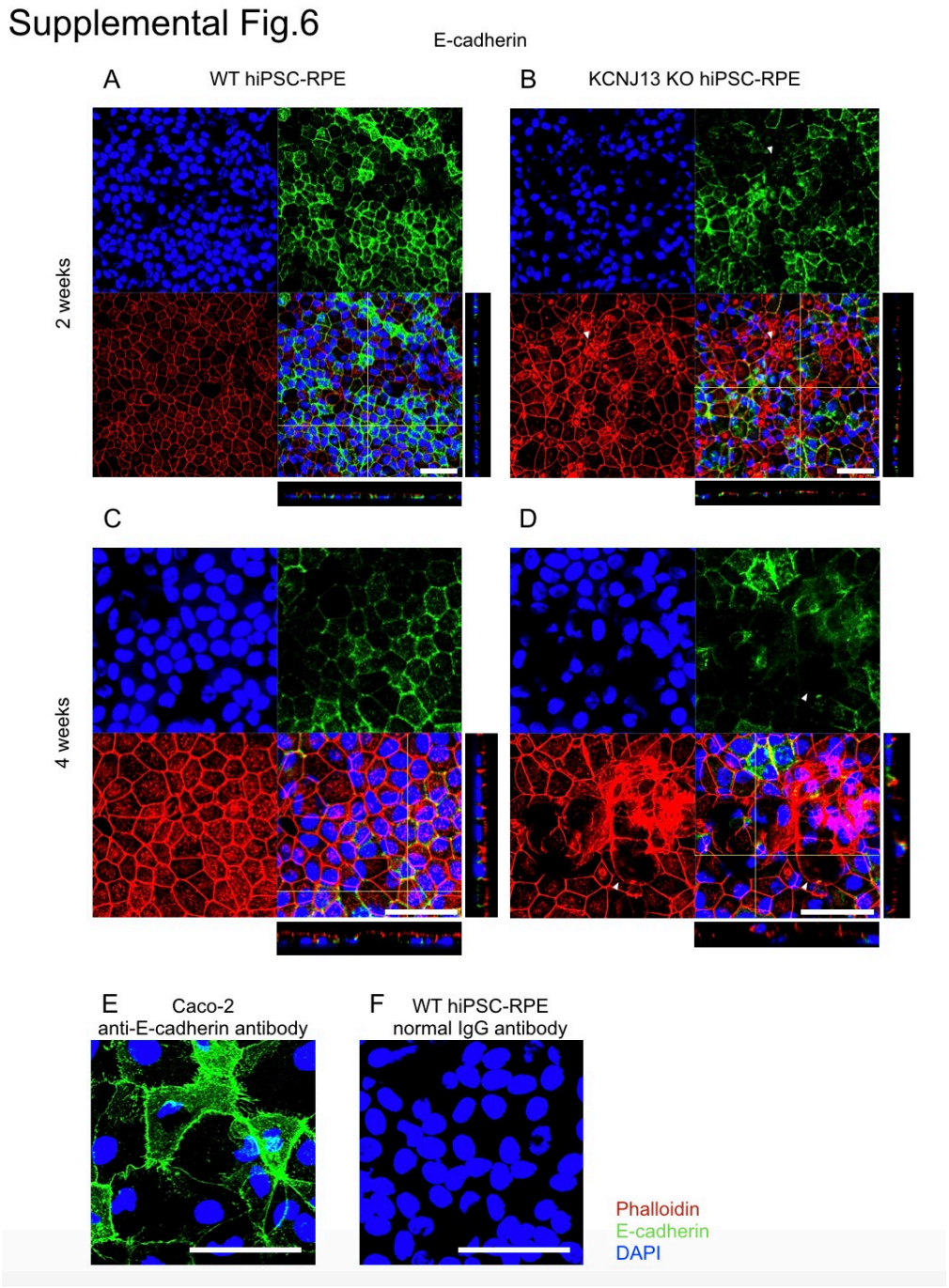
Transmission electron microscopic images of WT (**A**) and *KCNJ13* KO (**B, C**) hiPSC-RPE cells. (**A**) Monolayer of WT hiPSC-RPE cells with apical microvilli. (**B**) These *KCNJ13*-KO hiPSC-RPE cells are also single-layered and have apical microvilli. (**C**) Double-layered *KCNJ13*-KO hiPSC-RPE cells and an exploded cell with debris. Cell contents are released basolaterally (arrowheads) to the living RPE cells. Nu, nucleus.

