Goblet Cell Differentiation Potential in Human Corneal Limbal Epithelial Progenitor Cells In Vitro

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PURPOSE. The existence of goblet cells has been regarded as a critical differential point to distinguish conjunctival epithelium from corneal epithelium in vivo. We tested differentiation potential of single progenitor cells from corneal limbal epithelium with growth factors in vitro.

METHODS. Dissociated single cells from corneal limbal epithelium were cultured in the serum- and feeder cell–free medium containing B27 and various growth factors using nontissue culture dishes. Specific marker expression was examined in the colonies stimulated with growth factors. Differentiation of some mucosal epithelia was tested.

RESULTS. Adherent single cells from dissociated single cells in corneal limbal epithelium did not proliferate in the serum- and feeder cell–free medium containing B27 only and formed corneal epithelium with B27 plus epidermal growth factor, while they gave rise to goblet cell with periodic acid Schiff–positive mucin and cytokeratin-3 and-12 expressing corneal epithelium with fibroblast growth factor (FGF)2 stimulation. Colonies stimulated with FGF2 expressed goblet cell specific MUC5AC and cytokeratin-7 mRNA and protein. FGF receptor 1 was a functional receptor for the differentiation to goblet cells and corneal epithelium.

CONCLUSIONS. Single corneal limbal progenitor cells give rise to goblet cells and corneal epithelium by FGF2 stimulation via FGF receptor 1 in vitro.

Keywords: corneal limbal epithelium, FGF2, goblet cells, progenitor cells, human

The conjunctival epithelium has mucin-secreting goblet cells, but goblet cells do not exist on the corneal epithelium in the normal condition of ocular surface.[1,2] This is a critical differential point to distinguish conjunctival epithelium from corneal epithelium in vivo.[3–5] The presence of goblet cells on the clear cornea has been shown in corneal limbal stem cell deficiencies,[3] which are caused by chemical burns, ocular pemphigoid, aniridia, or Stevens Johnson syndrome.[6,7] Goblet cells are distributed in not only conjunctival epithelium, but also various mucosal epithelia that line the upper and lower respiratory tract, gastrointestinal tract, inner ear, and sinuses.[8–12]

We introduced a new isolation technique of progenitor cells from human corneal limbal epithelium using nontissue culture dishes and hydrophilic tubes for cell collection.[13] By using this technique, we tested various growth factors for differentiation and proliferation of human corneal limbal epithelium in the serum-free culture condition. In this study, we demonstrate surprising data on corneal limbal epithelium by fibroblast growth factor 2 (FGF2) stimulation among growth factors tested in vitro.

MATERIALS AND METHODS

This study was conducted in accordance with the Declaration of Helsinki. The Institutional Review Board of Tokyo University approved this study.

Donor Corneas

Donor corneas were obtained from the SightLife and Rocky Mountain Lions Eye Bank at five to seven days after harvesting. The age of the donors was 53 to 68 years.

Separate Isolation of Corneal Limbal Epithelium and Conjunctival Epithelium

After removing the borderline area between corneal limbal epithelium and conjunctival epithelium to prevent contamination by conjunctival epithelium, corneal limbal tissues, and conjunctival tissues were separately harvested. The human peripheral bulbar conjunctival tissues were obtained with a margin of 3 mm from the corneal limbus. These

tissues were cut into small pieces about 1 to 2 mm in diameter, which were incubated overnight at 37°C in basal culture medium (Dulbecco's modified Eagle medium [DMEM]–F12, 1:1; Thermo Fisher Scientific, Tokyo, Japan) containing 0.02% Type IA collagenase (Sigma-Aldrich Japan, Tokyo, Japan), B27 (Thermo Fisher Scientific),[13,14] 100 units/mL of penicillin, and 100 µg/mL of streptomycin. Cells were collected into hydrophilic tubes (Sumilon Stem Full; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) during all experimental procedures, because these tubes are essential to avoid cell adhesion to the inside of tube as described previously.[13] Cells are incubated in 0.05% trypsin/0.02% ethylenediamine tetra-acetic acid (EDTA) for 10 minutes at 37°C and dissociated into a single cell suspension by pippeting. Then the cells were resuspended in basal medium (DMEM-F12). Cell numbers were determined using a Coulter counter.

