Original Article

Taiwan J Ophthalmol 2017;7:205-212

Access this article online

Quick Response Code:

Website:
www.e-tjo.org

DOI:
10.4103/tjo.tjo_101_17

Effect of air-lifting on the stemness, junctional protein formation, and cytokeratin expression of *in vitro* cultivated limbal epithelial cell sheets

Lily Wei Chen[1], Yan-Ming Chen[1,2], Chia-Ju Lu[1], Mei-Yun Chen[1], Szu-Yuan Lin[3], Fung-Rong Hu[1,4], Wei-Li Chen[1,4]

Abstract:

PURPOSE: The aim of this study is to evaluate the effects of air-lifting on the stemness, junctional protein formation, and cytokeratin expression of rabbit limbal stem cells cultivated *in vitro,* and to find out the proper timing of air-lifting before transplantation as limbal epithelial cell sheets for the treatment of limbal insufficiency.

MATERIALS AND METHODS: Limbal epithelial cells were isolated from the limbus of New Zealand white rabbits and cultivated *in vitro.* After the cells became confluent, different durations of air-lifting (0, 1, 2, 4, and 7 days) were performed. At the end of cultivation, immunohistochemistry on cryosections was performed and observed by fluorescein microscopy and *in vitro* confocal microscopy for cytokeratins (K3, K10, K12, K13, and K14), junctional and structural proteins (ZO-1, p120, and actin) and stem cell markers (ABCG2 and P63). Scanning electron microscopy was used for observing the microstructure of superficial cells. Transepithelial electrical resistance (TEER) was used to measure the transepithelial permeability.

RESULTS: The expression of K3, K10, K12, K13, K14, and ABCG2 showed no differences in pattern and location among different groups of airlifting. A time-dependent increase in corneal epithelial thickness was found after air-lifting. *In vitro* confocal microscopy demonstrated that K3, p120, and ZO-1 were expressed on the apical cell layer, whereas P63 and ABCG2 were expressed more on the basal epithelial layer. Scanning electron microscopy of the superficial layer demonstrated that airlifting induced time-dependent increase in the size of surface epithelial cells and triggered cellular differentiation. TEER results demonstrated a time-dependent increase of transepithelial electric resistance.

CONCLUSIONS: During limbal epithelial cell expansion *in vitro*, air-lifting can increase cellular stratification, enlarge surface cells, trigger cellular differentiation, and increase the transepithelial barrier. However, the expression of cellular junctional, stem cell and cytokeratin markers seems to have no obvious differences in pattern and localization.

Keywords:

Airlifting, cultivated cell sheets, cytokeratin, limbus, stem cell

[1]Department of Ophthalmology, National Taiwan University Hospital, [3]Department of Ophthalmology, Cathay General Hospital, [4]Center of Corneal Tissue Engineering and Stem Cell Biology, National Taiwan University Hospital, Taipei, [2]Department of Ophthalmology, E-Da Hospital, I-Shou University, Kaohsiung, Taiwan

Address for correspondence:
Prof. Wei-Li Chen, Department of Ophthalmology, National Taiwan University Hospital, 7 Chung-Shan South Road, Taipei, Taiwan.
E-mail: chenweili@ntu.edu.tw

Submission: 04-10-2017
Accepted: 24-10-2017

Introduction

Normal ocular surface structures, including the cornea, limbus, and conjunctiva, are covered by epithelial cells that maintain their integrity along with a stable preocular tear film. The limbus is particularly important since this is where stem cells of the cornea are located, and serves as the ultimate source for constant corneal epithelial renewal[1-5] Similar to the stem cells in other tissues, limbal stem cells are supported by a unique stromal microenvironment called the

How to cite this article: Chen LW, Chen YM, Lu CJ, Chen MY, Lin SY, Hu FR, *et al*. Effect of air-lifting on the stemness, junctional protein formation, and cytokeratin expression of *in vitro* cultivated limbal epithelial cell sheets. Taiwan J Ophthalmol 2017;7:205-12.

stem cell niche, which consists of various extracellular matrix components, cell membrane-associated molecules, and unique cytokine dialogs.[2-4] Severe damage to the limbal epithelial cells, the so-called limbal stem cell deficiency (LSCD) from chemical or thermal burns, Stevens–Johnson syndrome, ocular cicatricial pemphigoid, contact lens wear, severe microbial infections, or surgical procedures, may cause characteristic of clinical features. These include chronic inflammation and "conjunctivalization" of the cornea with vascularization, ingrowth of fibrous tissue, corneal opacification, an irregular and unstable epithelium, persistent epithelial defects, and appearance of goblet cells on the cornea.[6,7]