Supplemental Fig.5



Supplementary Figure S5.

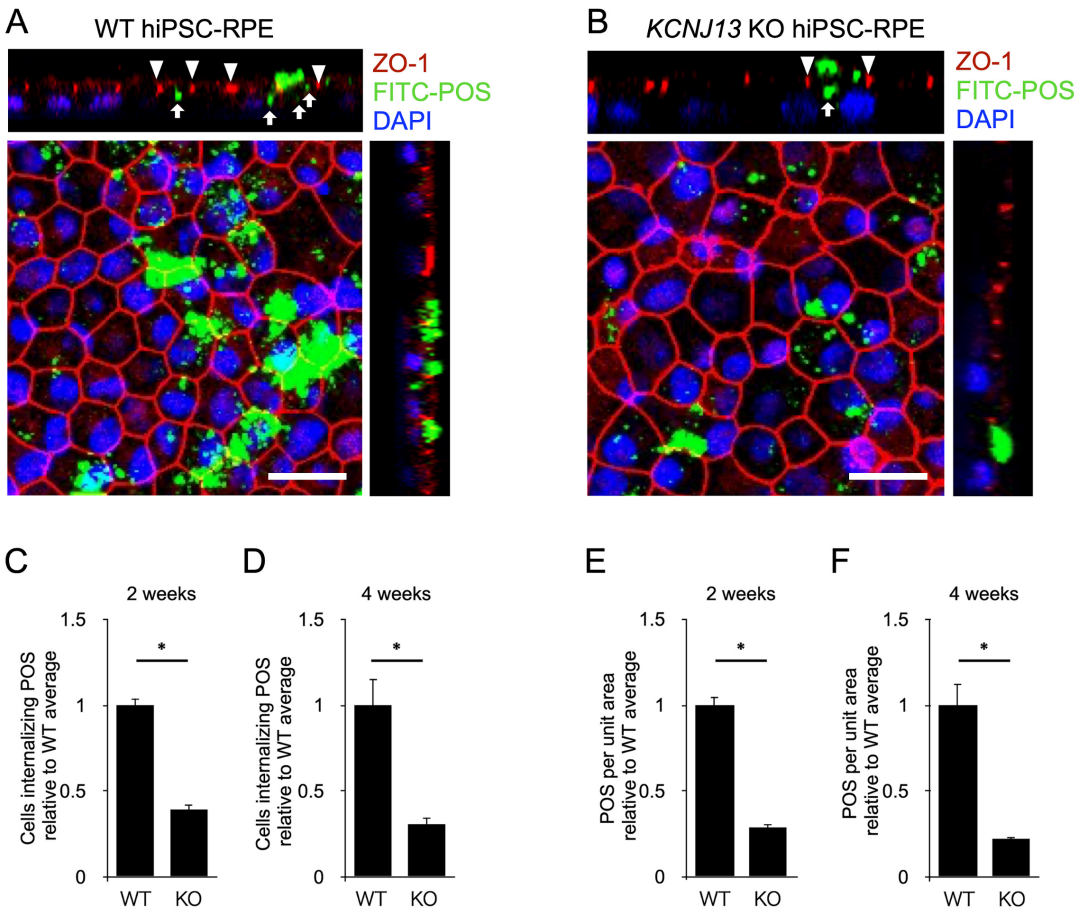
Immunofluorescence of Fibronectin as a mesenchymal marker. Fibronectin is not detected in the WT hiPSC-RPE cells at 2 weeks (**A**) and 4 weeks (**C**) after seeding. Fibronectin is detected in the *KCNJ13*-KO hiPSC-RPE cells at 2 weeks (**B**) and 4 weeks (**D**) after seeding (arrowheads). (**E**) Localization of Fibronectin in ARPE-19 cells supplemented with TGF-β and TNF-α, shown as a positive control. Culture of ARPE-19 cells was performed according to Matoba et al[19]. (**F**) Negative control for immunofluorescence. Scale bar = 50 μm.



Supplementary Figure S6.

