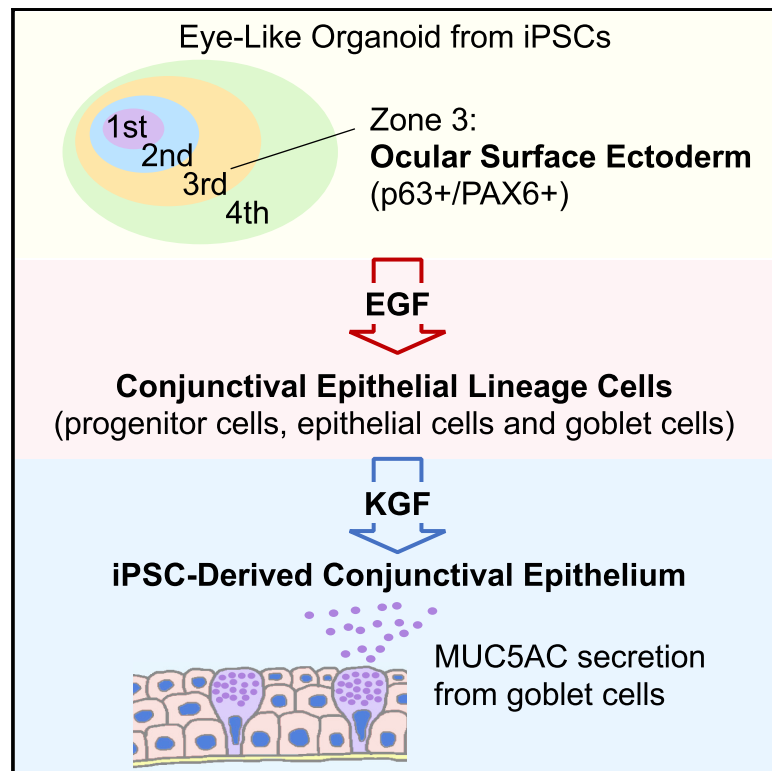Article

Generation of functional conjunctival epithelium, including goblet cells, from human iPSCs

Graphical Abstract

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In Brief

Nomi et al. generate conjunctival epithelial lineage cells from human iPSCs that have been expanded to form a two-dimensional, eye-like organoid. EGF and KGF work in conjunction to promote, respectively, the development and maturation of the iPSC-derived conjunctival epithelium, which contains mucin-producing goblet cells.

Highlights

- Human iPSCs can give rise to cells of a conjunctival epithelial lineage

- EGF signaling is required for conjunctival cells to develop from iPSCs

- KGF is needed for the maturation of the iPSC-derived conjunctival epithelium

- The iPSC-derived conjunctival epithelium contains mucin-producing goblet cells

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Article

Generation of functional conjunctival epithelium, including goblet cells, from human iPSCs

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SUMMARY

The conjunctival epithelium, which covers the sclera (the white of the eye) and lines the inside of the eyelids, is essential for mucin secretion and the establishment of a healthy tear film. Here, we describe human conjunctival development in a self-formed ectodermal autonomous multi-zone (SEAM) of cells that were derived from human-induced pluripotent stem cells (hiPSCs) and mimic whole-eye development. Our data indicate that epidermal growth factor (EGF) drives the generation of cells with a conjunctival epithelial lineage. We also show that individual conjunctival cells can be sorted and reconstituted by cultivation into a functional conjunctival epithelium that includes mucin-producing goblet cells. Keratinocyte growth factor (KGF), moreover, is necessary for the maturation of hiPSC-derived conjunctival epithelium—particularly the goblet cells—indicating key complementary roles of EGF and KGF in directing the differentiation and maturation, respectively, of the human conjunctival epithelium.

INTRODUCTION

A healthy ocular surface—comprising the conjunctival epithelium, corneal epithelium, lacrimal glands, accessory lacrimal glands, meibomian glands, and the tear film—is essential for good vision (Gipson, 2007). The conjunctiva, which sits on the sclera and lines the inside of the eyelids, is influential in regulating the ocular surface immune response and facilitating smooth eye movements. The conjunctival epithelium is also the source of mucins, which are vital for the stability of the tear film. Tear film instabilities invariably result in dry eye disease, which is manifested by physical discomfort and visual impairment (Lollett and Galor, 2018; Pflugfelder and de Paiva, 2017). Among the ocular surface epithelia, the corneal epithelium has been the most thoroughly studied, with the conjunctival epithelium mainly described in terms of cell morphology, proliferative capacity, lineage, and the presence of goblet cells. The differentiation and development of the conjunctival epithelium, however, has not been well characterized. Predominantly, this is because it is difficult to obtain conjunctival epithelial stem/progenitor cells from humans and because the properties of the conjunctival epithelium differ between humans and other animal species (Gipson, 2016). Furthermore, the conjunctival epithelium is composed of conjunctival epithelial cells and goblet cells, and an appropriate culture method that can maintain the hybrid
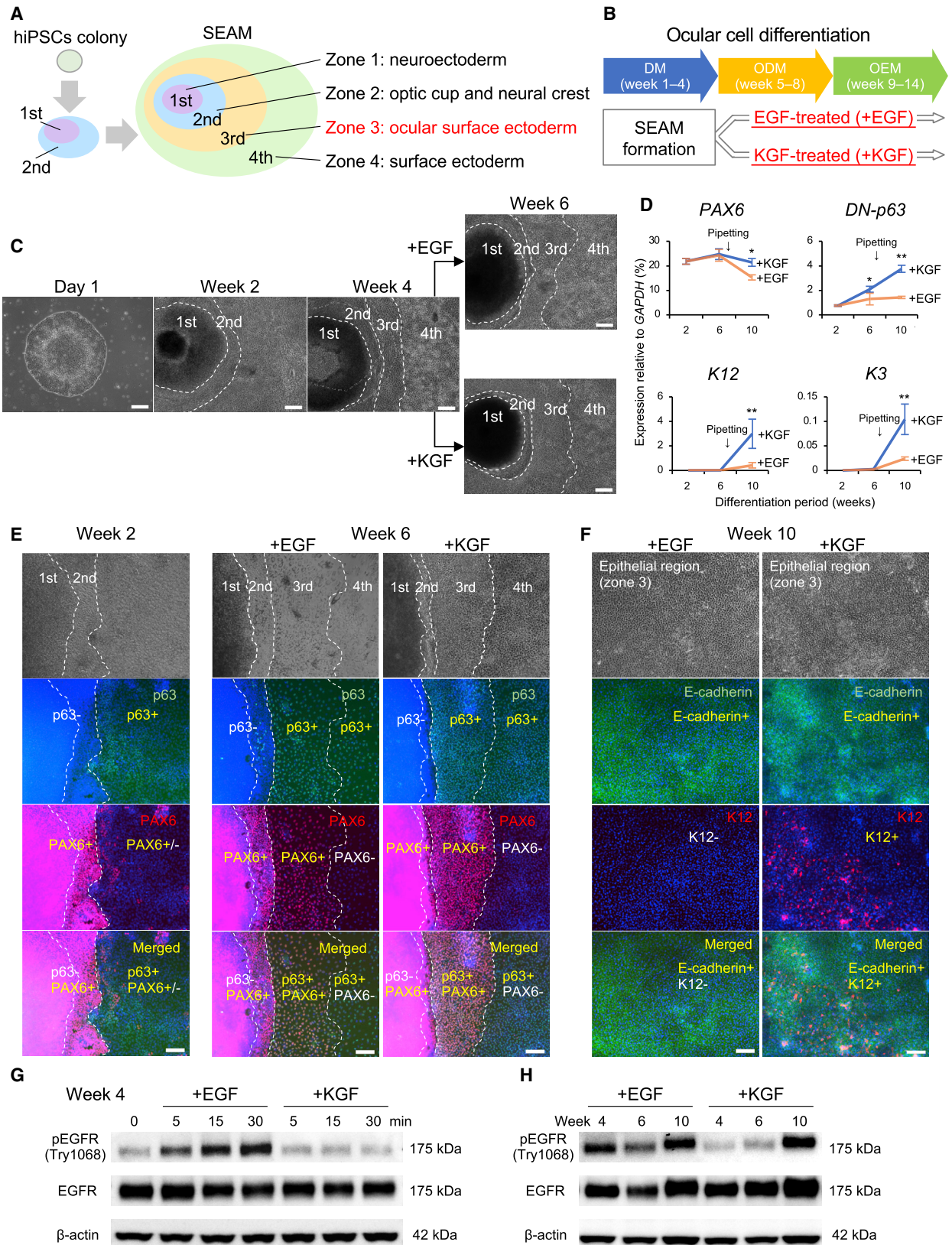
phenotype of the conjunctival epithelium has not been established, which hinders conjunctival research (Eidet et al., 2015).

Induced pluripotent stem cells (iPSCs) are PSCs that can be generated from somatic cells (Takahashi et al., 2007), and, in theory, it is possible to create any cell type found in the body using iPSCs (Eiraku et al., 2011; Zhong et al., 2014). Recently, we reported the *in vitro* generation of a self-formed ectodermal autonomous multi-zone (SEAM) of eye-like cells in which we were able to differentiate human iPSCs (hiPSCs) to mimic whole-eye development (Hayashi et al., 2016, 2017). We also succeeded in isolating and differentiating corneal epithelial stem/progenitor cells from hiPSC-derived SEAMs to fabricate functional corneal epithelia. These epithelial cell sheets were able to successfully repair the corneal surface in an experimentally induced animal model of corneal epithelial dysfunction, and hiPSC/SEAM-derived corneal epithelia are now being investigated as a transplant option for corneal epithelial failure in humans. The SEAM approach also represents a valuable resource for the study of corneal epithelial development, differentiation, and homeostasis and has the potential to similarly shed light on conjunctival development in the human eye.

Previously, we reported that human corneal epithelial cells at the periphery of the cornea, in a region of the tissue known as the limbus, exhibit conjunctival epithelium-like gene expression patterns when cultured in medium containing epidermal growth

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factor (EGF) (Yoshihara et al., 2017). Other researchers have also found that EGF has an important role in maintaining the phenotype of the conjunctival epithelium (Chung et al., 2007; Hodges et al., 2012; Shatos et al., 2009; Tan et al., 2004). Our studies further indicated that corneal epithelial cells maintain the gene expression patterns of the corneal epithelium when cultured in medium containing keratinocyte growth factor (KGF) (Yoshihara et al., 2017). Here, we investigate the potential generation of conjunctival epithelial lineage cells from hiPSC-derived SEAMs under the regulatory control of EGF and KGF.

RESULTS

EGF prohibits the differentiation of cells within a hiPSC-derived SEAM into a corneal epithelial phenotype

In a conventional SEAM, hiPSCs differentiate into four concentric zones: cells in the innermost zone 1 most closely resemble neuroectoderm; cells in zone 2 are similar to those of the optic cup and neural crest; cells in zone 3 have characteristics of the ocular surface ectoderm and lens; and cells in the outermost zone 4 are akin to surface ectoderm (Figure 1A). In SEAM zone 3, hiPSCs are driven toward a predominantly corneal epithelial cell phenotype by the addition of KGF 5 weeks after the start of differentiation. Phase-contrast micrographs obtained during hiPSC differentiation, as outlined in Figures 1B and S1A, indicate that a SEAM of four concentric zones emerges when EGF is added to the hiPSCs after 5 weeks of differentiation, similar to the results obtained with KGF (Figure 1C). A gene expression analysis of corneal epithelium-related markers revealed that, at week 10, the expression levels of paired box 6 (PAX6, an ocular cell marker), deltaN (DN)-p63 (an epithelial progenitor cell marker), keratin (K) 12, and K3 (corneal epithelial differentiation cell markers) were significantly downregulated in the EGF-treated SEAM (Figure 1D). Immunostaining for p63 and PAX6 at weeks 2 and 6 of differentiation (Figure 1E) showed that, in line with previous reports (Hayashi et al., 2016), the innermost first and second zones of the SEAM were p63$^-$/PAX6$^+$, the third zone was p63$^+$/PAX6$^+$, and the outer fourth zone was p63$^+$/PAX6$^-$. The EGF-treated SEAM exhibited a staining pattern similar to that of the KGF-treated SEAM, although the cell density in zone 3

was significantly less in the former case (Figures 1E, week 6, and S1D). Immunostaining images of the epithelial regions in zone 3 of the SEAM at week 10 of differentiation (pipetting was performed at week 7) are shown in Figure 1F, with E-cadherin used as an epithelial cell marker. K12 expression was observed in the KGF-treated SEAM derivatives (+KGF) but was not detected in the EGF-treated SEAM derivatives (+EGF), consistent with the results of the gene expression analysis (Figure 1D, K12). These findings indicate that SEAM formation can be induced by EGF and KGF, but that differentiation into corneal epithelial cells does not take place in the EGF-treated SEAM.

Western blotting results for the phosphorylated EGF receptor (pEGFR) and EGFR at week 4 of differentiation are shown in Figure 1G and disclose an increase in pEGFR levels immediately after the addition of EGF. At week 6, some phosphorylation of EGFR was observed, even in the KGF-treated SEAM, although the degree of phosphorylation was greater in the EGF-treated SEAM (Figure 1H, pEGFR). At week 10, the amount of EGFR phosphorylation was essentially the same in the EGF- and KGF-treated SEAMs, even though EGF was not added to the KGF-treated SEAM. The source of EGF in the KGF-treated SEAMs was mainly the cells in zones 3 and 4, as clarified by gene expression analysis of EGF in each zone of the EGF- and KGF-treated SEAMs at week 6, 8, and 10 (Figure S1C). These findings suggest that the activation of EGF signaling, especially at weeks 4–6 of differentiation, is critical for SEAM development.

Conjunctival epithelial lineage cells predominate in the EGF-treated SEAM

SEAMs were interrogated 10–14 weeks after the start of differentiation. Specifically, EGF-treated SEAMs were stained with anti-CD200, anti-stage-specific embryonic antigen-4 (SSEA-4), and anti-integrin subunit beta 4 (ITGB4) antibodies, followed by fluorescence-activated cell sorting (FACS) analysis, as described previously (Hayashi et al., 2018; Shibata et al., 2018, 2020). The CD200$^-$ fraction was extracted because CD200 is not expressed in human conjunctival epithelium (Figure S2B). Our analysis showed that SSEA-4 was expressed in both human conjunctival and corneal epithelium; however, the staining intensities were different, with the conjunctival epithelium

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Figure 1. Characterization of the EGF-treated SEAM

(A) Schematic of the SEAM structure.

(B) A schematic of the differentiation method for hiPSCs. EGF or KGF was added 5 weeks after the start of differentiation. DM, differentiation medium; ODM, ocular surface differentiation medium; OEM, ocular surface epithelium maintenance medium.

(C) Time-lapse phase-contrast microscopy for differentiating hiPSCs. The top panel shows the EGF-treated SEAM (+EGF) and the bottom panel shows the KGF-treated SEAM (+KGF) at week 6. Data are representative of 10 independent experiments. Scale bars, 200 μm.

(D) Gene expression analysis for PAX6, DN-p63, K12, and K3 in the entire SEAM at weeks 2, 6, and 10 after the start of differentiation. Weeks 2 and 6, n = 7; week 10, n = 10. *p < 0.05, **p < 0.01 (Mann-Whitney U test). Error bars: SEMs.

(E) Immunostaining for p63 (green) and PAX6 (red) in the SEAM at weeks 2 and 6 after the start of differentiation. In panels for week 6, the EGF-treated SEAM (+EGF) results are shown on the left, and the KGF-treated SEAM (+KGF) results are on the right. Data are representative of three independent experiments. Nuclei, blue. Scale bars, 100 μm.

(F) Immunostaining for E-cadherin (green) and K12 (red) in the SEAM at week 10 after the start of differentiation. The results for the EGF-treated SEAM (+EGF) are shown on the left, and those for the KGF-treated SEAM (+KGF) are on the right. Data are representative of three independent experiments. Nuclei, blue. Scale bars, 100 μm.

(G) Western blotting for phosphorylated EGF receptor (pEGFR) at Try1068, EGFR, and β-actin. Samples were collected before treatment (i.e., 0 min) and at 5, 15, and 30 min after treatment with EGF or KGF at week 4 of differentiation. Data are representative of three independent experiments.

(H) Western blotting for pEGFR at Try1068, EGFR, and β-actin. Samples were collected at weeks 4, 6, and 10 of differentiation. Data are representative of three independent experiments.

