Stem Cell Reports
Article
OPEN ACCESS

Small-Molecule Induction Promotes Corneal Epithelial Cell Differentiation from Human Induced Pluripotent Stem Cells

Alexandra Mikhailova,[1,2] Tanja Ilmarinen,[1,2] Hannu Uusitalo,[3,4] and Heli Skottman[1,2,*]
[1]Institute of Biomedical Technology, University of Tampere, Tampere 33520, Finland
[2]BioMediTech, Tampere 33520, Finland
[3]SILK, Department of Ophthalmology, University of Tampere, Tampere 33520, Finland
[4]TAUH Eye Center, Tampere University Hospital, Tampere 33520, Finland
*Correspondence: heli.skottman@uta.fi
http://dx.doi.org/10.1016/j.stemcr.2013.12.014

SUMMARY

Human induced pluripotent stem cells (hiPSCs) offer unique opportunities for developing novel cell-based therapies and disease modeling. In this study, we developed a directed differentiation method for hiPSCs toward corneal epithelial progenitor cells capable of terminal differentiation toward mature corneal epithelial-like cells. In order to improve the efficiency and reproducibility of our method, we replicated signaling cues active during ocular surface ectoderm development with the help of two small-molecule inhibitors in combination with basic fibroblast growth factor (bFGF) in serum-free and feeder-free conditions. First, small-molecule induction downregulated the expression of pluripotency markers while upregulating several transcription factors essential for normal eye development. Second, protein expression of the corneal epithelial progenitor marker p63 was greatly enhanced, with up to 95% of cells being p63 positive after 5 weeks of differentiation. Third, corneal epithelial-like cells were obtained upon further maturation.

INTRODUCTION

The cornea is a multilayered, transparent, and avascular structure forming the anterior part of the eye. Its outermost layer, the corneal epithelium, is exposed to the external environment and thereby needs to be rapidly regenerating and stratified. It is renewed by limbal stem cells, a type of tissue-specific stem cell located in specialized niche areas in the corneoscleral junction called limbus (Echevarria and Di Girolamo, 2011). Diseases affecting the cornea are a major cause of blindness worldwide and one of the leading causes of vision loss after cataract, with nearly 70% of corneal blindness being due to limbal stem cell deficiency (LSCD)—a disease characterized by abnormal corneal epithelial maintenance, resulting in conjunctivalization of the corneal surface (Ahmad, 2012). LSCD may be caused by acute trauma, such as chemical or thermal injury, or various chronic or genetic conditions (Notara et al., 2010; Osei-Bempong et al., 2013). Several different surgical techniques have been implemented to treat LSCD. One approach is to use cultivated limbal epithelial transplantation (CLET). However, this method is only possible if enough healthy limbal tissue is available, and long-term results show a considerable amount of variation in success rates. This is especially true in case of allogeneic transplantation, which also requires the use of long-term systemic immunosuppression (Baylis et al., 2011).

In search of novel therapies for corneal disorders, alternative cell sources have been investigated, including hair-follicle stem cells, mesenchymal stem cells, and umbilical-cord-lining stem cells (Blazejewska et al., 2009; Reinshagen et al., 2011; Reza et al., 2011). One of the techniques enabling the use of autologous cells, cultivated oral mucosal epithelial transplantation (COMET), has been extensively studied, giving promising results for stabilization of the ocular surface. Generally, the main problems with COMET, as with CLET, include variation in success rates, use of serum and animal-derived materials in the culture protocols, and peripheral corneal neovascularization (Chen et al., 2009a, 2012; Hirayama et al., 2012; Kolli et al., 2010; Nishida et al., 2004; Satake et al., 2011; Sotozono et al., 2013). Thus, it is important to further develop functional cell-based modes of treatment for corneal defects.

Human pluripotent stem cells (hPSCs) have a wider differentiation potential than tissue-specific stem cells, providing an unlimited source of cells. Human induced pluripotent stem cells (hiPSCs) in particular provide exciting new possibilities in the field of personalized medicine and disease modeling (Takahashi et al., 2007). The first study to successfully differentiate corneal epithelial-like cells from hPSCs used medium conditioned by limbal fibroblasts as a way of replicating the corneal stem cell niche (Ahmad et al., 2007). Since then, a few other studies have been published, all relying on various undefined or animal-derived components, such as feeder cells, amniotic membrane, or conditioned medium, alone or in combinations (Hanson et al., 2013; Hayashi et al., 2012;

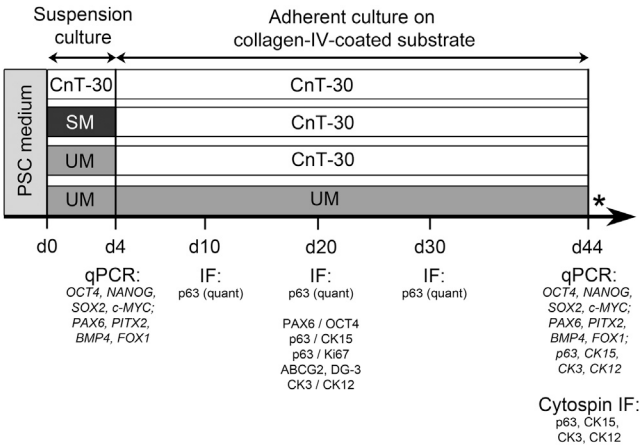

Figure 1. Schematic Outline of the Study

The culture conditions, duration for each stage, and main analyses are shown. The asterisk (*) represents the control. All analyses were carried out on adherent cultures, except d4 qPCR, for which cell aggregates were used. Endpoint analyses included qualitative and quantitative immunocytochemistry and qPCR. IF, immunofluorescence; PSC, pluripotent stem cell; SM, supplemented medium; UM, unsupplemented medium.

Hewitt et al., 2009; Shalom-Feuerstein et al., 2012). Using defined differentiation conditions free from animal-derived products and serum would diminish batch-to-batch variation, thereby minimizing the potential risk of animal pathogen transmission, immune reactions, and graft rejection (Kaur et al., 2013; Martin et al., 2005). Consequently, the repeatability and consistency of differentiation, as well as the safe use of the resulting cell populations in patients, would improve.

In this study, we developed a directed two-stage differentiation protocol for hiPSCs, without the use of feeder cells or serum. To do so, we replicated early developmental mechanisms by blocking the transforming growth factor β (TGF-β) and Wnt- signaling pathways with small-molecule inhibitors and activating fibroblast growth factor (FGF) signaling. We used this method to generate relatively pure populations of corneal epithelial-like progenitor cells capable of terminal differentiation toward mature corneal epithelial-like cells.

RESULTS

Inhibition of TGF-β and Wnt Signaling Together with FGF Activation Directs hiPSC Differentiation by Downregulating Pluripotency Markers and Upregulating Transcription Factors Active during Early Eye Development

The experimental design of this study is schematically presented in Figure 1. Differentiation of hiPSCs was initiated in suspension culture in one of the three induction media: commercial CnT-30 corneal epithelium medium; RegES^{-} medium supplemented with TGF-β inhibitor SB-505124, Wnt inhibitor IWP-2, and bFGF (SM); or the unsupplemented RegES^{-} medium (UM). To study the effects of induction medium on early stage differentiation, expression of several genes was studied using quantitative PCR (qPCR). After the 4-day induction period in suspension culture, expression of the undifferentiated stem cell markers *OCT4, NANOG, SOX2,* and *c-MYC* decreased in all conditions (Figure 2A). This was coupled with an increase in the expression of several genes important for eye development generally and corneal development in particular, namely *PAX6, PITX2, BMP4*, and *FOX1* (Figure 2B). The downregulation of *OCT4*, *NANOG*, *c-MYC*, and *PAX6* was significantly more pronounced in cells that had undergone induction in SM as compared to induction in CnT-30 medium or UM ($p < 0.05$; determined with Mann-Whitney U tests), suggesting higher extent of differentiation under these conditions. Relative gene expression of *OCT4* and *PAX6* was also analyzed for four additional cell lines, showing the same expression patterns, with varying orders of magnitude (Figure S1 available online).