Dealing with LSCD is a challenge to ophthalmologists. Among the popular treatment strategies, transplantation of limbal epithelial cells expanded *in vitro* has proven to be efficient and highly successful.[8,9] Several different protocols have been proposed for the culturing process, and issues regarding the need for feeder cells, the type of carriers, the choice of media for cultivation have all been debated.[10] Among which, the need for air-lift procedures after the cells become confluent and the proper duration of air-lifting have seldom been systemically investigated. Some studies favored the method of air-lifting due to rapid cell proliferation. During experimentation, limbal epithelial cell layers cultured with air-lifting increased dramatically from day 4 to day 14 to >15 cell layers in some areas while cells cultured without air-lifting remained mostly single-layered.[11,12]

Air-lifting is a common maneuver to induce epithelial stratification in organotypic cultures of epidermal keratinocytes. Under the air-lift condition, such an increase of epithelial stratification is thought to be caused by the upregulation of keratinocyte growth factor expression by fibroblasts and the release of IL-1 by keratinocytes in co-cultures.[13-16] With limbal explants cultured for ocular surface reconstruction, the cultivated cell sheets need to maintain both their normal corneal epithelial cell function and their stem cell phenotype. The cell sheets also need to be strong enough to prevent damage during the transplantation process and the early postoperative period. Li *et al*. have demonstrated that air-lifting induced abnormal epidermal differentiation without intrinsic alteration of stem cells in the limbus. Such abnormal epidermal differentiation was evidenced by positive expression of K10 keratin in the suprabasal cells and filaggrin in the superficial cells. Clones generated from epithelial cells that were harvested from airlift cultures only expressed K12 keratin and not K10 keratin. As early as 2 days in airlift cultures, p38 expression emerged in limbal basal epithelial cells and gradually extended to the cytoplasm and nuclei.[17] They also demonstrated that limbal tissues preserved under hypothermic airlift conditions maintained the intact structure, normal phenotype, high viability, and stem cell pool of limbal epithelia. Accordingly, they suggested that such a method may be used in eye bank tissue processing and corneal epithelial tissue engineering.[18] However, these results focused on the *ex vivo* preservation of limbal tissue instead of the *in vitro* culturing of limbal epithelial cell sheets for transplantation. Properly controlling the duration of airlift to obtain the most suitable cell products for transplantation can be important for cell therapy in the treatment of LSCD.

In this study, we aimed to evaluate the effect of air-lifting duration on the culturing result of rabbit limbal epithelial cell sheets for ocular surface reconstruction. We focused on the thickness of the cell products and the expression of stem cell markers, specific cytokeratins, and junctional proteins. We also evaluated the cellular differentiation by transepithelial permeability and the microscopic structure of superficial cells by scanning electron microscopy (SEM). Through this study, we aim to set up a suitable protocol for cultivating limbal epithelial cell sheets for treating patients with LSCD.

Materials and Methods

Chemicals and antibodies

The K3 and K12 antibodies that recognize cornea-specific keratin 3 and keratin 12 were purchased from Progen (AE5; Heidelberg, German). The K10 antibody, which recognizes epidermal keratinocyte-specific intermediate filament keratin 10, was purchased from Chemicon (Temecula, CA, USA). The K13 antibodies that recognize conjunctiva-specific keratin 3 were purchased from Leica Microsystems Inc., (Bannockburn, Il, USA). K14 expression was detected in epithelial cells, which purchased from Chemicon (Temecula, CA, USA). The ABCG2 antibody, which recognizes putative marker of corneal epithelial progenitor cells, was purchased from Chemicon (Temecula, CA, USA). The antibody for stem cell marker P63 was purchased from DakoCytomation (Carpinteria, CA, USA). The P120 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies for junctional and cytoskeletal protein markers ZO-1 and actin were purchased from Zymed (San Francisco, CA, USA). Fluorescent conjugates of phalloidin used to label actin filaments were purchased from Invitrogen (Life Technologies Corp., Carlsbad, CA, USA).

Animals

New Zealand albino rabbits (3.0–3.5 kg, 6-month-old) were used in this study. Treatment of all animals followed the regulations of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental

procedures were approved by the Committee for Animal Research of the National Taiwan University Hospital.

