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1 **Research paper**

2

3 **A TALEN-mediated, p63 knock-in application in human induced**  
4 **pluripotent stem cells**

5

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7

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18

19 **Highlights**

- 20 ● A p63 knock-in cell line in human iPS cells was generated by TALEN gene  
21 editing.

- 1 ● Visualizing p63+ cells facilitated to trace the epithelial differentiation.
- 2 ● Isolating p63+ cells during differentiation enabled epithelial stem cell research.
- 3 ● p63 knock-in human iPS cells apply for the studies of epithelial cell lineages.

4

## 5 **Keywords**

6 iPS cells, TALEN, Knock-in, p63, stem cells, epithelial stem cells

## 7 **Abstract**

8 The p63 is a biomarker whose expression in surface ectodermal cells during  
9 developmental processes of the cornea, skin, oral mucosa, and olfactory  
10 placodes and is indispensable for understanding the mechanisms of self-renewal  
11 and maintenance of epithelial stemness. Here, we generated a p63 knock-in (KI)  
12 human induced pluripotent stem (hiPS) cell line by using TALEN, where p63  
13 expression was visualized via enhanced green fluorescent protein expression.  
14 The established cells maintained pluripotency, and were found to be specifically  
15 targeted at the stem cell marker gene,  $\Delta Np63\alpha$ . The KI-hiPS cells successfully  
16 differentiated into corneal epithelial cells, and the functional epithelial stem cells  
17 through p63 expression were used to evaluate reconstruction of the corneal  
18 epithelium. This novel approach enables the tracing of cell lineages expressing

1 p63 from the early differentiation stage throughout epithelial development.  
2 Therefore, our data indicated that the p63-hiPS KI cells can be used as an  
3 effective tool extending beyond studies of epithelial cell lineages.

4

## 5 **1. Introduction**

6 p63 is expressed at surface ectodermal cells beginning in early development to  
7 facilitate formation of the epithelium, and to eventually maintain a stratified  
8 epithelial structure (Barbieri et al. 2006; Senoo et al. 2007; Melino et al. 2015) in  
9 the cornea, skin, oral mucosa, and olfactory placodes. p63 has two major  
10 isoforms, namely the transactivating (TAp63) and N-terminally truncated  
11 ( $\Delta$ Np63) isoforms, each of which is driven by a different promoter and is  
12 alternatively spliced at the C-terminus to generate the alpha, beta, and gamma  
13 forms (Figure 1A). Of these,  $\Delta$ Np63 $\alpha$  is a well-established as a marker of  
14 epithelial stem cells, and is abundantly expressed in the basal layers of the  
15 corneal limbus and epidermis, where stem cells with high proliferative capability  
16 are present (Barbieri et al. 2006; Kawasaki et al. 2006; Robertson et al. 2008).

17 Genome editing technology is sufficiently advanced as to potentially  
18 enable genetic modification for gene therapy, stem cell-based therapy, and

1 research into the biological mechanisms of intractable diseases (Byrne et al.  
2 2014). These technologies are based on zinc-finger nucleases (ZFNs) and  
3 transcription activator-like effector nucleases (TALENs) (Ochiai et al. 2010;  
4 Hockemeyer et al. 2011), which are engineered molecular scissors consisting of  
5 a site-specific DNA-binding domain fused to the non-specific nuclease domain  
6 of the restriction enzyme FokI. The clustered regularly interspaced short  
7 palindromic repeats (CRISPR) / Cas9 nuclease cleaves double-stranded DNA  
8 with the help of the RNA-guided Cas9 nuclease (Cong et al. 2013; Doudna and  
9 Charpentier. 2014). We have now used the TALEN system, which offers high  
10 cleavage efficiency and specificity (Guilinger et al. 2014) and infrequent off-  
11 target events (Gabriel et al. 2011; Ding et al. 2013; Yusa 2013), to establish a  
12 p63 knock-in (KI) human induced pluripotent (hiPS) cell line. These cells  
13 enable, for the first time, tracking of corneal epithelial differentiation *in vitro*.  
14 Several reports have shown that gene edited KI-hiPS cells were terminally  
15 differentiated (Cerbini et al. 2015; Wu et al. 2016), however, tracking epithelial  
16 stem cell markers using KI cell lines has not been previously reported.

17       Generation of the self-formed ectodermal autonomous multi-zone  
18 (SEAM), as achieved by Hayashi et al. (2016), demonstrated corneal epithelial

1 induction from hiPS cells. The ocular surface ectoderm generated by SEAM  
2 formation may potentially approach medical treatment for recovering corneal  
3 functions. The SEAM structure perfectly mimicked whole-eye development, and  
4 ocular surface ectodermal cells appeared in specific zones within 2–3 weeks,  
5 although the developmental timing and other mechanistic details remained  
6 unclear. The purpose of this study was to identify epithelial lineages by tracking  
7 p63 expression via an enhanced green fluorescence protein (EGFP) - fusion  
8 protein during differentiation. p63 represents an attractive candidate protein for  
9 elucidating the developmental mechanism of human epithelial stem cells, as it is  
10 expressed from the early developmental stage. We used this strategy to  
11 establish, for the first time, p63 KI-hiPS cells that enable tracing of epithelial  
12 development *in vitro*.

