Epidermal Differentiation and Loss of Clonal Growth Potential of Human Limbal Basal Epithelial Progenitor Cells during Intrastromal Invasion

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PURPOSE. Intrastromal invasion by limbal basal epithelial progenitor cells in explant cultures is associated with epithelial-mesenchymal transition. It remains unclear whether intrastromal invasion is contingent on culturing conditions and whether invaded cells retain their progenitor status and original lineage.

METHODS. Human limbal explants were cultured on various culture substrates, with or without air-lifting (AL), and subjected to hematoxylin and eosin staining and immunostaining to pan-cytokeratins, p63 α , Δ Np63, Pax6, CK10, and CK12. Single cells obtained by trypsin/EDTA from dispase-isolated epithelial sheets from both the outgrowth and the surface epithelium, or by collagenase from the remaining stroma, were seeded on 3T3 feeder layers.

RESULTS. Intrastromal invasion was verified in all seven explant cultures by positive pan-cytokeratin staining. Immunofluorescence staining revealed that invaded epithelial cells were positive for p63 α and Δ Np63, with or without nuclear staining of Pax6. Double immunostaining to CK10 and CK12 revealed that squamous metaplasia induced by AL was noted on the surface epithelium but not in intrastromally invaded epithelial cells. On 3T3 feeder layers, both the outgrowth and the surface epithelium yielded significant numbers of holoclones and meroclones positive to Δ Np63 but negative to CK10 and CK12. In contrast, intrastromally invaded epithelial cells generated only paraclones negative to Δ Np63 and CK12 but positive to CK10 regardless of culturing conditions.

CONCLUSIONS. Intrastromal invasion by limbal basal epithelial progenitor cells is universal in all explant culture conditions, explaining why there is a gradual decline of outgrowth potential. Alteration of the limbal stromal niche leads invaded epithelial cells to adopt an epidermal fate. (*Invest Ophthalmol Vis Sci.* 2011;52:4534–4545) DOI:10.1167/iovs.10-6376

Substantial experimental and clinical data support the notion that corneal epithelial progenitors, including stem cells (SCs), are located at the limbal basal layer,^{1,2} where they are supported by a unique stromal microenvironment known as

the niche.³ Studies in the past two decades have shown that limbal SCs share characteristics in common with adult somatic SCs, such as small cell size,⁴ high proliferative potential in different cultures,^{5–8} slow cycling, and, thus, label retaining.⁹ The in vivo microenvironment that regulates the SCs is mediated by both its neighboring cells and the extracellular matrix.¹⁰ This SC-niche interaction is of particular interest for researchers in the field of regenerative medicine because a better understanding of the regulatory mechanism should further improve the current ex vivo expansion protocol for human limbal epithelial progenitors.³ The fate of these limbal epithelial progenitors after ex vivo expansion conceivably dictates the ultimate success of subsequent human transplantation.

To date, there are at least six different ex vivo expansion protocols for engineering a surgical graft with human limbal epithelial progenitors for corneal surface transplantation.^{11–18} They all begin by obtaining a limbal biopsy specimen from a healthy eye as an explant, but the protocols vary greatly regarding whether limbal epithelial cells are isolated from the limbal explant, whether epithelially denuded amniotic membrane (dAM) or intact cryopreserved AM (iAM) is used as a carrier substrate, whether a murine 3T3 fibroblast feeder layer is used for cocultivation, and whether air-lifting (AL) is subsequently used to promote stratification. Regarding the protocols in which limbal epithelial cells are not isolated from the explant,^{13,14,17,18} our previous study has noted that human limbal basal epithelial cells migrate out onto iAM and invade the limbal stroma of the explant, resulting in a progressive decline of proliferative potential.¹⁹ The latter observation, which we termed “intrastromal invasion,” was also reported in rabbit limbal explants only when they were cultured by AL.²⁰ Hence, it remains unclear whether intrastromal invasion depends on culturing conditions of limbal explants being on iAM or AL. Recently, we also reported that AL, a common maneuver used by others to promote stratification during ex vivo expansion,^{15–18} also effectively induces squamous metaplasia on the surface epithelia of limbal explants when cultured on plastic coated with collagen I and that such squamous metaplasia is mediated through activation of the p38 MAPK signaling pathway.²¹ Therefore, it remains unclear whether AL-induced squamous metaplasia may also occur in epithelial progenitor cells invading the limbal stroma.