Primary culture of rabbit limbal epithelial cells

Rabbit limbal epithelial cells were co-cultured with mitomycin C (MMC) inactivated 3T3 fibroblasts, and denuded amniotic membrane was used as the matrix. Confluent 3T3 fibroblasts were treated with 4 μg/ml MMC for 2 h at 37°C under 5% CO_2 to inactivate their proliferative activity. Then, 3T3 cells were rinsed with phosphate-buffered saline (PBS) to remove MMC, trypsinized with 0.25% trypsin/0.53 mM EDTA, and plated onto plastic dishes at a density of 2×10^4 cells/cm^2. The human amniotic membrane was collected by the National Taiwan University Hospital tissue bank from consenting healthy mothers. The amniotic epithelial cells were removed with scrapers, and the membrane was placed on the bottom of the culture plate inserts (Corning, Inc., Corning, NY) with the basement membrane facing up. These inserts were placed in dishes containing MMC-treated 3T3 fibroblasts. With a surgical blade, limbal explants were carefully dissected from healthy rabbit eyes. These explants measured approximately 2 mm inside and outside palisade of Vogts, and were 200 μm thick with the epithelium intact. The corneal explants were incubated with 0.8U/ml Dispase II for 5 h at 4°C. Corneal stroma was then removed, and the epithelial tissue was treated with 0.05% trypsin/EDTA for 10 min at room temperature. Epithelial cells were dissociated and seeded on transwells with the denuded amniotic membrane. The culture medium was changed every 3 days. The flow chart for cell culturing is illustrated in Figure 1a.

Air-lift after cell sheets became confluent

After rabbit limbal epithelial cells reached confluency (usually within 14 days), the groups with airlifting were treated with different periods of air-lifting (0, 2, 4, and 7 days). The culture media on the upper wells were simply removed until the wells became semi-dry, and hence, the cells on the amniotic membrane would not dry out. Confluent cell sheets not subjected to air-lifting were used as the control [Figure 1b].

Immunohistochemistry

Cell sheets cultured under different conditions of airlifting were taken out from transwell insert, embedded in optimal cutting temperature compound, and cut into frozen sections with 8 um thickness. The sections were rinsed three times with 1 × PBS and fixed with acetone for 5 min. The fixed samples were permeabilized and blocked with 0.1% Triton X-100 for 10 min and 2% goat serum for 60 min. The samples were then incubated overnight in a moist chamber at 4°C with the following primary antibodies: anti-keratin 3, anti-keratin 10, anti-keratin 12, anti-keratin 13, anti-keratin 14, anti-ZO-1, anti-ZO-2, anti-p120, anti-ABCG2, and anti-p63. These were rinsed with PBS followed by incubation for 60 min with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Negative controls were obtained by omitting the primary antibodies. The samples were then mounted and examined with the ZEISS Axiovert 100TV microscope (Carl Zeiss MicroImaging, NY, USA).

Whole mount immunocytochemical staining with *in vitro* confocal microscopy

The cell sheets under different treatment conditions were fixed with acetone, then permeabilized and blocked with 0.1% Triton X-100 f and 2% goat serum. The samples were incubated with the following primary antibodies: anti-keratin 3, anti-ZO-1, anti-p120, anti-ABCG2, anti-p63, and anti-actin. The cell sheets were then rinsed and incubated with FITC-conjugated secondary antibodies. Negative controls were obtained by omitting the primary antibody. Cell sheets were then mounted and examined with the *in vitro* confocal microscope. Z-stack images were taken by 1 um sections from the apical cell layer to the basal cell layer. The images captured were analyzed using the Zen software. A Leica TCS SP2 confocal microscope was used for the imaging.

Scanning electron microscopy

Cell sheets under different treatment conditions were washed with 0.1M cacodylate buffer and fixed in 2.5% glutaraldehyde solution. The samples were then fixed with 1% OsO4, dehydrated in graded alcohol and dried in a drier. The samples were then coated with gold in a JFC-1100 unit and observed under a SEM (Jeol, JSM-5410, SEM).