See also Figures S1, S2, and S7–S10, and Table S1.
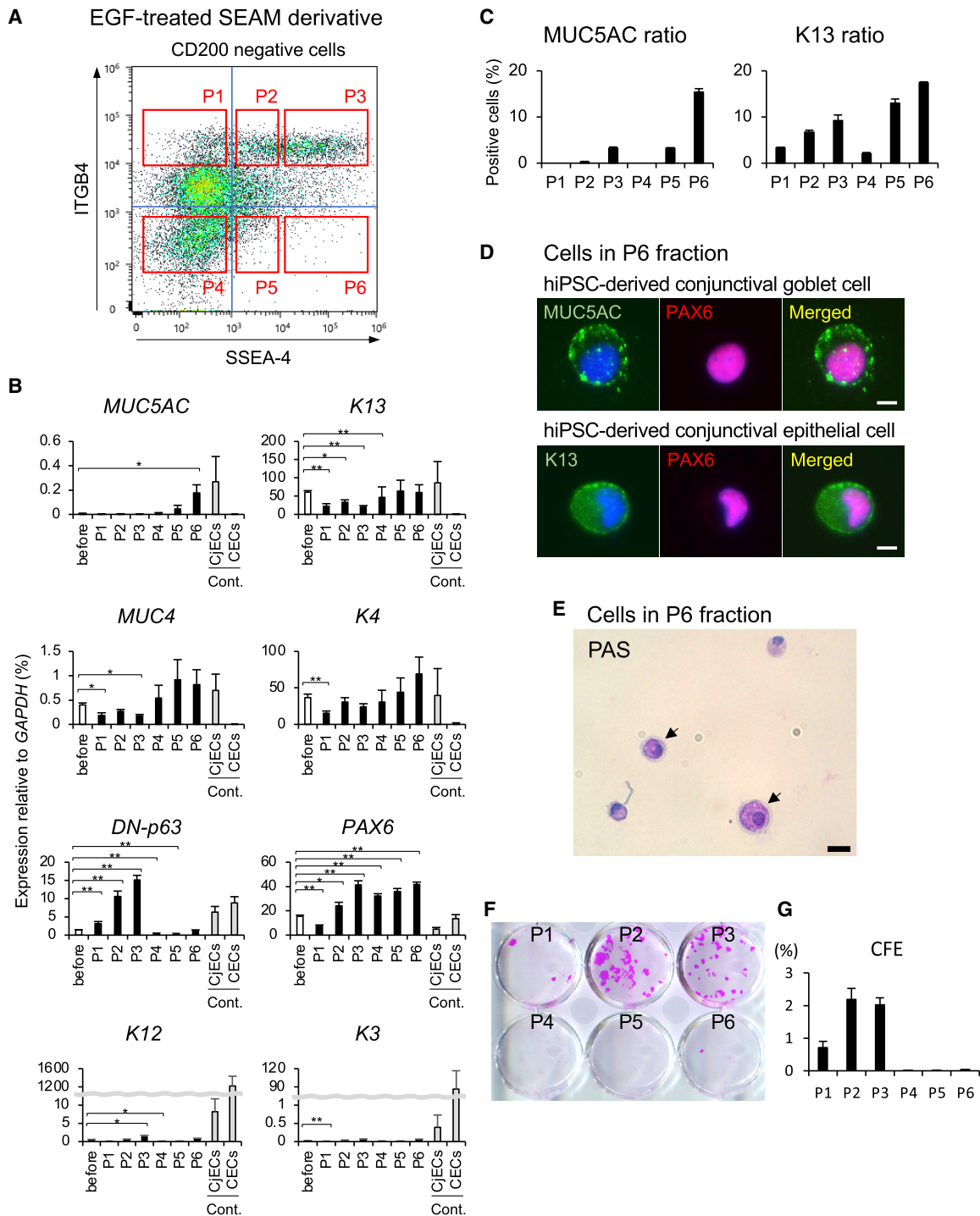
Figure 2. Analyses of the EGF-treated SEAM derivative

(A) FACS analysis of CD200, SSEA-4, and ITGB4 for the EGF-treated SEAM derivative after 10–14 weeks of culture. CD200⁻ cells were extracted and analyzed based on SSEA-4 and ITGB4 expression. The six fractions were defined as P1–P6. Data are representative of 10 independent experiments.

(B) Gene expression analysis of ocular surface epithelium-related markers for EGF-treated SEAM-derived cells before and after isolation by FACS. n = 11. *p < 0.05, **p < 0.01 (Steel's test). Error bars: SEMs. CECs, human native corneal epithelial cells; CjECs, human native conjunctival epithelial cells; cont., controls.

(C) The ratios of MUC5AC⁺ and K13⁺ cells in the P1–P6 fractions for EGF-treated SEAM derivatives. n = 4. Error bars: SEMs.

(D) Immunostaining for MUC5AC (green), PAX6 (red), and K13 (green) in the P6 fraction cells of the EGF-treated SEAM derivative. Data are representative of four independent experiments. Nuclei, blue. Scale bars, 5 μm.

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demonstrating milder staining (Figure S2B). Hence, SSEA-4⁺ cells were divided into a weak positive fraction and a strong positive fraction. In combination with ITGB4, six fractions were, therefore, examined (Figure 2A, P1–P6). KGF-treated SEAMs (i.e., those generated by the conventional SEAM method [Hayashi et al., 2016, 2017]) were similarly treated and used for comparison (Figure S1E).

Gene expression analysis revealed that conjunctival differentiation markers (i.e., *MUC5AC* [a goblet cell marker], *K13* and *K4* [mucosal epithelial cell markers], and *MUC4* [a membrane mucin marker]) were highly expressed in the SSEA-4⁺/ITGB4⁻ fractions (Figure 2B, P5–P6). Additionally, *K12* and *K3* expression levels were low in all fractions derived from the EGF-treated SEAM. Because the gene expression levels of conjunctival epithelium-related markers were decreased when CD200⁺ cells were included in each fraction, the gating of FACS is clearly relevant (Figure S3A). Immunostaining of cytospin specimens identified the phenotypes of conjunctival goblet cells (MUC5AC⁺ and PAX6⁺) and conjunctival epithelial cells (K13⁺ and PAX6⁺), especially in the P6 fraction of the EGF-treated SEAM (Figures 2C, 2D, S4A, and S4B). Periodic-acid-Schiff-positive (PAS⁺) cells were also confirmed in the same fraction (Figures 2E and S4C). The differentiated conjunctival cells were present in zone 3 of the EGF-treated SEAM; K13⁺, MUC4⁺, and K4⁺ cells in zone 4 were PAX6⁻ and were not, therefore, conjunctival cells (Figure S2A). Cells in the ITGB4⁺ fractions (P1–P3) exhibited elevated expression levels of *DN-p63* (an epithelial stem/progenitor cell marker). The expression levels of the conjunctival epithelial differentiation markers, *MUC5AC*, *K13*, *MUC4*, and *K4*, were also found to be less in the ITGB4⁺ fractions than they were in the ITGB4⁻ fractions (P4–P6). This suggests that the P1–P3 fractions contain undifferentiated cells, i.e., stem/progenitor cells with high proliferative potential. To test that hypothesis, we conducted colony-forming assays (CFAs) for each fraction of cells and confirmed the high-colony-forming efficiency (CFE) in P1–P3 (Figures 2F and 2G). Taken together, these findings imply that the P1–P3 fractions contain conjunctival stem/progenitor cells. We excluded most cells captured by FACS, which were defined as P7 (Figure S5A), because they did not have a conjunctival phenotype, had low expression levels of conjunctival epithelium-related markers (Figure S5B; *MUC5AC*, *K13*, and *p63*), and had insignificant proliferative capacity on culture dishes (Figure S5C).

Next, we aimed to assess the effects of EGF and KGF on the maturation of conjunctival progenitor cells. For that purpose, cells in the P1–P3 fractions, which we confirmed to have a high CFE, were further cultivated in maturation media (MMs) containing either EGF (EGF-supplemented medium [EGFm]) or KGF (KGF-supplemented medium [KGFm]) (Figure 3A). After 3–4 weeks, epithelial-like tissue was generated under both conditions (Figure 3B), although cell sheets derived from the P1 fraction did not contain uniform cells after cultivation in either

medium. Non-epithelial cells (p63⁻) were also apparent in the sheets (Figures 3B, arrows, and S6). Cells derived from the P2 and P3 fractions, however, formed homogeneous sheets. In parallel, we monitored the expression levels of various ocular surface epithelium-related markers, including those of native conjunctival and corneal epithelial cells, both before and after cultivation (Figure 3C). Unexpectedly, the goblet cell marker, *MUC5AC*, was significantly upregulated only in P2-derived cells cultured in KGFm. Neither P2-derived cells cultured in EGFm nor P3-derived cells cultured in either medium showed an upregulation (p < 0.01). Furthermore, under the same culture conditions, the expression levels of *K13* (a mucosal epithelial cell marker) and *MUC4* (a membrane mucin marker) were maintained. Expression of *K4* (another mucosal epithelial cell marker) was highest after incubation in maturation culture. *PAX6* was significantly downregulated, but a constant expression level was maintained. Among the four culture conditions examined, KGFm cultivation of P2-derived cells resulted in a gene expression pattern for various conjunctival epithelium-related markers that was most similar to the gene expression levels in native conjunctival epithelial cells. Expression levels of other conjunctival epithelium-related genes are shown in Figure S2C. When the P2-derived cells were cultivated in EGFm, the expression levels of *K13* and *MUC4* were maintained, but the expression level of *MUC5AC* was low. The EGF-treated SEAM derivatives were used in the above treatments, but we also confirmed similar findings in the KGF-treated SEAM derivatives. In the KGF-treated SEAM, MUC5AC⁺ cells appeared after 10 weeks of differentiation, as they did in the EGF-treated SEAM (Figure S1B). Gene expression levels of *MUC5AC* in the KGF-treated P6 fraction were far greater than were those of the EGF-treated SEAM derivatives (Figure S1F). However, the phenotype of the conjunctival epithelium was not observed in P2 cells from the KGF-treated SEAM derivatives, despite KGFm maturation culture (Figure S1G). Taken together, these findings indicate that EGF is able to maintain the phenotype of immature conjunctival lineage cells and that KGF is required for the maturation of hiPSC/SEAM-derived conjunctival goblet cells.

KGFm maturation culture of cells in the P2 fraction obtained from EGF-treated SEAMs can reconstitute a functional conjunctival epithelium that includes goblet cells

Because promising results were obtained when P2-derived cells were acquired from EGF-treated SEAMs cultivated in KGFm, these cells were expanded into cells sheets and further investigated using various conjunctival epithelial markers. En face observations of the cell sheets disclosed the presence of MUC5AC, K13, and PAX6 (Figure 3D), and we ascertained that MUC5AC⁺ cells were detected only when the P2-derived cells were cultivated in KGFm (Figure 3E). These findings are consistent with the results of gene expression analyses (Figure 3C).

(E) PAS staining of cells in the P6 fraction for the EGF-treated SEAM derivative. PAS⁺ cells are indicated with arrows. Data are representative of three independent experiments. Nuclei were stained with hematoxylin. Scale bar, 10 μm.

(F) CFA of sorted cells from the P1–P6 fractions derived from the EGF-treated SEAM. Data are representative of eight independent experiments.

(G) The CFE of sorted cells from the P1–P6 fractions. n = 8. Error bars: SEMs.

See also Figures S1–S5 and S7–S10, and Table S1.
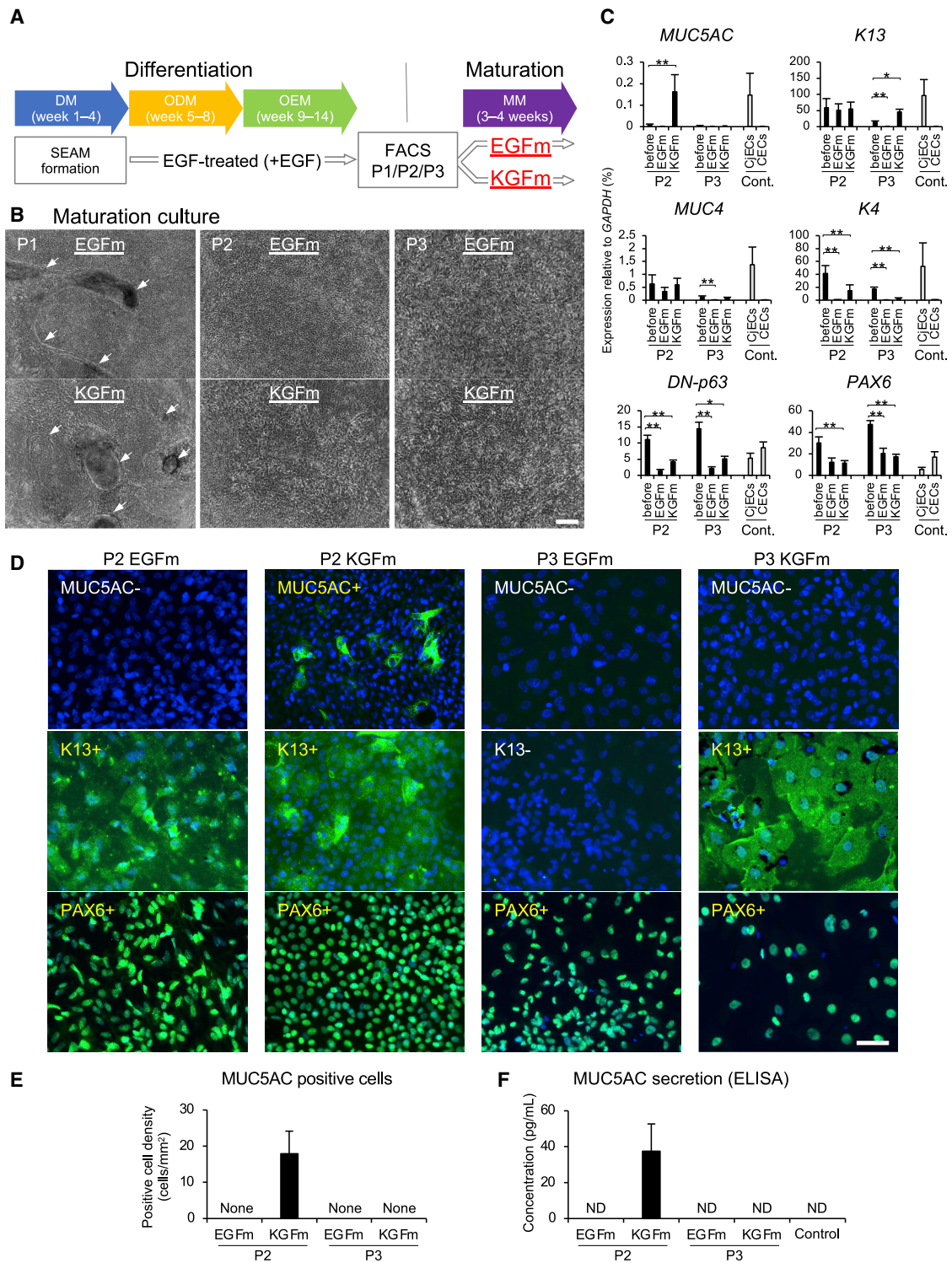
Figure 3. Generation of epithelial tissues from the EGF-treated SEAM derivative

(A) A schematic of the reconstitution method of epithelial tissues from the EGF-treated SEAM. EGFm, EGF-supplemented medium; KGFm, KGF-supplemented medium; MM, maturation medium.

(B) Phase-contrast images after maturation culture of the sorted P1–P3 cells. Arrows show non-epithelial cells. Data are representative of nine independent experiments. Scale bar, 200 μm.

Enzyme-linked immunosorbent assays (ELISAs) of the culture supernatant confirmed that MUC5AC was secreted from the P2-derived KGFm-cultured cell sheet (Figure 3F), adding credence to the idea that this hiPSC/SEAM-derived conjunctival epithelium was functional. Goblet-like cells were also observed in the conjunctival epithelial cell sheet (Figure 4A; enlarged view of MUC5AC). Expression of the conjunctival epithelial cell markers K13, K4, and K19; the membrane-bound mucin MUC4; and the unicellular gland marker K7—all of which are characteristic of native conjunctival epithelium (Figure 4A, right)—was also noted. PAS$^+$ cells were present as well, indicating the presence of mucin (Figures 4B and 4C, arrows). K12 and K10, which are not expressed in the conjunctival epithelium, were not detected (positive controls are shown in Figure S2D). The reported results were obtained using the 1383D2 hiPSC line, but we have confirmed, using lines 201B7 and YZWJs524, that a hiPSC/SEAM-derived conjunctival epithelium can be generated from EGF-treated, SEAM-derived P2 cells if incubation in maturation culture with KGFm is performed (Figures S7A–S7D).

To examine the effects of EGF on the SEAM in detail, hiPSCs were differentiated without the inclusion of EGF or KGF in the culture medium. This revealed that gene expression levels of conjunctival epithelium-related markers were less in cells from the P2 fraction obtained from untreated, compared with EGF-treated, SEAMs (Figure S8). This led us to conclude that the addition of EGF to a SEAM is a prerequisite when aiming to form a conjunctival epithelium from hiPSCs. Regarding the influence of EGF concentration on SEAM formation (Figure S9A), it was evident that, of the cells in the P2 fractions that had been obtained from SEAMs treated with various EGF concentrations, only those treated at 10 ng/mL of EGF resulted in upregulated *MUC5AC* expression (Figures S9B–S9E). This EGF concentration was also able to maintain other conjunctival epithelium-related markers and is thus considered the optimal concentration to be added to the SEAM. Regarding possible mechanisms at play in the generation of hiPSC-derived cells of a conjunctival phenotype, we note that EGF acts as a ligand, binding to EGFR (Singh et al., 2016). Accordingly, we examined whether other EGFR ligands, such as transforming growth factor (TGF)-α or amphiregulin (Areg), could promote the differentiation of hiPSCs into a conjunctival epithelial lineage. This revealed that, when TGF-α or Areg were added to the SEAM, a hiPSC-derived conjunctival epithelium, including goblet cells, was formed (Figures S10A–S10D). Overall, therefore, these results suggested that EGF ligands and/or EGFR signaling pathways are key to the differentiation of conjunctival epithelial stem/progenitor cells from hiPSCs via a SEAM.

