·Basic Research·

Comparative study of four culture methods to engineer murine corneal epithelial sheet

Xiao-Li Ma[1], Jun Kong[2], Han-Qiang Liu[1], Jiang-Yue Zhao[2], Jin-Song Zhang[2]

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[1] Department of Ophthalmology,the First Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China
[2] Department of Ophthalmology, the Fourth Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China
Correspondence to: Jin-Song Zhang. Department of Ophthalmology, the Fourth Affiliated Hospital of China Medical University, Shenyang 110001, Liaoning Province, China. zjsl@21cn.com
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Abstract

• AIM: To investigate the roles of feeder cells in stratification of murine corneal epithelial cells and build an ideal method to engineer stratified epithelial sheet.

• METHODS: Using contact feeder culture, separated feeder culture, compound feeder culture and culture without feeder cells by air-lifting method in Transwell chamber culture system, tissue engineered corneal epithelium was reconstructed. Corneal sheets were stained with hematoxylin and eosin (HE) for histological observation. The expression of p63 and keratin 19 (K19) and involucrin (IVL) was investigated by immunocytochemistry analysis.

• RESULTS: Stratification was limited to three to four layers in the contact feeder group, whereas separate feeder sheets were slightly more stratified. The compound feeder group produced a stratified epithelium with five to seven layers of cells. The group without 3T3 feeder cells formed only two to three layers of cells. Immunostaining images in the compound feeder group showed expression of progenitor markers p63 and K19 in the basal and suprabasal layer, as well as differentiation marker involucrin in all layers.

• CONCLUSION: The remarkable stratification as well as the limbal phenotype makes the compound feeder system a candidate tool for cultivating transplantable epithelial sheets.

• KEYWORDS: mouse; cornea; epithelium; cell culture; tissue engineering

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INTRODUCTION

Cultivated epithelial cell sheets are used clinically for reconstructing the ocular surface in blinding diseases that destroy the corneal epithelial stem cell niche located in the limbus [1,2]. The preparation of epithelial sheets requires feeder cells to expand progenitor cells and to produce stratified sheets. Using feeder cells is believed to reproduce several aspects of the stem cell niche, although molecular mechanisms involved in interactions between epithelium and feeder cells are unclear. Direct cell-to-cell contact and soluble factors secreted by viable feeder cells both seem to be involved in promoting the proliferation and differentiation of epithelial cells and the formation of stratified epithelial sheets. In the current study, we sought to find an ideal method to use feeder cells to engineer a fully stratified epithelial sheet with a limbal phenotype. Therefore, we compared several culture conditions with or without 3T3 feeder cells and devised a novel "compound feeder system" to achieve the optimal cultivated sheets.

MATERIALS AND METHODS

Tissue Preparation and Cell Culture C57BL/6 mice (CLER, Tokyo, Japan), aged 8-10 weeks, were handled according to the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eye globes were enucleated from the mice with forceps after death, washed profusely in phosphate buffered saline (PBS). Eyes from each animal were kept to separate throughout the culture procedure. Corneal buttons including the limbus were cut from the eye and cleaned of extraneous tissue (e.g. iris, ciliary body, etc.). Primary cell culture was performed using explants culture method similar to Hazlett *et al* [3]. Briefly, the button was cut in half and each explant with epithelium side up was plated flat on 6-well plates, one piece per well. After approximately 5-10 minutes to allow for attachment of the explant, serum-free low-Ca^{2+} medium(defined keratinocyte serum-free medium, KSFM; Invitrogen, Carlsbad, CA) consisting of 10ng/mL human recombinant EGF (Invitrogen), 100ng/mL cholera toxin (Calbiochem; Merck KGaA,

Darmstadt, Germany), antibiotics, and growth supplement supplied by the manufacturer was supplemented. The cultures were incubated at 37℃, under 95% humidity and 50mL/L CO_2 with the medium changed every 3 to 4 days. Within 10 days, the explant was carefully transferred to a new dish and cultured as described above.

Subculture The epithelial sheets were subcultured by TrypLE Express (Invitrogen) at 1∶3 split after small cells reached subconfluence until Passage 4 (P4) cultures. From P5, cells after subconfluence were subsequently serially passaged at a density of 5×10^4 per $75cm^2$ flask, 7-10 days per passage. The cultures were incubated at 37℃, under 95% humidity and 50mL/L CO_2 with the medium changed every 3 to 4 days.

Preparation of Feeder Cells NIH/3T3 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured with Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) containing 100mL/L fetal bovine serum (FBS). Confluent cells were treated with mitomycin C (4mg/mL, Sigma-Aldrich) at 37℃ for 2 hours. Dissociated cells were cryopreserved until use.