13       The use of TALEN technology facilitated the production of p63 KI-hiPS  
14 cells, which served as a robust and versatile tool for investigating cell  
15 differentiation and for applications in corneal epithelial development.

16

## 17 **2. Materials and methods**

### 18 *2.1 hiPS cell culture*

1 The human iPS cell line 1383D2 was provided by the Center for iPS Cell  
2 Research and Application (CiRA), Kyoto University. The cells were grown in  
3 StemFit medium (Ajinomoto, Tokyo, Japan) on dishes coated with Laminin  
4 511E8 (nippi iMatrix-511, Wako), and passaged every 7 days, with a medium  
5 change performed every 2 days (Nakagawa et al. 2014). All experimental  
6 procedures using recombinant DNA were approved by the Recombinant DNA  
7 Committee of Osaka University.

8

## 9 *2.2 Construction of the donor vector and TALEN plasmids*

10 The donor vector was constructed such that a P2A-EGFP-neomycin cassette  
11 was flanked by human p63 $\alpha$  homology arms (Fasmac, Kanagawa, Japan). A  
12 pair of hp63 $\alpha$  TALENs was synthesized in GeneART® Precision TALs (Thermo  
13 Fisher Scientific, MA) and subcloned into the pEF5/FRT/V5-DEST vector  
14 (Thermo Fisher Scientific) using Gateway LR Clonase II (Thermo Fisher  
15 Scientific). Recombinant DNA and genome-editing protocols were approved by  
16 the research ethics committee of Osaka University.

17

## 18 *2.3 Cleavage efficiency of p63 TALENs*

1 The TALEN plasmids were evaluated by performing an SSA assay (Sakuma et  
2 al. 2013). The pGL4-SSA reporter vector, containing an overlapped homologous  
3 luciferase gene was used to construct the pGL4-SSA-hp63 $\alpha$  vector. As a  
4 positive control, pGL-SSA-HPRT1 was used with either HPRT1 TALENs or  
5 HPRT1-NC TALENs (kindly provided by T. Yamamoto, Hiroshima University).  
6 The TALEN plasmids, the pGL4-SSA reporter vector and the pRL-CMV  
7 reference vector (Promega, Madison, WI) were transfected into human  
8 embryonic kidney HEK293T cells by using Lipofectamine LTX (Thermo Fisher  
9 Scientific) in a 96-well plate. Luciferase activity was measured in a multilabel  
10 counter (ARVO MX, Perkin Elmer, MA) at 24 h post-transfection, using the  
11 Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's  
12 instructions.

13

#### 14 *2.4 Establishment of hp63 KI hiPS cells*

15 Y-27632 (10  $\mu$ M, Wako) was added to 1383D2 cells 3 h prior to electroporation.  
16 Electroporation conditions for 1383D2 cells were first optimized from 7 different  
17 protocols, using the pmax GFP plasmid as the test transgene. The most  
18 efficient protocol was selected based on the transfection efficiency. The donor



1 vector (5  $\mu$ g) and TALEN plasmids (2.5  $\mu$ g each) were mixed in Nucleofector  
2 solution P3 with supplement (4D-Nucleofector X Kit L, Lonza) and  
3 electroporated into  $1 \times 10^6$  cells using the CB150 run program, according to the  
4 manufacturer's recommended protocol. Cells were then plated on dishes coated  
5 with Laminin 511E8 and cultured in Stem Fit medium containing 10  $\mu$ M Y-  
6 27632. The medium was replaced on the following day with fresh StemFit™  
7 medium containing 40  $\mu$ g/mL neomycin. The optimal antibiotic concentration for  
8 clone selection was then determined by exposing untransfected cells to 0–100  
9  $\mu$ g/mL neomycin 1 day after passage, and by selecting the minimum  
10 concentration that killed all cells in 7 days. After 12 to 14 days, neomycin-  
11 resistant colonies were trypsinized, and seeded at 250 to 750 cells per 10-cm  
12 dish, pre-coated with Laminin 511-E8. Subsequently, individual colonies were  
13 picked and expanded in 12-well plates after 14 to 16 days. Genomic DNA from  
14 individual clones was extracted using the NucleoSpin® Tissue (Takara, Japan)  
15 and genotyped by PCR (T100 Thermal cycler, Bio-Rad, CA), using the primers  
16 hp63-external-F (5'-TGAGGATGCCCTAAGTCCCT-3' and hp63-external-R (5'-  
17 AATCACCCCAACGTGAACCA-3'). Targets were amplified by performing a hot  
18 start at 98°C for 2 min and 35 cycles of denaturation at 98°C for 10 s,