DISCUSSION

By using growth-factor-assisted cultivation strategies, we were able to generate functional mucin-producing conjunctival epithelial cell sheets from hiPSCs (Figure 5). First, we showed that conjunctival epithelial lineage cells developed in EGF-treated hiPSC-derived SEAMs and that they could be isolated from EGF-treated SEAM derivatives. We then succeeded in reconstituting the conjunctival epithelium, including goblet cells, from the isolated cells under the influence of KGF. The SEAM concept is a technology for the differentiation of hiPSCs that mimics whole-eye development using feeder-free and serum-free media in a two-dimensional culture system (Hayashi et al., 2016, 2017). One of the characteristic features of SEAM formation is the emergence of a concentric four-zone configuration (zones 1 to 4, inner-to-outer) in which cells in different zones exhibit distinct morphologies and immunostaining patterns. Ocular surface epithelial stem/progenitor cells that express p63 and PAX6 are present in zone 3 in a conventional SEAM, but these are primarily of a corneal epithelial lineage. To date, the isolation of conjunctival epithelial lineage cells has proven elusive.

EGF promotes growth, maturation, and proliferation in various types of epithelial cell, including the conjunctival epithelium (Chung et al., 2007; Hodges et al., 2012; Shatos et al., 2009; Tan et al., 2004). Regarding the ocular surface, the involvement of EGF signaling in the development of the cornea and eyelid—but not the conjunctiva—has been suggested (Luetteke et al., 1993, 1999; Dong et al., 2017). In this study, we differentiated conjunctival lineage cells from hiPSCs by adding EGF, TGF-α, or Areg to SEAMs; all of which commonly bind to EGFR and activate EGFR signaling pathways. We further demonstrated that EGFR becomes specifically phosphorylated at an early stage after EGF augmentation and that the phosphorylation of EGFR at late stages fails to reconstitute the conjunctival epithelium from hiPSCs. Hence, we conclude that early activation of EGFR pathways is important for the differentiation and maintenance of conjunctival stem/progenitor cells in SEAMs. EGFR activates three main signal cascades: the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), the Ras/Raf/mitogen-activated protein kinase, and the phosphatidylinositol 3-kinase/Akt pathways (Scaltriti and Baselga, 2006). STAT3 is involved in the differentiation of mucosal-stratified squamous epithelia (Wu et al., 2003), whereas KGF has been reported not to activate the JAK/STAT pathway in the corneal epithelium (Liang et al., 1998). Therefore, our results are consistent with the concept that activation of the JAK/STAT pathway in the early stages of SEAM formation is likely to be instrumental in the differentiation of conjunctival cells. On the other hand, EGF signaling

(C) Gene expression analysis of ocular surface epithelium-related markers for sorted cells from the P2 and P3 fractions before and after maturation culture. Cells in the P2 and P3 fractions, n = 9; controls, n = 6. *p < 0.05, **p < 0.01 (Steel's test). Error bars: SEMs.

(D) Immunostaining for MUC5AC, K13, and PAX6 (green) in the same cell sheets as those for the en face images. Data are representative of four independent experiments. Nuclei, blue. Scale bar, 50 μm.

(E) The density of MUC5AC$^+$ cells from the P2 and P3 fraction cells after maturation culture. n = 4. Error bar: SEM.

(F) ELISA for MUC5AC in culture supernatants of the P2 and P3 fraction cells after maturation culture. Control: culture supernatant incubated without cells. n = 8. Error bar: SEM. ND, not detected.

See also Figures S1–S3 and S5–S10, and Table S1.

A hiPSC-derived conjunctival epithelium   Native conjunctival epithelium

B

C
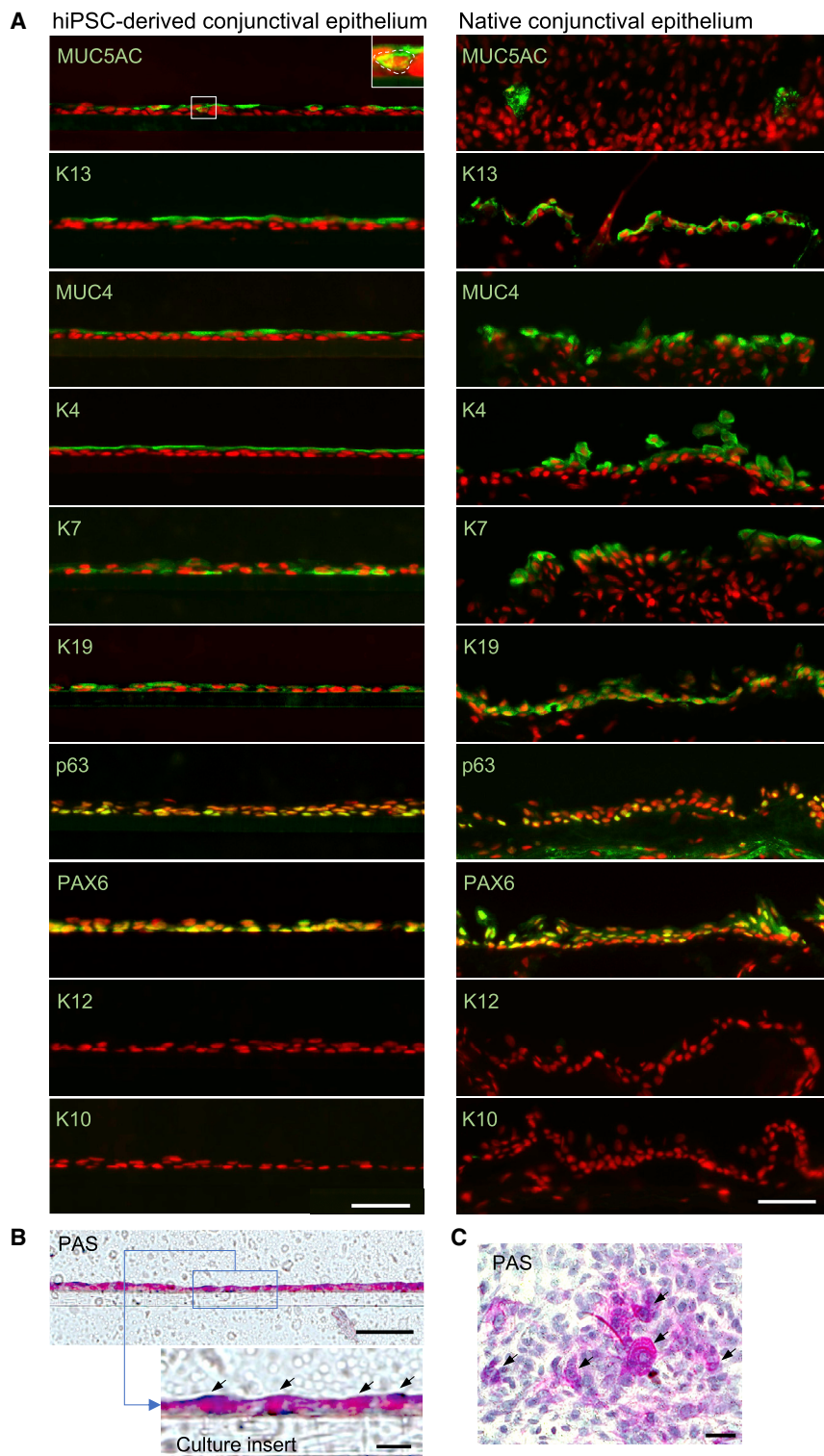
Figure 4. Characterization of the hiPSC/SEAM-derived conjunctival epithelium

(A) Immunostaining for conjunctival epithelial functional proteins and ocular surface epithelium-related markers (green) in the hiPSC/SEAM-derived conjunctival epithelium (left panels). Human native conjunctival epithelium is shown on the right for comparison. Data are representative of three independent experiments. Nuclei, red. Scale bars, 50 μm.

(B) PAS staining of the same cell sheets as those shown in (A). PAS^{+} cells are indicated with arrows. Data are representative of three independent experiments. Nuclei were stained with hematoxylin. Scale bars, 50 μm (top image) and 20 μm (enlarged image).

(C) PAS staining of the same cell sheets as those for the en face images. Data are representative of three independent experiments. Nuclei were stained with hematoxylin. Scale bar, 50 μm.

See also Figures S2, S7, and S10.

by the identification of corneal abnormalities in TGF-α-null mice and EGF/TGF-α/Areg-triple-null mice (Luetteke et al., 1993, 1999). Accordingly, we plan to further investigate the development of corneal/conjunctival cells in SEAMs with various EGFR/EGF-signaling inhibitors.

Several studies have reported the differentiation of PSCs into heterogeneous populations containing conjunctival cells; however, it is difficult to separate and analyze conjunctival epithelial lineage cells from derivatives containing other cell types (Hayashi et al., 2016; Susaimanickam et al., 2017). In this study, we succeeded in isolating conjunctival epithelial lineage cells in the living state from hiPSC derivatives by the use of FACS and specific cell surface markers. SSEA-4 is weakly expressed in native conjunctival epithelium, whereas ITGB4 is expressed in the basement membrane of the conjunctival epithelium. This leads to the notion that the SSEA-4^{+}/ITGB4^{−} population contains differentiated conjunctival cells and that the SSEA-4^{+}/ITGB4^{+} population contains undifferentiated conjunctival cells. The fact that cells that strongly express SSEA-4 could not mature into goblet cells suggests that the expression intensity of SSEA-4 may indeed reflect the degree of differentiation of hiPSC/SEAM-derived conjunctival cells.

One of the major challenges in studies of the conjunctival epithelium has been that the maintenance of the phenotype, particularly for the presence of conjunctival goblet cells, had not been achieved in vitro (Gipson, 2016). In this study, we

pathways may have a role for the later differentiation of corneal cells in the SEAM because EGFR was clearly phosphorylated in the KGF-treated SEAM at week 10, despite EGF not being included in the culture medium. This hypothesis is supported
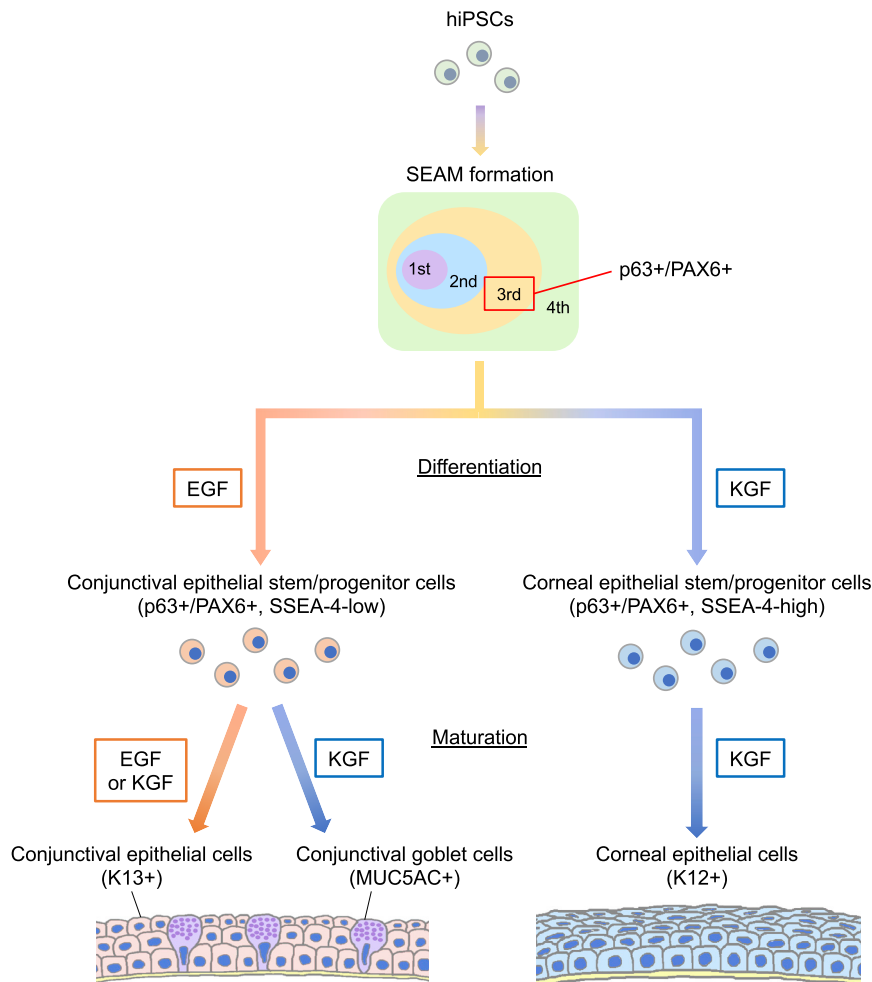
Figure 5. Schematic of conjunctival epithelial cell generation

hiPSCs were differentiated to form SEAMs. Ocular surface epithelial stem/progenitor cells, which express p63 and PAX6, are present in SEAM zone 3. In differentiation culture, conjunctival epithelial lineage cells were predominantly seen in the EGF-treated SEAM derivatives, whereas corneal epithelial lineage cells predominated in the KGF-treated SEAM derivatives. KGF was necessary for maturation of the hiPSC/SEAM-derived conjunctival epithelium, particularly for the goblet cells. See also Figures S1, S2, and S7–S10.

succeeded in reconstituting conjunctival epithelia, including goblet cells, *in vitro* by cultivating hiPSC/SEAM-derived conjunctival stem/progenitor cells. Interestingly, expression of the goblet cell marker *MUC5AC* was upregulated when KGF, but not EGF, was added to the MM. This indicates that KGF has an important role in the maturation of conjunctival goblet cells, consistent with other reports that have shown that KGF promotes the differentiation and proliferation of goblet cells in the intestinal tract (Fernández-Estívariz et al., 2003; Iwakiri and Podolsky, 2001). It has also been found that, in some epithelial cells, KGF can maintain a more-natural cell state than EGF is able to (Marchese et al., 1990; Miyashita et al., 2013; Yoshihara et al., 2017). Our discovery suggested that the difficulties associated with stably maintaining conjunctival goblet cells *in vitro* by conventional methods using EGF can be overcome with the use of KGF. The take-home message from the work described herein is that EGF is instrumental in promoting the differentiation of hiPSC/SEAM-derived ocular surface ectoderm into a conjunctival epithelial stem/progenitor cell lineage and that KGF is essential for the maturation of the conjunctival epithelium, particularly for the maturation of the goblet cells. This finding, aligned to the use of SEAM technology, has a range of potential applications to help us better understand human conjunctival development, as well as aiding drug discovery research targeting conjunctival cells and regenerative medicine applications to treat ocular surface disease.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

K. Nomi, R.H., and K. Nishida designed the study. Y.I. and Y.K. supported experimental procedures. K. Nomi conducted experiments and analyzed the data. Y.K. and T.K. maintained the hiPSC cultures. A.J.Q. supervised the project. K. Nomi, A.J.Q., and R.H. wrote the paper. R.H. and K. Nishida obtained financial support.

DECLARATION OF INTERESTS

K. Nomi, R.H., and K. Nishida have submitted a patent application on "Method for inducing differentiation into pluripotent stem cell-derived conjunctival cells." R.H. and K. Nishida have submitted a patent application on "Method for inducing differentiation of corneal epithelial cells from pluripotent stem cells."

REFERENCES

Chung, S.H., Lee, J.H., Yoon, J.H., Lee, H.K., and Seo, K.Y. (2007). Multi-layered culture of primary human conjunctival epithelial cells producing MUC5AC. Exp. Eye Res. *85*, 226–233.

Dong, F., Call, M., Xia, Y., and Kao, W.W.Y. (2017). Role of EGF receptor signaling on morphogenesis of eyelid and meibomian glands. Exp. Eye Res. *163*, 58–63.

Eidet, J.R., Dartt, D.A., and Utheim, T.P. (2015). Concise review: comparison of culture membranes used for tissue engineered conjunctival epithelial equivalents. J. Funct. Biomater. *6*, 1064–1084.

Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., Sekiguchi, K., Adachi, T., and Sasai, Y. (2011). Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature *472*, 51–56.

Fernández-Estívariz, C., Gu, L.H., Gu, L., Jonas, C.R., Wallace, T.M., Pascal, R.R., Devaney, K.L., Farrell, C.L., Jones, D.P., Podolsky, D.K., and Ziegler, T.R. (2003). Trefoil peptide expression and goblet cell number in rat intestine: effects of KGF and fasting-refeeding. Am. J. Physiol. Regul. Integr. Comp. Physiol. *284*, R564–R573.

Gipson, I.K. (2007). The ocular surface: the challenge to enable and protect vision: the Friedenwald lecture. Invest. Ophthalmol. Vis. Sci. *48*, 4390–, 4391–4398.

Gipson, I.K. (2016). Goblet cells of the conjunctiva: a review of recent findings. Prog. Retin. Eye Res. *54*, 49–63.

Hayashi, R., Ishikawa, Y., Sasamoto, Y., Katori, R., Nomura, N., Ichikawa, T., Araki, S., Soma, T., Kawasaki, S., Sekiguchi, K., et al. (2016). Co-ordinated ocular development from human iPS cells and recovery of corneal function. Nature *531*, 376–380.

Hayashi, R., Ishikawa, Y., Katori, R., Sasamoto, Y., Taniwaki, Y., Takayanagi, H., Tsujikawa, M., Sekiguchi, K., Quantock, A.J., and Nishida, K. (2017). Coordinated generation of multiple ocular-like cell lineages and fabrication of functional corneal epithelial cell sheets from human iPS cells. Nat. Protoc. *12*, 683–696.

Hayashi, R., Ishikawa, Y., Katayama, T., Quantock, A.J., and Nishida, K. (2018). CD200 facilitates the isolation of corneal epithelial cells derived from human pluripotent stem cells. Sci. Rep. *8*, 16550.

Hodges, R.R., Bair, J.A., Carozza, R.B., Li, D., Shatos, M.A., and Dartt, D.A. (2012). Signaling pathways used by EGF to stimulate conjunctival goblet cell secretion. Exp. Eye Res. *103*, 99–113.