Transepithelial electrical resistance

Rabbit limbal epithelial cells were cultured in 24-well transwell plates on filters with a pore size of 0.4 μm. Until cells reached confluency, cells were treated with different air-exposure durations. Resistance was measured with the Millipore Millicell Electrical Resistance System meter (Millipore, Billerica, MA), and transepithelial electrical resistance (TEER) (Ω/cm^2) and calculated by multiplying the measured resistance by the area of the transwell filter. Background resistance caused by the filter alone was subtracted from the experimental values.

Data evaluation and statistical methods

The comparison of corneal epithelial thickness as evaluated by reconstructed *in vitro* confocal microscopy was analyzed with Student's *t*-test for statistical significance ($P < 0.05$).

Results

Immunohistochemical study

Compared to cells cultivated without airlifting, a time-dependent increase in cell layers and thickness was

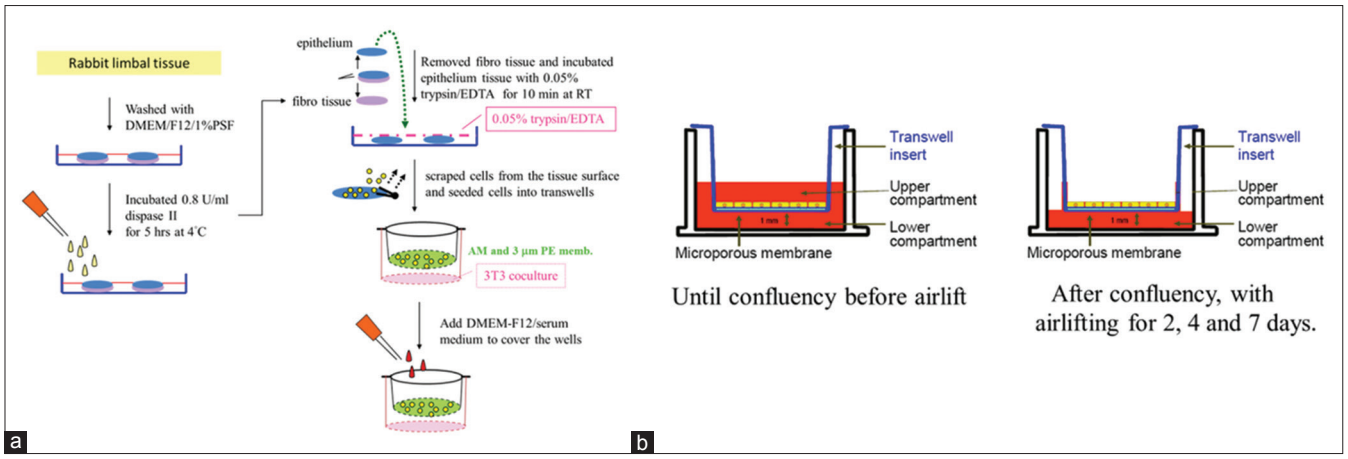

Figure 1: (a and b) Schematic drawing of rabbit limbal explant cultures cultivated with or without air-lifting. The procedure of cultivating rabbit limbal epithelial cells on transwells until confluency before airlifting. After rabbit limbal epithelial cells reached confluency (usually within 14 days), the upper wells containing confluent cells were airlifted for 2, 4, and 7 days

found from 2 days to 7 days after airlifting [Figure 2]. The expression of K3, K10, K12, K13, K14, ABCG2 all showed similar patterns, although thickness of epithelial layers differed in the four groups. The expression pattern demonstrated that cell sheets in the four groups had the phenotype of corneal epithelial cells but not conjunctival or dermal epithelial cells. The expression of ABCG2 in all four groups also demonstrated that cells in all four conditions expressed stem cell phenotypes.

Whole mount immunocytochemical staining with *in vitro* confocal microscopy

Figure 2a demonstrates the reconstructed corneal epithelial structure by *in vitro* confocal microscopy. On day 7 after air-lifting, we found a statistically significant increase of corneal epithelial thickness compared to the cells sheets with no airlifting. We found that ZO-1, p120, actin, K3, ABCG2, and P63 expressed on both groups. ZO-1, p120, actin, K3, and ABCG2 expressed more on the superficial layers in the group with air-lifting, whereas P63 expressed more on the basal layer in both groups.

To prove the immunostaining result of the reconstructed image in Figure 2a, Figure 3 demonstrates the layer by layer immunocytochemical staining result of K3, ZO-1, P120, and actin by *in vitro* confocal microscopy. The results were consistent with Figure 2, and demonstrated that K3, ZO-1, p120, and actin expressed more on the superficial cellular layers compared to the basal layers. K3 expressed mainly in the cytosol while ZO-1, P120, and actin expressed more on the cell membrane. The staining results are similar between the two groups, with or without air-lifting.