In this study, we noted that intrastromal invasion by limbal basal epithelial progenitor cells in human explants is a universal phenomenon because it occurs under different culturing conditions. In addition, these invading epithelial progenitor cells lose their clonal growth potential and adopt epidermal differentiation regardless of AL. The significance of these findings in ex vivo expansion of human limbal epithelial progenitors is further discussed.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, Ham's/F12 medium, amphotericin B, gentamicin, fetal bovine serum, newborn calf serum, mouse epidermal growth factor, 0.05% trypsin-0.53 mM EDTA (T/E), anti-Connexin 43 antibody (71-0700), anti-mouse Alexa 488 (A21202), and anti-rabbit Alexa 555 (A31572) were purchased from Invitrogen (Carlsbad, CA). Six-well plate (3335; CellBIND A) was obtained from Corning (Lowell, MA). Collagen I (354236) was purchased from BD Biosciences (San Jose, CA). Hydrocortisone, dimethyl sulfoxide, cholera toxin, insulin-transferrin-sodium selenite media supplement, mitomycin C, bovine serum albumin (BSA), Triton X-100, and Hoechst 33342 dye were purchased from Sigma-Aldrich (St. Louis, MO). Neutral protease grade II (Dispase II) and collagenase A were obtained from Roche (Indianapolis, IN). Mouse NIH 3T3 fibroblasts (CRL-1658) were from ATCC (Manassas, VA). Harris hematoxylin (HT532), eosin (HT110132), anti-mouse pan-cytokeratin (PanCK; C2562), and anti-mouse FITC (F9006) were from Sigma-Aldrich. Anti-rabbit FITC antibody (F6005), antibody diluent, and diaminobenzidine were from Dako Cytomation (Carpinteria, CA). Anti-p63 α antibody (4892) was from Cell Signaling (Danvers, MA). Anti- Δ Np63 antibody (SC8609) and anti-cytokeratin 12 (CK12) antibody (SC17099) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-cytokeratin 10 (CK10) antibody (MAB3230) and anti-BCRP1 (ABCG2) antibody (MAB4155) were from Chemicon (Temecula, CA). Anti-Pax6 antibody (PRB2780) was from Covance (Berkeley, CA). Anti-goat Texas Red (711-075-147) and anti-goat FITC antibodies (705-095-003) were from Jackson ImmunoResearch (West Grove, PA). ABC kit (Vectastain Elite) for rabbit IgG, mounting medium (Vectashield), and anti-rabbit IgG (BA1400) were from Vector Laboratories (Burlingame, CA). Optimal cutting temperature (OCT; Tissue-Tek) compound was from Sakura Finetek (Torrance, CA).

Human Tissue Preparation

Human tissue was handled according to the Declaration of Helsinki. Cryopreserved human AM was kindly provided by Bio-Tissue (Miami, FL). Fresh full-term human placentas were procured from healthy mothers after elective caesarean delivery approved by the institutional review board of the Baptist Hospital in Miami. Corneoscleral tissues from human donor eyes were obtained from the Florida Lions Eye Bank (Miami, FL) immediately after the central corneal button had been used for corneal transplantation. The tissue was rinsed three times with DMEM containing 50 μ g/mL gentamicin and 1.25 μ g/mL amphotericin B. After careful removal of excess sclera, conjunctiva, iris, and corneal endothelium, the remaining tissue was placed in a culture dish in the supplemented hormonal epithelial medium (SHEM)¹⁹ at 37°C under humidified 5% CO₂ for 10 minutes. After three rinses with SHEM for 10 minutes each, each corneoscleral rim was trimmed to obtain 12 limbal explant tissues of one clock-hour width (i.e., approximately 1 $\times$ 1.5 $\times$ 2.5 mm).

Human Limbal Explant Cultures

Human limbal explants were cultured in SHEM under seven (four submerged and three airlifted) experimental conditions: on plastic culture dish, on collagen I-coated plastic (PL) with AL, on plastic with 25 μ g/mL AME in SHEM (AME), on iAM, on iAM with AL, on dAM, and on dAM with AL. The preparation of AME was carried out aseptically using frozen human AM containing fetal and placental portions obtained from Bio-Tissue, Inc. (Miami, FL), as recently reported.²² To prepare iAM or dAM, thawed AM with the intact epithelium or the epithelium denuded by 0.1% EDTA for 30 minutes was fastened to a culture insert with the basement membrane side facing up, as reported.²³ On the center of each insert or plastic well, a human limbal explant was cultured in SHEM and incubated at 37°C in 5% CO₂ and 95% air; the medium was changed every 2 days. Explant samples cultured without AL were completely submerged in their respective conditions. In AL cultures, 2 mL SHEM was added to the level so that the explant stroma

was submerged while the epithelium was exposed to air. The outgrowth area was measured by scanning the border of the outgrowth. Some explants were removed and embedded in OCT compound for cryosectioning, whereas others were removed for epithelial colony formation assay.