Iwakiri, D., and Podolsky, D.K. (2001). Keratinocyte growth factor promotes goblet cell differentiation through regulation of goblet cell silencer inhibitor. Gastroenterology *120*, 1372–1380.

Kawasaki, H., Suemori, H., Mizuseki, K., Watanabe, K., Urano, F., Ichinose, H., Haruta, M., Takahashi, M., Yoshikawa, K., Nishikawa, S., et al. (2002). Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. Proc. Natl. Acad. Sci. USA *99*, 1580–1585.

Liang, Q., Mohan, R.R., Chen, L., and Wilson, S.E. (1998). Signaling by HGF and KGF in corneal epithelial cells: Ras/MAP kinase and Jak-STAT pathways. Invest. Ophthalmol. Vis. Sci. *39*, 1329–1338.

Lollett, I.V., and Galor, A. (2018). Dry eye syndrome: developments and lifitegrast in perspective. Clin. Ophthalmol. *12*, 125–139.

Luetteke, N.C., Qiu, T.H., Peiffer, R.L., Oliver, P., Smithies, O., and Lee, D.C. (1993). TGF α deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell *73*, 263–278.

Luetteke, N.C., Qiu, T.H., Fenton, S.E., Troyer, K.L., Riedel, R.F., Chang, A., and Lee, D.C. (1999). Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. Development *126*, 2739–2750.

Marchese, C., Rubin, J., Ron, D., Faggioni, A., Torrisi, M.R., Messina, A., Frati, L., and Aaronson, S.A. (1990). Human keratinocyte growth factor activity on proliferation and differentiation of human keratinocytes: differentiation response distinguishes KGF from EGF family. J. Cell. Physiol. *144*, 326–332.

Miyashita, H., Yokoo, S., Yoshida, S., Kawakita, T., Yamagami, S., Tsubota, K., and Shimmura, S. (2013). Long-term maintenance of limbal epithelial progenitor cells using rho kinase inhibitor and keratinocyte growth factor. Stem Cells Transl. Med. *2*, 758–765.

Miyazaki, T., Futaki, S., Suemori, H., Taniguchi, Y., Yamada, M., Kawasaki, M., Hayashi, M., Kumagai, H., Nakatsuji, N., Sekiguchi, K., and Kawase, E. (2012). Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. Nat. Commun. *3*, 1236.

Nakagawa, M., Taniguchi, Y., Senda, S., Takizawa, N., Ichisaka, T., Asano, K., Morizane, A., Doi, D., Takahashi, J., Nishizawa, M., et al. (2014). A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. Sci. Rep. *4*, 3594.

Pflugfelder, S.C., and de Paiva, C.S. (2017). The pathophysiology of dry eye disease: what we know and future directions for research. Ophthalmology *124* (*11S*), S4–S13.

Scaltriti, M., and Baselga, J. (2006). The epidermal growth factor receptor pathway: a model for targeted therapy. Clin. Cancer Res. *12*, 5268–5272.

Shatos, M.A., Hodges, R.R., Oshi, Y., Bair, J.A., Zoukhri, D., Kublin, C., Lashkari, K., and Dartt, D.A. (2009). Role of cPKCalpha and nPKCepsilon in EGF-stimulated goblet cell proliferation. Invest. Ophthalmol. Vis. Sci. *50*, 614–620.

Shibata, S., Hayashi, R., Okubo, T., Kudo, Y., Katayama, T., Ishikawa, Y., Toga, J., Yagi, E., Honma, Y., Quantock, A.J., et al. (2018). Selective laminin-directed differentiation of human induced pluripotent stem cells into distinct ocular lineages. Cell Rep. *25*, 1668–1679.e5.

Shibata, S., Hayashi, R., Kudo, Y., Okubo, T., Imaizumi, T., Katayama, T., Ishikawa, Y., Kobayashi, Y., Toga, J., Taniguchi, Y., et al. (2020). Cell-type-specific adhesiveness and proliferation propensity on laminin isoforms enable purification of iPSC-derived corneal epithelium. Stem Cell Reports *14*, 663–676.

Singh, B., Carpenter, G., and Coffey, R.J. (2016). EGF receptor ligands: recent advances. F1000Res. *5*, 2270.

Susaimanickam, P.J., Maddileti, S., Pulimamidi, V.K., Boyinpally, S.R., Naik, R.R., Naik, M.N., Reddy, G.B., Sangwan, V.S., and Mariappan, I. (2017).

Generating minicorneal organoids from human induced pluripotent stem cells. Development *144*, 2338–2351.

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.

Tan, D.T.H., Ang, L.P.K., and Beuerman, R.W. (2004). Reconstruction of the ocular surface by transplantation of a serum-free derived cultivated conjunctival epithelial equivalent. Transplantation *77*, 1729–1734.

Wu, R., Sun, S., and Steinberg, B.M. (2003). Requirement of STAT3 activation for differentiation of mucosal stratified squamous epithelium. Mol. Med. *9*, 77–84.

Yoshihara, M., Sasamoto, Y., Hayashi, R., Ishikawa, Y., Tsujikawa, M., Hayashizaki, Y., Itoh, M., Kawaji, H., and Nishida, K. (2017). High-resolution promoter map of human limbal epithelial cells cultured with keratinocyte growth factor and rho kinase inhibitor. Sci. Rep. *7*, 2845.

Zhong, X., Gutierrez, C., Xue, T., Hampton, C., Vergara, M.N., Cao, L.H., Peters, A., Park, T.S., Zambidis, E.T., Meyer, J.S., et al. (2014). Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat. Commun. *5*, 4047.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-p63	Santa Cruz Biotechnology	Cat# sc-8431; RRID: AB_628091; clone 4A4
Rabbit polyclonal anti-PAX6	Covance Research Products Inc	Cat# PRB-278P; RRID: AB_291612
Rabbit polyclonal anti-E-cadherin	Santa Cruz Biotechnology	Cat# sc-7870; RRID: AB_2076666
Goat polyclonal anti-K12	Santa Cruz Biotechnology	Cat# sc-17098; RRID: AB_639266
Mouse monoclonal anti-MUC5AC	Santa Cruz Biotechnology	Cat# sc-33667; RRID: AB_627973; clone CLH2
Mouse monoclonal anti-K13	Abcam	Cat# ab16112; RRID: AB_302267; clone AE8
Mouse monoclonal anti-MUC4	Abcam	Cat# ab52263; RRID: AB_881163; clone 8G-7
Mouse monoclonal anti-K4	Abcam	Cat# ab9004; RRID: AB_306932; clone 6B10
Mouse monoclonal anti-K7	Abcam	Cat# ab9021; RRID: AB_306947; clone RCK105
Rabbit polyclonal anti-K19	Abcam	Cat# ab15463; RRID: AB_2281021
Mouse monoclonal anti-K10	Dako	Cat# M7002; clone DE-K10
Rabbit monoclonal anti-phospho-EGF receptor (Tyr1068)	Cell Signaling Technology	Cat# 3777; RRID: AB_2096270; clone D7A5
Rabbit monoclonal anti-EGF receptor	Cell Signaling Technology	Cat# 4267; RRID: AB_2246311; clone D38B1
Mouse monoclonal anti-β-actin	Sigma-Aldrich	Cat# 5441; RRID: AB_476744; clone AC-15
Mouse monoclonal anti-SSEA-4 PE conjugate	BioLegend	Cat# 330406; RRID: AB_1089206; clone MC-813-70
Mouse monoclonal anti-CD104 Alexa Fluor 647 conjugate	BD Biosciences	Cat# 624024; clone 450-9D
Mouse monoclonal anti-CD200 PE-Cy7 conjugate	BD Biosciences	Cat# 624052; clone MRC OX-104
Donkey anti-mouse IgG Alexa Fluor 488 conjugate	Life Technologies	Cat# A-21202; RRID: AB_141607
Donkey anti-rabbit IgG Alexa Fluor 594 conjugate	Life Technologies	Cat# A-21207; RRID: AB_141637
Donkey anti-goat IgG Alexa Fluor 568 conjugate	Life Technologies	Cat# A-11057; RRID: AB_142581
Donkey anti-rabbit IgG horseradish peroxidase conjugate	GE Healthcare	Cat# NA934-100UI; RRID: AB_772206
Sheep anti-mouse IgG horseradish peroxidase conjugate	GE Healthcare	Cat# NA931-100UI; RRID: AB_772210
Biological samples		
Research-grade human corneoscleral tissue	SightLife	https://sightlife.org/
Chemicals, peptides, and recombinant proteins		
iMatrix-511 (LN511E8)	Nippi	Cat# 892012
FBS	Life Technologies	Cat# 12483-020
Mitomycin C	Kyowa Hakko Kirin	N/A
Dispase II, powder	Life Technologies	Cat# 17105-041

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TrypLE™ Express Enzyme (1X), phenol red	Thermo Fisher Scientific	Cat# 12605010
KnockOut™ Serum Replacement	Life Technologies	Cat# 10828-028
Sodium pyruvate	Life Technologies	Cat# 11360-070
Non-essential amino acids	Life Technologies	Cat# 11140-050
L-glutamine	Thermo Fisher Scientific	Cat# 25030081
Penicillin-streptomycin solution	Life Technologies	Cat# 15140-122
2-Mercaptoethanol	Life Technologies	Cat# 21985-023
Monothioglycerol	Wako	Cat# 195-15791
Y-27632	Wako	Cat# 034-24024
Recombinant human EGF	R&D Systems	Cat# 236-EG
Recombinant human KGF	Wako	Cat# 112-00813
Recombinant human TGFa	R&D Systems	Cat# 239_A
Recombinant human Amphiregulin	R&D Systems	Cat# 262-AR
B27™ supplement	Life Technologies	Cat# 17504-044
QIAzol Lysis Reagent	QIAGEN	Cat# 79306
4% paraformaldehyde phosphate buffer solution	Wako	Cat# 163-20145
Normal donkey serum	Jackson ImmunoResearch	Cat# 017-000-121
Triton™-X 100	Sigma-Aldrich	Cat# T8787
Hoechst 33342	Wako	Cat# 346-07951
RIPA buffer (10x)	Nacalai Tesque	Cat# 08714-04
PhosSTOP™ (phosphatase inhibitor)	Sigma-Aldrich	Cat# 4906845001
StemPro™ Accutase™ Cell Dissociation Reagent	Life Technologies	Cat# A11105-01
Methanol	Wako	Cat# 131-01826
10% formaldehyde neutral buffer solution	Wako	Cat# 062-01661
Rhodamine B	Wako	Cat# 180-00132
Critical commercial assays		
SuperScript™ III First-Strand Synthesis System for qRT-PCR	Life Technologies	Cat# 18080051
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23227
ECL™ Prime Western Blotting Detection Reagent	GE Healthcare	Cat# RPN2236
BD Cytofix/Cytoperm™ Fixation/ Permeabilization Solution Kit	BD Biosciences	Cat# 554714
PAS staining kit	MERCK KGaA	Cat#101646
ELISA Kit for Mucin 5 Subtype AC (MUC5AC)	USCN	Cat# SEA756Hu
Experimental models: cell lines		
Human: iPS cell line 1383D2	Center for iPS Cell Research and Application	N/A
Human: iPS cell line 201B7	RIKEN Bio Resource Center	Cat# HPS0063; RRID: CVCL_A324
Human: iPS cell line YZWJs524	Center for iPS Cell Research and Application	N/A
Mouse: NIH 3T3 cell line	N/A	N/A
Oligonucleotides		
TaqMan probes were listed in Table S1	Life Technologies	Cat# 4331182
Software and algorithms		
AxioVs40 version 4.8.2.0	Carl Zeiss	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
7500 Software version 2.0.6	Thermo Fisher Scientific	RRID: SCR_014596; https://www.thermofisher.com/us/en/home/technical-resources/software-downloads/applied-biosystems-7500-real-time-pcr-system.html
WorkOut 2.5 version 1.0.0.0	Dazdaq Solutions	N/A
Image Lab Software version 4.1	Bio-Rad	RRID: SCR_014210; http://www.bio-rad.com/en-us/sku/1709690-image-lab-software?ID=1709690
Cell Sorter Software version 2.1.5	SONY	N/A
JMP pro version 14.1.0	SAS	RRID: SCR_014242; https://www.jmp.com/en_us/software/predictive-analytics-software.html

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the Lead Contact, Ryuhei Hayashi (ryuhei.hayashi@ophthal.med.osaka-u.ac.jp).

Materials availability
Unique materials generated in this study are available from the Lead Contact upon reasonable request following the signing of a Materials Transfer Agreement.

Data and code availability
This study did not generate any datasets or code.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Cell lines
hiPSC lines 1383D2 and YZWJs524 were provided by the Center for iPS Cell Research and Application, Kyoto University (Nakagawa et al., 2014), while the hiPSC line 201B7 was provided by RIKEN Bio Resource Center (Tsukuba, Japan) (Takahashi et al., 2007). All hiPSCs were maintained in StemFit medium (Ajinomoto, Tokyo, Japan) on laminin 511E8 fragment (LN511E8; Nippi, Tokyo, Japan)-coated dishes (0.5 μg/cm^2) at 37°C (Miyazaki et al., 2012; Nakagawa et al., 2014). All experiments using recombinant DNA were approved by the Recombinant DNA Committees of Osaka University and were conducted according to our institutional guidelines. Mouse NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) at 37°C. For use as feeder cells in CFAs, NIH 3T3 cells were treated with 8 μg/mL mitomycin C for 2 h at 37°C.

Preparation of human native conjunctival and corneal epithelial cells
Research-grade corneoscleral tissues were obtained from SightLife (Seattle, WA, USA) and bulbar conjunctiva was excised using surgical microscissors. The excised conjunctiva and the remaining corneoscleral tissue were incubated separately in DMEM containing dispase (Thermo Fisher Scientific) for 1 h at 37°C. Subsequently, the conjunctival epithelial layer was scraped with forceps under a dissecting microscope and dissociated using TrypLE™ Express Enzyme (Thermo Fisher Scientific) for 20 min at 37°C.

METHODS DETAILS

SEAM formation from hiPSCs
The differentiation protocol for hiPSCs is illustrated in Figures 1B and S1A, which is as previously described (Hayashi et al., 2016, 2017) with some modifications. The hiPSCs were seeded on LN511E8-coated dishes at 350–700 cells/cm^2, after which they were cultured in StemFit medium for 8–12 days at 37°C. The culture medium was then changed to differentiation medium (DM; GMEM [Thermo Fisher Scientific] supplemented with 10% KnockOut™ Serum Replacement [Thermo Fisher Scientific], 1 mM sodium pyruvate [Thermo Fisher Scientific], 0.1 mM nonessential amino acids [Thermo Fisher Scientific], 2 mM L-glutamine [Thermo Fisher Scientific], 1% penicillin-streptomycin solution [Thermo Fisher Scientific], and 55 μM 2-mercaptoethanol [Thermo Fisher Scientific]

or monothioglycerol [Wako, Osaka, Japan]) (Kawasaki et al., 2002). After approximately four weeks of culture in DM at 37°C, the medium was changed to ocular surface differentiation medium (ODM; DM and Cnt-PR [without EGF or fibroblast growth factor 2; CELLnTEC Advanced Cell Systems, Bern, Switzerland], 1:1, containing 10 μM Y-27632 [Wako] and 1% penicillin-streptomycin solution) at 37°C. As needed, 1, 10, or 100 ng/mL EGF (R&D Systems, Minneapolis, MN, USA); 10 ng/mL KGF (Wako); 10 ng/mL TGF-α (R&D Systems); and/or 10 ng/mL Areg (R&D Systems) were added to the medium. During ODM culture, at week 7 after the start of differentiation, non-epithelial cells were removed by manual pipetting under a microscope. After pipetting, the medium was changed to fresh ODM. After approximately four weeks culture in ODM, the medium was changed to ocular surface epithelium maintenance medium (DMEM/F12 [2:1; Thermo Fisher Scientific] containing 10 μM Y-27632, 2% B27™ supplement [Thermo Fisher Scientific], and 1% penicillin-streptomycin solution) for 2–6 weeks at 37°C. As needed, 1, 10, or 100 ng/mL EGF; 10 ng/mL KGF; 10 ng/mL TGF-α; and/or 10 ng/mL Areg were added to the medium. Phase-contrast microscopic observations were performed with an Axio Observer.D1 (Carl Zeiss, Jena, Germany) and analyzed with AxioVs40 software (Carl Zeiss). Cells in each zone of a SEAM at weeks 6, 8, and 10 of differentiation were manually separated and collected under a microscope as needed.

qRT-PCR

Total RNA was extracted from cells using QIAzol Lysis Reagent (QIAGEN, Venlo, Netherlands). RT was performed using the Super-Script™ III First-Strand Synthesis System for qRT-PCR (Thermo Fisher Scientific) according to the manufacturer's protocol, and the generated cDNA was then used as a template for PCR. qRT-PCR was performed using the ABI Prism 7500 Fast Sequence Detection System (Thermo Fisher Scientific) in accordance with the manufacturer's instructions and analyzed with 7500 software (Thermo Fisher Scientific). The TaqMan MGB used in this study is shown in Table S1. The thermocycling program was performed with an initial cycle at 95°C for 20 s, followed by 45 cycles at 95°C for 3 s and 60°C for 30 s.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 20 min at 4°C or cold methanol (Wako) for 20 min at −20°C as needed. They were then washed with Tris-buffered saline (TBS; TaKaRa Bio, Shiga, Japan) or phosphate-buffered saline (PBS) three times for 5 min and incubated with TBS containing 5% donkey serum and 0.3% Triton™ X-100 (Sigma-Aldrich, Saint Louis, MO, USA) for 1 h at room temperature to block nonspecific reactions. The cells were then incubated with various antibodies (shown in Key resources table) overnight at 4°C, after which they were washed three times with TBS for 5 min and then incubated with a 1:200 dilution of Alexa Fluor 488-, 568-, or 594-conjugated secondary antibodies (Thermo Fisher Scientific) and a 1:100 dilution of Hoechst 33342 (Wako) for 1 h at room temperature. Stained samples were observed using a fluorescence microscope (Axio Observer.D1). The density of SEAM zone 3 was calculated as the average of the p63+/PAX6+ cells per field in 10 different random fields from three different samples. To assess the density of goblet cells in the hiPSC-derived conjunctival sheet, MUC5AC+ cells were counted per unit area, defined as a quarter area of a 24 well cell insert.