Corneal Epithelial-like Cells Possessing Appropriate Cell Morphology and Protein Expression Are Obtained via Directed Differentiation of hiPSCs

After the 4-day induction period in suspension culture, cell aggregates were plated down onto collagen-IV-coated well-plates for further differentiation in adherent culture using CnT-30 corneal epithelium medium. Additionally, UM was used as control in order to assess the efficiency of spontaneous differentiation. The propensity of cell aggregates to attach to collagen-IV-coated well-plates was quantified after 3 days in adherent culture, with clear differences between the culture conditions (Figure 2C). The highest degree of adhesion was observed after induction with SM, with around 80% of cell aggregates attaching ($p < 0.005$; determined with one-way ANOVA). Similar yet less prominent differences in adhesion were observed between culture conditions for an additional hiPSC line (Figure S1).

Cell outgrowths from the attached aggregates were observed already at the day 7 time point, possessing predominately fibroblast-like cell morphology regardless of the induction medium (Figure 3). Cells maturated in CnT-30 medium obtained a polygonal epithelial morphology with a certain degree of stratification observed at the endpoint of the study (Figures 3A–3C). Differences between conditions became clearer upon reaching confluency. Specifically, cells that had undergone induction in SM were fastest to reach confluency and therefore stratified the most (Figure 3B). On the other hand, cells differentiated under spontaneous conditions in UM (control)

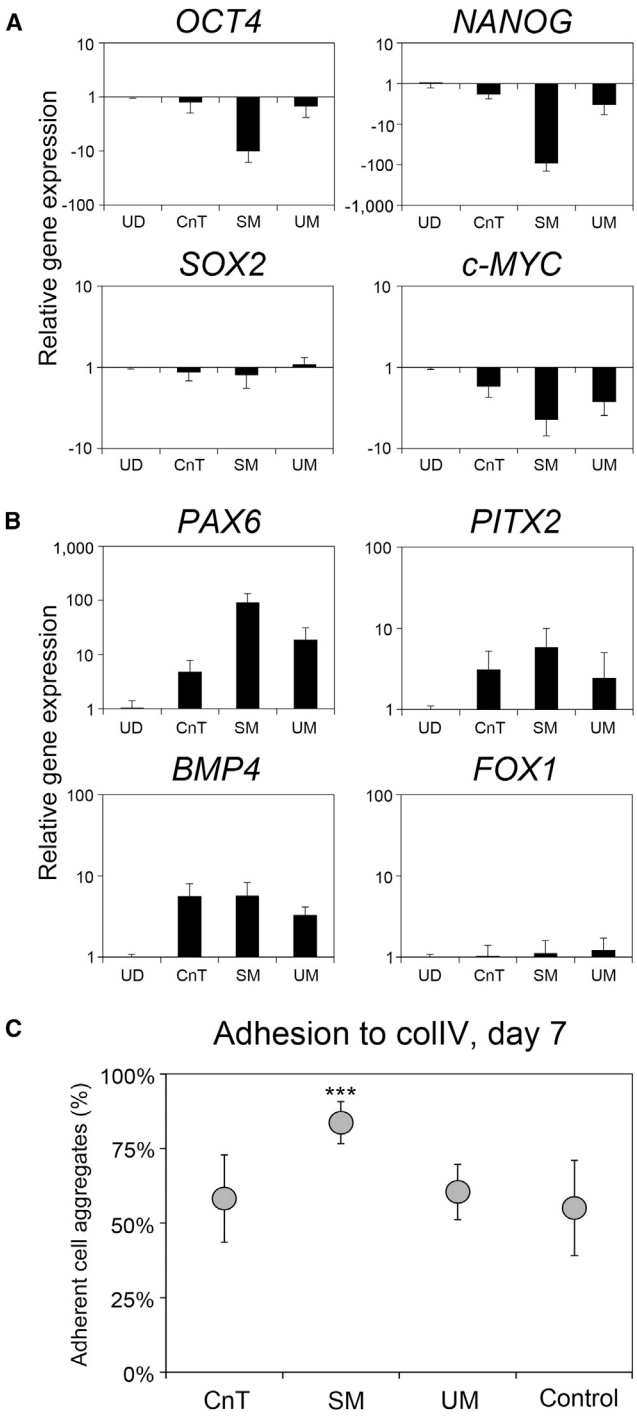

Figure 2. Small-Molecule Induction Enhances Early Stage Differentiation

(A and B) Relative gene expression levels of pluripotency markers (A) and transcription factors involved in early eye development (B) after the 4-day induction stage.

(C) Ratios of cell aggregates attached to collagen-IV-coated substrate at day 7. Statistical significance determined using one-way ANOVA (***$p < 0.005$).

proliferated rapidly, but their morphology was very heterogeneous, with mostly elongated and fibroblast-like cells forming thick layers (Figure 3D). Differentiation under spontaneous conditions was therefore not sufficient for the generation of corneal epithelial-like cells within the timeframe of the study. Moreover, areas of pigmented cells resembling retinal pigment epithelium (RPE) were often observed under spontaneous differentiation conditions, as expected based on the fact that RPE differentiation is fairly efficient in spontaneous conditions (Vaajasaari et al., 2011; Schwartz et al., 2012). In accordance with our results regarding the efficiency and precision of our differentiation protocol, cell cultures maturated in CnT-30 medium did not yield RPE-like cells.

To verify that differentiation was progressing toward corneal epithelium, cells were analyzed with immunocytochemistry at several time points during differentiation (see Figures S2A and S2B for staining verification of key markers using human corneal epithelial cell line). After 20 days in differentiation culture, cells maturated in CnT-30 medium expressed PAX6, but not OCT4 (Figure 4A). Moreover, corneal epithelial progenitor marker p63 was coexpressed with Ki67, a marker of proliferating cells (Figure 4B). The putative corneal epithelial progenitor markers CK15, ATP-binding cassette subfamily G member 2 (ABCG2), and desmoglein 3 (DG-3) were also expressed at this time point (Figures 4C and 4D). Notably, p63 and CK15 were coexpressed in most cells (Figure 4C). Expression of proteins specific to terminally differentiated corneal epithelium, namely cytokeratins 3 and 12, was also detected already at this time point, especially in cells that had undergone induction in SM (Figures 4E and S2). Conversely, cells that were differentiated under spontaneous conditions in UM (control) did not express any of the abovementioned corneal epithelial markers (Figure S2E). Cell morphology and protein expression were assessed for all studied cell lines, with similar results (Figure S1).

Furthermore, cells that had undergone SM induction were chosen to be subjected to a small-scale stratification assay. After 1 week of culture at the air-liquid interface, cell stratification was induced to a certain extent, with two clearly separate cell layers obtained, and CK12 expression limited to the apical surface of the construct (Figure S3).

Small-Molecule Induction Improves Corneal Epithelial Differentiation Efficiency and Reproducibility

Protein expression of the commonly used corneal epithelial progenitor marker p63 was analyzed at 10-day intervals

Data represent the mean ± SD from three independent biological replicates. UD, undifferentiated. See Figure S1 for gene expression in four additional cell lines.