Culture of Adherent Cells in Nontissue Culture Dishes

Immediately after being resuspended in the basal medium, completely disaggregated single cells were cultured overnight at a density of 500 cells/cm² in medium containing B27 and 5 ng/mL FGF2 in nontissue culture dishes for floating culture. We used B27 supplement for culture because we suggested that serum-, feeder-, and bovine pituitary extract–free medium containing B27 using nontissue culture dish was an efficient method for enriching progenitor cells from human corneal limbal epithelium.[13] The appropriate concentration of FGF2 was determined in the concentration gradient test. FGF2 was selected because in preliminary studies we found that, unlike the other FGF5, keratinocyte growth factor, insulin-like growth factor, transforming growth factor-beta, and hepatocyte growth factor, FGF2 produced different type cells other than epithelium-like cells. Dishes were washed three times with basal culture medium containing B27 to remove nonadherent cells, and then culture of epithelium was done in basal medium containing B27 only, B27 plus 20 ng/mL of EGF (Sigma-Aldrich), or B27 plus 5 ng/mL of FGF2.

Antibodies (Abs)

The following Abs were used: mouse anti-cytokeratin-3/2p monoclonal antibody (mAb; AE-5, 1:100; Santa Cruz Biotechnology, Inc, Dallas, TX, USA), mouse anti-human cytokeratin-4 mAb (6B10, 1:300; Abcam, Cambridge, UK), rabbit monoclonal [EP1599Y] anti-human cytokeratin 4 (1:200, Abcam), mouse anti-human cytokeratin-7 mAb (RCK105, 1:10000; Millipore, Billerica, MA, USA), mouse anti-human cytokeratin-13 mAb (1:20; American Research Products, Inc., Palos Verdes, CA, USA), mouse anti-human cytokeratin-12 mAb (N-16; 1:500; Santa Cruz Biotechnology), rabbit anti-cytokeratin 12/K12 mAb (1:200, EPR17882, Abcam), rabbit anti-human MUC5AC polyclonal Ab (H-160, 1:100, Santa Cruz Biotechnology), anti-FGFR1 mAb (1:200, 133105; R&D Systems, Minneapolis, MN, USA), anti-human FGFR2 (blocking) mAb (1:200, 98725; R&D Systems), anti-FGFR3 mAb (1:200, 135334; R&D Systems), anti-FGFR4 mAb (1:200, 137114; R&D Systems), and anti-FGFR1 blocking mAb (VBS1; Chemicon, Temecula, CA, USA). As a control, mouse immunoglobulin G (IgG; Sigma-Aldrich), mouse immunoglobulin M (IgM; Sigma-Aldrich) or normal rabbit serum (Agilent Technology, Tokyo, Japan) was used instead of the primary Ab. The secondary Abs used were fluorescence-labeled goat anti-mouse IgG (Alexa Fluor 488,

1:200; Thermo Fisher Scientific), fluorescence-labeled goat anti-rabbit IgG (Alexa Fluor 594, 1:400; Thermo Fisher Scientific), donkey anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody (Alexa Fluor 488, 1:500, Thermo Fisher Scientific), and donkey anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody (Alexa Fluor 647, 1:500, Thermo Fisher Scientific).