1 annealing at 66°C for 30 s, and extension at 72°C for 4 min and 45 s, followed  
2 by final extension at 72°C for 10 min. PCR products were analyzed by  
3 electrophoresis on 0.8% agarose gels. Fragments of apparent size of 4,247 bp  
4 (KI) and 1,604 bp (WT) were sequenced, using an Applied Biosystems 3100  
5 Genetic Analyzer (Thermo Fisher Scientific). Clones were genotyped as wild  
6 type (WT), heterozygous (WT/KI), and homozygous (KI/KI). Only a homozygous  
7 KI clone was used for further analysis, with a WT clone used as a control.  
8 Frozen stocks were prepared in STEM-CELLBANKER cryoprotectant (Nippon  
9 Zenyaku Kogyo, Japan) at  $2 \times 10^5$  live cells per tube.

10

### 11 *2.5 Western blot analysis of 2A peptide cleavage*

12 p63 KI-hiPS cells were harvested at 10 weeks of differentiation and analyzed by  
13 western blotting to assess 2A peptide cleavage between endogenous hp63 $\alpha$   
14 and the EGFP insert. After cells were lysed in RIPA lysis buffer (Nacalai, Japan),  
15 20  $\mu$ g total protein was loaded on 4–12% Tris-Glycine SDS-PAGE gels, which  
16 were then blotted and probed with monoclonal antibodies against p63 (4A4,  
17 Santa Cruz, CA) and GAPDH (6C5, Santa Cruz), or with a polyclonal antibody  
18 against GFP (FL, Santa Cruz).

1

2 *2.6 Differentiation of hiPS cells into ocular ectodermal cells*

3 hiPS cells were induced to differentiate into ocular ectodermal cells, as  
4 previously described (Hayashi et al. 2016). Briefly, cells were seeded in dishes  
5 pre-coated with Laminin 511E8, and grown in StemFit™ medium for 8 to 12  
6 days, starting on day 0. For ocular differentiation, the medium was then  
7 replaced with differentiation medium (DM) based on Glasgow-MEM (Thermo  
8 Fisher Scientific) supplemented with 10% knockout serum replacement  
9 (Thermo Fisher Scientific), 1 mM sodium pyruvate (Thermo Fisher Scientific),  
10 0.1 mM non-essential amino acids (Thermo Fisher Scientific), 2 mM L-  
11 glutamine (Thermo Fisher Scientific), 1% penicillin-streptomycin, and 55 µM 2-  
12 mercaptoethanol (Thermo Fisher Scientific) or monothioglycerol (Wako Osaka,  
13 Japan). After 4 weeks, cells were cultured for another 4 weeks in CDM (diluted  
14 1:1 in DM and Cnt-PR (without EGF and FGF2) (CELLnTEC Advanced Cell  
15 Systems, Bern, Switzerland) supplemented 20 ng/mL KGF (Wako), 10 µM Y-  
16 27632 (Wako), and 1% penicillin-streptomycin. After differentiation for 6 - 8  
17 weeks, non-epithelial cells were removed by manual pipetting under a  
18 microscope. To further differentiate cells into corneal epithelium, they were then

1 cultured for an additional 4 weeks in corneal epithelium maintenance medium  
2 (CEM; DMEM/F12 [2:1]), Thermo Fisher Scientific) containing 2% B27  
3 supplement (Thermo Fisher Scientific), 20 ng/ml KGF, 10  $\mu$ M Y-27632, and 1%  
4 penicillin-streptomycin.

5

## 6 *2.7 Flow cytometry and cell sorting*

7 KI-hiPS cells were differentiated for 10–12 weeks, dissociated with Accutase  
8 (Thermo Fisher Scientific) for 60 to 90 min at 37°C, harvested through a 40- $\mu$ m  
9 cell strainer (BD Biosciences, San Diego, CA), and collected into corneal  
10 epithelium maturation medium (KCM medium, a 3:1 mix of DMEM without  
11 glutamine and Nutrient Mixture F-12 Ham; Thermo Fisher Scientific)  
12 supplemented with 5% fetal bovine serum (Japan Bio Serum, Hiroshima,  
13 Japan), 0.4  $\mu$ g/mL hydrocortisone succinate (Wako), 2 nM 3,3',5-triiodo-l-  
14 thyronine sodium salt (MP Biomedicals, Santa Ana, CA), 1 nM cholera toxin  
15 (List Biological Laboratory, Campbell, CA), 2.25  $\mu$ g/mL bovine transferrin HOLO  
16 form (Thermo Fisher Scientific), 2 mM L-glutamine, 0.5% insulin transferrin  
17 selenium (Thermo Fisher Scientific), and 1% penicillin-streptomycin). Cells were  
18 washed in phosphate-buffered saline, and stained with antibodies against