Stratification Ability Analysis To evaluate the effect of direct cell-to-cell contact with feeder cells, 3T3 fibroblasts feeder cells were seeded on a cell culture inserts (Transwell, cat no 3 450, Corning, Corning, NY) at a density of $2.5\times10^4/cm^2$. On the following day, 3×10^5 epithelial cells were seeded on the insert. After epithelial cells reached confluence, cells were airlift cultured for an additional week to allow stratification (Contact feeder culture, Figure 1A).

To evaluate the effect of soluble factors secreted by feeder cells, 3×10^5 epithelial cells were seeded on the insert. After epithelial cells reached confluence, $2.5\times10^4/cm^2$ 3T3 feeder cells were seeded in the bottom of the well. On the following day, epithelial cells were airlift cultured for an additional week to allow stratification (Separate feeder culture, Figure 1B).

To evaluate the effect of both direct cell-to-cell contact with feeder cells and soluble factors secreted by feeder cells, 3T3 fibroblasts feeder cells were seeded on a cell culture inserts at a density of $2.5\times10^4/cm^2$. On the following day, 3×10^5 epithelial cells were seeded on the insert. After epithelial cells reached confluence, $2.5\times10^4/cm^2$ 3T3 feeder cells were seeded again in the bottom of the well. On the following day, epithelial cells were airlift cultured for an additional week to allow stratification (Compound feeder culture, Figure 1C).

As a control, 3×10^5 epithelial cells were seeded on the insert. After epithelial cells reached confluence, cells were airlift cultured for an additional week to allow stratification (Culture without feeder cells, Figure 1D).

Histological Observation and Immunostaining Fresh frozen cryosections were fixed with formalin and stained with hematoxylin and eosin (HE) for histological observation.

For immunohistochemistry, fresh frozen cryosections were fixed with 40g/L paraformaldehyde (PFA, Wako Ltd. Osaka, Japan). PFA-fixed cells were permeabilized with 3g/L Triton X-100 (Sigma-Aldrich, St. Louis, MO). After background staining was blocked with 100mL/L normal donkey serum, the cells were treated with the following monoclonal primary antibodies: anti-p63 (1∶100, 4A4; Santa Cruz biotechlogy, inc, Santa Cruz, CA), anti-K19 (NeoMarkers For Lab Vision Corporation, Fremont, CA), and anti-involucrin (1∶100, Covance, Emeryville, CA). The cells were then treated with Cy3-conjugated secondary antibodies(Chemicon International, Inc., Temecula, CA). The nuclei were counterstained with 4', 6'-diamino-2-phenylindole(1mg/mL, DAPI; Dojindo Laboratories, Tokyo, Japan).

RESULTS

Stratification was limited to three to four layers in the contact feeder group (Figure 2A), whereas separate feeder sheets were slightly more stratified (Figure 2B). However, the compound feeder group revealed marked stratification and produced a stratified epithelium with five to seven layers of cells (Figure 2C). As a control, the group without 3T3 feeder cells was the least stratified and formed only two to three layers of cells (Figure 2D).

Immunostaining images in the compound feeder group showed expression of progenitor markers p63 (Figure 3A) and K19 (Figure 3B) in the basal and suprabasal layer, as well as differentiation marker involucrin (Figure 3C) in the whole layer.

DISCUSSION

Epithelial-mesenchymal interactions control epidermal growth and differentiation and regulate tissue homeostasis in the epidermis[4,5]. Feeder cells support the serial subculture of epithelial cells though several passages [6], allow colony formation by epithelial cells [4,7], inhibit the growth of contaminating fibroblasts [6], and maintain human telomerase reverse transcriptase expression [8] and Sp1/Sp3 activity [9]. This interplay is based on two basic processes: production of

