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Comparsion of cell-suspension and explant culture of mouse corneal epithelial cells in mice

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Abstract

• AIM: To compare cell-suspension and explant culture of mouse corneal epithelial cells (MCEC).

• METHODS: MCEC were cultured by cell-suspension culture and explant culture, respectively. Colony forming efficiency (CFE) and cell proliferation were determined. The expression of corneal epithelial progenitor cell marker p63 and K19, as well as differentiation marker K12 was investigated by Western blotting.

• RESULTS: Twenty of 25 (80%) cornea explant were successfully subcultured to passage1 (P1), while only 12% cell-suspension culture were successfully subcultured to P1. There were statistical significance between explant culture and cell-suspension culture ($P<0.01$). Up to 55% of P1 cells in explant culture were passaged over P10 and were stably subcultured though at least 25 passages. However, cells cultured in suspension culture never achieved confluence in P2. CEF of P1 in explant culture was higher than P1 in cell-suspension culture ($P=0.02$) and CEF of P20 in explant culture was higher than P1 in explant ($P=0.001$). Immunostaining images showed expression of p63 and K19 in cell-suspension culture P1 and explant culture P1 and P20. K12 was expressed in P1 of both cell-suspension culture and explant culture, however, there was not K12 expressed in P20 of explant culture.

• CONCLUSION: In MECE culture, compared with cell-suspension culture, the explant culture is a preferable option.

• KEYWORDS: cornea; epithelium; cell culture; explant culture; cell-suspension culture

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INTRODUCTION

The corneal epithelium is a rapidly regenerating stratified squamous epithelium. The epithelial progenitor cells, including limbal stem cell (LSC) and transient amplifying cell (TAC) with extensive proliferative potential, are crucial for maintaining the homeostasis of corneal epithelium, which are influenced by many exogenous factors, including gradients of diffusible molecules, cell or substrate adhesion contacts, and direct cell-cell communication. Understanding of such a delicate balance depends upon *in vivo* experiments and on the establishment of adequate *in vitro* models that allow the manipulation of cells under controlled conditions. However, the limited life span, insufficient cell yields and quick differentiation of these cells greatly hinder progress of such research. Repeated isolation of cells from corneo-limbal tissue and time consuming primary culture are required. Therefore, establishing long-term culture of corneal epithelial cells would facilitate studies of proliferation, differentiation and regeneration of corneal epithelium.

Methods of culture and subculture of human corneal epithelial cells have been well documented [1]. However, there have been no such cases for mouse corneal epithelial cells. There are 2 approaches for the primary culture of corneal epithelial cells *in vitro*, cell-suspension culture and explant culture [2]. With explant culture method, Hazlett *et al*[3] cultured mouse corneal epithelial cells firstly, but failed in subculturing the cells over three passages. With cell-suspension culture, Kawakita *et al*[4] reported a method to successfully established a mouse corneal epithelial line, but more than 200 mice were used. Until 2009, Ma *et al*[5] firstly reported reproducible establishment of long-term mouse corneal cell culture from one single cornea with explant culture method. Herein, we compared cell-suspension and explant culture of mouse corneal epithelial cells to investigate optimum culture condition.

MATERIALS AND METHODS

Materials Serum-free low-Ca^{2+} medium (defined keratinocyte serum-free medium, KSFM; Invitrogen, Carlsbad, CA) was supplemented with 10μg/L human recombinant EGF (Invitrogen), 100μg/L cholera toxin (Calbiochem; Merck KGaA, Darmstadt, Germany), antibiotics, and growth supplement supplied by the manufacturer.