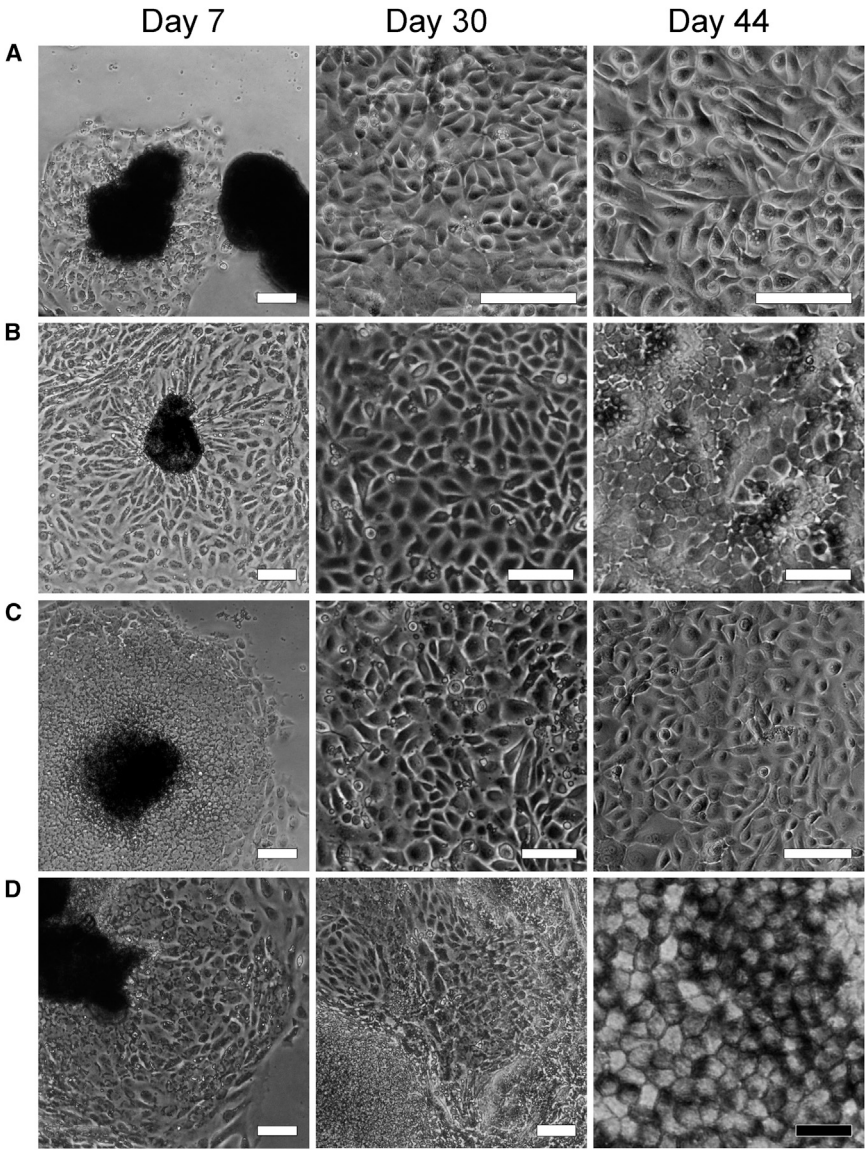

Figure 3. Cell Morphology at Different Stages of Differentiation

Representative images of cells cultured in CnT-30 medium after induction with CnT-30 (A), SM (B), or UM (C) and under spontaneous culture conditions in UM (D).

White scale bars represent 100 μm and black scale bar represents 25 μm. See Figure S1 for representative images of four additional cell lines.

by means of immunofluorescent staining. The first of the three time points was after 6 days in adherent culture, a total of 10 days in differentiation culture, giving the cell aggregates time to properly adhere to the collagen-IV-coated substrate. The next time point was at day 20 and the last one at day 30. Expression of p63 was not detected in control cells cultured under spontaneous conditions in UM throughout the course of the study (Figure 5A).

Cells maturated in CnT-30 medium expressed p63 at each time point, and expression levels were clearly affected by the induction medium (Figure 5A). In order to quantify the differences between the culture conditions, amounts of p63-positive cells were counted at each time point (Figure 5B). After induction with CnT-30, the number of p63-positive cells varied between biological replicates, masking the overall differences between time points. In contrast, after induction with SM, the number of p63-positive cells increased with time, roughly by 40% from day 10 to day 30 ($p = 0.034$), with less variation between biological replicates. Induction with UM proved to be less efficient in producing p63-positive cells, even upon maturation in CnT-30 medium, with an average of around 25%–35% of cells being p63 positive. Protein expression of p63 was also analyzed for an additional hiPSC line, showing less prominent differences between culture conditions, with the least interreplicate variation for the SM condition (Figure S1).

Based on the DAPI staining, total numbers of cells were determined at each time point to assess cell proliferation overtime (Figure 5C). Regardless of the induction medium, a slight decrease in cell numbers was observed at day 20,

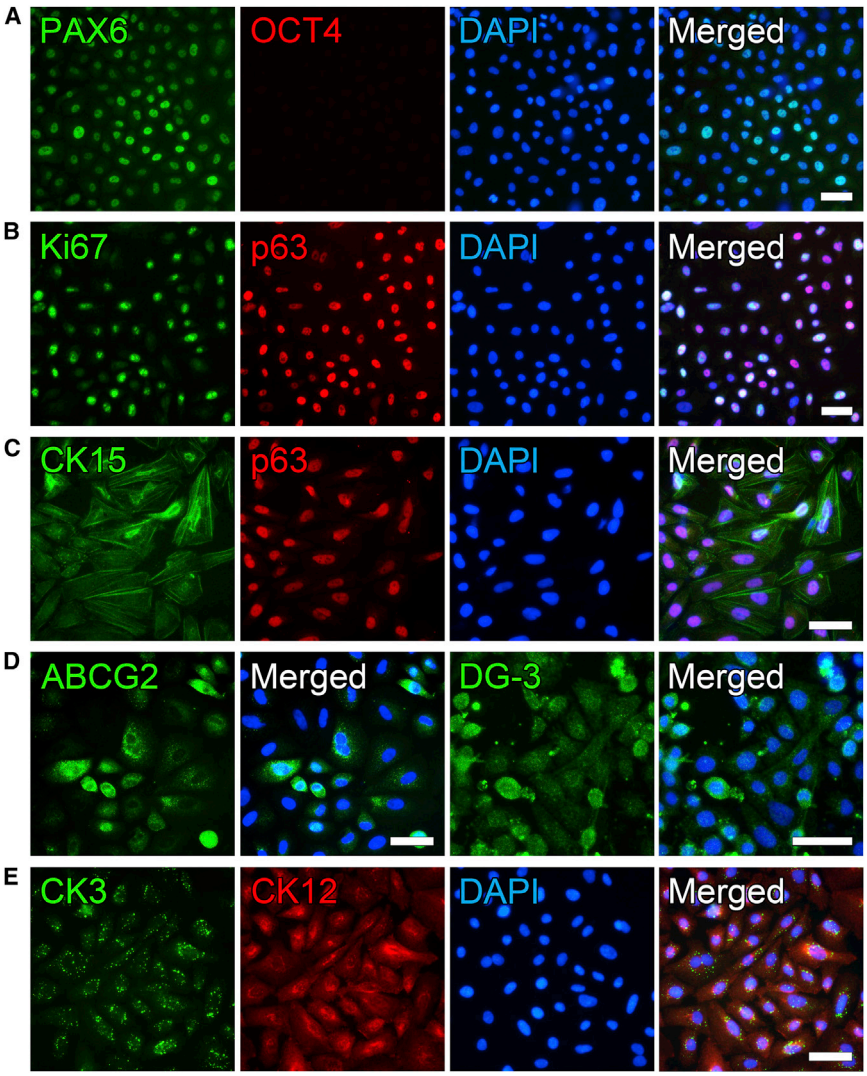

Figure 4. Protein Expression during Differentiation of Corneal Epithelial-like Cells

(A–E) Representative images of cells cultured in CnT-30, after induction with SM. After a total of 20 days in differentiation culture, cells expressed PAX6, but not the pluripotency marker OCT4 (A). Several putative corneal epithelial progenitor markers (B–D), as well as the two markers of terminally differentiated corneal epithelium (E), were expressed at this time point. The scale bars represent 50 μm.

See Figure S1 for representative images of an additional cell line. See Figure S2 for control stainings of p63, CK15, CK3, and CK12 and for representative images of HEL24.3 cell line cultured in the remaining test conditions. See Figure S3 for the stratification assay demonstrating that hiPSC-derived corneal epithelial cells have the capability for terminal differentiation.

increasing again by day 30. The differences between culture conditions were negligible, with roughly similar cell numbers at each time point.

Because induction medium affected the subsequent yield of p63-positive epithelial progenitor cells, expression of several corneal epithelial markers was studied also at the endpoint of the study. After a total of 44 days in differentiation culture, gene expression of pluripotency markers, early eye development transcription factors, and key markers of the corneal epithelium were analyzed using qPCR. The pluripotency markers *OCT4*, *NANOG*, and *SOX2* were downregulated in all culture conditions (Figure 6A). Expression of *PAX6* was highly upregulated, and changes in expression of *PITX2*, *BMP4*, and *FOX1* were minor (Figure 6B). Expression levels of *p63* were similar in all three study conditions, whereas *CK15*, *CK3*, and *CK12* were expressed at a higher level after induction with SM (Figure 6C). When compared to the native human corneal tissue, expression levels of *p63* and *CK15* were similar, but both *CK3* and *CK12* were expressed at much higher levels in the primary tissue. To confirm the results of the gene expression analysis, the four corneal epithelial markers were analyzed on the protein level using immunofluorescently stained cytospin samples (Figures 6D and 6E). Expression of all four proteins was affected by the induction medium, with consistently highest amounts of positive cells obtained after induction with SM. The same trend was observed in each of the separate biological replicates and on average. As evidenced by double stainings, p63 and CK3 were for the most part mutually exclusive, whereas cytokeratins 15 and 12 were often coexpressed. Expression of these four proteins was negligible in control cells cultured under spontaneous conditions in UM (data not shown).