Western blotting

Cells were lysed in RIPA buffer (Nacalai Tesque, Kyoto, Japan) consisting of 50 mM Tris-HCl buffer [pH 7.6], 150 mM NaCl, 1% (w/v) nonidet P40 substitute, 0.5% (w/v) sodium deoxycholate, protease inhibitor cocktail, and 0.1% (w/v) SDS supplemented with phosphatase inhibitor (Sigma-Aldrich). Protein concentrations were measured using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific) and analyzed with WorkOut 2.5 software (Dazdaq Solutions, Brighton, UK). Protein samples (30 μg) were separated by SDS-PAGE using 3%–8% gradient Tris-acetate gels and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with 5% skim milk (BD Biosciences, San Diego, CA, USA) for 1 h at room temperature to block nonspecific reactions, and incubated with antibodies against pEGFR (3777; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were washed three times with TBS-T for 5 min and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (NA934-100UL; GE Healthcare, Little Chalfont, UK) secondary antibodies for 1 h at room temperature. The membranes were washed three times with TBS-T for 5 min, after which immunoblotting bands were visualized by ECL™ Prime Western Blotting Detection Reagent (GE Healthcare). The membranes were scanned using a ChemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA) and analyzed with Image Lab software (Bio-Rad). The membranes were washed three times with distilled water for 10 min, and incubated with stripping buffer containing of 62.5 mM Tris-HCl, 2% SDS, and 0.7% 2-mercaptoethanol for 30 min at 50°C. The membranes were then washed four times with TBS-T for 10 min, incubated with 5% skim milk for 1 h at room temperature, and incubated with antibodies against EGFR (4267; Cell Signaling Technology) or β-actin (A5441; Sigma-Aldrich) overnight at 4°C or for 1 h at room temperature. The membranes were washed three times with TBS-T for 5 min and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (NA931-100UL; GE Healthcare) secondary antibodies for 1 h at room temperature. The membranes were washed three times with TBS-T for 5 min, immunoblotting bands were visualized by ECL prime reagent, scanned using a ChemiDoc XRS+ imaging system, and analyzed with Image Lab software.

FACS analysis

Differentiated hiPSCs were analyzed by FACS as described previously (Hayashi et al., 2017, 2018). Cells were then dissociated using StemPro™ Accutase™ Cell Dissociation Reagent (Thermo Fisher Scientific) and resuspended in cold KCM medium (DMEM without glutamine and Nutrient Mixture F-12 Ham [3:1; Thermo Fisher Scientific] supplemented with 5% FBS [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, USA], 1 nM cholera toxin [List Biological Laboratory, Campbell, CA, USA], 2.25 μg/mL bovine transferrin HOLO form [Thermo Fisher Scientific], 2 mM L-glutamine, 0.5% insulin transferrin selenium solution [Thermo Fisher Scientific], and 1% penicillin-streptomycin solution). The harvested cells were filtered with a cell strainer (40 μm; BD Biosciences) and stained with antibodies against CD200 (BD Biosciences), SSEA-4 (BioLegend, San Diego, CA, USA), and CD104 (ITGβ4; BD Biosciences) for 30 min on ice. Stained cells were washed twice with PBS, and cell sorting was performed using a SH800 instrument (SONY, Tokyo, Japan). In all FACS experiments, differentiated hiPSCs were also stained with nonspecific isotype IgG as controls (BioLegend and BD Biosciences). Data were analyzed using Cell Sorter software (SONY).

Cytospin experiment
Cells were fixed with BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences) for 20 min at 4°C and/or cold methanol for 20 min at −20°C as needed. The fixed cells were suspended in KCM at 2×10^5 cells/mL. 200 μL of the cell suspension was centrifuged at 1,000 rpm (123 × g) for 5 min in a Cytospin 4 Cytocentrifuge (Thermo Fisher Scientific) and attached to glass slides.

PAS staining
Differentiated hiPSCs were fixed with cold methanol for 20 min at −20°C, 10% formaldehyde neutral buffer solution (Nacalai Tesque) for 1 h at room temperature, or BD Cytofix/Cytoperm™ for 20 min at 4°C. PAS staining was performed with a PAS staining kit (MERCK KGaA, Darmstadt, Germany) according to the manufacturer's protocol. The stained samples were observed with an Axio Imager.A2 (Carl Zeiss).

CFA
Cells sorted by FACS were seeded onto mitomycin C-treated NIH 3T3 feeder cells at 2000–5000 cells/well in 12-well plates. They were then cultured in KCM containing 20 ng/mL KGF and 10 μM Y-27632 for 10–14 days at 37°C. The colonies were fixed with 10% formaldehyde neutral buffer solution for 1 h at room temperature and then stained with rhodamine B (Wako) for 2 h at room temperature. Colony formation was assessed using a dissecting microscope, and the CFE was calculated.

Maturation culture of sorted hiPSC
Sorted hiPSCs were seeded on LN511E8-coated (0.5 μg/cm²) cell culture inserts without cell passaging. Cells were then cultured in MM (DMEM/F12 containing 10 μM Y-27632, 2% B27 supplement, 1% penicillin-streptomycin solution, and 10–20 ng/mL EGF or 10-20 ng/mL KGF) until confluence (about 2–4 weeks) at 37°C.

ELISA for MUC5AC
Culture supernatants were collected from mature hiPSC-derived epithelia, filtered using syringe filters (pore size 0.22 μm) and stored at −80°C. ELISAs were performed with an ELISA kit for MUC5AC (USCN, Wuhan, China) according to the manufacturer's protocol. The optical density at wavelengths of 450 and 570 nm was measured using a microplate reader (ARVO X4; PerkinElmer, Waltham, MA, USA) and analyzed with WorkOut 2.5 software.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were conducted with JMP Pro version 14.1.0. Data are presented as means ± standard errors of the means (SEMs); n represents biological repeats. Statistical analyses were performed by Mann-Whitney U tests for comparisons of two groups and by Steel's tests for multiple comparisons. Differences with p values of less than 0.05 were considered statistically significant. Further details are provided in the context of each specific assay in the relevant section of the Figure legends.
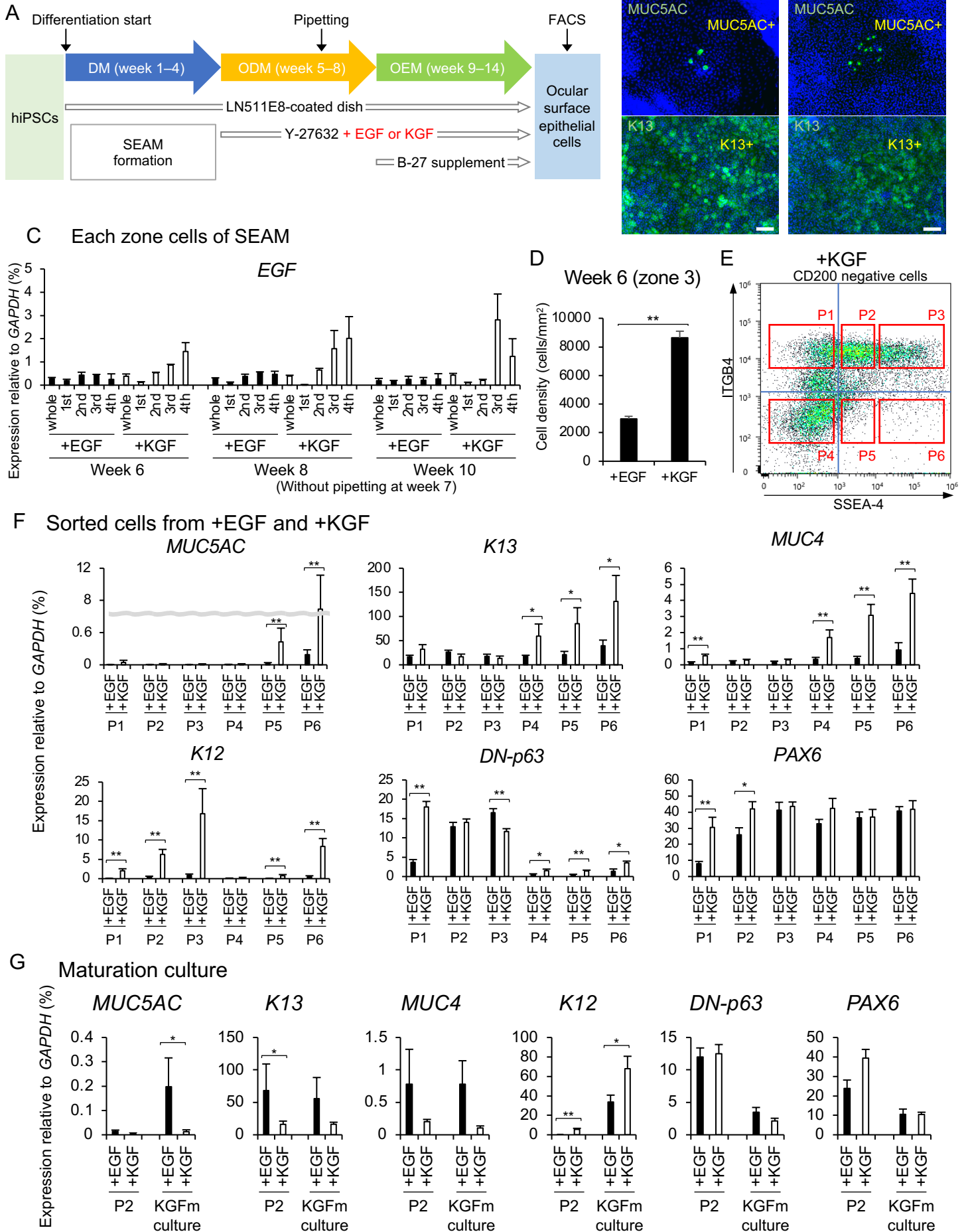
Supplemental Information

Generation of functional conjunctival epithelium,

including goblet cells, from human iPSCs

Kimihito Nomi, Ryuhei Hayashi, Yuki Ishikawa, Yuki Kobayashi, Tomohiko Katayama, Andrew J. Quantock, and Kohji Nishida
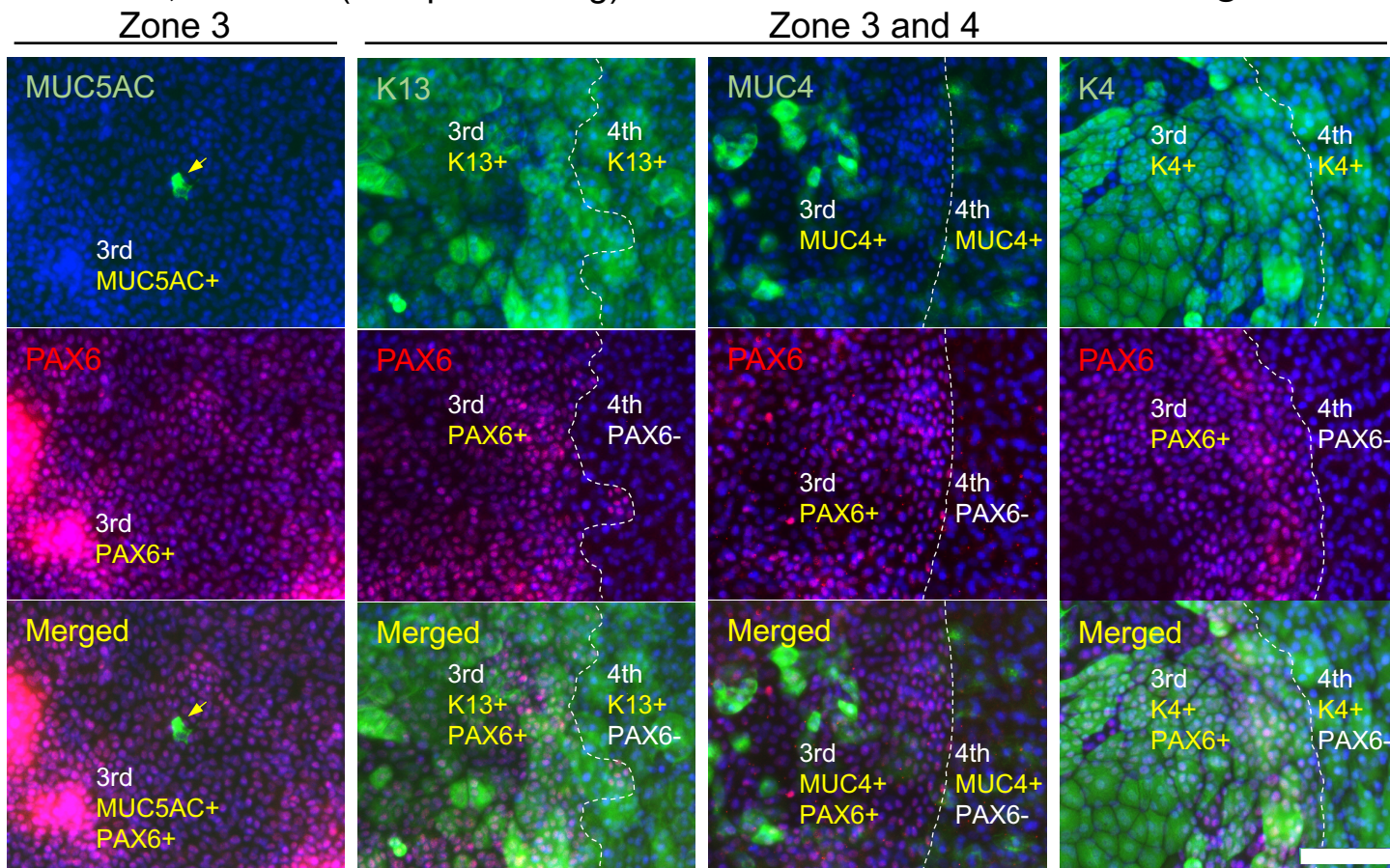
Figure S1

Figure S1. Comparison of the EGF- and KGF-treated SEAM derivatives, related to Figures 1–3, and 5.

(A) A detailed schematic of the differentiation method for hiPSCs. EGF or KGF was added five weeks after the start of differentiation. LN511E8, laminin511 E8 fragment. (B) Immunostaining for MUC5AC and K13 (green) in the SEAM at week 10 after the start of differentiation. The results for the EGF-treated SEAM (+EGF) are shown on the left, and those for the KGF-treated SEAM (+KGF) on the right. Data are representative of three independent experiments. Nuclei, blue. Scale bars, 100 µm. (C) Gene expression analysis for *EGF* in each zone of the EGF- and KGF-treated SEAM at weeks 6, 8, and 10 after the start of differentiation. These results were obtained using the 201B7-hiPSC line. N = 2. Error bars: SEMs. (D) The cell density in zone 3 of the EGF- and KGF-treated SEAM at week 6 after the start of differentiation. N = 10. **$p < 0.01$ (Mann-Whitney U tests). Error bars: SEMs. (E) FACS analysis of CD200, SSEA-4, and ITGB4 from the KGF-treated SEAM derivative after 10–14 weeks of culture. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. The six fractions were defined as P1–P6. Data are representative of seven independent experiments. (F) Gene expression analysis for ocular surface epithelium-related markers for the EGF- and KGF-treated SEAM-derived fractions. N = 7. *$p < 0.05$, **$p < 0.01$ (Mann-Whitney U tests). Error bars: SEMs. (G) Gene expression analysis of ocular surface epithelium-related markers for sorted cells from the P2 fraction before and after maturation culture in KGFm. Comparison of cells from the EGF- and KGF-treated SEAM. N = 6. *$p < 0.05$, **$p < 0.01$ (Mann-Whitney U test). Error bars: SEMs.