Immunocytochemistry of Adherent Colonies

Cells were fixed with methanol (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) in phosphate buffered saline solution (PBS) for 10 minutes. After washing in PBS, the cells were incubated for 30 minutes with 3% bovine serum albumin (BSA) in PBS containing 0.3% Triton × 20 (BSA/PBST with Tween 20 (PBST)) to block nonspecific staining. Then the cells were incubated for two hours at room temperature with specific primary Abs diluted in BSA/PBST. After further washing in PBS, the cells were incubated for one hour at room temperature with the appropriate secondary Abs diluted in BSA/PBST. Nuclei were counterstained with Hoechst 33342 (1:2000; Thermo Fisher Scientific) or DAPI (5 µM, Abcam). After further washing in PBS, examination was done under a fluorescence microscope (model BH2-RFL-T3 and BX50; Olympus, Tokyo, Japan).

Histochemistry

To determine whether in vivo goblet cells marker, acidic glycoconjugates are present in the isolated colonies, periodic acid Schiff (PAS) staining was performed with PAS Staining Kit (Muto Pure Chemicals Co., Ltd, Tokyo, Japan) as per the manufacturer's instruction.

Preparation of RNA and RT-PCR

Corneas from donors aged 47 and 61 years were used for Reverse transcription-Polymerase chain reaction (RT-PCR). Total RNA was isolated with a kit (Isogen; Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized using a Reverse Transcription System (Promega Corporation, Madison, WI, USA) and 1 µg of total RNA in a 20-µL reaction mixture. Then PCR was performed with cDNA polymerase (Ampli Taq Gold; Applied Biosystems, Foster City, CA, USA) in a 50-µL reaction mixture. After incubation at 95°C for nine minutes, amplification was done at 94°C for 30 seconds and then at 60°C for 30 seconds using a thermal cycler (i-Cycler; Bio Lad Laboratories, Hercules, CA). Products were separated on 2% agarose gel and stained with ethidium bromide. The primer sequences and estimated product sizes are listed in Table. Human corneal endothelium from donor corneas and human brain cDNA (BioChain Institute, Inc, Hayward, CA, USA) were positive controls for FGFR1 to 4. The total RNAs without reverse transcription (RT) were negative controls.

Western Blotting

Samples were subjected to Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) on 7% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). The membranes were blocked for 60 minutes at RT in blocking solution (20 millimolar [mM] Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20, and 5% skim milk) and then incubated overnight

TABLE. FGFR, Fibroblast Growth Factor Receptor

Gene Name		Size (bp)	Cycles (n)
MUC5AC			
	5'-TCCACCATATACCGCCACAGA-3'	103	35
	5'-ATGGTGCCGTTGTAATTTGTTGT-3'		
Cytokeratin 7			
	5'-CAGGAACTCATGAGCGTGAA-3'	346	35
	5'-GGGTGGGAATCTTCTTGTGA -3'		
FGFR1			
	5'-TACCACCGACAAAGAGATGG -3'	287	35
	5'-CTGGCTGTGGAAGTCACTCT -3'		
FGFR2			
	5'-TGGAGCGATCGCCTCACCG -3'	352	35
	5'-CTTCCAGGCGCTGGCAGAACTGT -3'		
FGFR3			
	5'-CACCACCGACAAGGAGCTA -3'	433	30
	5'-GCTCGAGCTCGGAGACATT -3'		
FGFR4			
	5'-GGGTCCTGCTGAGTGTGC -3'	406	30
	5'-GGGGTAACTGTGCCTATTCG -3'		

at 4°C with each diluted mAb. After washing, incubation was done with horseradish peroxidase-conjugated donkey anti-rabbit Ig (1:500 dilution) (Amersham Biosciences, Buckinghamshire, UK), and bound Abs were detected by a chemiluminescence assay (ECL; Amersham Biosciences).