Harvesting of Epithelial Sheets from the Outgrowth and Surface Epithelium of the Explant

Limbal epithelial sheets were isolated directly from the surfaces of limbal explants after cultivation, based on our reported digestion method using 10 mg/mL neutral protease grade II (Dispase II; Roche) in SHEM at 4°C for 16 hours.²⁴ Epithelial cells were harvested from the outgrowth on plastic by T/E at 37°C for 15 minutes and from that on iAM or dAM by first making a 7-mm diameter trephine cut, including the underlying AM, and then incubating it in 10 mg/mL neutral protease grade II (Dispase II; Roche) in SHEM at 37°C for 15 minutes. The epithelial sheet was separated under the dissecting microscope using the technique described.¹⁹

Epithelial Clonal Growth on 3T3 Fibroblast Feeder Layers

Epithelial sheets harvested from the surface epithelium of the limbal explant and from the outgrowth on different substrates were treated with T/E into single cells. The remaining limbal explant stroma was digested with 1 mg/mL collagenase A in SHEM at 37°C for 16 hours to isolate cells remaining in the stroma. The colony formation method reported by Barrandon²⁵ for epidermal keratinocytes was modified and adopted by us for limbal epithelial progenitor cells.²⁶ In brief, Swiss albino 3T3 fibroblasts, grown in DMEM containing 10% newborn calf serum at 80% subconfluence, were treated with 5 μ g/mL mitomycin C for 2 hours and then trypsinized and plated at a density of 2 $\times$ 10⁴ cells/cm² in six-well plates. Epithelial cells were isolated from the surface epithelium and the outgrowth, whereas entire cells isolated from the remaining stroma were seeded at a density of 30 cells/cm² in six-well plates on 3T3 fibroblast feeder layers in SHEM. Cultures were incubated at 37°C under 5% CO₂ and 95% humidity; the medium was changed every 2 days. Colonies were fixed by 50% methanol and 50% acetone 10 days later and stained with crystal violet, PanCK, and double stained to either Δ Np63-Pax6 or CK10-CK12.

Histology and Immunostaining

Frozen sections 6- μ m thick obtained from the explants were fixed in acetone at -20°C for 10 minutes and stained with hematoxylin and eosin (H&E). For immunofluorescence staining, fixed sections or epithelial colonies were incubated in 0.2% Triton X-100 for 30 minutes. After three rinses with PBS for 15 minutes and preincubation with 2% BSA to block nonspecific staining for 30 minutes, sections were incubated with primary antibodies for 16 hours. After three washes with PBS for 5 minutes each, the sections were incubated with secondary antibodies for 1 hour. Nuclei were counterstained with Hoechst 33342, and the sections were rinsed with PBS for 15 minutes. For double staining between Δ Np63 and Pax6, the sections were incubated with both primary antibodies (Δ Np63 at 1:50 and Pax6 at 1:300) for 16 hours and then FITC anti-goat IgG at 1:50 and biotinylated anti-rabbit IgG at 1:100 for 1 hour. Images of Δ Np63 were first taken under an epifluorescence microscope before the immunohistochemistry staining for Pax6, which was performed by blocking the endogenous peroxidase activity by 0.6% hydrogen peroxide for 10 minutes, three washes with PBS for 15 minutes, incubation with ABC reagent for 30 minutes, final incubation with diaminobenzidine for 1 minute, and counterstaining with Hoechst 33342. Images were photographed at the limbal region with either confocal or epifluorescence microscopy.

Statistical Analysis

Summary data are reported as the mean $\pm$ SD. Group means were compared using the appropriate version of Student's unpaired *t*-test. Test results are two-tailed, where $P < 0.05$ is considered statistically significant.

RESULTS

Intrastromal invasion was first noted in submerged human limbal explants cultured on iAM¹⁹. To determine whether this phenomenon was restricted only to iAM, we prepared submerged cultures by seeding human limbal explants on four different substrates (PL, AME, iAM, and dAM).