A +EGF, Week 10 (multiple staining)

Zone 3

Zone 3 and 4
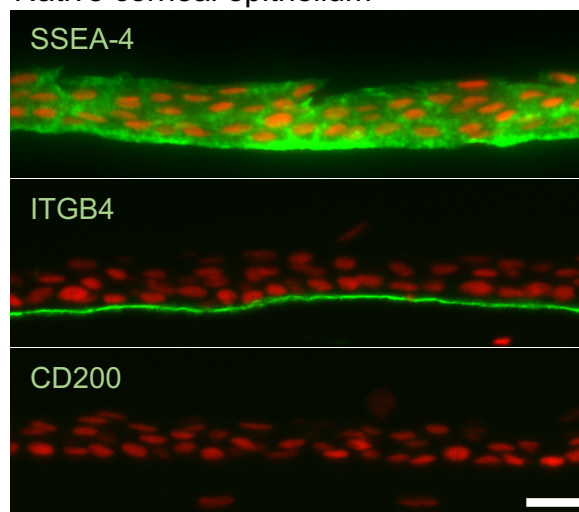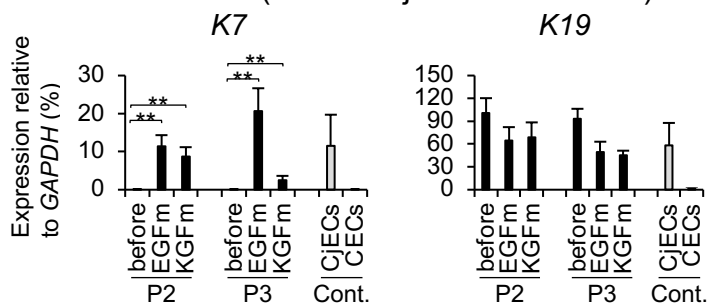
Figure S2

B Ocular surface cells markers using FACS

Native conjunctival epithelium
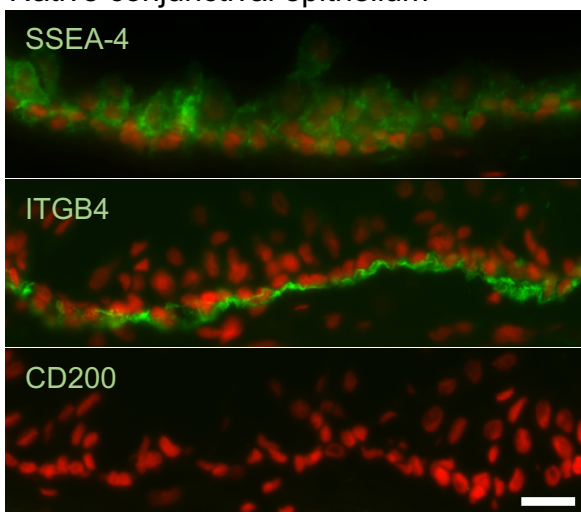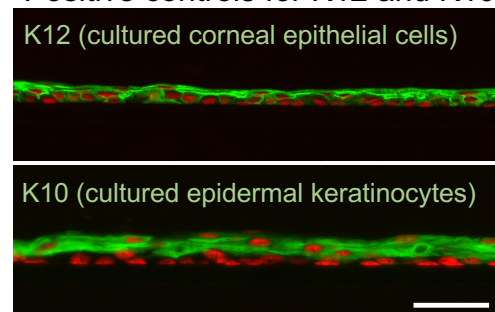
Native corneal epithelium

C Maturation culture (other conjunctival markers)
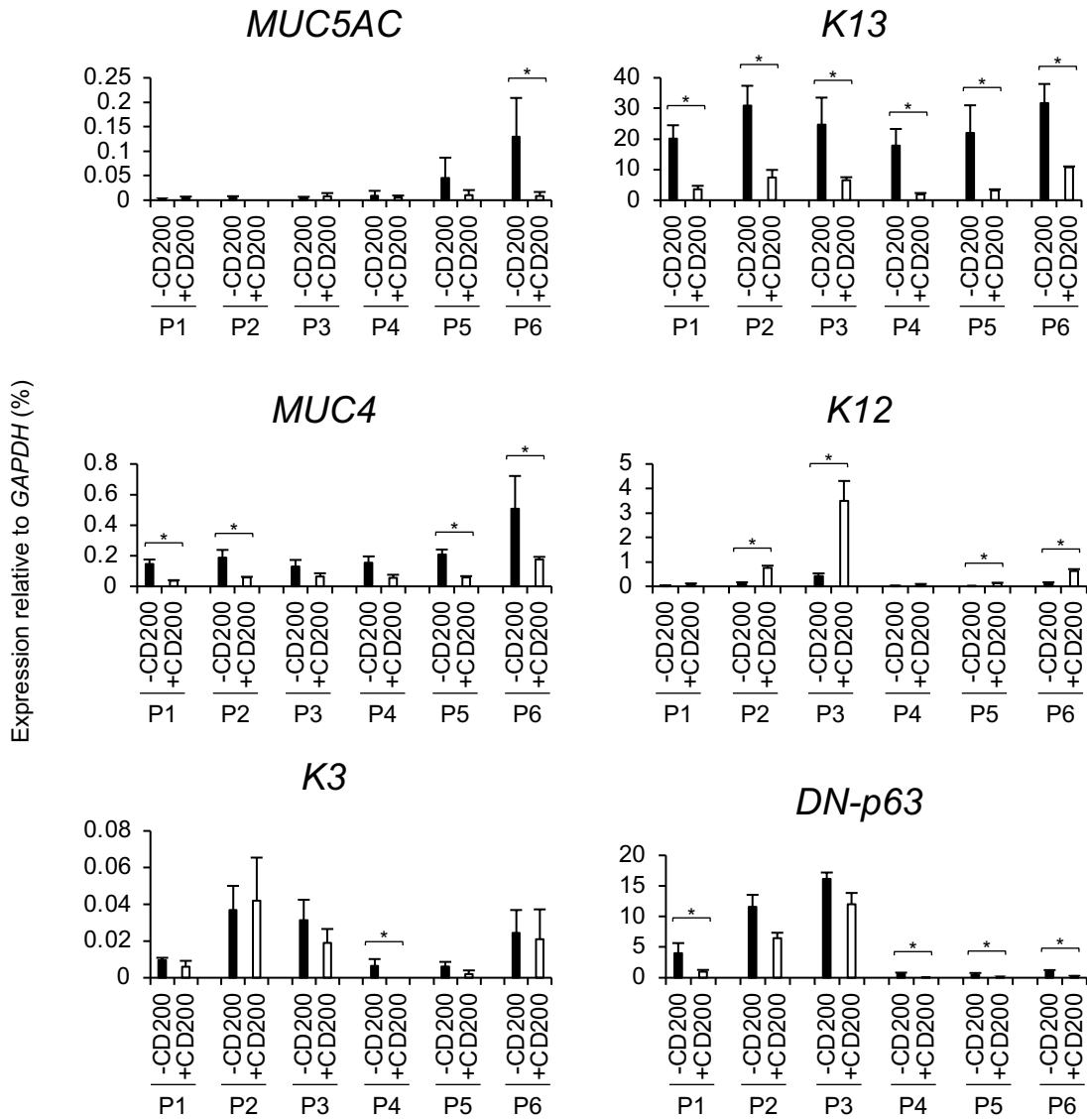
D Positive controls for K12 and K10

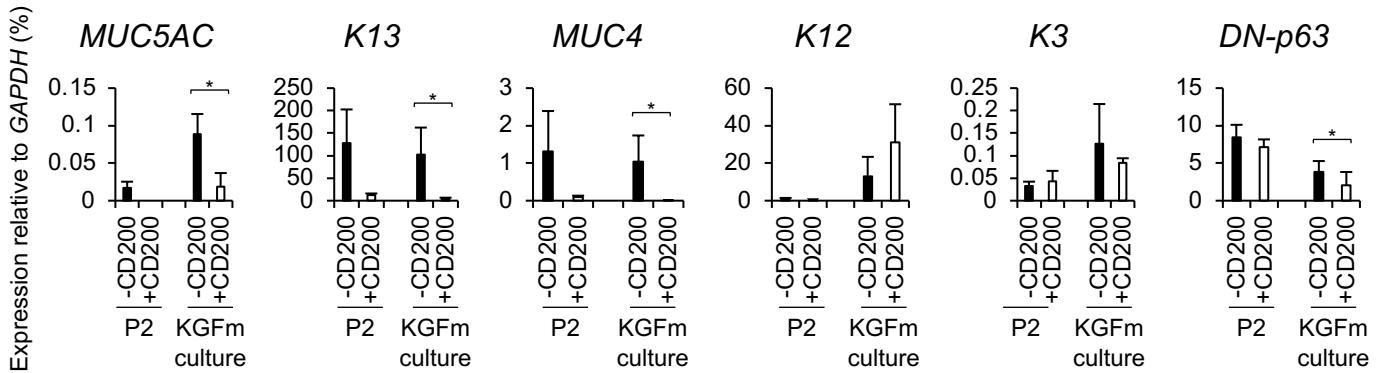**Figure S2. Expression of the ocular surface epithelium-related markers, related to Figures 1–5.**
(A) Immunostaining for conjunctival epithelium-related markers in zone 3 and 4 of the EGF-treated SEAM at week 10 after the start of differentiation. Data are representative of three independent experiments. Nuclei, blue. Scale bar, 100 μm. (B) Immunostaining for CD200, SSEA-4, and ITGB4 in human native conjunctival and corneal epithelium. Nuclei, red. Scale bars, 20 μm. (C) Gene expression analysis for *K7* and *K19* for sorted cells of the P2 and P3 fractions from the EGF-treated SEAM before and after maturation culture. Cells in the P2 and P3 fractions, n = 9; controls, n = 6. **$p < 0.01$ (Steel's test). Error bars: SEMs. CECs, human native corneal epithelial cells; CjECs, human native conjunctival epithelial cells; cont., controls. (D) Immunostaining for K12 in cultured human corneal epithelial cells and for K10 in cultured human epidermal keratinocytes. Shown as positive controls for Figure 4A. Nuclei, red. Scale bar, 50 μm.

Figure S3

A — Sorted cells from +EGF

MUC5AC

K13

MUC4

K12

K3

DN-p63

Expression relative to *GAPDH* (%)

B — Maturation culture

MUC5AC

K13

MUC4

K12

K3

DN-p63

Expression relative to *GAPDH* (%)

Figure S3. Comparison of CD200-negative cells and CD200-positive cells from the EGF-treated SEAM derivatives, related to Figures 2 and 3.

(A) Gene expression analysis for ocular surface epithelium-related markers for CD200-negative cells (-CD200) and CD200-positive cells (+CD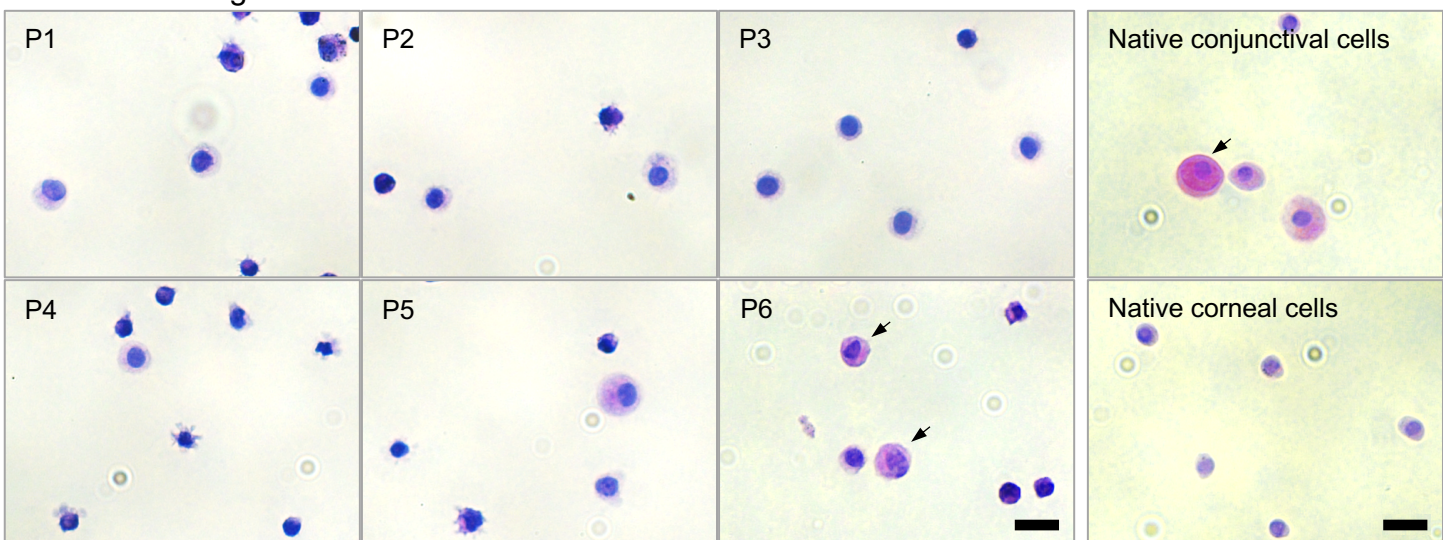200) from the EGF-treated SEAM-derived fractions. N = 3. *p < 0.05 (Mann-Whitney U tests). Error bars: SEMs. (B) Gene expression analysis for ocular surface epithelium-related markers for sorted cells from the P2 fraction before and after maturation culture in KGFm. Comparison of CD200-negative cells (-CD200) and CD200-positive cells (+CD200) from the EGF-treated SEAM-derived P2 fractions. N = 3. *p < 0.05 (Mann-Whitney U test). Error bars: SEMs.

Figure S4

A MUC5AC/PAX6/Hoechst (multiple staining)   Controls
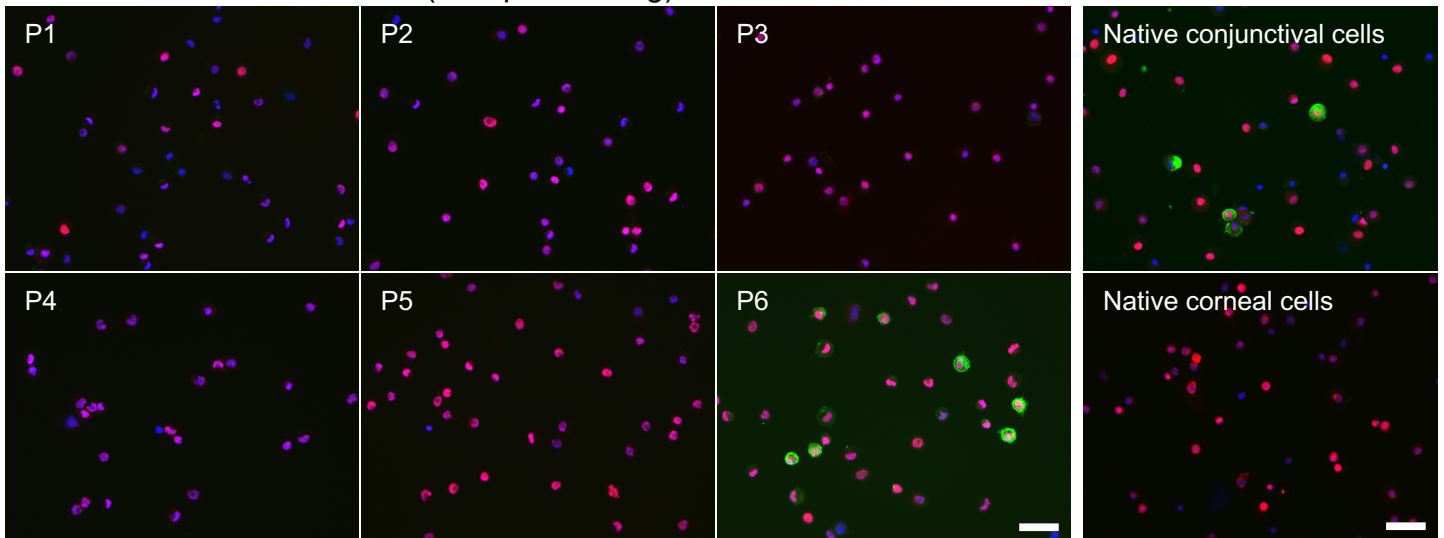
B K13/PAX6/Hoechst (multiple staining)   Controls


C PAS staining   Controls
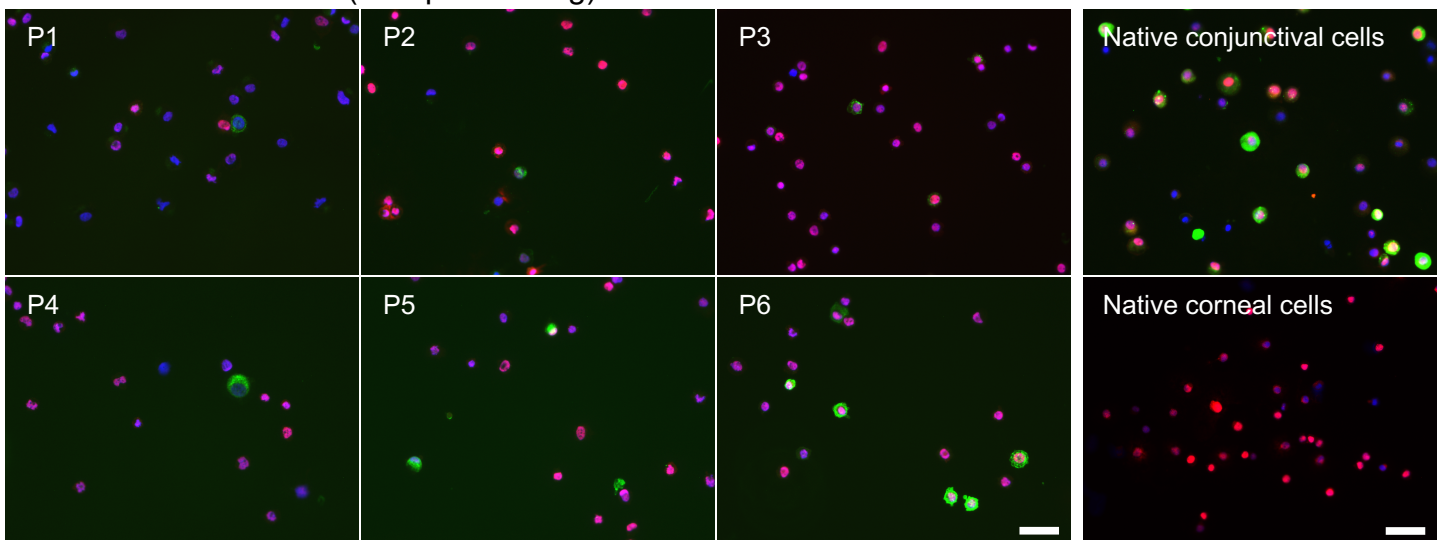
Figure S4. Comparison of the P1–P6 fraction cells derived from the EGF-treated SEAM, related to Figure 2.