Scanning electron microscopy

Figure 4 demonstrates the results of using the SEM for observing the morphology of the superficial layer of apical epithelial cells under different conditions of airlifting. Figure 4a-d demonstrate a time-dependent increase in cell size and the differentiation of cells with more prominent cellular borders and increased expression of microvilli on the cell surface when the duration of air-lifting increased.

Transepithelial electrical resistance

The results of TEER, which represented the differentiation of epithelial cell sheets, demonstrated a time-dependent increase in transepithelial resistance [Figure 5].

Discussion

Nowadays, corneal transplantation with autologous cultivated limbal epithelial cell sheets is the standard strategy for treating LSCD for many corneal specialists. Different cell culture techniques have been proposed with various successes.[19-21] During *in vitro* culturing, whether cell sheet products need to receive air-lifting is an important issue that has not been well-evaluated before. It is well-known that air-lifting can improve cellular stratification and improve cellular differentiation with the formation of epithelial tight junctions *in vitro*. Exposure to the air-fluid interface by air-lifting has been a common maneuver to induce epithelial stratification in organotypic cultures of epidermal keratinocytes.[13-15,17,22] The cell products that received air-lifting may be more convenient for surgeons since these may theoretically be stronger and less vulnerable to surgical damage. However, cellular differentiation may inevitably lead to the loss of cellular stemness, which may decrease the therapeutic effects of transplanting limbal epithelial cells sheets to treat limbal insufficiency. How to balance these factors and provide the best culturing condition is a major challenge for cell product manufacturing.

Air-lifting was originally developed to make skin cell culture sheets for transplantation. The cell product is

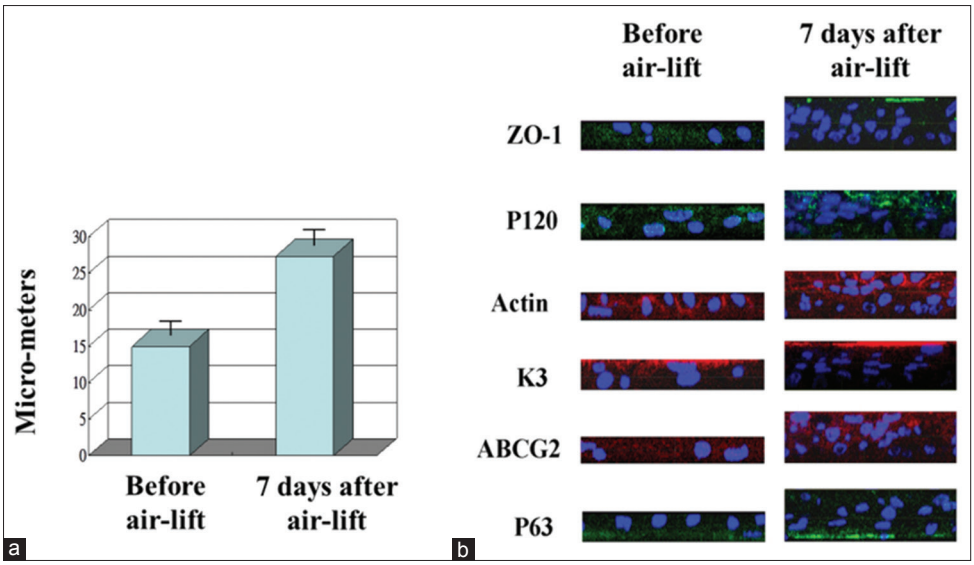

Figure 2: (a and b) Reconstructed picture of immunocytochemical staining result of cultivated cells sheets before and after airlifting for 7 days by *in vitro* confocal microscopy. The thickness of cultivated epithelial cell sheets demonstrated a significant increase of corneal epithelial thickness before and 7 days after airlifting ($P < 0.05$). The staining pattern clearly demonstrated that ZO-1, P120, and K3 mainly expressed on the superficial layer of the cell sheets. ABCG2 expressed on whole thickness, while P63 expressed more on the basal layer of cultivated cell sheets. Green: Fluorescein isothiocyanate staining of ZO-1, P120, actin, K3, ABCG2 and P63. Red: Propidium iodide staining of actin, K3 and ABCG2. Blue: 4', 6-diamidino-2-phenylindole counterstaining of nucleus

