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Human corneal limbal organoids maintaining limbal stem cell niche function

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ABSTRACT

Corneal epithelial stem cells reside in the limbal area between the cornea and conjunctiva. We examined the potential use of limbal organoids as a source of transplantable limbal stem cells. After treating tissue with collagenase, limbal cells were seeded onto Matrigel and cultivated using limbal phenotype maintenance medium. After 1-month, approximately 500 organoids were formed from one donor cornea. Organoids derived from vertical sites (superior and inferior limbus) showed large colony forming efficiency, a higher ratio of slow cycling cells and N-cadherin-expressing epithelial cells compared to horizontal sites. The progenitor markers Keratin (K) 15 and p63 were expressed in epithelial sheets engineered form a single organoid. Organoids transplanted in the limbus of a rabbit limbal deficiency model confirmed the presence of organoid-derived cells extending on to host corneas by immunohistochemistry. Our data show that limbal organoids with a limbal phenotype can be maintained for up to 1 month *in vitro* which can each give rise to a fully stratified corneal epithelium complete with basal progenitor cells. Limbal organoids were successfully engrafted *in vivo* to provide epithelial cells in a rabbit limbal deficiency model, suggesting that organoids may be an efficient cell source for clinical use.

1. Introduction

The corneal epithelium is a stratified layer of non-keratinized cells that form the outer most layer of the ocular surface. Due to the rapid turnover of corneal epithelial cells, stem cells are believed to exist in a unique microenvironment, or a "niche", that supports the self-renewal and differentiation of stem cells as reported in skin, brain and hematopoietic system (Tumbar et al., 2004; Alvarez-Buylla and Lim, 2004; Zhang et al., 2003). Corneal epithelial cells are supplied from stem cells located in the corneal limbus surrounding the peripheral cornea (Cotsarelis et al., 1989; Schermer et al., 1986). Several markers such as N-cadherin (N-cad) and melanocytes have been identified in the human limbal epithelial stem cell niche (Hayashi et al., 2007). Rigorous digestion of limbal tissue with collagenase has been reported to maintain the limbal architecture for *in vitro* studies compared to milder dispase digestion (Chen et al., 2011). We also showed how niche-like cells beneath the limbal epithelial basement membrane directly interact with N-cad positive limbal basal epithelial cells using collagenase digestion (Higa et al., 2013). These findings indicate that unique niche cells exist in the corneal limbus that regulate epithelial progenitors cells. Recently, three-dimensional (3D) culture of epithelial organoids with cellular components such as fibroblasts and collagen has allowed the study of niche interactions *in vitro* (Pastula et al., 2016; Sato et al., 2009). In this study, we cultured limbal epithelial organoids that maintain human limbal epithelial niche function by preserving corneal epithelial stem cells for up to 1 month. Moreover, single limbal organoids were shown to produce a robust, stratified epithelial sheet that include progenitor cells. Transplanted organoids also functioned to produce epithelial cells in a rabbit stem cell deficiency model.

Abbreviations: N-cad, N-cadherin; K, keratin; SHEM, supplemental hormonal epithelial medium; LPMM, limbal phenotype maintenance medium; EGF, epidermal growth factor; KGF, keratinocyte growth factor; CFE, colony-forming efficiency; V (vertical axis), the superior or inferior side of limbal tissue; H (horizontal axis), the temporal or nasal side of limbal tissue; BrdU, 5′-bromo-2′-deoxyuridine

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2. Material and methods

2.1. Antibodies

Mouse monoclonal antibodies (mAbs) for N-cadherin, keratin (K) 15, p63, K3, 5′-bromo-2′-deoxyuridine (BrdU), human nuclei and laminin were purchased from Invitrogen (3B9, Carlsbad, CA), Lab Vision (LHK15, Fremont, CA), Santa Cruz Biotechnology Inc. (4A4, Santa Cruz, CA), PROGEN (AE5, Heidelberg, German), Dako Cytomation (MIB-1, Glostrup, Denmark), Abcam (mabcam8226, Cambridge, UK), Abcam (ab191181) and CosmoBio Co., LTD (NU-01-LA3, Tokyo, Japan), respectively. Rabbit and goat polyclonal antibody for collagen type IV, tenascin-C and K12 were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL), Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and TransGenic Inc. (Kumamoto, Japan), respectively. FITC-, rhodamine- and Cy3-conjugated secondary antibodies were purchased form Jackson Immuno Research Laboratories and Invitrogen.

2.2. Organoid culture

Donor corneas were obtained from the U.S. eyebank eyes (CorneaGen, Seattle, WA). Experiments were done using limbal tissue after central corneal buttons were used for transplantation. Consent for the use of human tissue in research was obtained by the eye bank at the time of tissue processing. Limbal rims of corneoscleral tissue were prepared by careful removal of excess sclera, iris, and corneal endothelial tissue. Limbal tissue was divided into equal parts, then treated with either 1.2U/ml Dispase II (Roche, Mannheim, Germany) or 2 mg/ml collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in F12/DMEM at 37 °C for 60 min, respectively. Cell clusters were obtained by physically scraping the limbal area using a gill corneal knife (Bausch & Lomb Surgical, Inc. St. Louis, MO), divided into equal volumes, suspended in Matrigel (Corning Inc. Life Sciences, Tewksbury, MA) and then cultured on cell culture inserts (Corning Enterprises, Corning, NY) with supplemental hormonal epithelial medium (SHEM, consisting of F12/DMEM (Invitrogen), 4% FBS, 10 ng/ml epidermal growth factor (EGF, Invitrogen), 5 μg/ml human recombinant insulin (Wako), 1.3 mg/ml tri-iodo-thyronine (Sigma-Aldrich, St. Louis, MO), 0.25 μg/ml isoproterenol hydrochloride (Sigma-Aldrich), 0.5 μg/ml hydrocortisone (Sigma-Aldrich) and antibiotics) or limbal phenotype maintaining medium (LPMM) (SHEM changed from EGF to 10 ng/ml keratinocyte growth factor (KGF, PeproTech, Rocky Hill NJ) and 10 μg/ml Y27632 (Rho kinase inhibitor, Wako)), respectively (Miyashita et al., 2013).

In another experiment, we mimicked an autologous source of tissue by performing organoid cultivation with a 2.5 mm-diameter tissue punch (KAI Industries Co., Ltd., Gifu, Japan). Tissue was procured at the superior or inferior side (vertical axis) and temporal or nasal side (horizontal axis) of a donor cornea. Orientation was determined by the fact that corneal diameter in longest in the horizontal plane (Fig. 2A). The isolated tissue was culture as shown above.