(A) Immunostaining for MUC5AC (green) and PAX6 (red) in the P1–P6 fraction cells of the EGF-treated SEAM derivative. Human native conjunctival and corneal cells are shown on the right for comparison. Data are representative of four independent experiments. Nuclei, blue. Scale bars, 50 μm. (B) Immunostaining for K13 (green) and PAX6 (red) in the P1–P6 fractions cells of the EGF-treated SEAM derivative. Human native conjunctival and corneal cells are shown on the right. Data are representative of four independent experiments. Nuclei, blue. Scale bars, 50 μm. (C) PAS staining of cells in the P1–P6 fractions for the EGF-treated SEAM derivative. PAS-positive cells are indicated with arrows. Human native conjunctival and corneal cells are shown on the right. Data are representative of three independent experiments. Nuclei were stained with hematoxylin. Scale bars, 20 μm.

Figure S5

A

+EGF

CD200 negative cells

B   Sorted cells from +EGF

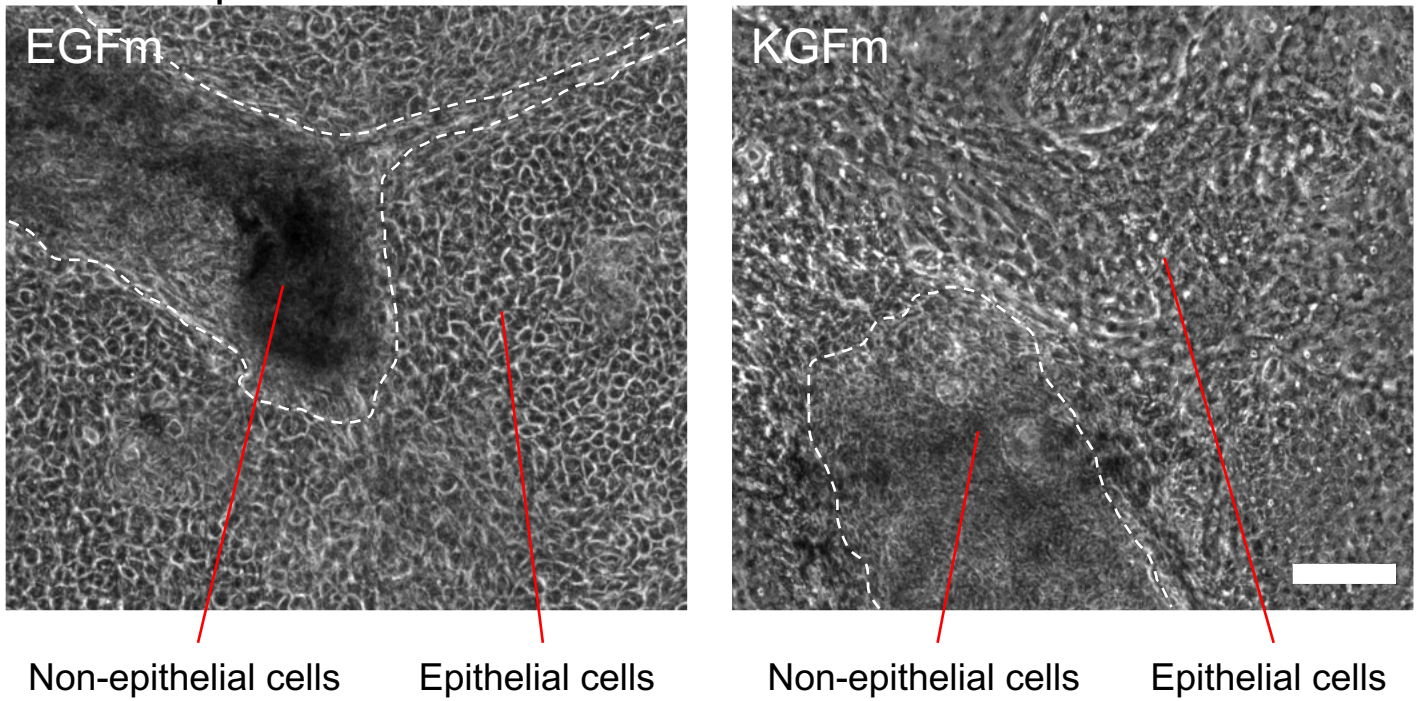
C   Maturation culture (P7)
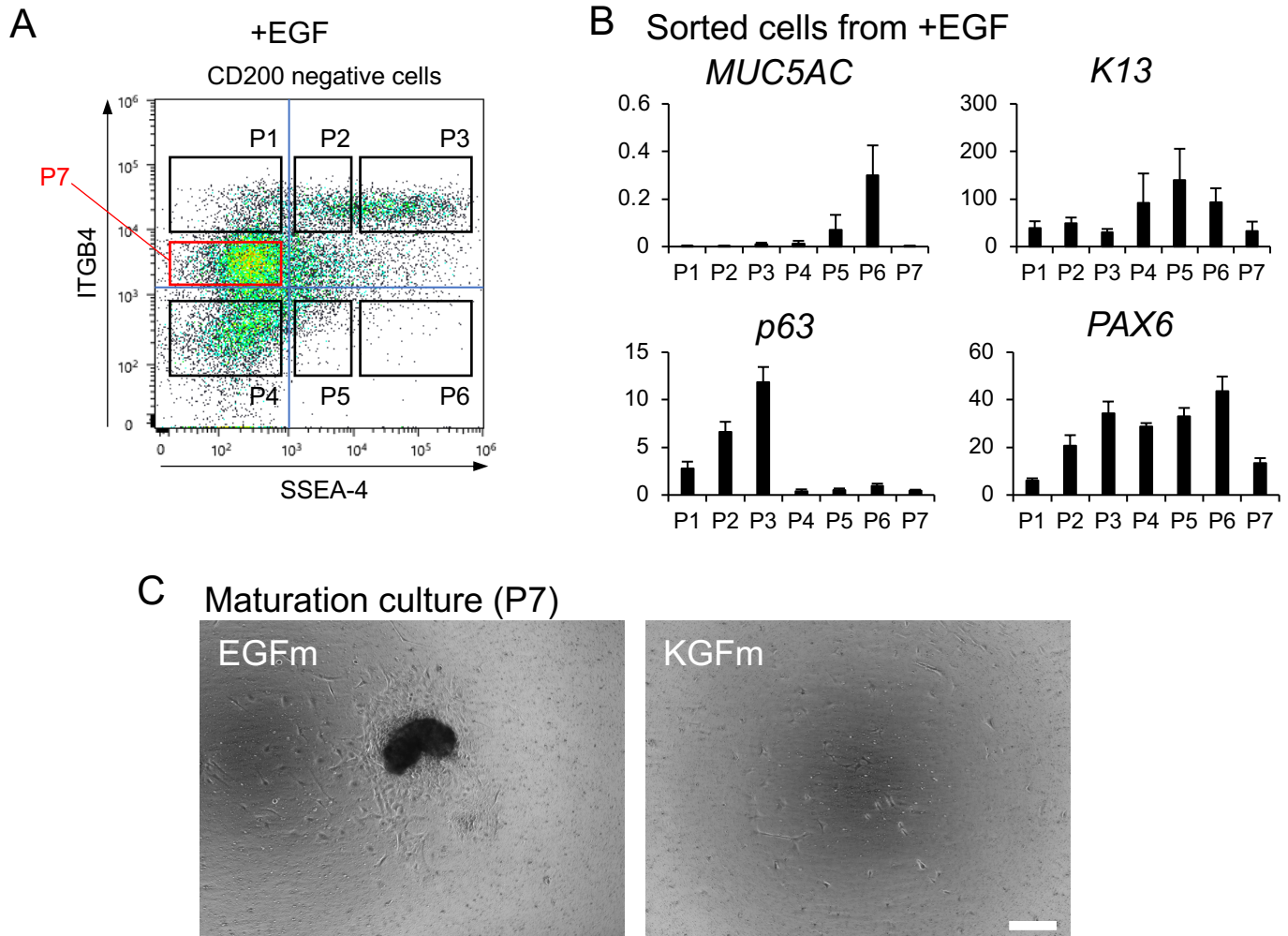
Figure S5. Analyses of the majority of cells captured by FACS in the EGF-treated SEAM derivatives, related to Figures 2 and 3.

(A) FACS analysis of CD200, SSEA-4, and ITGB4 from the EGF-treated SEAM derivative after 10–14 weeks of culture. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. The majority of cells captured by FACS were defined as P7. Data are representative of five independent experiments. (B) Gene expression analysis of conjunctival epithelium-related markers for EGF-treated SEAM-derived fractions. N = 5. Error bars: SEMs. (C) Phase-contrast images after maturation culture of the sorted P7 cells. They did not grow on the culture dish. Data are representative of two independent experiments. Scale bar, 200 μm.

Figure S6

A Non-epithelial cells of the P1-derived cells

Non-epithelial cells   Epithelial cells   Non-epithelial cells   Epithelial cells

## B Non-epithelial region
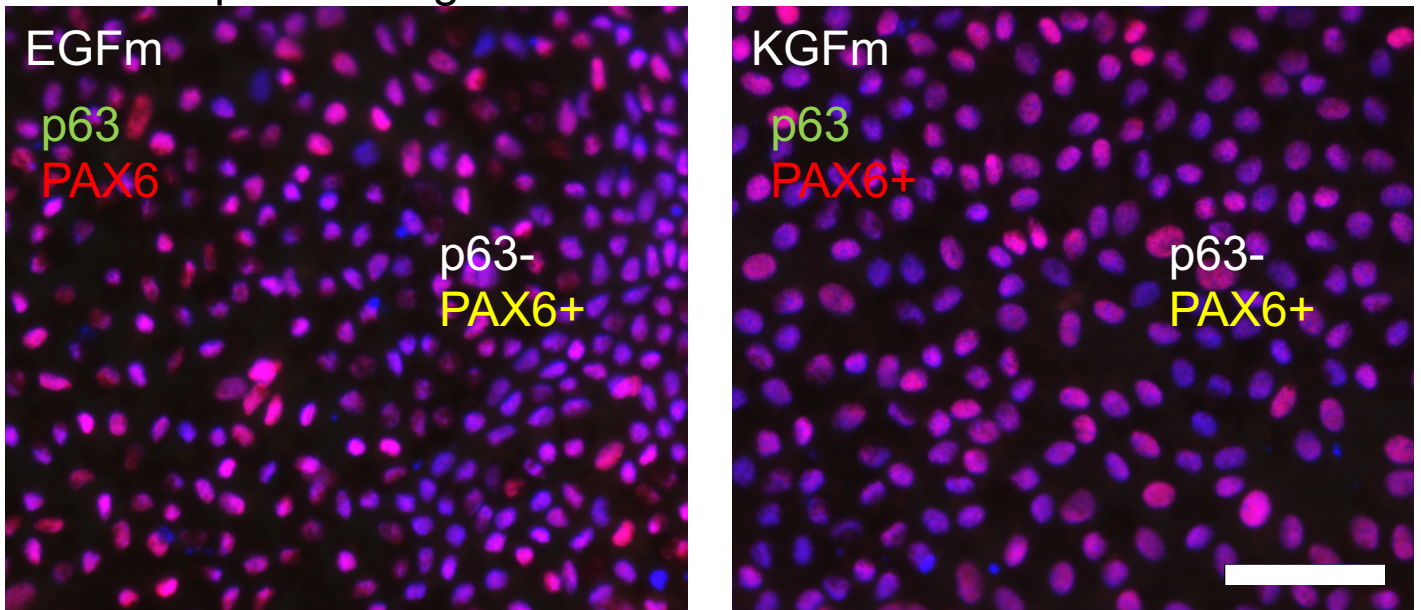
Figure S6. Non-epithelial cells in the P1-derived cells after maturation culture in EGFm and KGFm, related to Figure 3.

(A) Non-epithelial cells are indicated by dotted lines. Scale bar, 100 µm. (B) Immunostaining for p63 (green) and PAX6 (red) in the non-epithelial region of the P1-derived cells after maturation culture. Data are representative of three independent experiments. Nuclei, blue. Scale bar, 50 µm.

Figure S7

A   201B7 hiPSC

B

C   YZWJs524 hiPSC

D
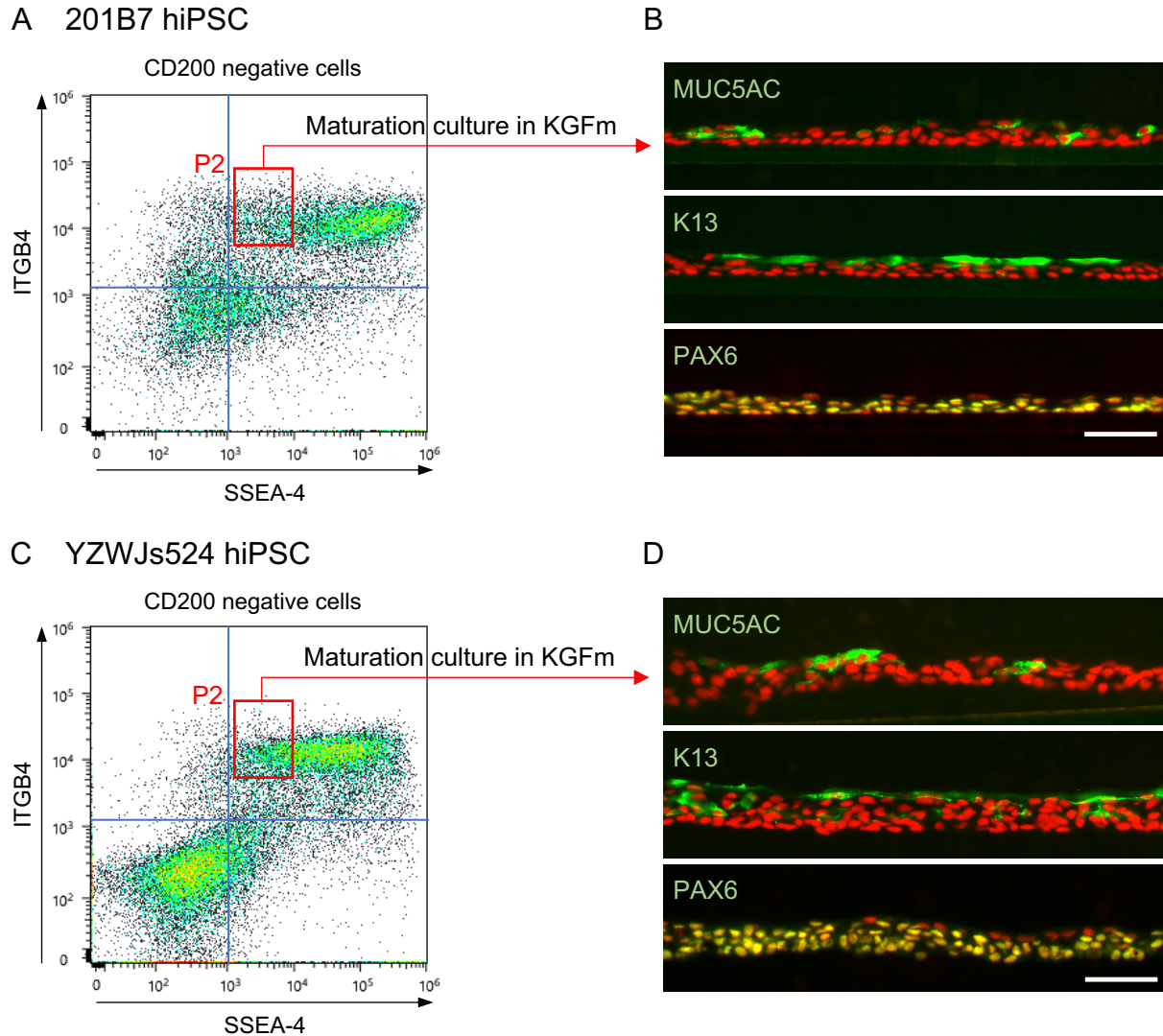
Figure S7. Reconstruction of conjunctival epithelium from different hiPSC lines, related to Figures 1–5.

(A) FACS analysis of CD200, SSEA-4, and ITGB4 from the 201B7 hiPSC EGF-treated SEAM derivative after 10–12 weeks of culture. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. Data are representative of three independent experiments. (B) Immunostaining for MUC5AC, K13, and PAX6 (green) for cells from the P2 fraction from the 201B7 hiPSC EGF-treated SEAM after maturation culture for 3–4 weeks in KGFm. Data are representative of three independent experiments. Nuclei, red. Scale bar, 50 μm. (C) FACS analysis of CD200, SSEA-4, and ITGB4 for the YZWJs524 hiPSC EGF-treated SEAM derivative after 10–12 weeks of culture. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. Data are representative of three independent experiments. (D) Immunostaining for MUC5AC, K13, and PAX6 (green) for cells from the P2 fraction from the YZWJs524 hiPSC EGF-treated SEAM after maturation culture for 3–4 weeks in KGFm. Data are representative of three independent experiments. Nuclei, red. Scale bar, 50 μm.