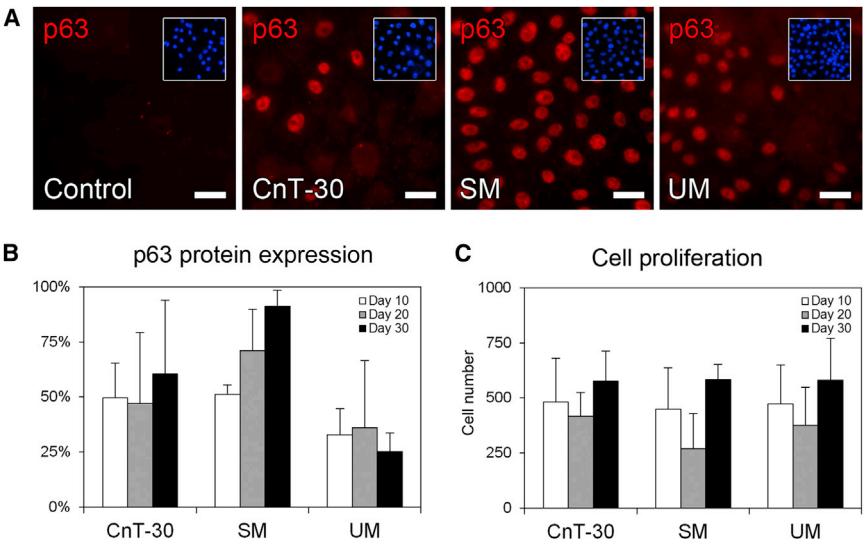

Figure 5. Corneal Epithelial Progenitor Marker p63 Expression Over Time in Different Culture Conditions

(A) Representative images taken at day 30 in different culture conditions (DAPI = blue). The scale bars represent 25 μm.

(B) Expression of p63 was quantified from four separate biological replicates at 10-day intervals.

(C) Cell proliferation was assessed via cell counting from four separate biological replicates at 10-day intervals. Data represent the mean ± SD from independent biological replicates.

DISCUSSION

In this study, we chose to examine the effects of a combination of two small-molecule inhibitors, SB-505124 and IWP-2, together with FGF, on differentiation of hiPSCs toward eye precursors and further toward corneal epithelial cells. During embryogenesis, corneal epithelium originates from the head/ocular surface ectoderm (Collomb et al., 2013; Wolosin et al., 2004). Although many of the developmental mechanisms and signaling routes remain elusive, it is known that blocking TGF-β/Nodal and Wnt/β-catenin-signaling pathways is required for head/ocular surface ectoderm development (Arkell and Tam, 2012; Dupont et al., 2005; Fuhrmann, 2008; Gage et al., 2008). Ectodermal placodes play a major part in the development of vertebrate cranial sensory organs and have been shown to emerge from a common preplacodal region (Streit, 2007). Preplacode induction in the naive ectoderm is promoted by a combination of FGF expression together with Wnt and bone morphogenetic protein (BMP) antagonists (Litsiou et al., 2005). Replication of these developmental cues by first blocking BMP signaling with Noggin to induce ectoderm formation, followed by activation of FGF signaling, was shown to initiate lens-progenitor cell differentiation from hESCs under chemically defined culture conditions (Yang et al., 2010). In a recently published study, a small-molecule inhibitor of the Src family kinases was shown to promote simple epithelial differentiation of hPSCs by downregulating canonical Wnt signaling (Lian et al., 2013a). Moreover, there is recent evidence showing that small-molecule inhibition of the TGF-β- signaling pathway improves ectodermal differentiation of hiPSCs (Shalom-Feuerstein et al., 2013).

It has been previously confirmed that SB-505124 selectively inhibits TGF-β and activin signaling more efficiently than its analog SB-431542 (DaCosta Byfield et al., 2004), which has been shown to induce neural differentiation when used in combination with Noggin (Chambers et al., 2009). IWP-2, on the other hand, functions as an inhibitor of the canonical Wnt pathway (Chen et al., 2009b) and was shown to induce cardiomyocyte differentiation when applied following a pretreatment with glycogen synthase kinase 3 inhibitor (Lian et al., 2013b). In our study, the initial induction of differentiation in the presence of these two factors, together with FGF, upregulated the expression of transcription factors active during early eye development, namely *PAX6, PITX2, BMP4*, and *FOX1*. Meanwhile, expression of the pluripotency markers *OCT4, NANOG, SOX2*, and *c-MYC* was downregulated. More importantly, the use of SB-505124, IWP-2, and bFGF during the induction stage of the protocol (SM condition) improved overall yields of p63-positive cells in each of the separate biological replicates when compared to induction without these factors (UM followed by maturation in CnT-30 medium). Expression of p63 peaked at the 30-day time point when up to 95% of the cells in SM condition were p63 positive and decreased to an average of 70% by the endpoint of the study (day 44). In the UM condition, the number of cells expressing p63 continued to increase to some extent after the 30-day time point, and by the endpoint of the study, approximately 60% of cells were p63 positive. In other words, corneal epithelial differentiation had progressed further in SM condition, marked by decreasing expression of p63 and increasing expression of CK3 and CK12, whereas differentiation in UM condition was less efficient in the given time frame. In contrast, differentiation under spontaneous conditions (in UM throughout

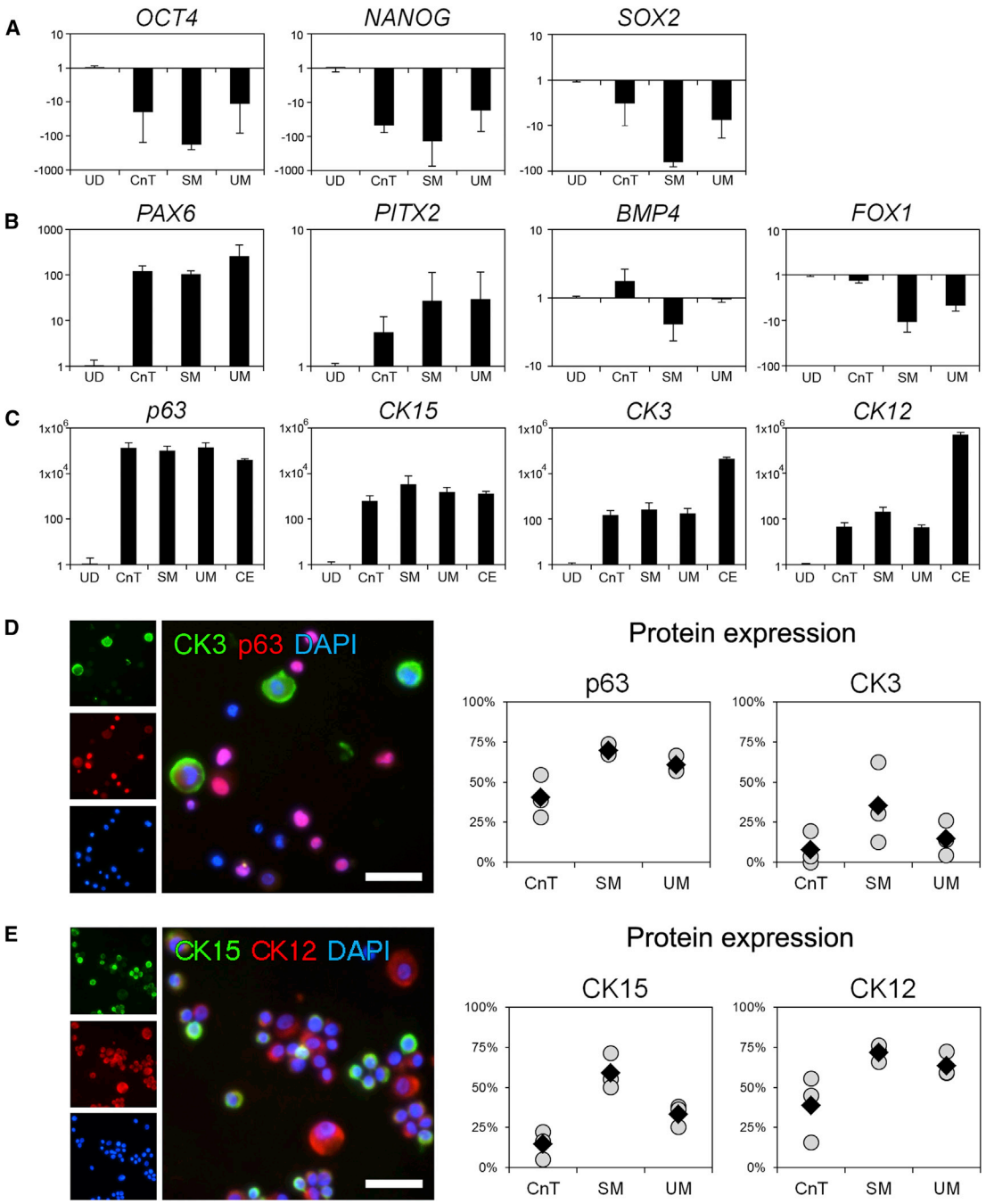

Figure 6. Expression of Key Markers at the Endpoint of the Study

(A–C) Relative gene expression levels of the pluripotency markers (A), transcription factors active during early eye development (B), and key corneal epithelial markers (C) after 44 days in differentiation culture. Data represent the mean ± SD from three independent biological replicates. CE, corneal epithelium.