Different Epithelial Outgrowth Rates among Four Submerged Cultures

Herein, we noted that dAM provided the fastest outgrowth rate among the four conditions, followed by PL, AME, and iAM (Fig. 1A). The finding that the outgrowth rate on dAM was

faster than that on iAM was consistent with what has been reported.²⁷ The difference in the outgrowth rate was supported by the total number of cells isolated from the outgrowth after 14 days of cultivation—that is, $1.23 \times 10^6 \pm 4 \times 10^5$, $1.20 \times 10^6 \pm 5 \times 10^5$, $1.09 \times 10^6 \pm 7 \times 10^5$, and $1.72 \times 10^5 \pm 9 \times 10^4$ cells ($n = 3$) for dAM, PL, AME, and iAM, respectively ($P = 0.0003$, $P = 0.007$, and $P = 0.04$, when iAM was compared with PL, AME, and dAM, respectively). Furthermore, the cell morphology observed after 14 days of cultivation was also different among these four submerged cultures. The size of outgrowth epithelial cells cultured under AME was smaller than that cultured on PL (Fig. 1C vs. 1B). The cells on iAM (Fig. 1D) and dAM (Fig. 1E) were more compacted and smaller than those on PL with or without AME (Figs. 1B, 1C).

Intrastromal Invasion of Limbal Basal Epithelial Cells

In addition to migration of the surface epithelium onto the substrates as an outgrowth, we noted an intrastromal invasion

FIGURE 1. Outgrowth rate (A) and cell morphology (B–E) among four submerged cultures. Outgrowth area (A) was measured every 2 days for limbal explants cultured on PL, AME, iAM, and dAM in a submerged manner. A representative morphology of the outgrowth cells was taken at day 14 revealing larger cells on PL (B) compared with AME (C), whereas the outgrowth cells of iAM (D) and dAM (E) revealed a homogeneous pattern of small, uniform cells, with the latter more uniform than the former. Scale bar, 200 μ m.

of limbal basal epithelial cells by H&E staining and immunostaining to PanCK. Compared with the control without cultivation, which only showed a stratified epithelium (Figs. 2A, 2B), clusters of epithelioid cells were found in the limbal stroma of all four submerged cultures after cultivation for 14 days. This evidence was better revealed under higher

magnification (Figs. 2E, 2H, 2K, 2M). These invading cells were confirmed to be epithelial cells by positive PanCK staining (Figs. 2F, 2I, 2L, 2O). The finding of intrastromal invasion on iAM was consistent with what we have recently reported.¹⁹ Herein, our results further showed that intrastromal invasion by human limbal epithelial cells was consis-

FIGURE 2. Intrastromal invasion by limbal epithelial cells. Cryosections of limbal explants before (A) and after 14 days culture on PL (D), AME (G), iAM (J), and dAM (M) were subjected to H&E staining (*left, middle*) and immunostaining to PanCK (*right*). The appearance of invaded epithelial cells was better appreciated at a higher magnification of the inset (*left*) shown in the middle column (B, E, H, K, N) and demonstrated by positive PanCK (*green*) staining (F, I, L, O, respectively) as opposed to the control explant before culture, which has no invaded epithelial cells (C). Scale bar, 200 μ m.

tently found in all four submerged culturing conditions (four explants for PL and AME, two explants for iAM and dAM).

Invading Limbal Epithelial Progenitor Cells Expressed Δ Np63 and Pax6

Our previous paper showed that invading stromal epithelial cells were derived from the limbal basal epithelial cells, as judged by the expression of p63 when limbal explants were cultured on iAM.¹⁹ Herein, we first performed double-immunofluorescence staining of PanCK and p63 α , a better marker for limbal basal progenitor cells,^{28,29} followed by double immunostaining to Δ Np63 and Pax6, a transcription factor specifically expressed by postnatal human ocular surface epithelial cells.^{30,31} Compared with the control explant (Figs. 3A–C), explants after culture (Figs. 3D–F) revealed invaded cells that were positive to both PanCK and p63 α . To determine the progenitor status of the invaded cells, subsequent staining with Δ Np63 and Pax6 was performed. Our results showed that invaded epithelial cells of all four submerged conditions, each with four explants (except dAM, with two explants) from three donors, were positive to Δ Np63 in the nucleus (Figs. 3G, 3K), suggesting that they were all derived from limbal basal progenitors. In all explants of the four submerged cultures, these Δ Np63-positive cells were also positive to Pax6 (Figs. 3H, 3L), indicating that they were derived from the ocular surface epithelium. However, we noted that some invading epithelial cells from 2 of 4 explants from PL cultures (Fig. 3J, arrow) and 1 of 4 explants from AME cultures (Fig. 3N, arrows) were Pax6 negative, indicating that some intrastromally invaded epithelial progenitor cells might have lost the expression of their ocular surface epithelial lineage.