To detect slow cycling cells, cell clusters were labeled with 10 mM 5′-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) during the first 3 days of culture. To detect transplanted organoids, cultivated organoids were labeled with 75 mg/ ml Ferucarbotran (Resovist, FUJIFILM Toyama Chemical Co., Ltd., Tokyo, Japan) on the last day of culture. After 1 month, organoids in Matrigel were analyzed for number and size (mm^2) using the Image J program (National Institutes of Health, Bethesda). Donor corneas used in the study ranged from 42 to 66 years of age and confirmed that age did not affect the number and size of organoids.

2.3. Epithelial sheet culture from a single organoid

Fibrin sealant was purchased from Fujisawa (Bolheal®, Osaka, Japan), and its reconstitution was performed as reported previously (Higa et al., 2007; Itabashi et al., 2005). In brief, a solution containing 40 mg of human fibrinogen and 0.18 U of thrombin was diluted with 7.5 ml saline, and 0.3 ml was spread rapidly onto the upper chambers of a 6-well Transwell culture insert (Corning). Two hours later, polymerized fibrin-coated culture inserts were obtained, and stored at 4 °C. An organoid was prepared by careful isolation from Matrigel using a tweezer, and then seeded onto a fibrin-coated culture insert with LPMM and co-cultured with mitomycin-C (MMC)-treated 3T3 fibroblasts for 3 weeks.

2.4. DNA cell cycle analysis

Organoids were dispersed using 2 mg/mL collagenase at 37 °C for 2 h. Cell suspensions were pelleted by centrifugation, and cells were resuspended in a solution containing 4 mM sodium citrate (Wako) (pH 7.6), 0.2% Nonidet P-40 (Calbiochem, San Diego, CA, USA), and 50 μg/mL propidium iodide (Wako). After incubation on ice for 30 min, cell suspensions were treated with 250 μg/mL RNase A (Fermentas, Waltham, MA, USA) for 15 min at 37 °C to remove double-stranded RNA. Cells were measured using flow cytometer (EPICS XL; Beckman Coulter, Miami, FL, USA). Data were analyzed using flow cytometry software FlowJo (Tree Star Inc., Ashland, OR, USA). Five independent replicates of the organoid culture and DNA cell cycle analysis were performed.

2.5. Colony-forming efficiency (CFE)

To evaluate the proliferative potential of organoids, dispersed cells were used in a CFE assay as described previously (Li et al., 2005; Kim et al., 2004; Tseng et al., 1996). NIH 3T3 fibroblasts in DMEM containing 10% FCS were treated with MMC (4 μg/mL) for 2 h at 37 °C, harvested using trypsin-EDTA, and plated at a density of 6×10^5 cells with supplemental hormonal epithelial medium (5% FCS) in 34-mm culture dishes. Single cells were prepared from organoids treated with Dispase II (Roche) for 90 min and TrypLE Express (Invitrogen) for 10 min. Each dish was seeded with 33 cells/ cm^2. Growth capacity was evaluated on day 14, when cultured cells were stained with rhodamine B (Wako Pure Chemical Industries, Ltd.) for 30 min. The number of colonies obtained from each sheet ($n = 5$) was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). CFE (%) was calculated using the following formula: CFE (%) = number of colonies/ number of seeded cells (3×10^2 cells/ well) × 100.

2.6. Organoid transplantation

To evaluate the limbal function of organoids, limbal deficiency model was prepared using female Japanese white rabbits (2.5 kg body weight, Shiraishi Experimental Animal Breeding Farm). Under intravenous anesthesia with a mixture of medetomidine hydrochloride (0.5 mg/kg, Domitor, Meiji Seika Kaisha, Tokyo, Japan), midazolam hydrochloride (2.0 mg/kg, Dormicam, Astellas Pharma, Tokyo, Japan), and butorphanol (0.5 mg/kg, Vetorphale, Meiji Seika Kaisha), the rabbit corneal and limbal epithelium were scraped using a grinder (Handy router, KOHNAN shoji Co., Ltd., Osaka, Japan) 3 times in 1 week. Pockets at 4 sites (superior, inferior, temporal, and nasal side) were made in the rabbit limbus using a microsurgery knife (Grieshaber, Alcon, Tokyo, Japan). A single organoid was inserted in each pocket and sealed with surgical tissue adhesives (Bolheal®). After transplantation, rabbits were followed by slit-lamp microscope and fluorescein staining, and rabbit limbal tissue sections were observed histologically at the end of the study (1 week).

2.7. Berlin blue staining

To detect transplanted organoids containing ferucarbotran labeled cells, rabbit limbal tissues were stained with Berlin blue. Tissue sections