(D and E) Cells positive for each corneal epithelial marker were quantified through immunostaining of cytospin samples. Separate biological replicates are presented in gray, and their mean in black. The scale bars represent 50 μm.

the course of the study) was not sufficient for generation of p63-positive cells. Furthermore, even though there were no significant differences between conditions with regard to proliferation rates, because p63 was shown to be expressed in most cells that had undergone induction with SM, these p63-positive cells are likely the ones proliferating.

In contrast, there seem to be other proliferating cell types present in the other culture conditions besides the p63-positive cells.

Although an entirely specific marker for corneal epithelial progenitor cells is yet to be identified, transcription factor p63, especially its isoform ΔNp63α, has been linked to stemness and is highly expressed in the basal layers of the corneal epithelium and in the limbus (Kawasaki et al., 2006; Robertson et al., 2008). Mutations in p63 cause severe abnormalities, such as syndromes of ectodermal dysplasia affecting various ectodermal tissues, including the cornea (Shalom-Feuerstein et al., 2013), highlighting the importance of this transcription factor for proper corneal development. More importantly, p63 expression also appears to be clinically relevant, as it was discovered that cell cultures used for limbal stem cell transplantation containing more than 3% of p63-positive cells were associated with a 78% success rate, whereas transplants containing 3% or less p63-positive cells were successful in only 11% of patients with LSCD (Rama et al., 2010). To this respect, the high differentiation rate of p63-positive cells obtained with our protocol could be sufficient to gain good success rate in clinical applications.

In our study, the adhesion to collagen-IV-coated substrate was significantly improved upon induction with SM. The ability to attach to collagen IV is considered to be important for epithelial cells, and several previously published differentiation studies have used this characteristic for selection of epithelial progenitor cells from limbal epithelial cell populations and, to a lesser extent, from differentiating pluripotent stem cells (Bian et al., 2010; Homma et al., 2004; Kumagai et al., 2010; Li et al., 2005). In addition, it has been shown that p63 is important for cell adhesion and migration in various epithelial cell lineages, including corneal epithelial cells (Carroll et al., 2006). Therefore, the high adhesion rate to collagen-IV-coated substrate observed in our study might be at least in part due to high expression of p63 after induction with SM.

To further verify the corneal progenitor state of cells maturated in CnT-30 medium after induction with SM, we confirmed the protein expression of ABCG2, CK15, and DG-3. These proteins have been previously established as putative progenitor markers of the corneal epithelium (Chen et al., 2004; Davies et al., 2009; Nieto-Miguel et al., 2011; Ordonez and Di Girolamo, 2012; Schlötzer-Schrehardt et al., 2007; Takács et al., 2009). In addition, cells differentiated under these conditions expressed the proliferation marker Ki67, whereas expression of the pluripotency marker OCT4 was not detected. Finally, expression of PAX6 is important all throughout corneal epithelial differentiation, due to its action as a coactivator of the *CK12* gene (Collomb et al., 2013). Overall, this expression profile confirms the epithelial and proliferative nature of the cells in question.

Upon further differentiation, corneal epithelial-like cells were obtained, possessing the appropriate cell morphology and expressing proteins specific to corneal epithelium. Expression of cytokeratins 3 and 12 serves to distinguish terminally differentiated corneal epithelium from the epidermis and other mucosal epithelia (Davies and Di Girolamo, 2010; Kurpakus et al., 1994; Tanifuji-Terai et al., 2006). Coexpression of CK15 and CK12 was often observed, suggesting these cells are less mature yet committed to terminal differentiation toward corneal epithelium. In order to improve the yields of mature corneal epithelial cells and to obtain a stratified cell sheet resembling the native corneal epithelium, a consistent and efficient stratification method would need to be employed. For instance, air-liquid interface culture has been shown to promote stratification of corneal epithelial cells in vitro (Ban et al., 2003; Ilmarinen et al., 2013; Toropainen et al., 2001). In our preliminary tests, we did observe a certain degree of uniform stratification after 1 week of cell culture at the air-liquid interface, supporting the conclusion that these cells are able to mature toward terminally differentiated and stratifying corneal epithelial cells. Further optimization of variables, such as the exposure time and medium supplementation, remains to be carried out if aiming for transplantation of fully maturated corneal epithelial sheets rather than cells at the progenitor stage capable of proliferating and differentiating on site.

Differentiation of corneal epithelial cells from hPSCs has proven to be rather challenging, with most of the previously published studies relying on the use of undefined factors, such as conditioned medium, PA6 feeder cells, Bowman's membrane, or amniotic membrane (Ahmad et al., 2007; Hanson et al., 2013; Hayashi et al., 2012; Hewitt et al., 2009; Shalom-Feuerstein et al., 2012). The few studies carried out in more defined conditions have been focused on differentiation of epithelial progenitor cell populations expressing CK14 (Metallo et al., 2008), CK14 and p63 (Sakurai et al., 2011), or CK8 and CK18 (Lian et al., 2013a). In general, the success in differentiation of p63-positive corneal epithelial progenitor cells has not been thoroughly examined or quantified, mainly concentrating on qualitative analysis methods. Moreover, these studies have been fairly unsuccessful in obtaining mature corneal epithelial-like cells expressing terminal differentiation markers CK3 and CK12. For instance, Lian et al. (2013a) describe a differentiation method yielding 90.7% CK14-positive cells within 2 weeks of differentiation, yet only 4.78% of these cells were found to express CK3, and Sakurai et al. (2011) reported colonies containing cells positive for p63 and CK14 within 5 weeks of differentiation. To this respect, our differentiation method is highly

efficient and consistent, giving rise to corneal epithelial-like cells within a relatively short time frame of 6 weeks. It is not uncommon to detect variation in differentiation propensity among different hiPSC and hESC lines (Osafune et al., 2008; Toivonen et al., 2013), with donor identity and gender being among potential sources of variation in the case of hiPSC lines (Boulting et al., 2011). Therefore, to further verify the efficacy of the differentiation method, two additional hiPSC lines (FiPS 5-7 and A116; Rajala et al., 2010; Toivonen et al., 2013) and three hESC lines (Regea08/017, Regea08/023, and Regea11/013; Skottman, 2010) were also tested to a lesser extent, in terms of appropriate cell morphology, gene, and protein expression, confirming our results.

Last, but not least, the differentiation method described here can be refined to be fully defined and xeno-free by substituting human serum albumin (HSA) present in RegES$^-$ medium with recombinant HSA (He et al., 2011) and using a higher certificate, xeno-free version of CnT-30 medium (CELLnTEC Advanced Cell Systems AG). These modifications would make the protocol more compliant to good manufacturing practice standards, providing a more valuable approach for possible clinical applications in the future.

In conclusion, the present study describes an efficient method for differentiating human pluripotent stem cells toward homogeneous populations of p63-positive epithelial cells, which have the potential of differentiating further toward mature corneal epithelial-like cells. With minor modifications, this method could be carried out in fully defined and xeno-free conditions, improving its reliability and the safety of resulting cell populations. Moreover, novel human cell-based tissue models could be developed for accurate, personalized, and reliable drug discovery for treating corneal diseases.