1 SSEA-4 (MC-81370; BioLegend) and ITGB-4 (CD104; 58XB4, BioLegend) as  
2 described (Hayashi et al. 2016). Isotype-matched IgG and IgM antibodies were  
3 used as negative controls. Cells were sorted on a SH800 Cell Sorter (Sony  
4 Biotechnology Inc., Japan), and the data were analyzed using SH800 and  
5 FlowJo software (TreeStar, San Carlos, CA). Cell expressing EGFP, SSEA-4,  
6 and ITGB-4 (hiCECs) were seeded in a temperature responsive-dish (CellSeed,  
7 Japan), and cultured to sub-confluency in CEM. To enhance stratification, cells  
8 were grown in KCM medium supplemented with 20 ng/mL KGF and 10  $\mu$ M Y-  
9 27632. Cells were cultured for a total of 16–18 days, and the hiCEC sheet was  
10 harvested by cooling to below 32°C for 30 min (Hayashi et al. 2010).

11

## 12 *2.8 Cytospin experiments*

13 For immunofluorescence staining, sorted cells were adjusted to a density of  $2 \times$   
14  $10^5$  cells/mL, and 200  $\mu$ L of the cell suspension was centrifuged at 1,000 rpm  
15 for 5 min in a Cytospin™ 4 Cyto centrifuge (Thermo Fisher Scientific).

16

## 17 *2.9 Immunofluorescence staining*

18 Cell sheets were embedded in Tissue-Tek® Optimal Cutting Temperature

1 compound (Sakura Fineteck, Japan) and sectioned at 10  $\mu\text{m}$ . To assess  
2 pluripotency and visualize p63 KI-hiPS cells, samples were fixed for 20 min at  
3 room temperature in 4% paraformaldehyde, and blocked for 1 h in Tris-buffered  
4 saline (TBS, Takara Bio) containing 5% donkey serum (Jackson  
5 ImmunoResearch Laboratories, Inc.) and 0.3% Triton X-100 (Wako). Samples  
6 were then probed overnight at 4°C with antibodies against NANOG (Abcam  
7 ab109250), OCT 3/4 (Abcam ab19857), SSEA-4 (Abcam ab16287) and TRA-1-  
8 60 (BD Pharmingen 560173) in TBS containing 1% donkey serum and 0.3%  
9 Triton X-100. Subsequently, samples were labeled for 1 h at room temperature  
10 with secondary antibodies conjugated to Alexa Fluor 488, 568, and 647  
11 (Thermo Fisher Scientific). Cells were counterstained with Hoechst 33342  
12 (Molecular Probes) to visualize the nuclei and imaged by fluorescence  
13 microscopy (Axio Observer. D1, Carl Zeiss).

14

### 15 *2.10 Hematoxylin and eosin staining*

16 hiCEC sheets derived from p63 KI-hiPS cells were fixed with 10% formaldehyde  
17 neutral buffer solution (Wako), sectioned in paraffin at 3  $\mu\text{m}$ , stained with  
18 hematoxylin and eosin following deparaffinization and hydration, and imaged

1 under a microscope (Axio Imager. A2, Carl Zeiss).

2

### 3 *2.11 Alkaline phosphatase staining*

4 Cells were stained for 10 min at room temperature with a chromogenic

5 substrate, which consisted of 4.5  $\mu$ L NBT (75 mg/mL) in 70%

6 dimethylformamide and 3.5  $\mu$ L BCIP (50 mg/mL) in 100% dimethylformamide,

7 diluted in 1 mL 100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 50 mM  $MgCl_2$ .

8 Staining was stopped by washing with phosphate-buffered saline, and cells

9 were fixed for 30 min at 4°C with 4% paraformaldehyde, air dried, and imaged

10 on an EVOS FL Auto imaging system (Thermo Fisher Scientific).

11

### 12 *2.12 qRT-PCR experiments*

13 Differentiated hiPS cells were harvested at specific time periods and lysed in

14 QIAzol reagent (Qiagen, Valencia, CA). Total RNA was extracted and reverse-

15 transcription was performed using the SuperScript III First-Strand Synthesis

16 System for qRT-PCR (Thermo Fisher Scientific), and synthesized cDNA was

17 amplified in duplicate on an ABI Prism 7500 Fast Sequence Detection System

18 (Thermo Fisher Scientific) using TaqMan Fast Universal PCR Master Mix

1 (Applied Biosystems) or SYBR Premix Dimer Eraser (Takara Bio). Targets were  
2 amplified with TaqMan MGB probes assays against  $\Delta$ Np63 (Hs00978339\_m1),  
3 p63 $\alpha$  (Hs00978338\_m1), TAp63 (Hs00978349\_m1), and GAPDH  
4 (HS99999905\_m1). EGFP was amplified using the EGFP-Fwd (5'-  
5 TATATCATGGCCGACAAGCA-3') and EGFP-Rev (5'-CTGGGTGCTCAGGTA  
6 GTGGT-3') primers and detected with SYBR Green. Thermocycling conditions  
7 for the TaqMan reactions consisted of an initial cycle at 95°C for 20 s, and 45  
8 cycles at 95°C for 3 s and 60°C for 30 s, while the SYBR green reactions  
9 consisted of an initial cycle at 95°C for 30 s, and 40 cycles at 95°C for 3 s, 65°C  
10 for 30 s, and 72°C for 30 s.