Clonal Growth Differed among Outgrowth Epithelium, Surface Epithelium, and Intrastromally Invaded Epithelium

We have successfully isolated epithelial cells from the outgrowth and from the surface epithelium of the limbal explants using our modified neutral protease grade II digestion method, and we have isolated epithelial cells from the remaining stroma by collagenase digestion.¹⁹ We also showed that the proliferative capacity of epithelial progenitor cells from the outgrowth and the surface epithelium exhibits a progressive decline on 3T3 feeder layers.¹⁹ At that time, we attributed such a decline to the epithelial-mesenchymal transition (EMT) of limbal basal epithelial progenitor cells. To illustrate whether such a decline was unique to iAM cultures, we examined and compared the clonal growth of epithelial progenitor cells isolated from these three sites in all four submerged culture conditions. Our results showed that epithelial cells from the surface epithelium yielded the highest number of clones in all four submerged cultures (Fig. 4A), followed by epithelial cells from the outgrowth and those from the remaining stroma. Using the criteria provided by an earlier report,²⁵ all clones could be classified into holoclone with a smooth border and small cells (Fig. 4E), meroclone with a less smooth border and larger cells (Fig. 4F), and paraclone with a highly irregular border and large differentiated cells (Fig. 4G). Interestingly, epithelial cells derived from outgrowth and surface epithelia generated both holoclones and meroclones (Figs. 4E, 4F), whereas those from the remaining stroma generated only paraclones (Fig. 4G). Furthermore, only 1 or 2 paraclones were generated from each remaining stroma in all four submerged culture conditions (Fig. 4A), suggesting that epithelial progenitors invading the limbal stroma have lost their clonal growth potential.

Limbal Epithelial Clones Expressed Δ Np63 and Pax6

Previously, the epithelial progenitor status of the resultant clones was studied only in rabbit limbal explants after AL, where they were found to express PanCK and p63.²⁰ To determine the epithelial progenitor status of the resultant clones, we performed double immunostaining between Δ Np63 and Pax6 in all four submerged culturing conditions. Our results showed that all epithelial clones were positive to Δ Np63 staining (Figs. 5A, 5D, 5G), whereas some were positive (Figs. 5B, 5C) and some were negative (Figs. 5E, 5F) to Pax6 staining. The positive Pax6 staining was indicated by the negative Hoechst 33342 nuclei staining because the former quenches the nuclear Hoechst staining (Figs. 5C, 5D). Only 2 of the 153 epithelial clones derived from the surface epithelium of explant cultured on PL were negative to Pax6 (Fig. 5E), as evidenced by the positive Hoechst nuclear staining (Fig. 5F). In contrast, all paraclones derived from the intrastromally invaded epithelial progenitors were negative to nuclear staining of Δ Np63 but positive to Pax6 staining, consistent with their highly differentiated, flattened, and irregular morphology (Figs. 5G–I). Taken together with what has been described, these results suggested that invaded epithelial progenitors lost their clonal growth potential and that some cells lost the ocular surface epithelial lineage.

We previously reported that AL promotes intrastromal invasion of rabbit limbal explants cultured on a polycarbonate membrane insert and that invaded epithelial cells eventually undergo EMT.²⁰ Our data shown in Figure 2 clearly demonstrated that AL was not a prerequisite when human limbal explants were tested. However, AL also promotes squamous metaplasia in the surface epithelium of human limbal explants cultured on collagen I-coated plastic.²¹ Therefore, it was important to determine whether AL might also promote intrastromal invasion in human limbal explants cultured on different substrates. Furthermore, we also sought to determine whether invaded epithelial cells might undergo squamous metaplasia. For these reasons, we cultured human limbal explants in an AL manner on PL coated with collagen I, iAM, and dAM.