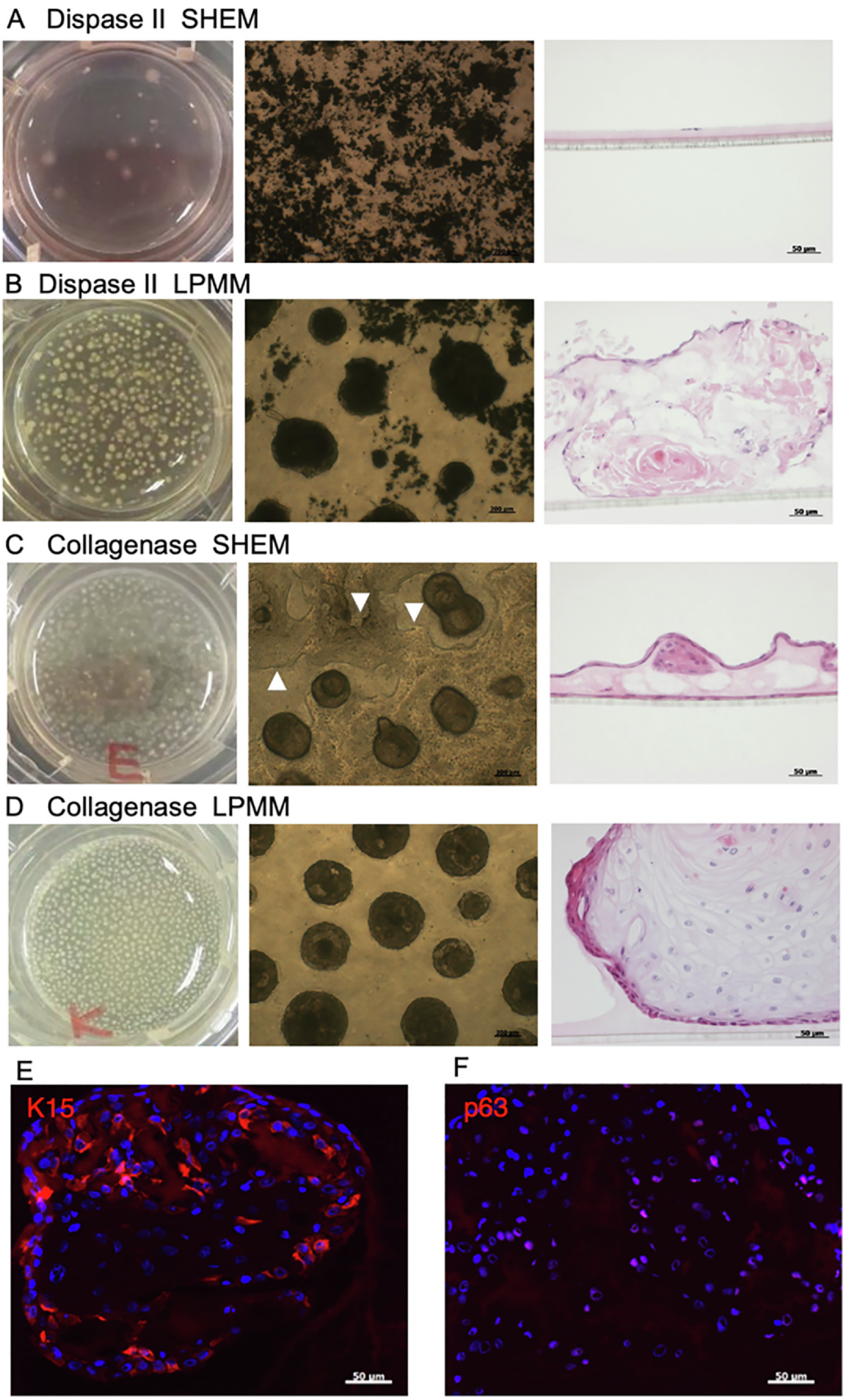

Fig. 1. Limbal organoid culture after 1 month. (A, B) Limbal tissue digested with dispase II and cultured with either SHEM medium (A) or with limbal phenotype maintaining medium (LPMM) (B). (C, D) Limbal tissue digested with collagenase and cultured with SHEM (C) or LPMM (D). Left panels: Macroscopic view of organoids on culture inserts. Middle panel: Micrograph of organoids. Right panel: Hematoxylin & Eosin (H.E.) stain of organoids. (E) Immunohistochemistry of K15 (red) and DAPI (blue). (F) Immunohistochemistry of p63 (red) and DAPI (blue). Scale bar: middle panel: 200 μm, right panel, E and F: 50 μm.

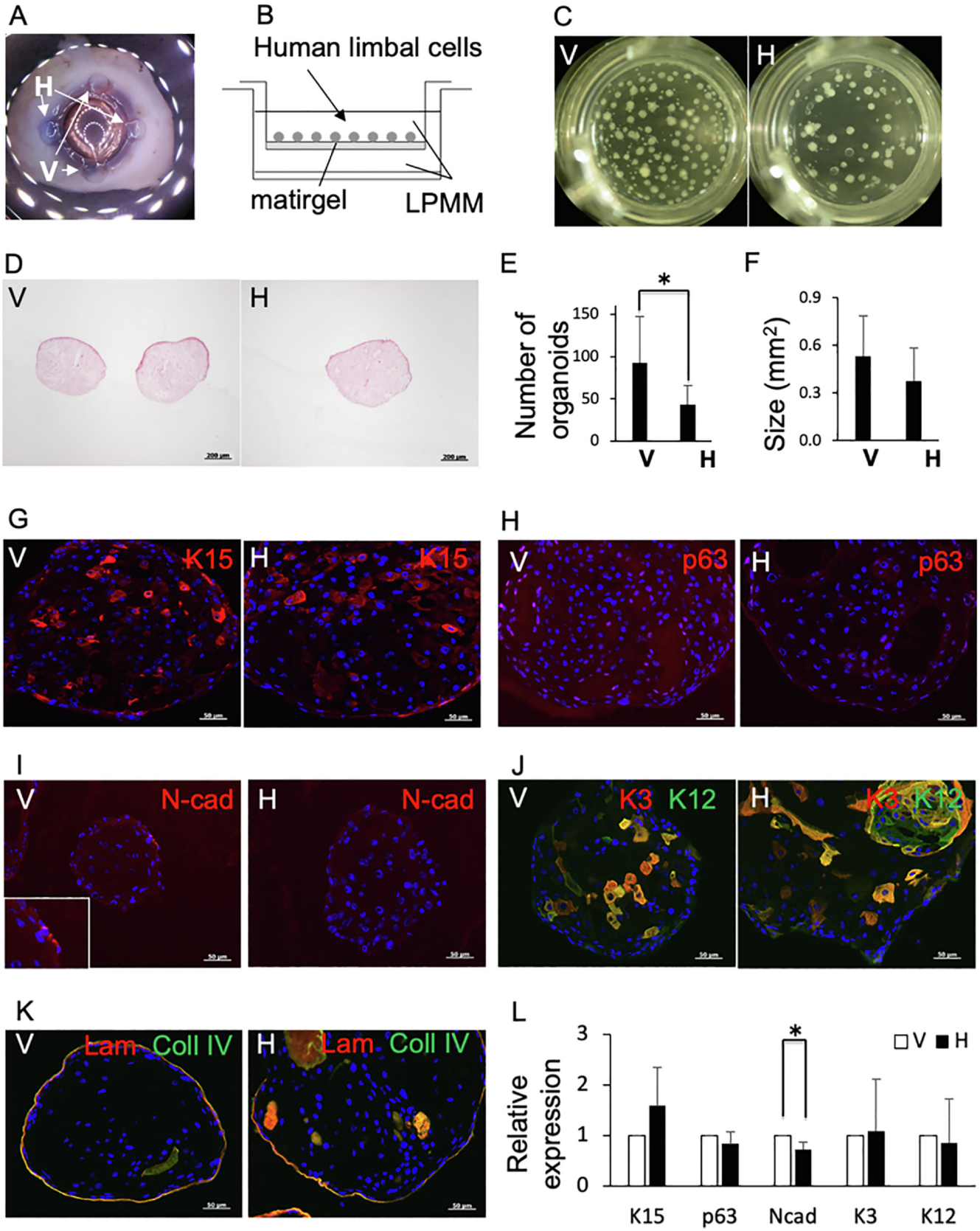

Fig. 2. Characterization of limbal organoids. (A) Small segments of limbal tissue were harvested and divided into either the superior and inferior areas (vertical axis: V) or the temporal and nasal areas (horizontal axis: H). Orientation was confirmed by placing the maximal corneal diameter in the horizontal position. (B) Schema of organoid culture technique. LPMM: limbal phenotype maintaining medium. (C) Organoids from the vertical (V) and horizontal (H) limbal tissue after 1 month culture. (D) H.E. staining of organoids. (E, F) Comparison of organoid formation (E) and size (F) from each site ($p < 0.05$, $n = 5$). (G-I) Immunohistochemistry of K15 (G), p63 (H), N-cad (I), K3 and K12 (J), and collagen type IV and laminin (K) in organoids from each site. Insert is an enlarged image of N-cad expression in the vertical group. DAPI nuclear counterstain (blue). Scale bar: 50 μm. (L) Real-time PCR of K15, p63, N-cad, K3, and K12. Y-axis indicates relative expression compared to organoids from vertical axis (V), which were normalized to 1. Error bars indicate SD ($p < 0.05$, $n = 5$).

