Research paper

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

Yuki Kobayashi[1], Ryuhei Hayashi[1,2*] Andrew J. Quantock[3] and Kohji Nishida[1*].

1 Department of Ophthalmology, Osaka University Graduate School of Medicine. 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
2 Department of Stem Cells and Applied Medicine, Osaka University Graduate School of Medicine, Suita, Osaka, 565-0871, Japan.
3 Structural Biophysics Group, School of Optometry and Vision Sciences, College of Biomedical and Life Sciences, Cardiff University, Cardiff, CF24 4HQ, Wales, UK.

Corresponding authors:

*Ryuhei Hayashi; ryuhei.hayashi@ophthal.med.osaka-u.ac.jp, *Kohji Nishida; knishida@ophthal.med.osaka-u.ac.jp

Highlights

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

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

Keywords

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

Abstract

The p63 is a biomarker whose expression in surface ectodermal cells during developmental processes of the cornea, skin, oral mucosa, and olfactory placodes and is indispensable for understanding the mechanisms of self-renewal and maintenance of epithelial stemness. Here, we generated a p63 knock-in (KI) human induced pluripotent stem (hiPS) cell line by using TALEN, where p63 expression was visualized via enhanced green fluorescent protein expression. The established cells maintained pluripotency, and were found to be specifically targeted at the stem cell marker gene, ΔNp63α. The KI-hiPS cells successfully differentiated into corneal epithelial cells, and the functional epithelial stem cells through p63 expression were used to evaluate reconstruction of the corneal epithelium. This novel approach enables the tracing of cell lineages expressing

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

1. Introduction

p63 is expressed at surface ectodermal cells beginning in early development to facilitate formation of the epithelium, and to eventually maintain a stratified epithelial structure (Barbieri et al. 2006; Senoo et al. 2007; Melino et al. 2015) in the cornea, skin, oral mucosa, and olfactory placodes. p63 has two major isoforms, namely the transactivating (TAp63) and N-terminally truncated (ΔNp63) isoforms, each of which is driven by a different promoter and is alternatively spliced at the C-terminus to generate the alpha, beta, and gamma forms (Figure 1A). Of these, ΔNp63α is a well-established as a marker of epithelial stem cells, and is abundantly expressed in the basal layers of the corneal limbus and epidermis, where stem cells with high proliferative capability are present (Barbieri et al. 2006; Kawasaki et al. 2006; Robertson et al. 2008).

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

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

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

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

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

2. Materials and methods

2.1 hiPS cell culture

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

2.2 Construction of the donor vector and TALEN plasmids

The donor vector was constructed such that a P2A-EGFP-neomycin cassette was flanked by human p63α homology arms (Fasmac, Kanagawa, Japan). A pair of hp63α TALENs was synthesized in GeneART® Precision TALs (Thermo Fisher Scientific, MA) and subcloned into the pEF5/FRT/V5-DEST vector (Thermo Fisher Scientific) using Gateway LR Clonase II (Thermo Fisher Scientific). Recombinant DNA and genome-editing protocols were approved by the research ethics committee of Osaka University.

2.3 Cleavage efficiency of p63 TALENs

The TALEN plasmids were evaluated by performing an SSA assay (Sakuma et al. 2013). The pGL4-SSA reporter vector, containing an overlapped homologous luciferase gene was used to construct the pGL4-SSA-hp63α vector. As a positive control, pGL-SSA-HPRT1 was used with either HPRT1 TALENs or HPRT1-NC TALENs (kindly provided by T. Yamamoto, Hiroshima University). The TALEN plasmids, the pGL4-SSA reporter vector and the pRL-CMV reference vector (Promega, Madison, WI) were transfected into human embryonic kidney HEK293T cells by using Lipofectamine LTX (Thermo Fisher Scientific) in a 96-well plate. Luciferase activity was measured in a multilabel counter (ARVO MX, Perkin Elmer, MA) at 24 h post-transfection, using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions.

2.4 Establishment of hp63 KI hiPS cells

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

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

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

2.5 Western blot analysis of 2A peptide cleavage

p63 KI-hiPS cells were harvested at 10 weeks of differentiation and analyzed by western blotting to assess 2A peptide cleavage between endogenous hp63α and the EGFP insert. After cells were lysed in RIPA lysis buffer (Nacalai, Japan), 20 μg total protein was loaded on 4–12% Tris-Glycine SDS-PAGE gels, which were then blotted and probed with monoclonal antibodies against p63 (4A4, Santa Cruz, CA) and GAPDH (6C5, Santa Cruz), or with a polyclonal antibody against GFP (FL, Santa Cruz).