Immunofluorescence of E-cadherin as an epithelial marker. E-cadherin is detected in the WT hiPSC-RPE cells at 2 weeks (**A**) and 4 weeks (**C**) after seeding. E-cadherin is detected in the *KCNJ13*-KO hiPSC-RPE cells at 2 weeks (**B**) and 4 weeks (**D**) after seeding. (**E**) Localization of E-cadherin in Caco-2 cells is shown as a positive control. Culture of Caco-2 cells was performed according to Fujita et al[25]. (**F**) Negative control for immunofluorescence. Scale bar = 50 μm.

Supplemental Fig.7



Supplementary Figure S7.

Phagocytic activity of WT and *KCNJ13*-KO hiPSC-RPE cells. (**A**) Confocal microscope images of WT hiPSC-RPE 4 weeks after seeding. The Z-stack image shows that FITC-POS (green, arrows) are internalized below ZO-1 (red, arrowheads). (**B**) Confocal microscope images of *KCNJ13*-KO hiPSC-RPE 4 weeks after seeding. The Z-stack image shows that FITC-POS are not taken up into cells below ZO-1 (arrowheads). (**C, D**) The ratio of cells phagocytosing FITC-POS relative to WT cells in 2 (**C**) or 4 (**D**) weeks after seeding (2 weeks, WT: 1.0 ± 0.037, KO: 0.39 ± 0.029, $p < 0.001$; 4 weeks, WT: 1.0 ± 0.15, KO: 0.31 ± 0.033, $p < 0.001$) (WT and KO, n = 6 for each). (**E, F**) The number FITC-POS per area relative to WT cells in 2 (**E**) or 4 (**F**) weeks (2 weeks, WT: 1.0 ± 0.049, KO: 0.29 ± 0.018, $p < 0.001$; 4 weeks, WT: 1.0 ± 0.13, KO: 0.23 ± 0.012, $p < 0.001$ (WT and KO, n = 6 for each). Data are shown as mean ± SE. Scale bar = 20 μm.

Supplementary Methods

Culture of human iPS cells and differentiation into RPE

Undifferentiated hiPSCs were maintained on mouse embryo fibroblast (MEF) feeder cells in Primate ES medium (ReproCELL, Yokohama, Japan) supplemented with 5 ng/ml basic fibroblast growth factor (βFGF; ReproCELL) and fresh medium was added daily. hiPSCs were passaged in small clumps after treatment with dissociation solution for iPSCs and replaced onto MEFs every 7 days. To directly differentiate into RPE cells, hiPSCs were cultured on gelatin-coated dishes in differentiation medium (Glasgow minimal essential medium [GMEM]; Thermo Fisher Scientific) supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol and 20% KnockOut^{TM} Serum Replacement (KSR) (Thermo Fisher Scientific) for 4 days, GMEM and 15% KSR for 6 days, and GMEM and 10% KSR for 18 days. At 4 weeks, pigmented cells with a typical RPE cobblestone appearance appeared focally and the differentiation medium was switched to serum-free RPE medium (SFRM) containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) [7:3] supplemented with GIBCO^{TM} B-27^{TM} Supplement (Thermo Fisher Scientific) and 2 mM L-glutamine for 7 days. Pure populations of pigmented cells were obtained by transferring pigmented colonies to SFRM supplemented with 10 ng/ml βFGF and 0.5 μM SB431542 (Cat. no. S4317; Sigma-Aldrich, St. Louis, MO). The differentiation medium was changed every three days.

Gene editing of hiPSCs via CRISPR-Cas9 system

Undifferentiated hiPSCs were cultured in feeder-free conditions. One hour before electroporation, Y-27632 was added to the medium at a final concentration of 10 μM. After washing in PBS, hiPSCs were dissociated with 0.25% trypsin. Then the medium was added and the cells were suspended into single cells. After centrifugation (120 *g*, 5 min) at room temperature, the supernatant was removed, the medium was added, and the cells were re-suspended. After a second centrifugation, the number of cells were counted and 1.0×10^6 cells were prepared in Opti-MEM, and subsequently transferred to a cuvette for electroporation. One μg of Cas9 protein (IDT) and 0.5 μg of each gRNA were added to the cells. Electroporation was performed by using a NEPA 21 electroporator (Nepa Gene, Ichikawa, Japan) under the conditions described by Li et al[18] (poring pulse: pulse voltage, 125 V; pulse width, 5 ms; pulse number, 2). After electroporation, the cells were placed onto the feeder-free plate containing medium with Y-27632 (10 μM) and subsequently passaged. The gene-edited iPSCs were cloned according to Park et al.[17]: Approximately 100 cells were cultured and each single cell was marked; when the diameter of colonies grown from marked