were fixed with 10 N formalin (Wako) for 20 min at room temperature, and were stained with berlin blue solution with a mixture or 2% potassium ferrocyanide (Wako) and 2% hydrochloric acid (Wako) for 20 min. The sections were incubated with 1 μg/ ml nuclear fast red (Wako) at R.T. for 15 min. The sections were dehydrated in graded ethanol, cleared in xylene and cover-slipped with mounting medium (Mount Quick, Daido sangyo Co., Ltd., Saitama, Japan). Images were observed using a microscope (Axioplan2 imaging, Carl Zeiss Inc.).

2.8. Histological analysis

Organoids, epithelial sheets engineered from a single organoid, and organoid-transplanted rabbit limbal tissues were frozen in O.C.T. compound (Sakura Finetek, Tokyo, Japan), and sliced into 5-μm-thick sections for hematoxylin and eosin staining and immunostaining. Sections were fixed for 5 min in 2% paraformaldehyde (Wako) or cold acetone (Wako) and were blocked by incubation with 10% normal donkey serum (Chemicon Int. Inc., Temecula, CA) and 1% bovine serum albumin (Sigma) for 1 h at room temperature (R.T.). Sections for BrdU staining were treated with the denaturing reagent containing BrdU immunohistochemistry system (Merck, Darmstadt, Germany) for 30 min at R.T. before blocking. Antibodies to Ncad (1:50), K15 (1:300), p63 (1: 50), K3 (1:150), K12 (1:300), collagen type IV (1:200), laminin (1:50), BrdU (1:50), tenascin-C (1:50) and human nuclei (1:50) were applied for 90 min at R.T., followed by incubation with FITC- or rhodamine- or Cy3-conjugated secondary antibody. After 3 washes with phosphate buffer saline (PBS), the sections were incubated with 0.5 μg/ ml 4′, 6-diamidino- 2-phenylindole (DAPI; Dojindo Laboratories, Tokyo, Japan) at R.T. for 5 min. Finally, sections were washed three times in PBS and cover-slipped using an aqueous mounting medium containing an anti-fading agent (Fluoromount/Plus, Diagnostic Biosystems, Pleasanton, CA). Images were observed using a florescence microscope (Axioplan2 imaging, Carl Zeiss Inc., Thornwood, NY).

2.9. Real-time PCR analysis

Total RNA was isolated from organoids using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the protocol provided by the manufacturer. Complementary DNA (cDNA) was prepared from total RNA with 0.25 M dithiothreitol, x5 reaction buffer, RNase inhibitor, and avian myeloblastosis virus reverse transcriptase (Takara Bio Inc., Shiga, Japan) by incubating a 25-μL mixture at 41 °C for 1 h. This cDNA was used as a template for real-time PCR amplification. Real-time PCR was carried out using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Probes and primers were purchased from Applied Biosystems. Reactions were performed according to manufacturer's methods and run in triplicate in five independent experiments. 18S rRNA was used as an internal control to normalize the variability in expression levels. Comparative expression ratios were calculated with the ΔΔCt method, using the following formula:

$$\text{Relative expression ratio} = 1/2^{(\Delta Ct\ (18S)\ -\ \Delta Ct\ (\text{each gene}))}$$

The values are expressed as the vertical axis (V) normalized to 1.

2.10. Statistical analysis

Statistical analysis was performed using the Student's *t*-test (Excel; Microsoft, Redmond, WA), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Organoid culture

Organoid formation after 1 month was observed in four different culture conditions. Organoids were uniformly round with collagenase treatment compared to the irregular morphology after dispase II treatment (Fig. 1). With collagenase digestion, extension of limbal epithelial cells from the organoid was observed in standard medium with EGF (SHEM), but was rarely observed in medium with KGF + Y27632 (LPMM) (Fig. 1C, D). Only with collagenase digestion and LPMM, organoids maintained the Keratin (K) 15 and p63-positive phenotype of limbal epithelial cells (Fig. 1E, F). Over 500 organoids were obtained from a single donor source with collagenase digestion (569.4 ± 403.2 organoids/ donor, n = 5).

3.2. Organoid culture from small samples of human limbal tissue

To establish an organoid culture protocol from a limited source of autologous tissue, we compared organoid formation from small 2.5 mm diameter samples of human limbal tissue (Fig. 2A). Uniformly small and round organoids were formed from these limited amounts of limbal tissue (Fig. 2C, D). Efficiency of organoid formation was different depending upon the site of the original limbus. More than 50 organoids were derived from tissue on the vertical axis (Fig. 2E). Organoid size (mm^2) tended to be larger in the vertical axis (0.53 ± 0.26 mm^2) compared to the horizontal axis (0.37 ± 0.21 mm^2), but the difference was not statistically different (Fig. 2E, F). Organoids derived from the vertical axis consisted of many p63 positive cells (Fig. 2H). N-cadherin (N-cad), which is sporadically expressed in limbal basal epithelial cells as a putative marker of epithelial progenitor cells, was only expressed at low levels (Fig. 2I insert). K3 and K12 expression was observed in both organoids (Fig. 2J), and collagen type IV (Coll IV) and laminin was expressed in the around of both organoids (Fig. 2K). Relative expression of immature cell markers (K15, p63, N-cad) and differentiation markers (K3, K12) was demonstrated by real-time PCR (Fig. 2L). Only N-cad expression was significantly higher in the organoids from vertical axis (V) compare to horizontal axis (H).