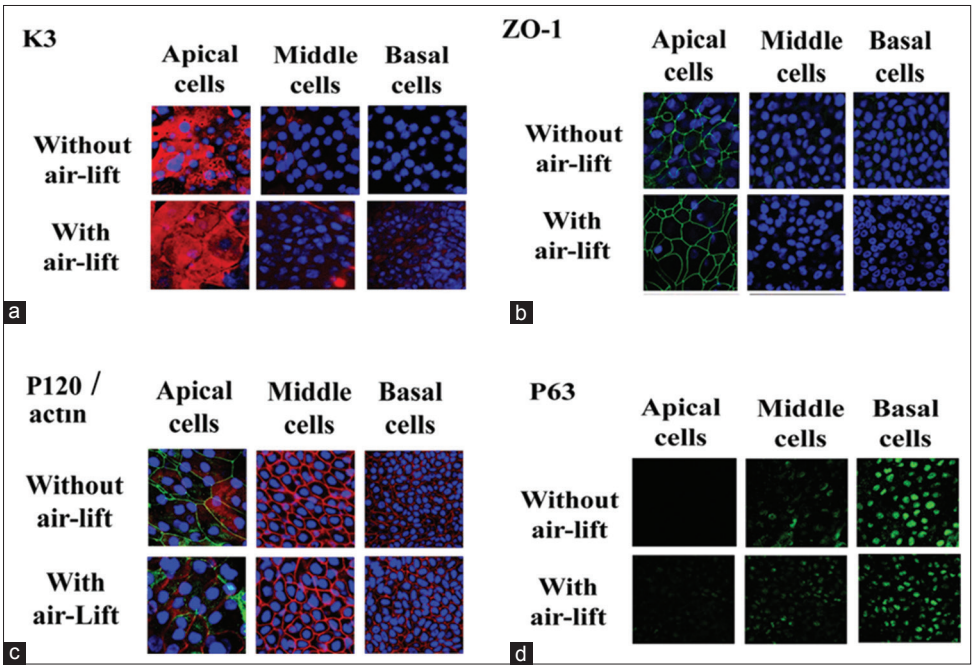

Figure 3: (a-d) *In vitro* confocal microscopic result of immunohistochemistry staining on cells in different layers. Immunocytochemical staining of K3. Only apical cells expressed K3 staining in the cytosol in both groups. Red: Propidium iodide staining of K3. Blue: 4', 6-diamidino-2-phenylindole counterstaining of nuclei. Immunocytochemical staining of ZO-1. ZO-1 expressed on the cell membrane. Only apical cells expressed ZO-1. Green: Fluorescein isothiocyanate staining of ZO-1. Blue: 4', 6-diamidino-2-phenylindole counterstaining of nuclei. immunocytochemical staining of p120 and actin. Only apical cells expressed p120 on the cell membrane while actin was found in all cell layers in both groups. Fluorescein isothiocyanate staining of p120. Red: Propidium iodide staining of actin. Blue: 4', 6-diamidino-2-phenylindole staining of nuclei. Immunocytochemical staining of P63. Basal cells expressed P63 in the nuclei, while the middle layer expressed less staining. No cells in the apical layer expressed P63. Green: fluorescein isothiocyanate staining of P63

initially submerged in the medium followed by exposure to an air-liquid interface by lowering of the medium level. Epidermal cell cultures made by air-lifting appear morphologically more similar to *in vivo* tissues. This seems to show that the closer the culturing conditions are to the natural tissue environment, the more closely the cultured epithelium mimics *in vivo* tissue.[23-25] Airlifting in the field of corneal epithelial culturing was developed by Zieske *et al.*, who first exposed rabbit cultured corneal epithelial cells to a perfect "dry" environment.[26] In