Blocking of FGFRs

To assess the role of the FGF2, anti-FGFR1 blocking mAb (1:200, VBS1, Millipore), anti-FGFR2 blocking mAb (1:200, 98725, R&D Systems), or nonimmunized Ig was added to basal medium with B27 and 5 ng/mL of FGF2. Concentration of FGF2 was set at the minimum concentration to promote goblet cell differentiation based on our preliminary study. The disaggregated single cells were cultured overnight at a density of 2000 per well on 24-well nontissue culture dishes in medium containing B27 and EGF. Dishes were washed twice with basal medium containing B27 to remove the nonadherent cells, and then culture was done in basal medium containing B27, FGF2, and anti-FGFR1 blocking mAb (1:200) or anti-FGFR2 blocking mAb (1:200); or B27, FGF2, and control Ig. Antibodies were added every other day, and PAS-positive colonies were counted six days after culture.

Statistical Analysis

One-way analysis of variance and Scheffe's multiple comparison tests were used to compare mean values. All analyses were performed using the Stat View statistical software package (Abacus Concepts, Berkeley, CA, USA). The level of significance was set at $P < 0.05$.

RESULTS

FGF2 to Promote the Differentiation of Corneal Limbal Epithelium-Derived Progenitor Cells Into Goblet Cells

After treatment with trypsin/EDTA, disaggregated corneal limbal epithelium were cultured overnight at a density of 5000 per dish (1 cell/µL) in nontissue culture dishes contain-
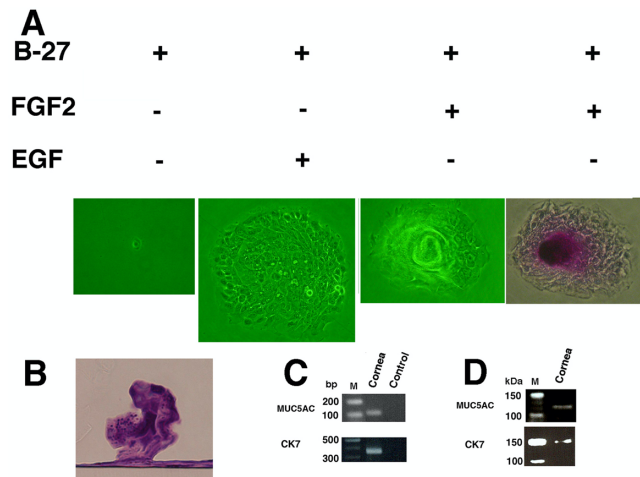
FIGURE 1. Differentiation of single cells in response to different growth factors. (**A**) Adherent single cells do not proliferate in basal medium containing B27 alone. Addition of EGF to B27 promotes differentiation into epithelium-like cells. Addition of FGF2 instead of EGF induces the production of amorphous material, which is PAS-positive (right). (**B**) PAS staining of a cross-section of a colony shows a goblet cell-like morphology. Expressions of goblet cell-specific MUC5AC and cytokeratin 7 are detected in the colonies by RT-PCR (**C**) and Western blotting (**D**). Similar results were obtained with repeated four experiments. M, size markers; CK, cytokeratin.

ing serum- and feeder cell-free basal medium with B27 only, B27 plus 20 ng/mL of EGF, or B27 plus 5 ng/mL of FGF2. Adherent single cells did not proliferate in the medium with B27 only (Fig. 1A left). Approximately 70 cells per 1000 cells adhered to noncoating dishes, and 20% of them formed round colonies in the medium containing B27 plus EGF (Fig. 1A second from left). Similarly, approximately 15 cells per 1000 cells from corneal limbal epithelium proliferated in medium containing B27 with FGF2 and the central cells of all proliferating round colonies secreted amorphous material without exception (Fig. 1A second from right), which was positive for mucin-like PAS stain (Fig. 1A right). Surprisingly, PAS staining of a cross-section of an adherent colony showed a goblet cell-like morphology of the cells, and the intracellu-