3.3. Cell cycle and colony formation by organoids

BrdU was stained in order to observe label-retaining cells in organoids as a means to indicate slow-cycling cells. The number of BrdU positive cells in the vertical axis was higher compared to the horizontal axis (Fig. 3A). To compare cell cycle of organoids from each site, we performed DNA cell cycle analysis by flow cytometer (Fig. 3B). Organoid cells from the vertical axis in the G0/G1 phase (80.1 ± 1.5%) were significantly higher compared to cells from the horizontal axis (77.4 ± 1.3%) (Fig. 3C, $P < 0.05$, n = 5). To estimate proliferative potential of organoids, we compared CFE and colony size in each organoid group (Fig. 3E). CFE of vertical axis cells (6.3 ± 2.9%) was higher than horizontal axis cells (3.2 ± 2.3%) (Fig. 3F, $P < 0.05$, n = 5). Colony area of vertical axis cells (2.8 ± 2.2 mm^2) was also higher than horizontal axis cells (0.6 ± 0.5 mm^2) (Fig. 3G, $P < 0.05$, n = 5). The data show that slow-cycling cells and proliferative potential was maintained more in organoids derived from the vertical axis compared to the horizontal axis.

3.4. Epithelial sheet culture from a single organoid

To examine the potential of organoids as a cell source for cultivated epithelial sheets, we cultured epithelial sheets from a single organoid using the limbal phenotype maintaining medium (LPMM) and 3 T3 feeder cells (Fig. 4A). The single organoid-derived epithelial sheet showed robust stratification (Fig. 4B), and basal cells within the sheet maintained the expression of Keratin (K) 15 and p63 indicating the corneal limbal phenotype (Fig. 4C, D). Basement membrane and extracellular matrix of the sheet also showed the expression of collagen type IV (coll IV), laminin (Lam) and tenascin-C (TNC) (Fig. 4E, F).

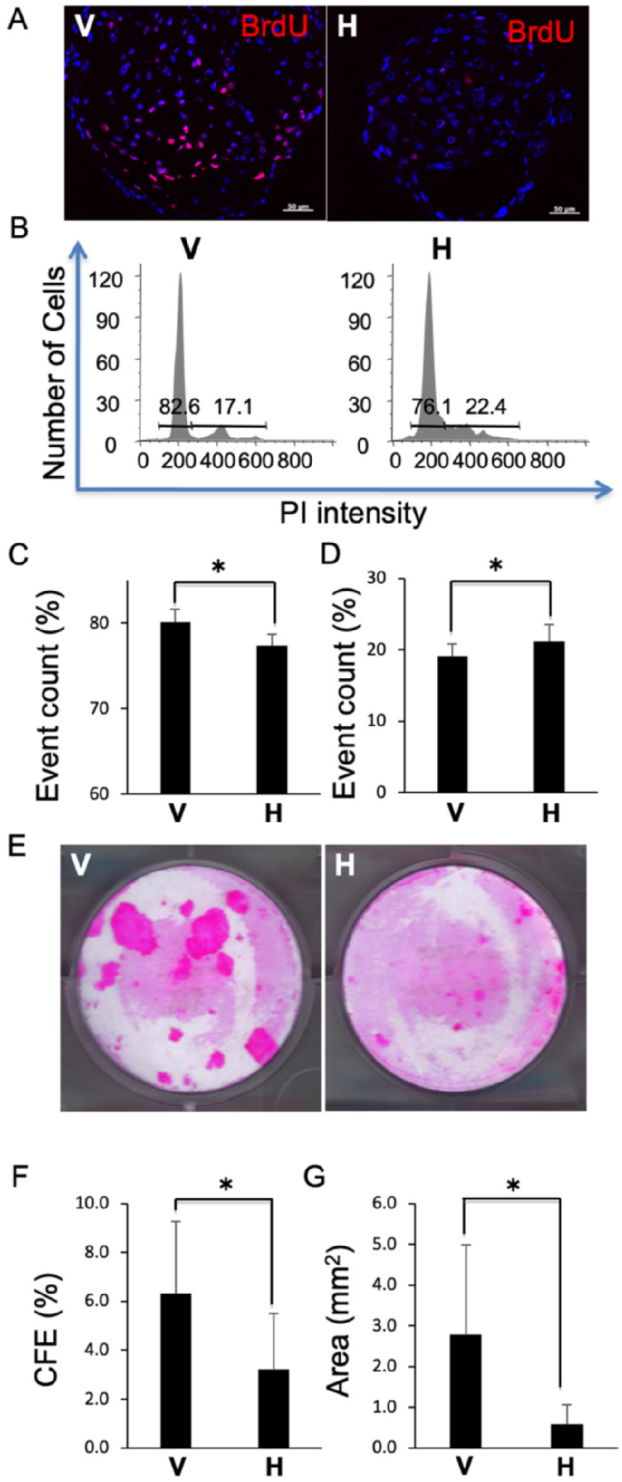

Fig. 3. Cell cycle and colony forming efficacy of organoids. (A, B) Immunostaining of BrdU (label retaining cells) (red) in organoids from the vertical axis (V) and horizontal axis (H) with DAPI nuclear counterstaining (blue). Scale bar: 50 μm. (B) DNA cell cycle analysis in organoids by flow cytometer. Left gate: G0/G1 phase, Right gate: S/G2-M phase. (C) Graphs (G0/G1 phase) of B indicating ratio of event count (%) ($p < 0.05$, $n = 5$). (D) Graphs (S/G2-M phase) of B indicating ratio of event count (%) ($p < 0.05$, $n = 5$). (E) Comparison of CFE in each organoid group. Colonies were stained with rhodamine B after 2 weeks. (F, G) Number (F) and size (G) of colonies obtained from each organoid group ($p < 0.05$, $n = 5$).

3.5. Organoid transplantation

To confirm the *in vivo* function of organoids, we transplanted human limbal organoids in a rabbit limbal deficiency model. Organoids derived from the pigmented human limbus retained pigments after 1 month in culture (Fig. 5A-C). Pigments of organoids were observed in the limbus of the non-pigmented rabbit limbal deficiency model immediately after transplantation (Fig. 5E). Epithelialization was observed in the peripheral cornea of the rabbit limbal deficiency model (Fig. 5F, G), and pigmentation in the transplantation site was retained after 1 week (Fig. 5H-K). Pigmented epithelial cells were observed extending toward the central cornea from the limbus after transplantation (Fig. 5L).

Ferucarbotran-labeled organoids were also observed in the transplanted sites shown by Berlin blue, and pigmented epithelial cells were detected in the transplanted site and epithelial cells extending into the host cornea (Fig. 6A-D). Immunohistochemistry against human nuclei confirmed the presence of organoid-derived cells extending on to host corneas (Fig. 6E-H). Interestingly, epithelium expanding form organoids toward the conjunctiva was not observed. The basement membrane of organoids strongly expressed type IV collagen compare to the basement membrane of host corneal and limbal epithelium (Fig. 6I-L).