EXPERIMENTAL PROCEDURES

Corneal Epithelial Differentiation

The experimental design of this study is schematically presented in Figure 1. To initiate corneal epithelial differentiation of hiPSCs, undifferentiated colonies were manually dissected and transferred to suspension culture in 6-well plates (Corning Ultra-Low Attachment; Corning Life Sciences). Cells were cultured as three-dimensional cell aggregates for 4 days, changing the culture medium daily. This induction phase was carried out in one of three culture conditions: (1) CnT-30 condition, using the commercially available defined and serum-free CnT-30 corneal epithelium medium (CELLnTEC Advanced Cell Systems AG) supplemented with the appropriate supplements provided with the medium and 50 U/ml penicillin/streptomycin; (2) UM condition, using the serum-free and xeno-free RegES$^-$ medium originally developed for the culture of undifferentiated stem cells (Rajala et al., 2010), but modified by omitting retinol, bFGF, and activin A; or (3) SM condition, using RegES$^-$ medium supplemented with 10 μM SB-505124 (catalog number S4696; Sigma-Aldrich), 10 μM IWP-2 (catalog number 681671; Merck) and 50 ng/ml human bFGF(PeproTech). For the full composition of the media, see Table S1.

After the 4-day induction period, cell aggregates were plated onto cell culture substrate coated with human placental collagen IV (5 μg/cm^2; catalog number C5533; Sigma-Aldrich) at a density of about 50 aggregates/cm^2. Either 24-well plates (Corning CellBIND) or 24-well hanging cell culture inserts (Merck Millipore; 1 μm pore size) were used. Cells were maintained in adherent culture for 40 more days in CnT-30 medium, replacing the culture medium three times a week. Additionally, spontaneous differentiation was tested with culture in unsupplemented RegES$^-$ medium throughout the course of the study—both in suspension and adherent stages. This condition is referred to as UM (control). Three days after plating the aggregates onto 24-well plates, a total of 7 days in differentiation culture, the adhesion ratios were quantified in each of the culture conditions. Cell growth on 24-well plates and cell-culture inserts was regularly monitored under Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe).

Corneal epithelial cell stratification was assessed for the most promising of the tested culture conditions. After 4 to 5 weeks of differentiation on cell-culture inserts, stratification was induced by aspirating the medium from inside the insert. Cells were cultured at the air-liquid interface for 1 week, replacing the medium beneath the inserts daily with fresh CnT-30 medium.

qPCR

Total RNA was extracted from undifferentiated hiPSCs from cell aggregates collected after the 4-day induction phase and from adherent cultures after 44 days of differentiation, using NucleoSpin RNA II kit (Macherey-Nagel). Additionally, RNA was extracted from native human corneal epithelium, isolated as described in the Supplemental Experimental Procedures. RNA concentration of each sample was determined using NanoDrop-1000 spectrophotometer (NanoDrop Technologies). From each RNA sample, 200 ng were used to synthesize cDNA using the High-Capacity cDNA RT kit (Applied Biosystems). The resulting cDNA samples were analyzed with qPCR using sequence-specific TaqMan Gene Expression Assays (Applied Biosystems) for *OCT4* (Hs00999632_g1), *NANOG* (Hs02387400_g1), *SOX2* (Hs01053049_s1), *c-MYC* (Hs00153408_m1), *PAX6* (Hs01088112_m1), *PITX2* (Hs04234069_mH), *BMP4* (Hs00370078_m1), and *FOX1* (Hs01125659_m1). In addition, the endpoint samples and the native human corneal epithelium were also analyzed with assays for *p63* (*TP63*; Hs00978339_m1), *CK15* (*KRT15*; Hs00267032_m1), *CK3* (*KRT3*; Hs00365074_m1), and *CK12* (*KRT12*; Hs00165015_m1). All samples and controls were run as triplicate reactions with the 7300 Real-Time PCR system (Applied Biosystems). Results were analyzed with the 7300 System SDS Software (Applied Biosystems) and Microsoft Excel. Based on the cycle threshold (C_T) values given by the software, the relative quantification of each gene was calculated by applying the $-2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Results were normalized to *GAPDH* (Hs99999905_m1), with the undifferentiated hiPSCs as the calibrator to determine the relative quantities of gene expression in each sample. The analysis was

repeated four times for all cell lines at the d4 time point and only for HEL24.3 cell line at the endpoint.

Immunocytochemistry

Expression of ABCG2, CK3, CK12, CK15, DG-3, Ki67, OCT4, p63, and PAX6 was qualitatively analyzed with immunofluorescent stainings at the d20 time point. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min and washed three times with PBS. Cell membranes were then permeabilized for 10 min with either 0.1% Triton X-100 or 0.5% saponin (both from Sigma-Aldrich) in the case of the primary antibody raised against ABCG2. Nonspecific binding sites were blocked with 3% BSA (Sigma-Aldrich) for 1 hr. Primary antibodies (Table S2) were appropriately diluted in 0.5% BSA and incubated with the cells for 1 hr at room temperature or overnight at +4°C. Primary antibody detection was done with the appropriate Alexa Fluor 568 or 488-conjugated secondary antibodies (Table S2) diluted 1:800 in 0.5% BSA for 1 hr at room temperature. Samples were mounted onto object glasses in mounting medium containing DAPI (VectaShield; Vector Laboratories) for visualization of nuclei. Images of stained cells were captured with Olympus IX51 fluorescence microscope or Zeiss LSM 700 confocal microscope (Carl Zeiss) and edited in Adobe PhotoShop.

Protein expression of the corneal epithelial progenitor marker p63 was analyzed and quantified at three time points (10, 20, and 30 days in differentiation culture) from cells cultured on collagen-IV-coated hanging inserts. Images of stained cells were captured with Olympus IX51 fluorescence microscope (Olympus) from multiple randomly selected areas. Total cell numbers were determined by counting DAPI-stained nuclei, and percentages of p63-positive nuclei were consequently quantified. This analysis was repeated four times for two hiPSC lines (HEL24.3 and A116), with a total of at least 1,000 cells counted for each time point and condition.

Cells of the HEL24.3 cell line cultured on 24-well plates were used for quantitative immunofluorescence analysis at the endpoint of the study (d44). Cells were rinsed with PBS and detached using TrypLE Select (Invitrogen) for 5–10 min at +37°C. Cell suspensions were strained through 40 μm cell strainers and centrifuged at 1,500 rpm for 5 min. Cell pellets were resuspended in cold PBS and single-cell suspension volumes adjusted to contain 50,000 cells/150 μl sample. Cells were then spun onto object glasses using the Cellspin II cytocentrifuge (Tharmac), and the resulting cytospin samples were used for quantitative analysis of key marker expression. Images of multiple randomly selected areas were captured with Olympus IX51 fluorescence microscope. Percentages of cells positive for each marker were quantified in relation to DAPI-stained cells in three separate biological replicates, with a total of at least 1,000 cells counted for each condition.

Statistical Analysis

All values are presented as mean of separate biological replicates, with error bars depicting SDs. Statistical analysis was carried out in the IBM SPSS Statistics Software, using one-way ANOVA for cell attachment, and Mann-Whitney U tests for gene expression and p63 protein expression data. Data normality was tested using Shapiro-Wilk normality test prior to statistical analysis. Results were considered significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2013.12.014.

ACKNOWLEDGMENTS

We would like to express our gratitude to Professor Timo Otonkoski's research group at University of Helsinki, Finland for providing the hiPSC lines used in this study. We also thank Hanna Koskenaho, Outi Heikkilä, and Outi Melin for technical assistance. The study was financially supported by the Academy of Finland (218050 and 133879), University of Tampere, and Tampere Graduate Program in Biomedicine and Biotechnology. The PAX6 antibody developed by Atsushi Kawakami was obtained from the Developmental Studies Hybridoma Bank developed under auspices of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and maintained by Department of Biology, The University of Iowa, Iowa City, IA 52242. University of Tampere has filed a patent application for the protocol reported herein.

Received: October 10, 2013
Revised: December 23, 2013
Accepted: December 23, 2013
Published: February 6, 2014

REFERENCES

Ahmad, S. (2012). Concise review: limbal stem cell deficiency, dysfunction, and distress. Stem Cells Transl. Med. *1*, 110–115.

Ahmad, S., Stewart, R., Yung, S., Kolli, S., Armstrong, L., Stojkovic, M., Figueiredo, F., and Lako, M. (2007). Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche. Stem Cells *25*, 1145–1155.

Arkell, R.M., and Tam, P.P. (2012). Initiating head development in mouse embryos: integrating signalling and transcriptional activity. Open Biol. *2*, 120030.