lar material and surface layer of the colony were PAS-positive (Fig. 1B). Expression of goblet cell-specific MUC5AC and cytokeratin 7[9] were both positive by RT-PCR (Fig. 1C) and Western blotting (Fig. 1D) in colonies derived from corneal limbal epithelium, indicating that the colonies with PAS-positive material contain goblet cells. Commercially available MUC5AC antibodies tested did not work for immunohistochemical study. Single cells from central corneal epithelium did not proliferate in the medium with B27 plus EGF and FGF2. Isolated single cells from human conjunctival epithelium also form epithelium-like round colonies with B27 plus EGF and small colonies bearing goblet cell-like PAS positive material with B27 plus FGF2 (Supplemental Fig. S1A). A cross-section of the colony from conjunctival epithelium showed typical goblet cell-like morphology (Supplemental Fig. S1B) and MUC5AC/CK7 expression by RT-PCR (Supplemental Fig. S1C) and Western blotting (Supplemental Fig. S1D), respectively. These findings indicate that conjunctival epithelium also showed similar differentiation and proliferation pattern with the same growth factors' stimulation.

Cytokeratin Expression of a Single Cell-Derived Colony Stimulated with EGF and FGF2

Adherent single cells obtained from the corneal limbal epithelium formed a colony on nontissue culture dishes in serum-free medium with B27-supplement and 20 ng/mL of EGF. After at least 10 days of culture, the cells formed a double layer. To test whether the isolated corneal limbal epithelium are mixed with conjunctival epithelium, 10 colonies were tested in each antibody. In contrast to negative staining with control mouse IgG (Fig. 2A), the upper layer of cells on the adherent basal cells expressed cytokeratin-3 (Fig. 2B) and cytokeratin-12 (Fig. 2C), which are markers of differentiated corneal epithelium, although differentiated conjunctival epithelium markers (cytokeratin-4 and cytokeratin-13) were not detected without exception. Next, colonies stimulated with B27 and FGF2 were stained with corneal epithelium expressing anti-cytokeratin-3 mAb and PAS. PAS-positive mucin-like material exists in the center of colony and adherent cells are cytokeratin-3-positive in the merged figure on the dish (Fig. 2D). Moreover, a cross-section of an adherent colony derived from corneal limbal epithelium showed cytokeratin-3-positive green and cytokeratin-12-positive red cells in a section including a goblet-like form cell (Fig. 2E). But we could not detect cytokeratin-4 positive red cells in a cross-section of an adherent colony derived from corneal limbal epithelium (Fig. 2F). On the other hand, a single cell from human conjunctival epithelium formed multilayered cells, which expressed cytokeratin-4 (Supplemental Fig. S2A) and cytokeratin-13 (Supplemental Fig. S2B). These cytokeratin-4 and -13 positive cells in conjunctival epithelium-derived cells are bigger than those of corneal limbal epithelium-derived cells. These findings demonstrate that single cells derived colonies isolated from corneal limbal area were all corneal epithelium unmixed with conjunctival epithelium and FGF2 promotes the differentiation of corneal limbal epithelium-derived cells to goblet cells and corneal epithelium, suggesting isolated single cells from corneal limbal epithelium are bi-potent corneal epithelium progenitor cells.
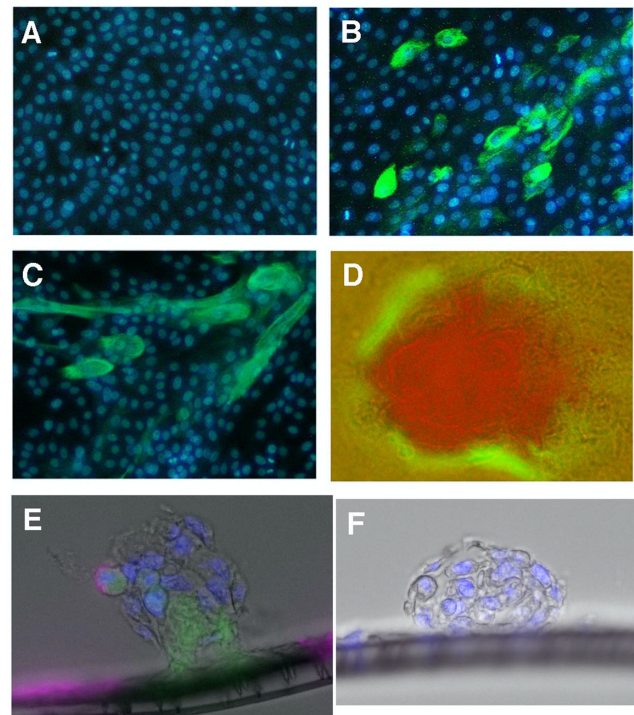
Figure 2. Marker expression in the corneal limbal epithelium-derived cells. No positive staining in the single cell derived cells cultured with B-27 and EGF are obtained with the control mouse IgG (**A**). Elongated and spindle-like cells growing on the basal layer express cytokeratin-3 (**B**) and cytokeratin-12 (**C**) after 10 days of culture, which are specific markers of differentiated corneal epithelium. Similar results were obtained with repeated experiments. (**D**) In the colony stimulated with B27 and FGF2, PAS and anti-cytokeratin-3 mAb staining were performed. *Merged figure* shows PAS-positive material in the center of colony and cytokeratin-3 expressing cells around the colony. (**E**) A cross-section shows the corneal epithelium specific cytokeratin-3 positive (*green*) and cytokeratin-12 positive cells (*red*) in an adherent colony including goblet cell-like form. (**F**) Cytokeratin-4 (*red*) is negative in the cross section of the adherent colony derived from corneal limbal epithelium. Nucleus was stained with DAPI.