4. Discussion

In this study, we demonstrated that corneal limbal organoids maintained the limbal progenitor phenotype for 1 month using collagenase digestion and LPMM medium. Collagenase digestion enables isolation of progenitor cells while maintaining the niche microenvironment including the basement membrane and niche mesenchymal cells (Chen et al., 2011; Higa et al., 2013). LPMM is a long-term maintenance medium that includes rho kinase inhibitor (Y-27632) and KGF, and has been shown to maintain homeostasis in limbal epithelial sheet for over a year (Miyashita et al., 2013, 2017). Similar stem cell-niche interactions in the mouse intestine were reconstructed by 3D organoid culture using isolated crypts and stroma cells (neurons and/or myofibroblasts) in Matrigel (Pastula et al., 2016). After 1 month culture, limbal stroma cells were located around the organoids (data not shown) and the limbal epithelial markers K15 and p63 were expressed in organoids (Fig. 1E, F), suggesting that corneal limbal progenitors/stem cells were isolated with their niche compartments, including the basement membrane and niche like stroma cells. Collagen type IV (Coll IV) and laminin were sometimes expressed in the inside of organoids after 1 month (Fig. 2K). Since we collected limbal cells using collagenase so that the niche structure is not completely destroyed, there is a possibility that original niche structures remained within organoids after 1 month. Organoid cells maintained the limbal phenotype after 1 month. Organoids cultured in SHEM (EGF containing) medium displayed outgrow of cells, while organoids in LPMM do not display such outgrow of cells (Fig. 1C, D). Since organoids were formed autonomously in Matrigel, we believe that KGF and Y-27632 in LPMM provides a stable balance of proliferation and differentiation, while EGF containing medium tips the balance towards proliferation. A similar balance of proliferation and differentiation was observed in our previous report on long-term culture of epithelial sheets with LPMM medium (Miyashita et al., 2017).

N-cad is expressed in limbal basal epithelial cells as a possible marker of putative epithelial progenitor cells, and is associated with melanocytes or niche-like cells that exist immediately beneath the limbal epithelial basement membrane through homotypic adhesion (Hayashi et al., 2007; Higa et al., 2013). N-cad is also a key component of the bone marrow stem cell niche where spindle-shaped N-cad expressing osteoblasts interact with long-term hematopoietic stem cells (Zhang et al., 2003; Zhu and Emerson, 2004; Nilsson et al., 2001; Whetton and Graham, 1999). In this study, we found sporadic N-cad expression in the edge of organoids from the vertical axis (Fig. 2I, L). We also showed that organoids from the vertical axis contained more

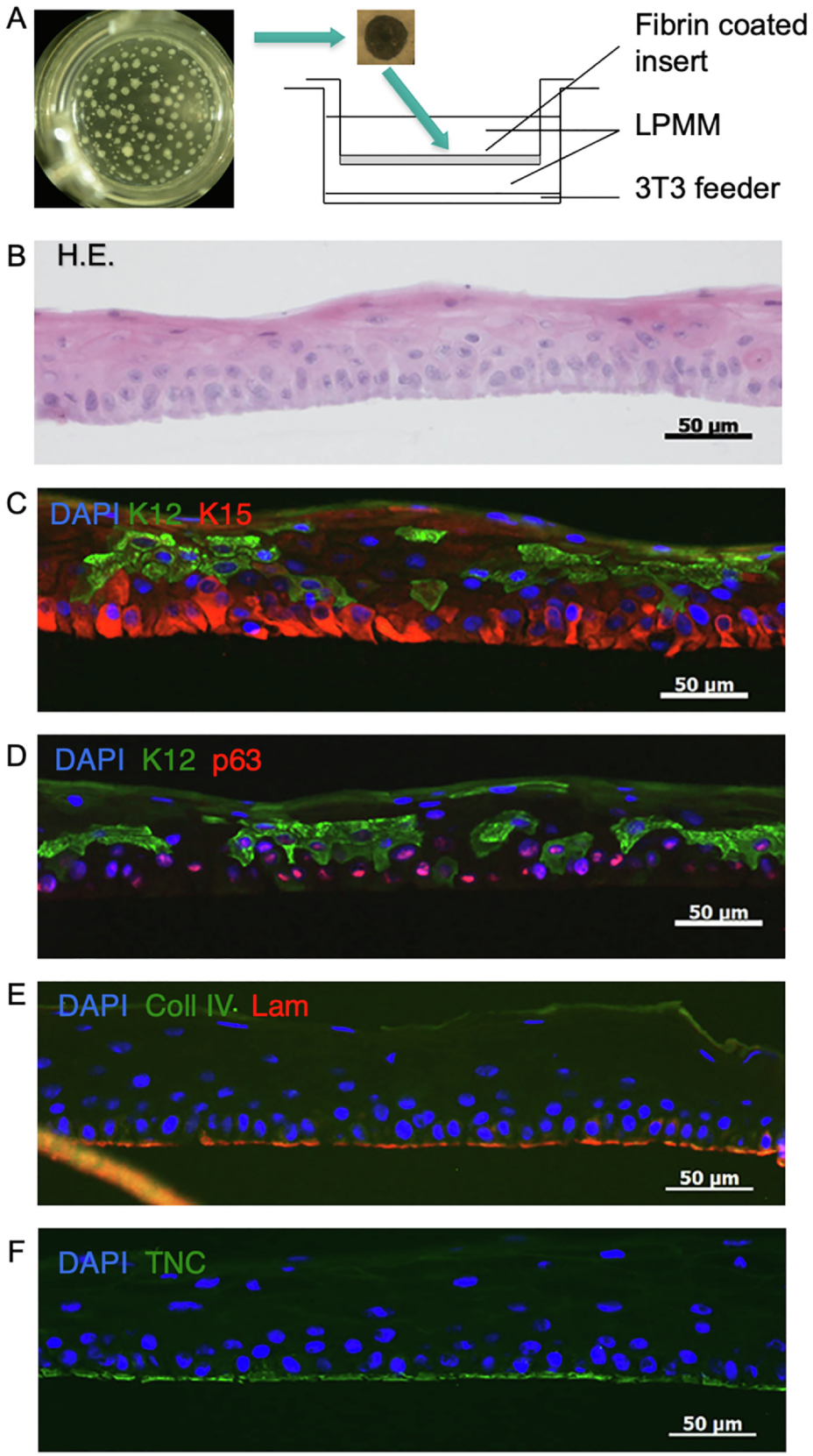

Fig. 4. Epithelial sheet culture from a single organoid. (A) Photograph of cultivated organoids after 1 month from which a single organoid was used to cultivate a stratified epithelial sheet co-culture with 3 T3 feeder cells. LPMM: limbal phenotype maintaining medium. (B) H.E. staining of epithelial sheet. (C) Immunostaining of K12 (green) and K15 (red). (D) Immunostaining of K12 (green) and p63 (red). (E) Immunostaining of collagen type IV (green) and laminin (red). (F) Immunostaining of tenascin-C (green). DAPI nuclear counterstain (blue). Scale bar: 50 μm.