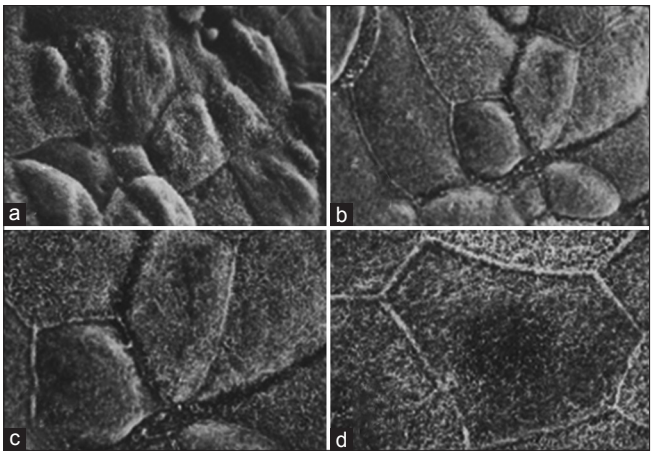

Figure 4: (a-d) Scanning electron microscopic result of the superficial layer of cultivated rabbit limbal epithelial cells, After cells became confluent but before airlifting, After airlifting for 2 days. After airlifting for 4 days. After airlifting for 7 days. Airlifting induced time-dependent increase in the size of surface epithelial cells and triggered cellular differentiation. Significant cellular junctions were found on day 4 and day 7 after air-lifting. Bar chart: 5 um

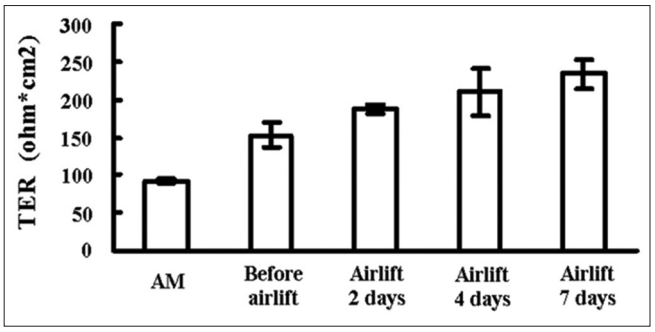

Figure 5: Transepithelial electrical resistance of rabbit limbal epithelial cells in different groups of air-lifting. AM: upper well of transwell coated with amniotic membrane but without cultivated cells. Before airlift: The confluent cultivated cell sheets before an airlift. Airlift 2 days, 4 days, and 7 days: The confluently cultivated cells received airlifting for 2, 4, and 7 days

that dry model, the cornea epithelium grew out more evenly and stratified to up to 20 cell layers, with multiple layers of enucleated squamous-like cells in the apical surface that resembled cornified cells in the epidermis. Interestingly, these authors' next model lowered the level of the medium to meet just the surface of the culture while still enabling the medium to wet the surface and allow tissue construct to remain moist on its apical side. They found that a rabbit corneal epithelial culture sheet maintained by this new air-lift method differentiated more than cultures grown in the submerged or perfectly dry environments. This model seems reasonable because the *in vivo* cornea is not maintained in a dry environment, but is always kept wet by the tear film. Accordingly, some recent studies favored air-lifting in cultivated limbal epithelial cell sheets for transplantation.[11,12,17,27] Recently, Li *et al*. found that air-lifting not only promoted epithelial stratification of the corneal epithelium but also induced pronounced epithelial migration.[17]

With the limbal epithelial sheets cultured for reconstruction of the ocular surface, the ideal cell products need to maintain their normal corneal epithelial phenotype. The present study demonstrated the expression of corneal epithelial specific cytokeratin K3 and K12, without the expression of dermal specific cytokeratin K10 and conjunctival epithelial specific cytokeratin K13 in groups with different durations of airlifting [Figure 6]. *In vitro* confocal microscopy [Figures 2 and 3] also demonstrated the expression of K3 on the superficial layer of cultivated cells before and after airlifting. The study finding demonstrated that even after air-lifting for 7 days, the cell products still maintained cornea-specific phenotype without the evidence of transformation into other epithelial cell types. Since the basal layer of the cell products in all groups expressed stem cell maker ABCG2 and P63 [Figures 2, 3 and 6], airlifting for <7 days seems to maintain the stemness of the cell products and may be applied in cell therapy for treating patients with LSCD. *In vitro* confocal microscopy [Figures 2 and 3] clearly demonstrated the localization of P63 positive cells to the basal layers in all groups, which was similar to the *in vivo* condition of having limbal stem cells reside in the basal layer of palisades of Vogts.

In this study, air-lifting was found to induce cellular stratification and trigger cellular differentiation without affecting the expression of cytokeratin markers. We found that air-lifting induced rapidly increased the thickness of cell sheets. Although there seemed to be no change in the expression pattern and location of junctional proteins p120 and ZO-1, SEM clearly showed that the cell size increased, the border of cellular junction matured, and the microvilli on cellular surface increased time-dependently after airlifting. Functionally, TEER also demonstrated that the transepithelial cellular barrier increased after airlifting. All these changes proved that airlifting could trigger the differentiation of cell products. Careful control of the differentiation process may be needed if we plan to create cell products with more stem cell phenotype.