2.6 Differentiation of hiPS cells into ocular ectodermal cells

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

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

2.7 Flow cytometry and cell sorting

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

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

2.8 Cytospin experiments

For immunofluorescence staining, sorted cells were adjusted to a density of 2×10^5 cells/mL, and 200 μL of the cell suspension was centrifuged at 1,000 rpm for 5 min in a Cytospin^{TM} 4 Cytocentrifuge (Thermo Fisher Scientific).

2.9 Immunofluorescence staining

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

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

2.10 Hematoxylin and eosin staining

hiCEC sheets derived from p63 KI-hiPS cells were fixed with 10% formaldehyde neutral buffer solution (Wako), sectioned in paraffin at 3 μm, stained with hematoxylin and eosin following deparaffinization and hydration, and imaged

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

2.11 Alkaline phosphatase staining

Cells were stained for 10 min at room temperature with a chromogenic substrate, which consisted of 4.5 µL NBT (75 mg/mL) in 70% dimethylformamide and 3.5 µL BCIP (50 mg/mL) in 100% dimethylformamide, diluted in 1 mL 100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$. Staining was stopped by washing with phosphate-buffered saline, and cells were fixed for 30 min at 4℃ with 4% paraformaldehyde, air dried, and imaged on an EVOS FL Auto imaging system (Thermo Fisher Scientific).

2.12 qRT-PCR experiments

Differentiated hiPS cells were harvested at specific time periods and lysed in QIAzol reagent (Qiagen, Valencia, CA). Total RNA was extracted and reverse-transcription was performed using the SuperScript III First-Strand Synthesis System for qRT-PCR (Thermo Fisher Scientific), and synthesized cDNA was amplified in duplicate on an ABI Prism 7500 Fast Sequence Detection System (Thermo Fisher Scientific) using TaqMan Fast Universal PCR Master Mix

(Applied Biosystems) or SYBR Premix Dimer Eraser (Takara Bio). Targets were amplified with TaqMan MGB probes assays against ΔNp63 (Hs00978339_m1), p63α (Hs00978338_m1), TAp63 (Hs00978349_m1), and GAPDH (HS99999905_m1). EGFP was amplified using the EGFP-Fwd (5′-TATATCATGGCCGACAAGCA-3′) and EGFP-Rev (5′-CTGGGTGCTCAGGTA GTGGT-3′) primers and detected with SYBR Green. Thermocycling conditions for the TaqMan reactions consisted of an initial cycle at 95℃ for 20 s, and 45 cycles at 95℃ for 3 s and 60℃ for 30 s, while the SYBR green reactions consisted of an initial cycle at 95℃ for 30 s, and 40 cycles at 95℃ for 3 s, 65℃ for 30 s, and 72℃ for 30 s.

3. Results

3.1 Establishing p63 KI-hiPS cells

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

occurred efficiently (Figure 1C). The most abundant isoform in corneal epithelium *in vivo* was detected ΔNp63α isoform by using a specific probe for ΔNp63α and TAp63α isoform only (Figure 1D) (Di Iorio et al. 2005; Krishnan et al. 2010). Therefore, the levels of *p63α* were essentially the same as that of *ΔNp63α* in corneal epithelium.

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

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

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

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

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

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

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

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

Quantitative real-time reverse-transcriptase PCR (qRT-PCR) results confirmed that p63 expression correlated with *EGFP* expression in KI-hiPS cells. As in parental cells, the predominant isoform in KI-hiPS cells was *ΔNp63*, which showed the same expression as *p63α*, whereas *TAp63* was barely detected (Figure 4B).

3.4 Isolation of p63 KI-hiPS ocular surface ectodermal cells

After culturing hiPS-derived ocular surface ectodermal cells in the 3rd and 4th

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

3.5 Cell sheets derived from p63 KI-hiPS cells

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

Author contributions

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

the paper. K.N. obtained financial support.

Acknowledgements

The authors wish to thank S. Kawasaki, R. Katori, and Y. Ishikawa of Osaka University for providing excellent technical support and scientific discussions. We are grateful to T. Yamamoto (Hiroshima University, Japan) for generously providing the HPRT1 vectors. This work was supported by grants from the Project for the Realization of Regenerative Medicine of The Japan Agency for Medical Research and Development (AMED).