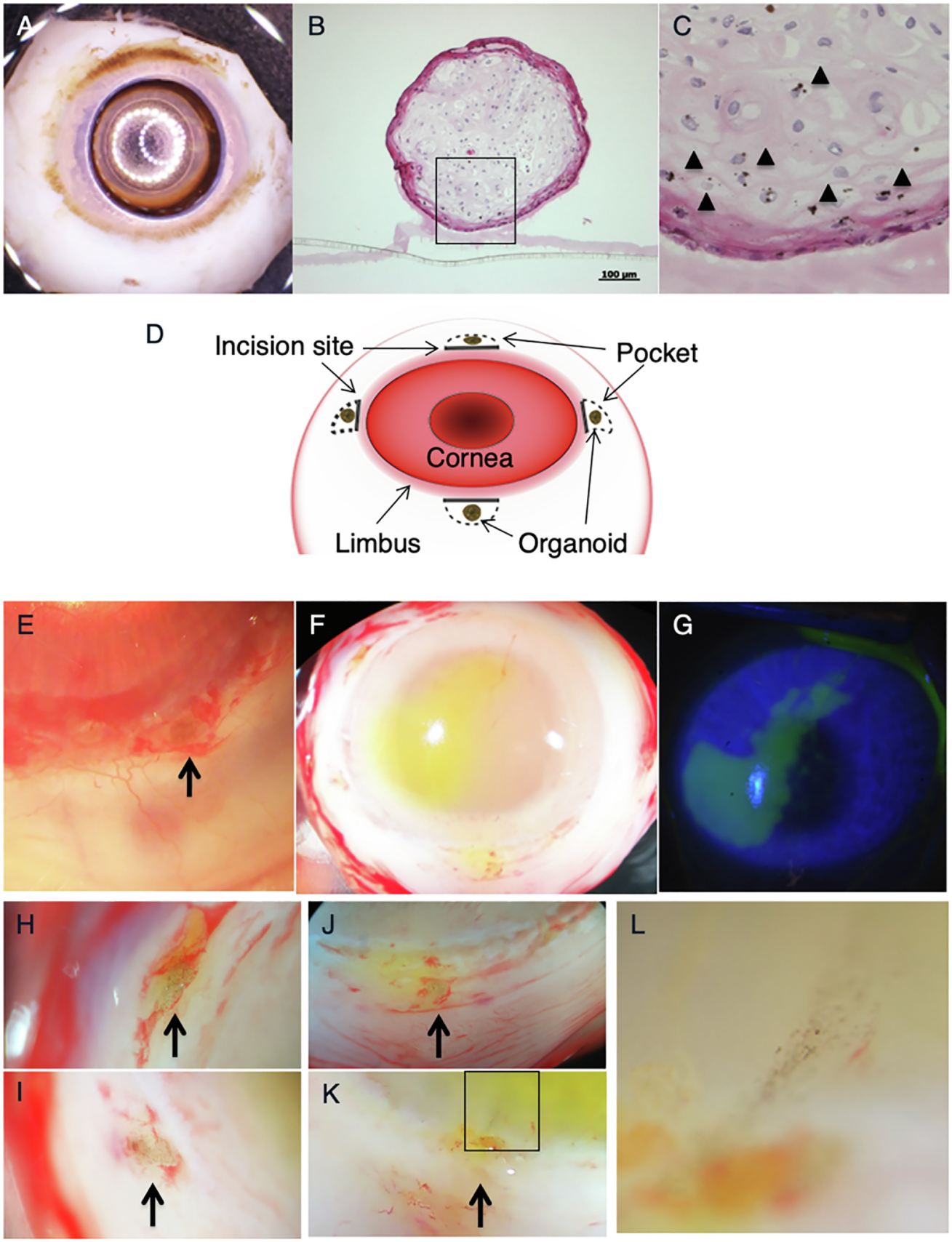

Fig. 5. Organoid transplantation in a rabbit limbal deficiency model. (A) Pigmented human limbal tissue. (B) H.E. staining of an organoid generated from pigmented human limbal tissue. (C) Enlarged image of square area in B. Arrowhead: pigments. (D) Location of the transplanted organoids in rabbit eye. (E) Rabbit limbus immediately after organoid transplantation. Arrow: transplanted organoid. (F) Rabbit eye 1 week after transplantation. (G) Fluorescein staining of rabbit eye 1 week after transplantation. (H-K) Transplanted site after 1 week. Arrow: Donor derived pigments in the transplanted sites. (L) Enlarged image of square area in K show pigmented epithelium expanding on to the cornea.