Figure S8

A Week 10 of differentiation

B Untreated SEAM derivative
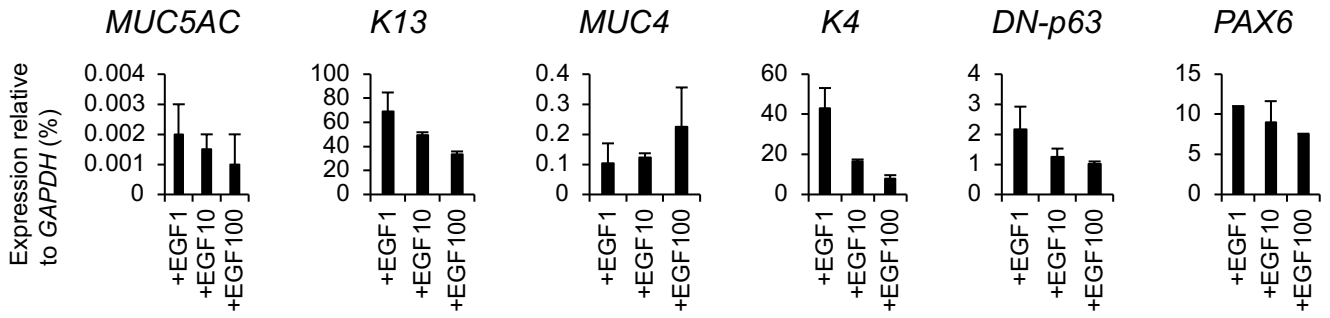

C Maturation culture
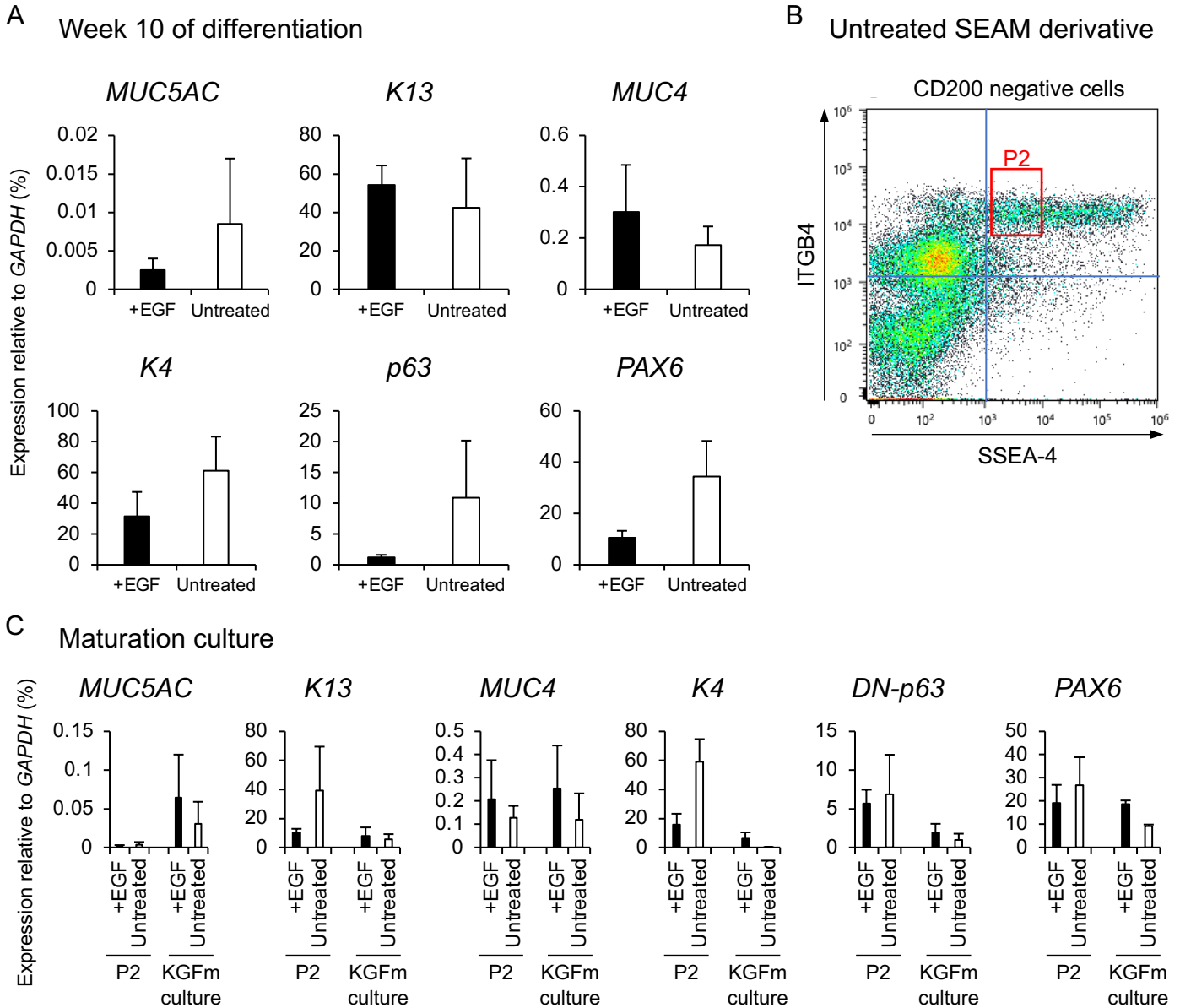
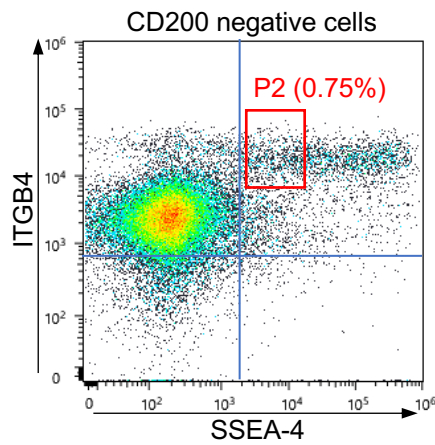**Figure S8. Verification of the EGF-treated and untreated SEAM derivatives, related to Figures 1–3, and 5.**

(A) Gene expression analysis for ocular surface epithelium-related markers in the whole SEAM at week 10 after the start of differentiation. Comparison of the EGF-treated and untreated SEAMs. N = 2. (B) FACS analysis of CD200, SSEA-4, and ITGB4 for the untreated SEAM derivative after 10 weeks of culture. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. (C) Gene expression analysis for ocular surface epithelium-related markers for the sorted cells from the P2 fraction before and after maturation culture in KGFm. Comparison of cells from the EGF-treated and untreated SEAMs. N = 2.
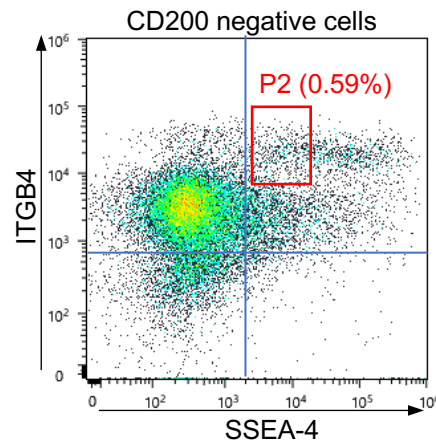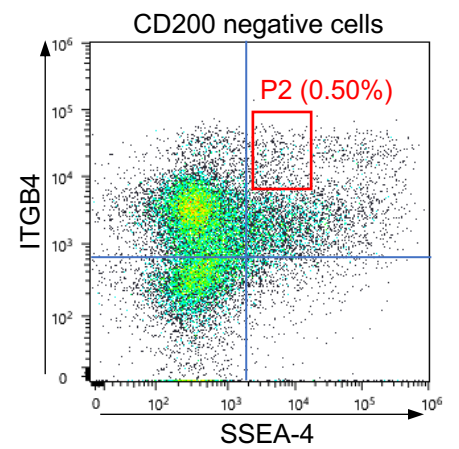
Figure S9

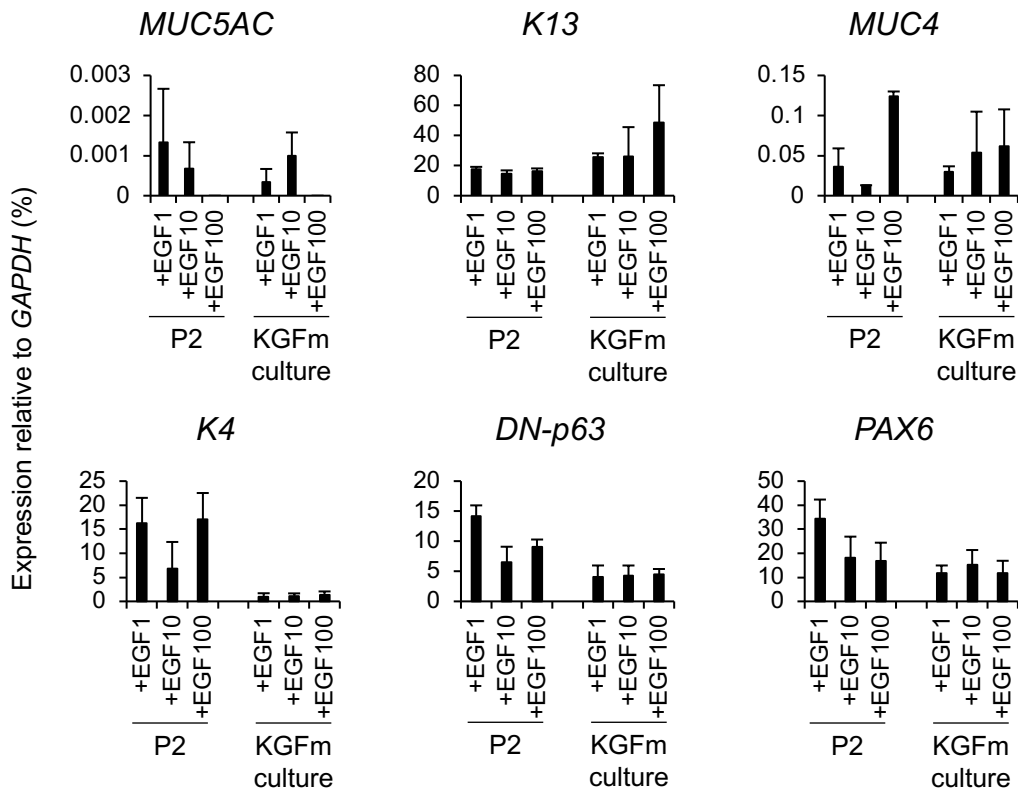## A Week 10 of differentiation

B +EGF 1 ng/mL
CD200 negative cells

C +EGF 10 ng/mL
CD200 negative cells

D +EGF 100 ng/mL
CD200 negative cells


E Maturation culture

Figure S9. Effects of EGF concentration on SEAM differentiation, related to Figures 1–3, and 5.
(A) Gene expression analysis of ocular surface epithelium-related markers in the whole SEAM at week 10 after the start of differentiation, with a range of EGF concentrations; +EGF1, 1 ng/mL EGF; +EGF10, 10 ng/mL EGF; +EGF100, 100 ng/mL EGF. N = 2. (B) FACS analysis of CD200, SSEA-4, and ITGB4 for the EGF-treated SEAM derivative after 10–12 weeks of culture. EGF was added at a concentration of 1 ng/mL. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. Data are representative of three independent experiments. (C) FACS analysis of CD200, SSEA-4, and ITGB4 for the EGF-treated SEAM derivative after 10–12 weeks of culture. EGF was added at a concentration of 10 ng/mL. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. Data are representative of three independent experiments. (D) FACS analysis of CD200, SSEA-4, and ITGB4 for the EGF-treated SEAM derivative after 10–12 weeks of culture. EGF was added at a concentration of 100 ng/mL. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. Data are representative of three independent experiments. (E) Gene expression analysis for ocular surface epithelium-related markers for the sorted cells from the P2 fraction before and after maturation culture in KGFm. Comparison of the P2 cells from EGF-treated SEAMs derived using various concentrations of EGF. N = 3. Error bars: SEMs.

Figure S10

A +TGF-α

B

C +Areg

D
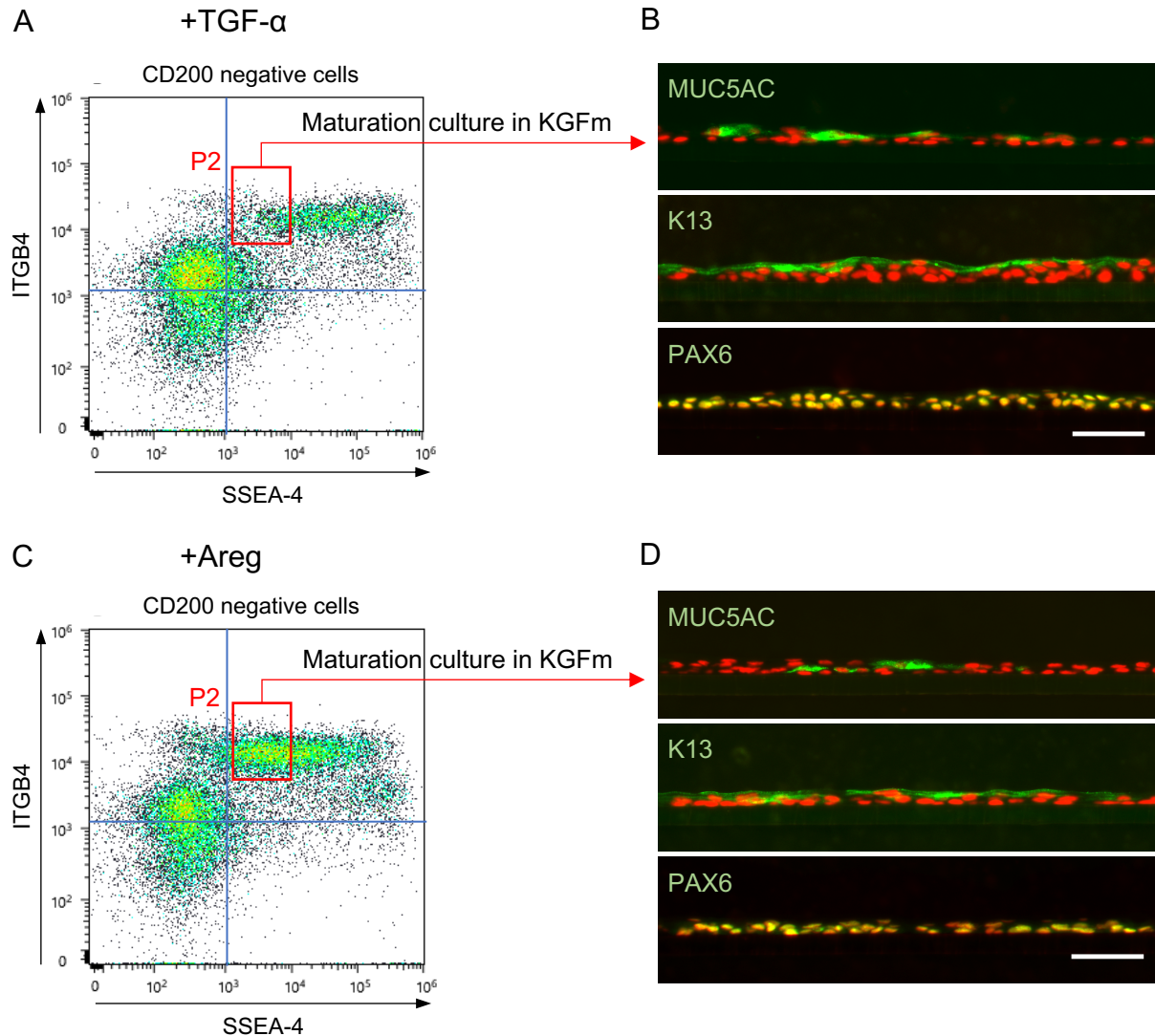
Figure S10. Reconstruction of hiPSC-derived conjunctival epithelium by adding other EGFR ligands to the SEAM, related to Figure 1–5.

(A) FACS analysis of CD200, SSEA-4, and ITGB4 for the SEAM derivative after 10–12 weeks of culture. TGF-α was added to the SEAM instead of EGF. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. Data are representative of three independent experiments. (B) Immunostaining for MUC5AC, K13, and PAX6 (green) for the TGF-α-treated SEAM-derived P2 cells after maturation culture for 3–4 weeks in KGFm. Data are representative of three independent experiments. Nuclei, red. Scale bar, 50 μm. (C) FACS analysis of CD200, SSEA-4, and ITGB4 for the SEAM derivative after 10–12 weeks of culture. Areg was added to the SEAM instead of EGF. CD200-negative cells were extracted and analyzed for SSEA-4 and ITGB4. Data are representative of three independent experiments. (D) Immunostaining for MUC5AC, K13, and PAX6 (green) for the Areg-treated SEAM-derived P2 cells after maturation culture for 3–4 weeks in KGFm. Data are representative of three independent experiments. Nuclei, red. Scale bar, 50 μm.

Table S1

Target genes	TaqMan probe ID
GAPDH	Hs99999905_m1
PAX6	Hs00240871_m1
DN-p63	Hs00978339_m1
K12	Hs00165015_m1
K3	Hs00365080_m1
MUC5AC	Hs01365616_m1
K13	Hs00999762_m1
MUC4	Hs00366414_m1
K4	Hs00361611_m1
EGF	Hs01099999_m1
K7	Hs00559840_m1
K19	Hs00761767_s1

Table S1. TaqMan probes used in this study, related to Figures. 1–3.