The study has several limitations. First, our culturing method was different from Li *et al*.'s study in that we used *in vitro* rabbit limbal stem cells instead of *ex vivo* human limbal explants,[9] which may explain some differences between the two studies. Second, other factors need to be considered for producing limbal epithelial products before transplantation, including the preparation of carriers, the usage of feeder cells, the protocol to isolate limbal epithelial cells, and the components of the culturing media. All these elements may affect the culturing results of epithelial cell products.

In summary, the study demonstrated that air-lifting may trigger cellular stratification and differentiation in limbal epithelial cell products designed to treat LSCD.

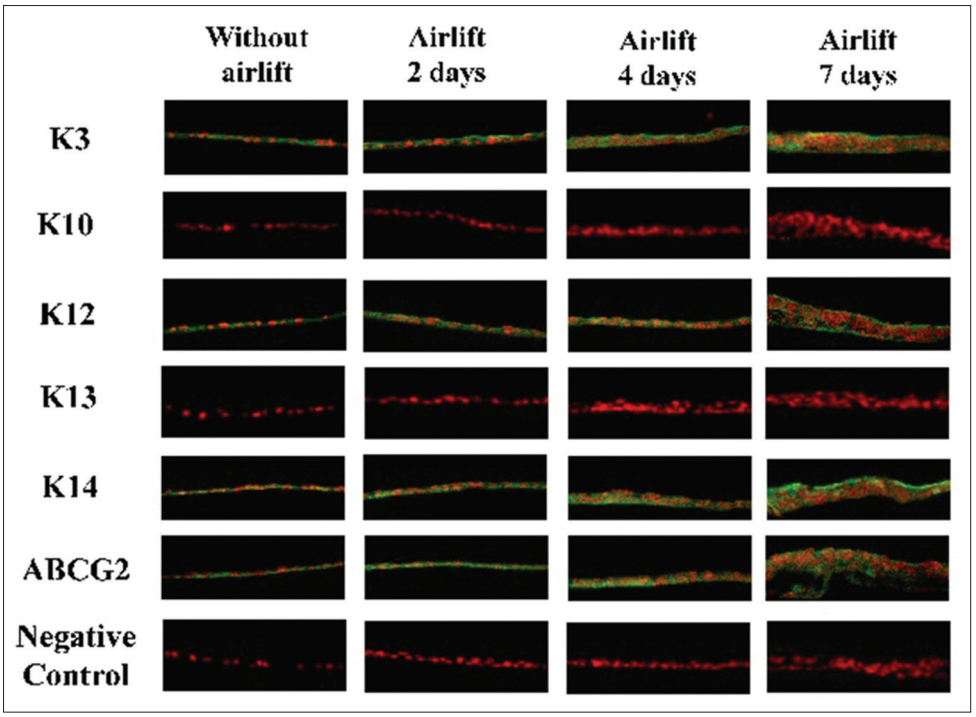

Figure 6: The immunocytochemical staining result of rabbit limbal epithelial cell sheets cultivated under different duration of airlifting. The expression pattern of K3, K10, K12, K13, K14 and ABCG2 were examined. The expression of K3, K10, K12, K13, K14 and ABCG2 showed no differences in pattern and location among different groups, while the epithelial thickness increased time-dependently. On day 7: significantly increased thickness of cell sheets was found. Green: fluorescein isothiocyanate staining of K3, K10, K12, K13, K14 and ABCG2. Red: Propidium iodide counterstaining of nucleus. Negative control: the staining result without primary antibodies

The expression pattern and localization of cytokeratins and stem cells markers were not affected by airlifting for at least 7 days. Cultivating limbal epithelial cell sheets for ocular surface reconstruction with short-term air-lifting appears effective and safe. Further studies are warranted to apply this finding into clinical practice for ocular surface reconstruction.

Financial support and sponsorship

The National Taiwan University Hospital Plan Asia One and the Department of Medical Research at the NTUH and the Institutional Grants of the E-Da Hospital and Taiwan University Hospital (EDAHP100008).

Conflicts of interest

The authors declare that there are no conflicts of interests of this paper.

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