Ban, Y., Cooper, L.J., Fullwood, N.J., Nakamura, T., Tsuzuki, M., Koizumi, N., Dota, A., Mochida, C., and Kinoshita, S. (2003). Comparison of ultrastructure, tight junction-related protein expression and barrier function of human corneal epithelial cells cultivated on amniotic membrane with and without air-lifting. Exp. Eye Res. *76*, 735–743.

Baylis, O., Figueiredo, F., Henein, C., Lako, M., and Ahmad, S. (2011). 13 years of cultured limbal epithelial cell therapy: a review of the outcomes. J. Cell. Biochem. *112*, 993–1002.

Bian, F., Liu, W., Yoon, K.C., Lu, R., Zhou, N., Ma, P., Pflugfelder, S.C., and Li, D.Q. (2010). Molecular signatures and biological pathway profiles of human corneal epithelial progenitor cells. Int. J. Biochem. Cell Biol. *42*, 1142–1153.

Blazejewska, E.A., Schlötzer-Schrehardt, U., Zenkel, M., Bachmann, B., Chankiewitz, E., Jacobi, C., and Kruse, F.E. (2009). Corneal limbal microenvironment can induce transdifferentiation

of hair follicle stem cells into corneal epithelial-like cells. Stem Cells *27*, 642–652.

Boulting, G.L., Kiskinis, E., Croft, G.F., Amoroso, M.W., Oakley, D.H., Wainger, B.J., Williams, D.J., Kahler, D.J., Yamaki, M., Davidow, L., et al. (2011). A functionally characterized test set of human induced pluripotent stem cells. Nat. Biotechnol. *29*, 279–286.

Carroll, D.K., Carroll, J.S., Leong, C.O., Cheng, F., Brown, M., Mills, A.A., Brugge, J.S., and Ellisen, L.W. (2006). p63 regulates an adhesion programme and cell survival in epithelial cells. Nat. Cell Biol. *8*, 551–561.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. *27*, 275–280.

Chen, Z., de Paiva, C.S., Luo, L., Kretzer, F.L., Pflugfelder, S.C., and Li, D.Q. (2004). Characterization of putative stem cell phenotype in human limbal epithelia. Stem Cells *22*, 355–366.

Chen, H.C., Chen, H.L., Lai, J.Y., Chen, C.C., Tsai, Y.J., Kuo, M.T., Chu, P.H., Sun, C.C., Chen, J.K., and Ma, D.H. (2009a). Persistence of transplanted oral mucosal epithelial cells in human cornea. Invest. Ophthalmol. Vis. Sci. *50*, 4660–4668.

Chen, B., Dodge, M.E., Tang, W., Lu, J., Ma, Z., Fan, C.W., Wei, S., Hao, W., Kilgore, J., Williams, N.S., et al. (2009b). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat. Chem. Biol. *5*, 100–107.

Chen, H.C., Yeh, L.K., Tsai, Y.J., Lai, C.H., Chen, C.C., Lai, J.Y., Sun, C.C., Chang, G., Hwang, T.L., Chen, J.K., and Ma, D.H. (2012). Expression of angiogenesis-related factors in human corneas after cultivated oral mucosal epithelial transplantation. Invest. Ophthalmol. Vis. Sci. *53*, 5615–5623.

Collomb, E., Yang, Y., Foriel, S., Cadau, S., Pearton, D.J., and Dhouailly, D. (2013). The corneal epithelium and lens develop independently from a common pool of precursors. Dev. Dyn. *242*, 401–413.

DaCosta Byfield, S., Major, C., Laping, N.J., and Roberts, A.B. (2004). SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. Mol. Pharmacol. *65*, 744–752.

Davies, S.B., and Di Girolamo, N. (2010). Corneal stem cells and their origins: significance in developmental biology. Stem Cells Dev. *19*, 1651–1662.

Davies, S.B., Chui, J., Madigan, M.C., Provis, J.M., Wakefield, D., and Di Girolamo, N. (2009). Stem cell activity in the developing human cornea. Stem Cells *27*, 2781–2792.

Dupont, S., Zacchigna, L., Cordenonsi, M., Soligo, S., Adorno, M., Rugge, M., and Piccolo, S. (2005). Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. Cell *121*, 87–99.

Echevarria, T.J., and Di Girolamo, N. (2011). Tissue-regenerating, vision-restoring corneal epithelial stem cells. Stem Cell Rev. *7*, 256–268.

Fuhrmann, S. (2008). Wnt signaling in eye organogenesis. Organogenesis *4*, 60–67.

Gage, P.J., Qian, M., Wu, D., and Rosenberg, K.I. (2008). The canonical Wnt signaling antagonist DKK2 is an essential effector of PITX2 function during normal eye development. Dev. Biol. *317*, 310–324.

Hanson, C., Hardarson, T., Ellerström, C., Nordberg, M., Caisander, G., Rao, M., Hyllner, J., and Stenevi, U. (2013). Transplantation of human embryonic stem cells onto a partially wounded human cornea in vitro. Acta Ophthalmol. (Copenh.) *91*, 127–130.

Hayashi, R., Ishikawa, Y., Ito, M., Kageyama, T., Takashiba, K., Fujioka, T., Tsujikawa, M., Miyoshi, H., Yamato, M., Nakamura, Y., and Nishida, K. (2012). Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblast and corneal limbal epithelium. PLoS ONE *7*, e45435.

He, Y., Ning, T., Xie, T., Qiu, Q., Zhang, L., Sun, Y., Jiang, D., Fu, K., Yin, F., Zhang, W., et al. (2011). Large-scale production of functional human serum albumin from transgenic rice seeds. Proc. Natl. Acad. Sci. USA *108*, 19078–19083.

Hewitt, K.J., Shamis, Y., Carlson, M.W., Aberdam, E., Aberdam, D., and Garlick, J.A. (2009). Three-dimensional epithelial tissues generated from human embryonic stem cells. Tissue Eng. Part A *15*, 3417–3426.

Hirayama, M., Satake, Y., Higa, K., Yamaguchi, T., and Shimazaki, J. (2012). Transplantation of cultivated oral mucosal epithelium prepared in fibrin-coated culture dishes. Invest. Ophthalmol. Vis. Sci. *53*, 1602–1609.

Homma, R., Yoshikawa, H., Takeno, M., Kurokawa, M.S., Masuda, C., Takada, E., Tsubota, K., Ueno, S., and Suzuki, N. (2004). Induction of epithelial progenitors in vitro from mouse embryonic stem cells and application for reconstruction of damaged cornea in mice. Invest. Ophthalmol. Vis. Sci. *45*, 4320–4326.

Ilmarinen, T., Laine, J., Juuti-Uusitalo, K., Numminen, J., Seppänen-Suuronen, R., Uusitalo, H., and Skottman, H. (2013). Towards a defined, serum- and feeder-free culture of stratified human oral mucosal epithelium for ocular surface reconstruction. Acta Ophthalmol. (Copenh.) *91*, 744–750.

Kaur, J., Tilkins, M.L., Eckert, R., and Boucher, S. (2013). Methods for culturing human embryonic stem cells in a xeno-free system. Methods Mol. Biol. *997*, 115–126.

Kawasaki, S., Tanioka, H., Yamasaki, K., Connon, C.J., and Kinoshita, S. (2006). Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. Exp. Eye Res. *82*, 293–299.

Kolli, S., Ahmad, S., Lako, M., and Figueiredo, F. (2010). Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. Stem Cells *28*, 597–610.

Kumagai, Y., Kurokawa, M.S., Ueno, H., Kayama, M., Tsubota, K., Nakatsuji, N., Kondo, Y., Ueno, S., and Suzuki, N. (2010). Induction of corneal epithelium-like cells from cynomolgus monkey embryonic stem cells and their experimental transplantation to damaged cornea. Cornea *29*, 432–438.

Kurpakus, M.A., Maniaci, M.T., and Esco, M. (1994). Expression of keratins K12, K4 and K14 during development of ocular surface epithelium. Curr. Eye Res. *13*, 805–814.

Li, D.Q., Chen, Z., Song, X.J., de Paiva, C.S., Kim, H.S., and Pflugfelder, S.C. (2005). Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness. Exp. Eye Res. *80*, 581–590.

Lian, X., Selekman, J., Bao, X., Hsiao, C., Zhu, K., and Palecek, S.P. (2013a). A small molecule inhibitor of SRC family kinases promotes simple epithelial differentiation of human pluripotent stem cells. PLoS ONE *8*, e60016.