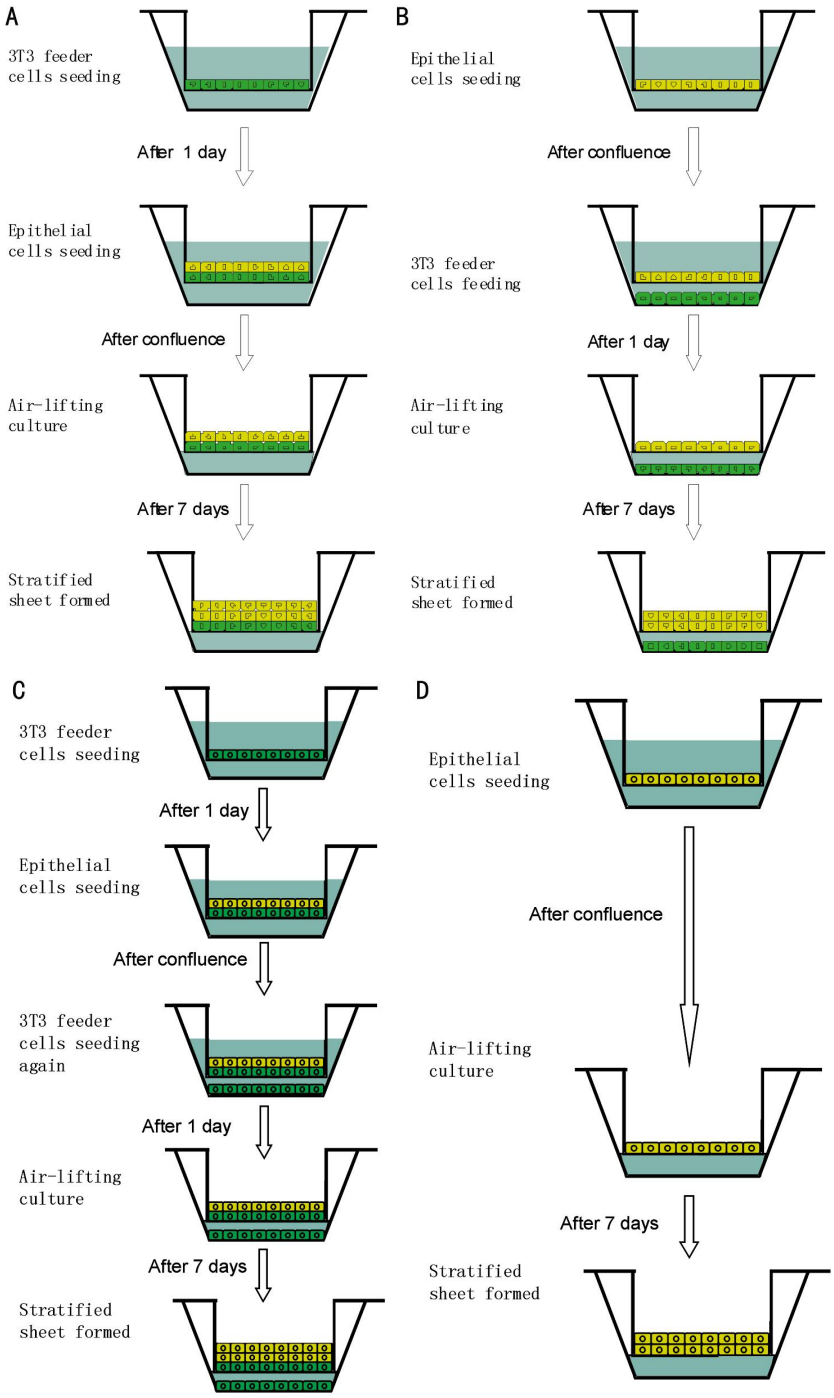

Figure 1 Methods to reconstruct the tissue engineered corneal epithelium A: contact feeder culture; B: separate feeder culture; C: compound feeder culture; D: culture without feeder cells

soluble factors displaying autocrine and paracrine activities[4], and direct cell-cell/matrix contact [10-12]. However, their functional significance in the dermal-epidermal interplay to regulate epithelial stratification and homeostasis is only poorly understood.

In our study, we compared four culture methods to investigate the roles of direct contact and soluble factors in stratification of murine corneal epithelial cells. The results showed that the compound feeder culture produced the most robust epithelial sheets with five to seven layers of cells (Figure 2C), both contact and separate feeder culture formed three to four layers (Figure 2A and 2B), however, the group without 3T3 feeder cells was the least stratified and limited to only two to three layers of cells (Figure 2D). The results