Air Lifting Induced Squamous Metaplasia in Surface Epithelia of Three Different Cultures

Limbal explant sections were then double immunostained with specific antibodies to epidermis-specific CK10³² and cornea-specific CK12³³ and were compared with those stained for PanCK to determine intrastromal invasion. As expected, the control before culturing (Fig. 6B) and the submerged dAM control (Fig. 6F) expressed only CK12 on the suprabasal cell layers of limbal epithelium. AL increased stratification, hyperproliferation, and abnormal epidermal differentiation, as judged by positive expression of CK10 in suprabasal and superficial cell layers in the explant cultured on PL coated with collagen I (Fig. 6I). AL-induced squamous metaplasia occurred not only on PL coated with collagen I, as previously reported,¹⁹ but also on the surface epithelia of explants cultured on iAM (Fig. 6M) and dAM (Fig. 6Q). Intrastromal invasion, as judged by positive PanCK staining in submerged cultures (Fig. 2), was also noted in all three AL conditions (Figs. 6L, 6P, 6T). Importantly, these invaded epithelial cells did not express CK10 or CK12 (Figs. 6K, 6O, 6S), further supporting that these invading epithelial progenitors remained undifferentiated.

FIGURE 3. Invaded limbal epithelial cells expressed p63 α , Δ Np63, and Pax6. Invaded limbal epithelial cells after culture with AME (D–F) that were positive to PanCK (D, green) were also positive for p63 α (E, red) compared with the control explant before culture (A–C), which revealed no invaded cells. To determine their progenitor status, immunofluorescence staining of Δ Np63 (G, K, green) was performed first, followed by immunohistochemical staining of Pax6 (H, L, brown) on cryosections of explants cultured under the four submerged conditions. The double immunostaining revealed that invaded limbal epithelial cells were all positive to Δ Np63, whereas some were negative to Pax6 (arrows) when cultured on PL (J) or in AME (N). All images were counterstained with Hoechst 33342 (blue) and overlaid with their respective staining (C, F, I, M). Scale bar, 200 μ m.

FIGURE 4. Epithelial clonal growth and morphology in four submerged culture conditions. Single epithelial cells obtained by trypsin/EDTA treatment of dispase-isolated outgrowth and surface epithelium, or by collagenase digestion of the remaining stroma were seeded on mitomycin-C treated 3T3 fibroblasts feeder layer. These epithelial colonies, designated as surface epithelium (B), outgrowth (C), and stroma (D), were cultured for 10 days before they were stained with crystal violet and were counted to determine the total number of clones (A) generated from PL, AME, dAM, and iAM. A representative epithelial morphology of the clone was taken from the surface epithelium of explant showing a holoclone (E), from the outgrowth showing a meroclone (F), and from the remaining stroma showing a paraclone (G). Scale bar, 200 μ m.

Epithelial Clones Derived from Intrastromally Invaded Epithelial Progenitors Expressed CK10 but Not CK12 in Both Submerged and Air-Lifted Cultures

Although AL induces squamous metaplasia on the surface epithelium, epithelial clones generated from such a surface epithelium on 3T3 fibroblast feeder layers maintained the expression of CK12, but not of CK10, indicating that the epithelial progenitors retain a normal fate.¹⁹ Herein, all clones generated from the outgrowth, the surface epithelium, and the stroma from both submerged and AL (data not shown) cultures were positive to PanCK (Figs. 7A2-F2), supporting their epithelial status. Double immunostaining to CK10 and CK12 was negative for all holoclones and meroclones generated from either the outgrowth (Figs. 7A, 7B) or the surface epithelium (Fig. 7C), suggesting that these epithelial cells remained undifferentiated. However, some clones derived from the surface epithelia of explants cultured on dAM in a submerged manner were positive to both CK10 (Fig. 7D4) and CK12 (Fig. 7D5). This finding correlates with our initial report that dAM promotes corneal epithelial differentiation.²⁷ The most notable finding was that all paraclones derived from the intrastromally invaded epithelial cells from both submerged and AL (data not shown) cultures were positive to CK10 (Figs. 7E4, 7F4) but not CK12 (Figs. 7E5,

7F5), suggesting that they adopted an epidermal lineage. Despite the findings that squamous metaplasia did not occur on the surface epithelia (in a submerged manner) (Figs. 6E-H) and that intrastromally invaded epithelial cells retained their progenitor status expressing p63 (Fig. 3) and remained undifferentiated (lack of CK12 and CK10 expression in Fig. 6), invaded epithelial cells adopted epidermal differentiation on clonal expansion on 3T3 feeder layers (Figs. 7E6, 7F6).

DISCUSSION

The present study provides strong evidence that the phenomenon of intrastromal invasion in the human limbal stroma is prevalent in all seven culturing conditions. This phenomenon is schematically depicted in Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6376/-/DCSupplemental>. Invaded epithelial cells expressed not only PanCK (Fig. 2), confirming their epithelial status, but also p63 α , Δ Np63, and Pax6 (Fig. 3), indicating their origin of basal epithelial progenitor cells^{34,35} derived from the ocular surface epithelium.^{30,36} Given that such a phenomenon occurs only in the stem cell-containing limbus, but not in the corneal epithelium even under AL stimulation,²⁰ we have speculated that intrastromal invasion is a unique experimental model with which to investigate how the limbal stromal niche might modulate self-renewal and fate decision of the limbal epithelial stem cells.