11

### 12 **3. Results**

#### 13 *3.1 Establishing p63 KI-hiPS cells*

14 A pair of TALENs was designed to target sites immediately upstream of the stop  
15 codon in the last exon (exon14) of p63 (Figure 1A, B). The cleavage efficiency,  
16 as measured by single-strand annealing (SSA) (Sakuma et al. 2013), was  
17 greater than that of the positive control HPRT1 and comparable to that of the  
18 positive control HPRT-NC, suggesting an adequate genome-cleavage event



1 occurred efficiently (Figure 1C). The most abundant isoform in corneal  
2 epithelium *in vivo* was detected  $\Delta Np63\alpha$  isoform by using a specific probe for  
3  $\Delta Np63\alpha$  and TAp63 $\alpha$  isoform only (Figure 1D) (Di Iorio et al. 2005; Krishnan et  
4 al. 2010). Therefore, the levels of  $p63\alpha$  were essentially the same as that of  
5  $\Delta Np63\alpha$  in corneal epithelium.

6 The donor vector, which encodes a p63-P2A peptide-EGFP-neomycin  
7 cassette, was electroporated with TALEN plasmids into 1383D2 hiPS cells (wild  
8 type ; WT) to insert the EGFP gene into the target region of p63 via homologous  
9 recombination. Transformants were selected on neomycin for 10–12 days and  
10 genotyped by PCR. Nine of 24 clones (37.5%) were heterozygous KIs, one of  
11 24 clones (4.2%) was a homozygous KI, and the remaining clones were either  
12 WT or were not successfully genotyped (Figure 1E, Table 1). The homozygous  
13 KI clone (clone #47), in which the p63-EGFP-neomycin cassette was knocked-  
14 in into both alleles, was verified by sequencing and expanded. In KI cells, p63  
15 was fused to EGFP via the self-cleaved 22 amino acid linker of the 2A peptide  
16 (Kim et al. 2011). Accordingly, p63 (63 kDa) fused to the 2A peptide was  
17 detectable by western blot analysis in these cells, along with free EGFP (27  
18 kDa, Figure 1F). Moreover, p63-2A-EGFP as an uncleaved band was not

1 detected. Collectively, these results indicated that the p63 TALENs were highly  
2 specific, that EGFP was integrated into the target site in p63, and that 2A  
3 peptide cleavage between p63 and EGFP was sufficiently efficient for detection  
4 purposes (Chan et al. 2011).

5

### 6 *3.2 Expression of pluripotent stem cell markers in p63 KI-hiPS cells*

7 Pluripotency was assessed by measuring alkaline phosphatase activity and by  
8 performing immunofluorescence staining. Both p63 KI-iPS cells and WT cells  
9 expressed alkaline phosphatase, suggestive of an undifferentiated state (Figure  
10 2A). In addition, the pluripotent stem cell markers OCT 3/4 and NANOG, as well  
11 as the cell surface markers SSEA-4 and tumor-related antigen (TRA) -1-60  
12 were all expressed in KI-hiPS cells (Figure 2B).

13

### 14 *3.3 Differentiation of p63 KI-hiPS cells into ocular surface ectodermal cells*

15 p63 KI-hiPS cells were formed within the 1<sup>st</sup> through 4<sup>th</sup> zones as the SEAM  
16 structure after 6 weeks of differentiation (Figure 3A). As assessed by  
17 immunofluorescence staining, p63 was clearly coexpressed with EGFP in  
18 epithelial cells in the 3<sup>rd</sup> and 4<sup>th</sup> zones (Figure 3B). The ocular cell marker PAX6

1 was also expressed in those zones after 6 weeks of differentiation (Figure 3C).

2 p63 expression was visualized via EGFP under a microscopy after 2  
3 weeks of differentiation. p63 expression was detectable via EGFP in an average  
4 of  $6.77 \pm 1.43\%$  cells at 2 weeks after differentiation ( $n = 17$ ) and in an  
5 average of  $2.30 \pm 1.00\%$  cells at 4 weeks ( $n = 8$ ). Between 7 and 8 weeks of  
6 differentiation, the 1<sup>st</sup> and 2<sup>nd</sup> zones in the SEAM were removed by manual  
7 pipetting to retain the epithelial cells only. After pipetting and culturing in corneal  
8 differentiation medium (CDM), the fraction of epithelial cells expressing p63  
9 then dramatically increased to an average of  $42.11 \pm 10.64\%$  at 10 weeks of  
10 differentiation ( $n = 7$ ) (Figure 4A).