cells was diameter of 300–400 μm, each colony was picked with a 1,000 μl micro-pipette tip. The isolated colonies were seeded onto a 3.5 cm-dish, expanded for DNA extraction, and genomic DNA was isolated for PCR analysis. After confirmation of targeted gene editing by sequencing (as described below), the cells were cultured on feeder cells.

Sequencing analysis

To confirm whether the *KCNJ13* gene was precisely edited in the target sites, the genomic DNA was examined by PCR. Genomic DNA from gene-edited hiPSCs was extracted with a commercial kit (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany) following manufacturer's protocol, and quantified using a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific). Eighty to 100 ng of isolated DNA were used for PCR analysis using primers shown in Table 1. By using a thermal cycler (T100; Bio-Rad, Hercules, CA), PCR was performed in the following conditions: denature at 98 °C for 10 sec, annealing at 55 °C for 5 sec, extension at 72 °C for 40 sec, 40 cycles). PCR products were resolved in 1% agarose gels in 1X TAE buffer (40 mM tris-acetate, 1 mM EDTA) with 0.025% ethidium bromide. DNA was visualized by a UV lamp and imaged using an electrophoresis imaging system (AE-6932GXES; ATTO, Tokyo, Japan). The DNA bands were cut and purified by Monarch DNA Gel Extraction Kit (New England Biolabs, Ipswich, MA) and directly sequenced. Also, the PCR products were subcloned into a plasmid, after transformation, colony PCR was performed using the resultant bacterial colonies, and processed for Sanger sequencing. Candidate off-target sites were similarly examined to confirm that no off-target editing occurred (Supplementary Figure 2).

Immunofluorescence

Briefly, after removing the medium, cells were fixed in 4% paraformaldehyde (PFA) for 30 min or 10min and washed with phosphate-buffered saline (PBS) for 5 min, three times. Cells were permeabilized with 0.3% Triton X-100 for 30 min. After washing with PBS for 5 min, three times, cells were treated with 1% bovine serum albumin (BSA) with or without 5% goat serum for 60 min, and incubated with primary antibodies at 4 °C overnight. Primary antibodies used in this study were as follows: anti-NANOG (diluted at 1:200; CST, Waltham, MA), anti-SSEA-4 (1:500; CST), anti-OCT4 (1:200; CST), anti-TRA-1-81(1:250; CST), anti-Kir7.1 (1:50, Cat. no. sc-398810; Santa Cruz Biotechnology, Dallas, TX), anti-ZO-1 (1:50; Thermo Fischer Scientific, Waltham, MA), anti-RPE65 (1:100; Abcam, Cambridge, UK), anti-ZO-1 (1:50; Thermo Fischer Scientific, Waltham, MA), anti-fibronectin (1: 3,000; Cat. no. ab2413, Abcam), anti-E-cadherin (1:100; Cat. no. 24E10, CST), anti-ezrin (1:500; Cat. no. E8897, Sigma-Aldrich, St. Louis, MO, USA) and normal rabbit IgG, Wako Pure Chemical Industries, Osaka,

Japan). After washing with PBS for 5 min, three times, cells were incubated with secondary antibodies for 30 min. Secondary antibodies used in this study were Alexa Fluor 488-conjugated goat anti-rabbit IgG (1: 1,000, Cat. no. A-11034; Thermo Fisher Scientific), Alexa Fluor 488-conjugated goat anti-mouse IgG (1: 1,000, Cat. no. A-11029; Thermo Fisher Scientific), and Alexa Fluor 568-conjugated goat anti-rabbit IgG (1: 1,000, Cat. no. A-11011; Thermo Fisher Scientific). After washing with PBS for 5 min, three times, cells were stained with DAPI for 15 min, with or without phalloidin for 45 min.