Initially, we were concerned that the phenomenon of intrastromal invasion might be an experimental artifact of what is known as limbal epithelial crypts. These crypts, which were first identified by Dua et al.,³⁷ are thought to be a unique digital extension of the limbal basal epithelium into the limbal stroma. Cells in the crypt are CK14+, CK3-, CK19+, ABCG-2+, CD34-, vimentin+, p63+, connexin 43+, and Ki67-.^{37,38} We ruled out this possibility by performing serial sectioning radially to two limbal explants without culturing from a single donor and did not detect any such crypt structure or evidence of intrastromal invasion (Supplementary Figs. S2A, S2B, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6376/-/DCSupplemental>). This finding is further substantiated by other radial sections of limbal explant before culture (CTL; Figs. 2, 6). However, tangential sectioning of three limbal explants from the same donor occasionally resulted in some islands of epithelial cells in the stroma (Supplementary Figs. S2C, S2D, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6376/-/DCSupplemental>). Furthermore, invaded epithelial cells were all connexin-43- (Supplementary Fig. S2K, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6376/-/DCSupplemental>), though some were also ABCG-2- (Supplementary Fig. S2I, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6376/-/DCSupplemental>). Nonetheless, intrastromal invasion was universally detected by random serial sectioning in the limbal stroma after 14 days' culturing from five donors under seven culturing conditions.

The present study further demonstrated that intrastromal invasion of limbal epithelial progenitor cells occurred when limbal explants were cultured not only on iAM, as previously reported,¹⁹ but also on dAM or plastic, with or without AME (Fig. 2). Without 3T3 feeder layers, the use of dAM as a substrate was inferior to that of iAM; we²⁷ and others^{28,39} have reported that dAM promotes corneal epithelial differentiation, whereas iAM maintains the limbal epithelial phenotype. Our study not only confirmed the fastest outgrowth rate on dAM when compared with iAM (Fig. 1A), as noted before,²⁷ it also disclosed that the outgrowth on plastic was intermediate with or without the addition of AME (Fig. 1A). Most important, the

FIGURE 5. Double immunostaining between Δ Np63 (green) and Pax6 (brown) of representative clones. Epithelial colonies generated from the outgrowth, the surface epithelium, and the remaining stroma were subjected to double immunostaining between Δ Np63 and Pax6. All epithelial clones from either the outgrowth or the surface epithelium of all four submerged cultures were positive to Δ Np63 and Pax6 (A–C; note that positive Pax6 staining obscures the blue nuclear counterstaining by Hoechst 33342). However, two clones from the surface epithelium cultured on PL were positive to Δ Np63 but negative to Pax6 (D–F; note that negative Pax6 staining was substantiated by the positive nuclear Hoechst staining). In contrast, epithelial clones generated from the remaining stroma were negative to nuclear staining of Δ Np63 but positive for Pax6 (G–I). Scale bar, 200 μ m.

clonal growth potential, judged by clone numbers and types on 3T3 feeder layers, was the worst in invaded epithelial progenitor cells compared with that of the outgrowth or the surface epithelium (Fig. 4). This finding supported our earlier assertion that the overall growth potential of limbal basal epithelial progenitors from the explant declines with time when cultured on iAM.¹⁹ Given that the loss of clonal growth potential in invaded epithelial progenitor cells also occurred in explants cultured on other substrates, such as dAM and plastic (Fig. 4), we concluded that intrastromal invasion is a universal mecha-

nism leading to the loss of epithelial clonal growth potential. Because limbal explants have been used to expand limbal epithelial progenitor cells on either iAM¹³ or dAM,^{12,14–18} our results suggest that these ex vivo expansion protocols might have the potential pitfall of losing limbal epithelial progenitors to intrastromal invasion.