Lian, X., Zhang, J., Azarin, S.M., Zhu, K., Hazeltine, L.B., Bao, X., Hsiao, C., Kamp, T.J., and Palecek, S.P. (2013b). Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. Nat. Protoc. *8*, 162–175.

Litsiou, A., Hanson, S., and Streit, A. (2005). A balance of FGF, BMP and WNT signalling positions the future placode territory in the head. Development *132*, 4051–4062.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods *25*, 402–408.

Martin, M.J., Muotri, A., Gage, F., and Varki, A. (2005). Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat. Med. *11*, 228–232.

Metallo, C.M., Ji, L., de Pablo, J.J., and Palecek, S.P. (2008). Retinoic acid and bone morphogenetic protein signaling synergize to efficiently direct epithelial differentiation of human embryonic stem cells. Stem Cells *26*, 372–380.

Nieto-Miguel, T., Calonge, M., de la Mata, A., López-Paniagua, M., Galindo, S., de la Paz, M.F., and Corrales, R.M. (2011). A comparison of stem cell-related gene expression in the progenitor-rich limbal epithelium and the differentiating central corneal epithelium. Mol. Vis. *17*, 2102–2117.

Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Yamamoto, K., Adachi, E., Nagai, S., Kikuchi, A., Maeda, N., Watanabe, H., et al. (2004). Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N. Engl. J. Med. *351*, 1187–1196.

Notara, M., Alatza, A., Gilfillan, J., Harris, A.R., Levis, H.J., Schrader, S., Vernon, A., and Daniels, J.T. (2010). In sickness and in health: Corneal epithelial stem cell biology, pathology and therapy. Exp. Eye Res. *90*, 188–195.

Ordonez, P., and Di Girolamo, N. (2012). Limbal epithelial stem cells: role of the niche microenvironment. Stem Cells *30*, 100–107.

Osafune, K., Caron, L., Borowiak, M., Martinez, R.J., Fitz-Gerald, C.S., Sato, Y., Cowan, C.A., Chien, K.R., and Melton, D.A. (2008). Marked differences in differentiation propensity among human embryonic stem cell lines. Nat. Biotechnol. *26*, 313–315.

Osei-Bempong, C., Figueiredo, F.C., and Lako, M. (2013). The limbal epithelium of the eye—a review of limbal stem cell biology, disease and treatment. Bioessays *35*, 211–219.

Rajala, K., Lindroos, B., Hussein, S.M., Lappalainen, R.S., Pekkanen-Mattila, M., Inzunza, J., Rozell, B., Miettinen, S., Narkilahti, S., Kerkelä, E., et al. (2010). A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PLoS ONE *5*, e10246.

Rama, P., Matuska, S., Paganoni, G., Spinelli, A., De Luca, M., and Pellegrini, G. (2010). Limbal stem-cell therapy and long-term corneal regeneration. N. Engl. J. Med. *363*, 147–155.

Reinshagen, H., Auw-Haedrich, C., Sorg, R.V., Boehringer, D., Eberwein, P., Schwartzkopff, J., Sundmacher, R., and Reinhard, T. (2011). Corneal surface reconstruction using adult mesenchymal stem cells in experimental limbal stem cell deficiency in rabbits. Acta Ophthalmol. (Copenh.) *89*, 741–748.

Reza, H.M., Ng, B.Y., Gimeno, F.L., Phan, T.T., and Ang, L.P. (2011). Umbilical cord lining stem cells as a novel and promising source for ocular surface regeneration. Stem Cell Rev. *7*, 935–947.

Robertson, D.M., Ho, S.I., and Cavanagh, H.D. (2008). Characterization of DeltaNp63 isoforms in normal cornea and telomerase-immortalized human corneal epithelial cells. Exp. Eye Res. *86*, 576–585.

Sakurai, M., Hayashi, R., Kageyama, T., Yamato, M., and Nishida, K. (2011). Induction of putative stratified epithelial progenitor cells in vitro from mouse-induced pluripotent stem cells. J. Artif. Organs *14*, 58–66.

Satake, Y., Higa, K., Tsubota, K., and Shimazaki, J. (2011). Long-term outcome of cultivated oral mucosal epithelial sheet transplantation in treatment of total limbal stem cell deficiency. Ophthalmology *118*, 1524–1530.

Schlötzer-Schrehardt, U., Dietrich, T., Saito, K., Sorokin, L., Sasaki, T., Paulsson, M., and Kruse, F.E. (2007). Characterization of extracellular matrix components in the limbal epithelial stem cell compartment. Exp. Eye Res. *85*, 845–860.

Schwartz, S.D., Hubschman, J.P., Heilwell, G., Franco-Cardenas, V., Pan, C.K., Ostrick, R.M., Mickunas, E., Gay, R., Klimanskaya, I., and Lanza, R. (2012). Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet *379*, 713–720.

Shalom-Feuerstein, R., Serror, L., De La Forest Divonne, S., Petit, I., Aberdam, E., Camargo, L., Damour, O., Vigouroux, C., Solomon, A., Gaggioli, C., et al. (2012). Pluripotent stem cell model reveals essential roles for miR-450b-5p and miR-184 in embryonic corneal lineage specification. Stem Cells *30*, 898–909.

Shalom-Feuerstein, R., Serror, L., Aberdam, E., Müller, F.J., van Bokhoven, H., Wiman, K.G., Zhou, H., Aberdam, D., and Petit, I. (2013). Impaired epithelial differentiation of induced pluripotent stem cells from ectodermal dysplasia-related patients is rescued by the small compound APR-246/PRIMA-1MET. Proc. Natl. Acad. Sci. USA *110*, 2152–2156.

Skottman, H. (2010). Derivation and characterization of three new human embryonic stem cell lines in Finland. In Vitro Cell. Dev. Biol. Anim. *46*, 206–209.

Sotozono, C., Inatomi, T., Nakamura, T., Koizumi, N., Yokoi, N., Ueta, M., Matsuyama, K., Miyakoda, K., Kaneda, H., Fukushima, M., and Kinoshita, S. (2013). Visual improvement after cultivated oral mucosal epithelial transplantation. Ophthalmology *120*, 193–200.

Streit, A. (2007). The preplacodal region: an ectodermal domain with multipotential progenitors that contribute to sense organs and cranial sensory ganglia. Int. J. Dev. Biol. *51*, 447–461.

Takács, L., Tóth, E., Berta, A., and Vereb, G. (2009). Stem cells of the adult cornea: from cytometric markers to therapeutic applications. Cytometry A *75*, 54–66.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861–872.

Tanifuji-Terai, N., Terai, K., Hayashi, Y., Chikama, T., and Kao, W.W. (2006). Expression of keratin 12 and maturation of corneal epithelium during development and postnatal growth. Invest. Ophthalmol. Vis. Sci. *47*, 545–551.

Toivonen, S., Ojala, M., Hyysalo, A., Ilmarinen, T., Rajala, K., Pekkanen-Mattila, M., Äänismaa, R., Lundin, K., Palgi, J., Weltner, J., et al. (2013). Comparative analysis of targeted differentiation of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells reveals variability associated with incomplete transgene silencing in retrovirally derived hiPSC lines. Stem Cells Transl. Med. *2*, 83–93.

Toropainen, E., Ranta, V.P., Talvitie, A., Suhonen, P., and Urtti, A. (2001). Culture model of human corneal epithelium for prediction of ocular drug absorption. Invest. Ophthalmol. Vis. Sci. *42*, 2942–2948.

Vaajasaari, H., Ilmarinen, T., Juuti-Uusitalo, K., Rajala, K., Onnela, N., Narkilahti, S., Suuronen, R., Hyttinen, J., Uusitalo, H., and Skottman, H. (2011). Toward the defined and xeno-free differentiation of functional human pluripotent stem cell-derived retinal pigment epithelial cells. Mol. Vis. *17*, 558–575.

Wolosin, J.M., Budak, M.T., and Akinci, M.A. (2004). Ocular surface epithelial and stem cell development. Int. J. Dev. Biol. *48*, 981–991.

Yang, C., Yang, Y., Brennan, L., Bouhassira, E.E., Kantorow, M., and Cvekl, A. (2010). Efficient generation of lens progenitor cells and lentoid bodies from human embryonic stem cells in chemically defined conditions. FASEB J. *24*, 3274–3283.