11 Quantitative real-time reverse-transcriptase PCR (qRT-PCR) results  
12 confirmed that p63 expression correlated with *EGFP* expression in KI-hiPS  
13 cells. As in parental cells, the predominant isoform in KI-hiPS cells was  $\Delta Np63$ ,  
14 which showed the same expression as *p63 $\alpha$* , whereas *TAp63* was barely  
15 detected (Figure 4B).

#### 16 17 *3.4 Isolation of p63 KI-hiPS ocular surface ectodermal cells*

18 After culturing hiPS-derived ocular surface ectodermal cells in the 3<sup>rd</sup> and 4<sup>th</sup>

1 zones for 10–12 weeks, corneal epithelial cells were isolated by fluorescence-  
2 activated cell sorting (FACS) based on positive expression of p63 based on  
3 EGFP fluorescence and the corneal epithelial markers SSEA-4 and ITGB-4. On  
4 average,  $11.66 \pm 5.03\%$  of the cells were human iPS cell-derived corneal  
5 epithelial cells (hiCECs) ( $n = 7$ ) (Figure 5A). Immunostaining confirmed  
6 coexpression of p63 and EGFP (Figure 5B). The isolated hiCECs were further  
7 cultured for tissue reconstruction of a hiCEC-derived cell sheet. During  
8 culturing, EGFP-positive cells were observed by fluorescence microscopy  
9 (Figure 5C). FACS analysis indicated that 48.2% of differentiated cells  
10 expressed both p63 and ITGB-4, with 94.3% of ITGB-4<sup>+</sup> cells also expressing  
11 p63. Conversely, 97.4% of p63<sup>+</sup> cells also expressed ITGB-4 (Figure S1A),  
12 although sorting based on SSEA-4 and ITGB-4 indicated that 92.3% and 44.0%  
13 of cells expressing or strongly expressing SSEA-4 also expressed p63 (Figure  
14 S1B).

15

### 16 *3.5 Cell sheets derived from p63 KI-hiPS cells*

17 Cell sheets were easily detached without any damage by reducing the  
18 temperature from 37°C to 20°C (Figure 6A). The cell sheets consisted of tightly

1 packed cobblestone-like structures, as observed in the corneal epithelium *in*  
2 *vivo*, and were visualized as EGFP-positive cells (Figure 6B). Hematoxylin and  
3 eosin staining revealed that the sheet had 3–4 layers of stratification, similar to  
4 corneal epithelial cells *in vivo* (Figure 6C). For immunohistochemical staining,  
5 EGFP-positive cells were present, in which p63 was coexpressed. The major  
6 corneal epithelial markers keratin 3 (K3/K76), keratin 12 (K12), and PAX6 were  
7 clearly expressed, although the conjunctival epithelial marker keratin 13 (K13)  
8 was barely detectable. The ocular surface marker MUC16 and the stratified  
9 epithelial marker keratin 14 (K14) were also strongly expressed. However, the  
10 epidermal marker keratin 10 (K10) was not expressed. ZO-1, a marker of tight  
11 junctions mediating cell-cell adhesion, was detected, suggesting that the cell  
12 sheet was capable of a barrier function (Figure 6D).

13

#### 14 **4. Discussion**

15 In this study, a p63 KI-hiPS cell line was successfully established by using  
16 TALEN-mediated genome editing. We confirmed that *EGFP* was coexpressed  
17 with *p63* as designed, which enabled us to track p63 expression via EGFP  
18 fluorescence. Labeling hiPS and human embryonic stem cells in this manner

1 was previously used to track differentiated and pluripotent cells (Eiraku et al.  
2 2011; Nakano et al. 2012; Sekine et al. 2014).

3 hiPS cells have potential to be differentiated into any type of cells. To  
4 evaluate KI-hiPS cells, it was induced differentiation into epithelial according to  
5 a protocol described by Hayashi et al. (2016). After differentiation, we observed  
6 approximately 11.66% of hiCECs, a level similar to the 14.1% observed using  
7 WT hiPS cells (Hayashi et al. 2016). This result indicated that KI-hiPS cells  
8 showed comparable differentiation potential relative to corneal epithelial.

9 Despite technical challenges, we succeeded in genetically manipulating  
10 feeder-free hiPS cells, noting that both hiPS cells and hES cells (Byrne et al.  
11 2014) exhibited specific characteristics in terms of growth rates, timing of  
12 passaging, morphology, differentiation tendency, antibiotic resistance, and  
13 optimal electroporation conditions. Maintaining the quality of hiPS cell  
14 pluripotency is essential for further induction experiments.

15 Previously, TRA-1-60 was used to separate undifferentiated hiPS cells  
16 from corneal epithelial cells by FACS (Hayashi et al. 2016). In contrast, TRA-1-  
17 60 is dispensable for isolating epithelium derived from KI-hiPS cells, since  
18 differentiated cells are conveniently labeled with EGFP. Of note, FACS analysis

1 suggested that p63-expressing cells could serve as surrogate cells for ITGB-4  
2 positive cells. On the other hand, sorting based on SSEA-4 and ITGB-4 clearly  
3 indicated that p63 was expressed at different levels. As p63 tended to be  
4 expressed in immature epithelial cells, fractions enriched in cells strongly  
5 expressing SSEA-4 should also be enriched in mature corneal epithelial cells. In  
6 addition, we noted that most SSEA-4+ cells also expressed the corneal  
7 differentiation marker keratin 12 (Hayashi et al. 2016), indicating that SSEA-4 is  
8 useful as a corneal differentiation marker. Thus, selecting SSEA-4+ and p63+  
9 cells from immature epithelium was more accurate and selective for isolating  
10 corneal epithelial cells.