The discovery that intrastromal invasion was found in all four submerged cultures also indicated that this phenomenon was not restricted to AL, as we first reported in rabbit limbal explants.²⁰ In fact, we also confirmed intrastromal

FIGURE 6. Immunofluorescence staining between CK10 and CK12 or PanCK on air-lifted explant cultures. Cryosections of human limbal explants cultured with AL for 14 days were subjected to immunostaining between CK10 (green) and CK12 (B, F, J, N, R, red) or PanCK (D, H, L, P, T, green). Expression of CK10 on the surface epithelium was absent in CTL (A) and submerged dAM (E) but present in all three AL cultures on PL coated with collagen I (I), iAM (M), and dAM (Q). Positive staining to PanCK (H, L, P, T) was detected in the stroma of all cultured explants. However, such invaded epithelial cells expressed neither CK12 nor CK10 (G, K, O, S).

FIGURE 7. Immunofluorescence staining of epithelial clones derived from outgrowth (A, B), surface epithelium (C, D), and stroma (E, F) to PanCK or between CK10 and CK12. All epithelial clones generated on mitomycin-C treated 3T3 fibroblasts feeder layers were first subjected to immunofluorescence staining with PanCK (green) and subsequent immunofluorescence staining between CK10 (green) and CK12 (red). All epithelial clones generated from the outgrowth and surface epithelium were negative for both CK10 (A4, B4, C4) and CK12 (A5, B5, C5) except for the latter from dAM, which was positive to both CK10 (D4) and CK12 (D5). In contrast, all epithelial clones (as evidenced by positive PanCK staining from E2, F2) generated from the stroma were CK10 positive (E4, F4) and CK12 negative (E5, F5). Scale bar, 200 μ m.

invasion in all three AL cultures (Fig. 6). The experimental maneuver of AL is commonly practiced to stimulate epithelial proliferation, stratification, and terminal differentiation in a number of epithelial cultures²⁰ and adopted to achieve the same purpose in several ex vivo expansion protocols.^{15–18} For the human limbal epithelium, we have previously reported²¹ that the epithelial changes induced by AL on the surface epithelium of the limbal explants are coupled with squamous metaplasia, as evidenced by the expression of CK10 and filaggrin. Herein, we noted that AL induced the same squamous metaplasia not only on the surface epithelia

of limbal explants cultured on plastic coated with collagen I²¹ but also on limbal explants cultured on iAM and dAM (Fig. 6), suggesting that the phenomenon of squamous metaplasia on the surface epithelium was also universal regardless of the substrate used. We also reported that AL-induced squamous metaplasia on the surface epithelium can be reversed by a small molecular weight inhibitor (SB203580) of the p38 MAPK pathway and reverted back to a normal phenotype expressing CK12 but not CK10 when subcultured on 3T3 feeder layers.²¹ Herein, all epithelial clones obtained from both submerged and AL cultures positively

expressed PanCK under the support of 3T3 feeder layers (Fig. 7). Surprisingly, for the first time, we noted that epithelial clones generated by intrastromally invaded epithelial cells gained sole expression of CK10 but not of CK12 (Fig. 7). Given that invaded epithelial cells already lost clonal growth potential, as evidenced by fewer paraclones (Fig. 4A), this finding further suggested that limbal basal epithelial progenitor cells adopted a permanent shift to an epidermal fate when invading the limbal stroma, regardless of whether their culture was submerged or air-lifted. Hence, besides undergoing EMT, as noted in our previous report (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6376/-DCSupplemental>),^{19,20} intrastromally invaded cells also adopt irreversible squamous metaplasia and together contribute to the loss of clonal growth potential when limbal explant culture is used.

The notion that invaded epithelial progenitor cells might have adopted an epidermal fate was further suggested by the discovery that some invaded epithelial progenitor cells also lost expression of Pax6 (Figs. 3I, 3M). The loss of Pax6 expression is an early sign for adult corneal epithelial cells to transdifferentiate into the epidermis when combined with the embryonic dermis.^{40,41} In this study, intrastromally invaded epithelial cells were still undifferentiated in the stroma and generated paraclones expressing CK10 only after subculturing on 3T3 feeder layers (Fig. 7) while keeping Pax6 nuclear staining (Fig. 5H). Future studies are needed to determine whether the loss of Pax6 expression precedes and controls the switch from the corneal to the epidermal lineage in invaded epithelial cells. Such studies will shed new light into the pathogenesis of pathologic squamous metaplasia in a number of cicatricial ocular surface diseases, in which there is a strong correlation with the loss of Pax6 expression.⁴² To this end, the model of culturing human limbal explants might be an ideal one for us to investigate how self-renewal and fate decision of limbal epithelial stem cells are regulated by their stromal niche. Better understanding of the niche regulation will promote the ultimate success of ex vivo expansion of human limbal epithelial progenitors, including stem cells, for subsequent transplantation.

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