Functional Blocking Against FGF Receptors

Among the possible FGF2 receptors (FGFR1-4), mRNAs for FGFR1 and FGFR2, but not FGFR3 and FGFR4 were detected in the colonies derived from corneal epithelium (Fig. 3A). Western blotting revealed bands for FGFR1 and FGFR2 (Fig. 3B), but not FGFR3 and FGFR4. The finding that colonies derived from corneal limbal epithelium contained goblet cells and expressed FGFR1 and FGFR2 suggests that these receptors may be functional for differentiation to goblet cell. Therefore we conducted blocking experiments of FGFR1 and FGFR2. PAS-positive colony number per 2×10^3 cells in the anti-FGFR1 mAb (n = 8), but not anti-FGFR2 mAb group (n = 8) significantly decreased as compared with that in the control Ig group in repeated experiments (Fig. 3C). Colonies with control IgG showed goblet-like cells in the center and epithelium-like cells (Fig. 3D), whereas those with anti-FGFR1 mAb suppressed proliferation of both types of cells (Fig. 3E). No apparent differences in the formation and number of colonies were detected between control IgG and anti-FGFR2 mAb treatment groups. These findings imply that FGF2 promotes the differentiation of corneal limbal
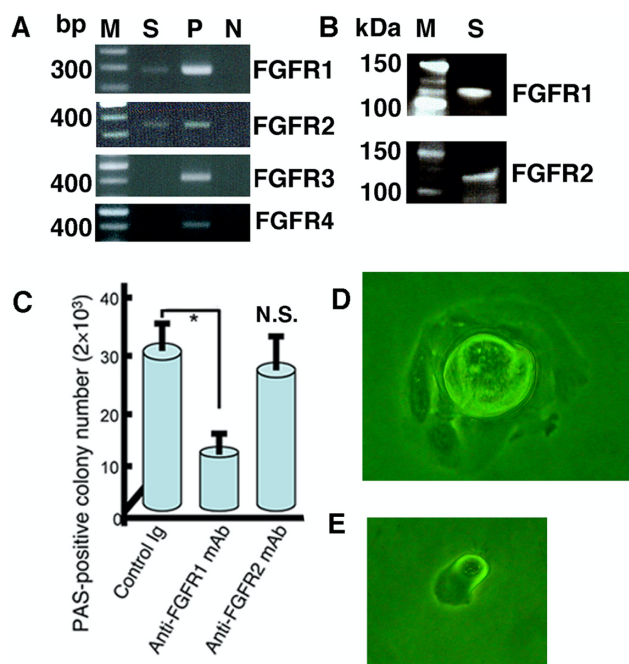
FIGURE 3. FGF receptor expression and effect of FGF receptor blockade in colonies derived from adherent single cells. (**A**) FGFR1 and FGFR2, but not FGFR3 and FGFR4, are detected in the adherent colonies by RT-PCR. (**B**) FGFR1 and FGFR2 expression is detected by Western blotting. (**C**) Effect of FGF receptor blockade was tested in colonies derived from adherent single cells. PAS-positive colony number in the anti-FGFR1 monoclonal antibody group significantly decreases as compared with that in the control Ig group. Treatment with an anti-FGFR2 blocking mAb does not affect the number of colony. (**D**) Colony with amorphous material and epithelium is observed with control IgG. (**E**) Anti-FGFR1 mAb suppresses the proliferation of both goblet-like cell and epithelium. Similar results were obtained with repeated three experiments. M, size markers; S, sample; P, positive control; N, negative control, N.S., not significant.