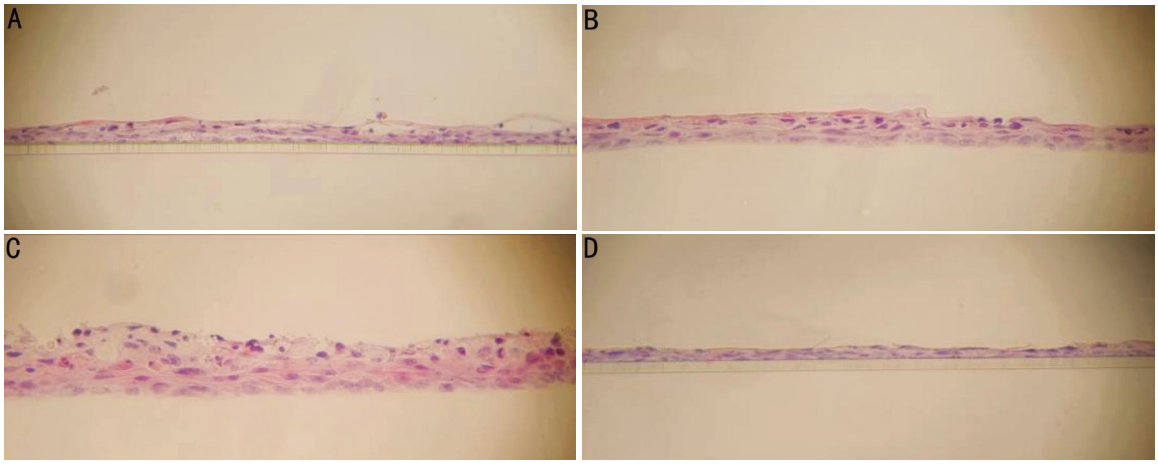

Figure 2 HE staining of the stratified corneal epithelial sheet A: contact feeder culture; B: separate feeder culture; C: compound feeder culture; D: culture without feeder cells

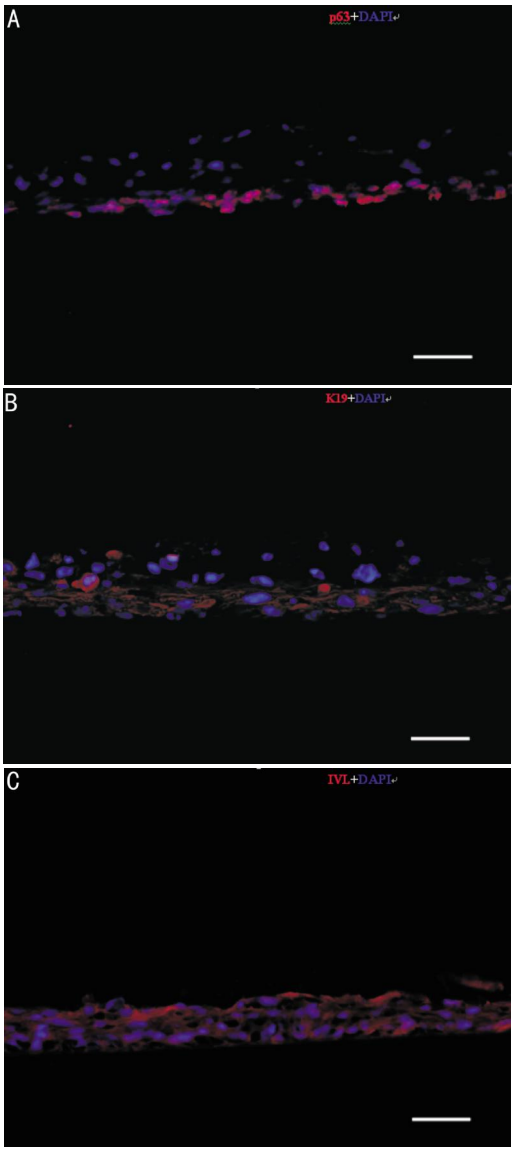

Figure 3 Immunostaining images of mouse corneal epithelial sheets Expression of progenitor markers p63 (A) and K19 (B) was located in the basal and suprabasal layer, as well as differentiation marker involucrin (C) in the whole layer. Scale bars, 50μm

suggested that direct contact as well as production of soluble factors play important roles in epithelial stratification and homeostasis. The compound feeder culture took the advantages of both of them and revealed marked stratification.

The expression of p63 [13,14] and K19 [15,16] was localized to progenitor cells with high proliferative capacity, including both limbal stem cells (LSCs) and transient amplifying cells (TACs) at present. Immunostaining showed in the compound feeder group the expression of progenitor markers p63 (Figure 3A) and K19 (Figure 3B) in the basal and suprabasal layer, which indicates that the cells in the basal and suprabasal layer of the sheet still maintained the phenotype of corneal epithelial progenitor cells and had high proliferative capacity. Involucrin [17] have been regarded as markers of differentiation of the corneal epithelial cells. The cells in the epithelial sheets expressed involucrine, which means that the cells retained the potential of differentiation.

In conclusion, the remarkable stratification as well as the limbal phenotype makes the compound feeder system a candidate tool for cultivating transplantable epithelial sheets.

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