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Figure legends

Figure 1. Generation of p63 KI-hiPS cells using TALEN-mediated genome editing. (A) Structure of the human p63 gene and TALEN recognition sites. p63 consists of an N-terminal transactivation domain (TA), a DNA-binding domain, a C-terminal oligomerization domain (Oligo), and other C-terminal domains including a second transactivation domain (TA), a sterile alpha motif domain (SAM), and a transactivation inhibitory domain (TID). Alternative splicing at the C-terminus generates the α, β, and γ isoforms. (B) Gene KI into the human p63α gene. The TALEN recognition sites for gene targeting were immediately upstream of the stop codon in the last exon of p63 (exon 14). The insert encoded a p63-2A peptide fused to an EGFP-neomycin cassette, which was flanked by a 550-bp homologous arms at both ends. (C) Cleavage efficiency of the p63 TALEN pairs in HEK293T cells, as measured by single-strand annealing. p63 TALENs had comparable genome-cleavage efficiency relative to those of the HPRT1 and HPRT1-NC TALENs. The data are shown as the mean ± SD (n = 3). (D) Expression of major p63 isoforms in the corneal epithelium *in vivo*. The major p63 isoform was ΔNp63α. The data are shown as the mean ±

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

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

Figure 3. Differentiation of p63 KI-hiPS cells into ocular epithelial cells. (A) Differentiation of p63 KI-hiPS cells into ocular cells in the SEAM structure. Representative image of undifferentiated hiPS cells at day 0 (left; scale bar, 200 μm), and representative SEAM structures with the 1st, 2nd, 3rd, and 4th zones after 6–7 weeks of differentiation (right; scale bar, 1000 μm). p63 expression

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

Figure 4. Expression of p63 isoforms in ocular cells differentiated from p63 KI-hiPS cells. (A) Fluorescence-activated cell sorting based on p63 expression at 0, 2, 4, and 10 weeks of differentiation. The plots are representative of 17, 8, and 7 independent samples at 2, 4, and 10 weeks of differentiation, respectively. The plots are indicated of KI (green) and WT (dotted black line) cells. (B) qRT-PCR of p63 isoforms and correlation with *EGFP*. *ΔNp63* and *p63α* were detected in $EGFP^+$ cells at 4 and 10 weeks of differentiation, but *TAp63* was barely expressed, suggesting that *ΔNp63α* is the predominant isoform. The data are shown as the mean ± SD (n = 5).

Figure 5. Isolation of corneal epithelial cells co-expressing p63 and EGFP. (A) Flow cytometric analysis of p63 and ITGB-4 at 10 weeks of differentiation. $p63^+$ cells were sorted based on ITGB-4 expression, and $p63^+$ $SSEA\text{-}4^+$ $ITGB\text{-}4^+$ were considered to be human iPS induced corneal epithelial cells (hiCECs). These cells were seeded in a temperature-responsive dish to culture a cell sheet. (B) Coexpression of p63 and EGFP in single epithelial cells. Cells were collected by performing a cytospin and then immunostained with an anti-p63 antibody (Scale bar, 100 μm). (C) Isolated $p63^+$ $SSEA\text{-}4^+$ $ITGB\text{-}4^+$ p63+ cells were visualized for green fluorescence at day 4 (Scale bar, 100 μm).

Figure 6. Characterization of cell sheets derived from p63 KI-hiPS cells. (A) Macro image of a harvested hiCEC sheet (Scale bar, 0.5 mm). (B) Phase-contrast imaging of (left) and $EGFP^+$ cells (right) in an hiCEC sheet grown on a temperature-responsive dish (Scale bar, 100 μm). (C) Cell sheet stained with hematoxylin and eosin (Scale bar, 50 μm). (D) Immunohistochemical analysis of EGFP (green), keratin 12, p63, PAX6, MUC16, keratin 14, keratin 3/76, keratin

13, ZO-1 and keratin 10 (red) expression. Nuclei are stained blue (Scale bar, 50 μm).

Supplemental information

Supplemental Figure 1. Coexpression of p63 and ITGB-4, and characterization of $p63^+$ cells. (A) Flow cytometric analysis of p63 and ITGB-4 after 10 weeks of differentiation. (B) $p63^+$ cells in $ITGB\text{-}4^+$ cells, and $ITGB\text{-}4^+$ cells in $p63^+$ cells. (C) Flow cytometric analysis of SSEA-4 and ITGB-4 after 10 weeks of differentiation, with cells in fractions 1 and 2 considered to express and strongly express SSEA-4, respectively. (D) Fraction of $p63^+$ cells in hiCEC fractions 1 (left) and 2 (right).