epithelium progenitor cells into goblet cells and epithelium mainly via FGFR1.

DISCUSSION

Our data show that isolated single corneal limbal epithelium-derived progenitor cells have the potential to differentiate into goblet cells and cytokeratin-3 and -12–expressing corneal epithelium by FGF2 stimulation in serum-free in vitro condition, implying that these limbal epithelia are bipotent progenitor cells. Among possible receptors for FGF2, FGFR1 is functionally relevant to differentiation into goblet cells and epithelium-like cells, indicating that FGF2 should be an inducer of goblet cell and epithelium differentiation via FGFR1 in corneal limbal epithelium.

In the culture medium with fetal bovine serum, EGF is reported as a key factor for differentiation of goblet cells,[9] whereas EGF did not promote goblet cell differentiation of conjunctival and corneal limbal epithelium in our serum-free culture condition. This discrepancy can be explained by modification of cellular differentiation due to fetal bovine serum, because fetal bovine serum contains a number of unknown growth factors and hormone. Evaluation of cellular proliferation and differentiation in unknown factor free culture system may contribute to disclosing direct effects of various growth factors on cell proliferation and differentiation in the field of cell biology.

Mouse corneal epithelial cells can give rise to both corneal epithelial and goblet cells,[15] but our findings are the first report to describe the bipotentiality to corneal epithelium and goblet cells in human corneal limbal progenitor cells. It is still unknown why corneal limbal progenitor cells do not produce goblet cells in vivo. FGF2 is expressed in the normal conjunctiva,[16,17] but not in the three layer of normal cornea. Diseased conjunctiva[18,19] and infiltrating cells of the monocytes/macrophage lineage produces higher levels of FGF2. Cultured human corneal epithelium, stromal fibroblasts, and endothelium express FGF2 protein.[20] These findings suggest inflammatory rather than normal conditions actively promote FGF2 expression in ocular surface and cornea, leading to goblet cell differentiation from limbal progenitor cells. Limbal adult stem cell deficiency is diagnosed if conjunctival goblet cells are found on the corneal surface.[3] The facts that FGF2 is abundantly expressed on the inflammatory condition and corneal limbal progenitor cells have potential to produce goblet cells by stimulation of FGF2 suggest that corneal limbal stem cell deficiency should not be diagnosed only by presence of goblet cells on the clear corneal surface.

In summary, cultured single corneal limbal progenitor cells can give rise to goblet cells and corneal epithelium when incubated with FGF2, while these cells did not proliferate in the medium with B27 only and formed corneal epithelium in the presence of EGF. FGFR1 is a functional receptor of FGF2 for differentiation into goblet cells and corneal epithelium. Our findings imply that bipotent progenitor cells from corneal limbal epithelium can directly give rise to goblet cells and corneal epithelium after stimulation by FGF2 via FGFR1 in vitro.

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