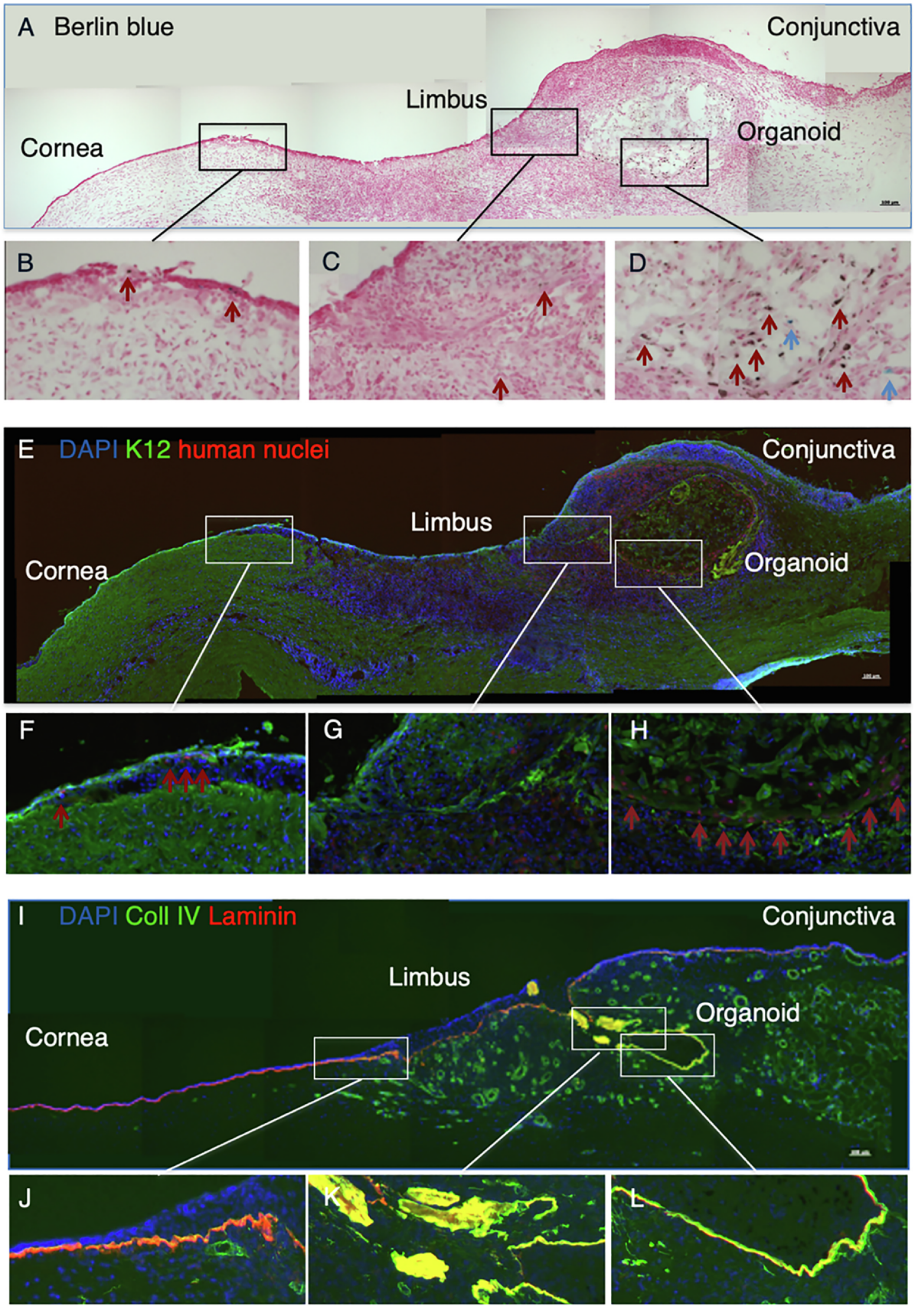

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Fig. 6. Histochemical analysis of rabbit limbal tissue transplanted with organoids. (A) Berlin blue staining of organoid-transplanted area of the limbus in a rabbit limbal deficiency model. Scale bar: 100 μm. (B-D) Enlarged image of A. Peripheral cornea (B), limbus (C), and organoid transplanted area (D). Blue arrowheads: berlin blue positive cells. Red arrowheads: pigment positive cells. (E) Immunohistochemistry of K12 and human nuclei in the organoid-transplanted limbus. Scale bar: 100 μm. (F-H) Enlarged image of E. Peripheral cornea (F), limbus (G), and organoid-transplanted area (H). Red arrow: human nuclei positive cells. (I) Immunohistochemistry of type IV collagen and laminin in the organoid-transplanted limbus. Scale bar: 100 μm. (J-L) Enlarged image of I. Peripheral cornea (J), limbus (K), and organoid-transplanted area (L).

slow cycling cells shown as BrdU positive label retaining cells and G0/G1 phase cells. Cells from the vertical axis showed higher colony forming efficiency (Fig. 3). These data suggest that organoids derived from the vertical axis maintained a more undifferentiated state compared to the horizontal axis. Our results support a previous report that showed how primary limbal epithelial cells derived from explants obtained from superior human limbus demonstrated the highest outgrowth success rate (Utheim et al., 2009).

Ocular surface diseases with limbal stem cell deficiency require replacement of corneal epithelial progenitors/stem cells, and epithelial sheets containing such immature cells are used for these patients (Nakamura et al., 2006; Nishida et al., 2004; Shimazaki et al., 2002; Rama et al., 2001; Pellegrini et al., 1997). Human limbal epithelial sheets were cultured by medium with supplement of Y27632 and KGF instead of EGF (LPMM) for at least 3 month while maintaining limbal epithelial phenotype (Miyashita et al., 2013) and homeostasis without dermal equivalents at least 1 year (Miyashita et al., 2017). Such long-term culture protocols of epithelial sheets may be more beneficial for patients due to the long shelf-life and availability on short notice. In this study, we successfully formed at least 500 organoids from a single limbus which were maintained for 1 month using LPMM. Furthermore, stratified epithelial sheets maintaining limbal epithelial phenotype with the basement membrane components were successfully engineered from a single organoid (Fig. 4). Collagen type IV and laminin are corneal and limbal epithelial basement membrane components, and tenascin-C is an extracellular matrix protein expressed in the basal layer of the normal limbus (Nakatsu et al., 2013; von Holst, 2008), which play an important role in maintaining the limbal epithelial phenotype in the engineering epithelial sheets. This implies that organoids derived from one donor can supply the stratified epithelial sheets containing progenitor cells for hundreds of patients.

Transplantation of donor limbal tissue is also useful for ocular surface diseases with limbal stem cell deficiency (Daya and Ilari, 108 (2001); Tan et al. (1996); Kenyon and Tseng (1989)). In this study, we successfully showed that organoids can endure engraftment to the limbus of a rabbit limbal deficiency model (Figs. 5, 6). Transplanted organoids were observed for 1 week in the rabbit, and human cornea epithelium was shown extending from transplanted organoids to host rabbit cornea. We used limbal epithelium with pigments since pigmented tissue can be easily distinguish from white rabbit tissue after transplantation since host tissue does not contain pigments. Type IV collagen and laminin are key components of the basement membrane for corneal and limbal epithelial cells to proliferate and differentiate (Ahmad et al., 2007; Homma et al., 2004), and type IV collagen was strongly maintained in the basement membrane of transplanted organoids (Fig. 6I-L). This suggests that an organoid functions as a niche unite that can supply corneal epithelial cells in the treatment of limbal stem cell deficiency. Although we demonstrated that transplanted organoid exhibited limbal properties, the observation period was short (1 week) due to the fact that the experiment was a xenograft (human in rabbit). Further studies to show efficacy and safety of organoid transplantation are required prior to clinical studies.

5. Conclusions

Our findings indicate that organoid culture maintained the limbal epithelial niche at least 1 month using collagenase isolation, LPMM and Matrigel. Transplantable epithelial sheets maintaining limbal epithelial phenotype were successfully engineered from a single organoid, and intact organoids were also shown to engraft to the limbus of a rabbit limbal deficiency model. Our protocol may further expand options for treating ocular surface diseases with limbal epithelial stem cell deficiency.

CRediT authorship contribution statement

Kazunari Higa: Conceptualization, Methodology, Investigation, Writing - original draft, Funding acquisition. **Junko Higuchi:** Investigation. **Reona Kimoto:** Formal analysis, Investigation. **Hideyuki Miyashita:** Methodology, Writing - review & editing. **Jun Shimazaki:** Writing - review & editing. **Kazuo Tsubota:** Writing - review & editing. **Shigeto Shimmura:** Conceptualization, Writing - original draft, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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