11       The eye is one of the 5 sensory organs, and is essential for vision.  
12 Corneal diseases such as limbal stem cell deficiency lead to blindness caused  
13 by the failure to maintain corneal transparency. Fortunately, such diseases are  
14 treatable, and vision can be restored as long as the retina and the optic nerve  
15 are functional. For example, cell sheets similar to the corneal epithelium and  
16 obtained hiPS cells can potentially be used in corneal transplants, although the  
17 fate of an iPS-derived graft after transplantation is largely unknown at present.  
18 By transplanting EGFP-labeled cell sheets to animal models, recovery can be

1 tracked long-term *in vivo* and *ex vivo*. Indeed, we anticipate that p63 KI-hiPS  
2 cells may prove useful in developing novel therapies such as ocular surface  
3 regeneration for translational research.

4 This is the first report describing the isolation of epithelial stem cells from  
5 differentiated hiPS cells. These stem cells formed functional tissues and have  
6 significant potential for further analysis of epithelial stem cell functions, epithelial  
7 development, and experimental transplantation. In particular, p63 KI-hiPS cells  
8 may enable further investigation of corneal epithelial differentiation, analysis of  
9 epithelial cell lineages, and identification of unknown p63 targets, since p63 is  
10 abundantly expressed during ectodermal and epidermal development, and in  
11 maintaining proliferative stem cells. For example, these cells may clarify how  
12 p63 integrates multiple signaling pathways such as those regulated by BMP,  
13 Wnt and TGF $\beta$  (Aberdam et al. 2007; Yoh and Prywes 2015; Zhang et al. 2015),  
14 in order to facilitate commitment and self-renewal during development.

15

#### 16 **Author contributions**

17 Y.K. performed the experiments and acquired the data. Y.K. and R.H. analyzed  
18 the data. R.H. and K.N. designed the experiments. Y.K. A.J.Q and R.H. wrote



1 the paper. K.N. obtained financial support.

2

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10

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1

2 **Figure legends**

3 **Figure 1.** Generation of p63 KI-hiPS cells using TALEN-mediated genome  
4 editing. (A) Structure of the human p63 gene and TALEN recognition sites. p63  
5 consists of an N-terminal transactivation domain (TA), a DNA-binding domain, a  
6 C-terminal oligomerization domain (Oligo), and other C-terminal domains  
7 including a second transactivation domain (TA), a sterile alpha motif domain  
8 (SAM), and a transactivation inhibitory domain (TID). Alternative splicing at the  
9 C-terminus generates the  $\alpha$ ,  $\beta$ , and  $\gamma$  isoforms. (B) Gene KI into the human  
10 p63 $\alpha$  gene. The TALEN recognition sites for gene targeting were immediately  
11 upstream of the stop codon in the last exon of p63 (exon 14). The insert  
12 encoded a p63-2A peptide fused to an EGFP-neomycin cassette, which was  
13 flanked by a 550-bp homologous arms at both ends. (C) Cleavage efficiency of  
14 the p63 TALEN pairs in HEK293T cells, as measured by single-strand  
15 annealing. p63 TALENs had comparable genome-cleavage efficiency relative to  
16 those of the HPRT1 and HPRT1-NC TALENs. The data are shown as the mean  
17  $\pm$  SD (n = 3). (D) Expression of major p63 isoforms in the corneal epithelium *in*  
18 *vivo*. The major p63 isoform was  $\Delta$ Np63 $\alpha$ . The data are shown as the mean  $\pm$

1 SD (n = 3). (E) PCR genotyping of p63 KI-hiPS cells. WT and KI p63 are 1,604  
2 bps and 4,247 bps, respectively. Clones 42, 53, and 48 were WT; clones 51, 54,  
3 and 60 were heterozygous KIs; and clone 47 was a homozygous KI. (F) 2A  
4 peptide cleavage in p63 KI-hiPS cells. The p63-2A fusion and EGFP were  
5 detected only in KI cells. GAPDH was used as a loading control.

6

7 **Figure 2.** Pluripotency of p63 KI-hiPS cells. (A) Alkaline phosphatase staining  
8 indicated pluripotency in both WT (left) and KI cells (right). The top panels are  
9 macro photographs, while the bottom panels show single, stained colonies  
10 (Scale bars, 1 cm (top) and 400  $\mu$ m (bottom)). (B) Immunohistochemical  
11 staining. The pluripotency markers OCT3/4, NANOG, TRA-1-60, and SSEA-4  
12 were detected in KI cells (Scale bar, 100  $\mu$ m).

13

14 **Figure 3.** Differentiation of p63 KI-hiPS cells into ocular epithelial cells.

15 (A) Differentiation of p63 KI-hiPS cells into ocular cells in the SEAM structure.  
16 Representative image of undifferentiated hiPS cells at day 0 (left; scale bar, 200  
17  $\mu$ m), and representative SEAM structures with the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> zones  
18 after 6–7 weeks of differentiation (right; scale bar, 1000  $\mu$ m). p63 expression

1 was detected in the 3<sup>rd</sup> zone. (B) Co-expression of p63 (red) and EGFP (green)  
2 in the 3<sup>rd</sup> and 4<sup>th</sup> zones in a SEAM after 6–7 weeks in culture. The top panels  
3 show a SEAM structure with all zones, and the bottom panels are the same  
4 structures show at a higher magnification (Scale bars, 100  $\mu$ m). (C) Co-  
5 expression of p63 (green) and PAX6 (red) after 6–7 weeks in culture. p63 was  
6 expressed in the 3<sup>rd</sup> and 4<sup>th</sup> zones, while PAX6 was expressed in the 2<sup>nd</sup> and 3<sup>rd</sup>  
7 zones. Cells expressing both p63 and PAX6 were fated to form corneal  
8 epithelium (Scale bar, 100  $\mu$ m).

9  
10 **Figure 4.** Expression of p63 isoforms in ocular cells differentiated from p63 KI-  
11 hiPS cells. (A) Fluorescence-activated cell sorting based on p63 expression at  
12 0, 2, 4, and 10 weeks of differentiation. The plots are representative of 17, 8,  
13 and 7 independent samples at 2, 4, and 10 weeks of differentiation,  
14 respectively. The plots are indicated of KI (green) and WT (dotted black line)  
15 cells. (B) qRT-PCR of p63 isoforms and correlation with *EGFP*.  $\Delta Np63$  and  
16 *p63 $\alpha$*  were detected in EGFP<sup>+</sup> cells at 4 and 10 weeks of differentiation, but  
17 *TAp63* was barely expressed, suggesting that  $\Delta Np63\alpha$  is the predominant  
18 isoform. The data are shown as the mean  $\pm$  SD (n = 5).

1

2 **Figure 5.** Isolation of corneal epithelial cells co-expressing p63 and EGFP. (A)

3 Flow cytometric analysis of p63 and ITGB-4 at 10 weeks of differentiation. p63<sup>+</sup>

4 cells were sorted based on ITGB-4 expression, and p63<sup>+</sup> SSEA-4<sup>+</sup> ITGB-4<sup>+</sup>

5 were considered to be human iPS induced corneal epithelial cells (hiCECs).

6 These cells were seeded in a temperature-responsive dish to culture a cell

7 sheet. (B) Coexpression of p63 and EGFP in single epithelial cells. Cells were

8 collected by performing a cytopsin and then immunostained with an anti-p63

9 antibody (Scale bar, 100  $\mu$ m). (C) Isolated p63<sup>+</sup> SSEA-4<sup>+</sup> ITGB-4<sup>+</sup> p63<sup>+</sup> cells

10 were visualized for green fluorescence at day 4 (Scale bar, 100  $\mu$ m).

11

12 **Figure 6.** Characterization of cell sheets derived from p63 KI-hiPS cells. (A)

13 Macro image of a harvested hiCEC sheet (Scale bar, 0.5 mm). (B) Phase-

14 contrast imaging of (left) and EGFP<sup>+</sup> cells (right) in an hiCEC sheet grown on a

15 temperature-responsive dish (Scale bar, 100  $\mu$ m). (C) Cell sheet stained with

16 hematoxylin and eosin (Scale bar, 50  $\mu$ m). (D) Immunohistochemical analysis of

17 EGFP (green), keratin 12, p63, PAX6, MUC16, keratin 14, keratin 3/76, keratin

1 13, ZO-1 and keratin 10 (red) expression. Nuclei are stained blue (Scale bar, 50  
2  $\mu\text{m}$ ).

3

#### 4 **Supplemental information**

5 **Supplemental Figure 1.** Coexpression of p63 and ITGB-4, and

6 characterization of p63<sup>+</sup> cells. (A) Flow cytometric analysis of p63 and ITGB-4

7 after 10 weeks of differentiation. (B) p63<sup>+</sup> cells in ITGB-4<sup>+</sup> cells, and ITGB-4<sup>+</sup>

8 cells in p63<sup>+</sup> cells. (C) Flow cytometric analysis of SSEA-4 and ITGB-4 after 10

9 weeks of differentiation, with cells in fractions 1 and 2 considered to express

10 and strongly express SSEA-4, respectively. (D) Fraction of p63<sup>+</sup> cells in hiCEC

11 fractions 1 (left) and 2 (right).