Cell-suspension Culture C57BL/6 mice (CLER, Tokyo, Japan), aged 8-10 weeks, were handled according to the Association for Research in Vision and Ophthalmology (ARVO) guidelines for animal care. Mouse corneal/limbal

epithelial sheets were isolated similar to Kawakita *et al*[6] method. In brief, eye globes were enucleated by forceps, washed profusely in PBS, stored in KSFM. These eyes were digested at 4℃ for 18h in KSFM containing 5mg/mL dispase Ⅱ. Subsequently, each eye was held in place by suction applied to the posterior pole using a transfer pipette and was gently shaken in KSFM to loosen the ocular surface epithelial sheet. Single cells were obtained from the above corneal/limbal epithelial sheets by TrypLE Express (Invitrogen) for 15minutes followed by vigorous pipetting which were seeded at a density of 20×10^3 cells/cm^2 on plastic containing KSFM. The cultures were incubated at 37℃, under 95% humidity and 50ml/L CO_2 with the medium changed every 3 to 4 days.

Explant Culture C57BL/6 mice, 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 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 Ma *et al*[7]. Briefly, the button was cut in half and each explant with epithelium side up was plated flat on 6-well plate, one piece per well. After approximately 5-10 minutes to allow for attachment of the explants, the cultures were incubated at 37℃, under 95% humidity and 5ml/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 mouse corneal epithelial cells (MCEC) were subcultured by TrypLE Express (Invitrogen) at 1∶3 split after small cells reached subconfluence until Passage 4 (P4) cultures. From P5, MCEC after subconfluence were subsequently passage at a density of 5×10^4 per 75cm^2 flask, 7-10 days per passage. The medium changed every 3 to 4 days.

Cell Proliferation The population doublings (PDs) were calculated as $\log_2(D/D_0)$, where D and D_0 were defined as the density of cells at the time of harvesting and seeding, respectively.

Colony Forming Efficiency MCEC were inoculated in 60mm dishes at 1000 cells/dish and cultured for 10 days. Cultured cells were stained with eosin for 1 hour. Colony forming efficiency (CFE) was calculated as the number of colonies/number of inoculated cells. Images were scanned and eosin-stained area was measured using Scion Image software (Scion Corp, Frederick, MD). Five independent experiments were performed.

Western Blotting Analysis MCEC were dissolved with lysis buffer (M-PER, Pierce, Rockford, IL). Same amount of proteins were loaded on a 100g/L Bis-Tris gel (Ready Gel; Bio-Rad Laboratories, Hercules, CA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were immunostained with primary antibodies against p63 (1∶200), K19 (1∶50) and K12 (1∶100), respectively. After the reaction with horseradish-peroxidase conjugated secondary antibody, protein bands were visualized by ECL (GE Healthcare, Buckinghamshire, UK) and X-ray film.

Statistical Analysis *Chi-square* tests and *Student's t*-test were used to evaluate the differences by SPSS 10.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

In cell-suspension culture, within 24 hours after seeding, some cells attached to plastic and started spreading but more than 70% of seeded cells did not attach. Cells were heterogenous population of small cobblestone-like cells and large squamous cells. The small cells surrounded by large cells formed a "cell nest" (Figure 1A). On confluence, cells were subcultured to passage 1 (P1). In P1, small cells decreased and large squamous cells were overwhelming majority. There was few "cell nests" of small cells (Figure 1C). Cells were cultured in reached confluence on day 10 in P0, but never achieved confluence in P2 (Figure 2A) on day 14 in P1.

In explant culture, MCEC began to grow out from the explant within 24 hours. The cells showed a cobblestone appearance, and epithelial cells were near the explants stratified (Figure 1B). In P1, cells were heterogenous population of small cobblestone-like cells and large squamous cells and small cells were overwhelming majority (Figure 1D). From P5, cells consisted of uniform small cells with typical cobblestone appearance and were stably subcultured through at least 25 passages without showing signs of replicative senescence (Figure 1E,2B).

Twenty of 25 (80%) cornea explant were successfully subcultured to P1, while only 12% ($n = 50$) cell-suspension cultures were successfully subcultured to P1. There were statistical significance between explant culture and cell-suspension culture ($\chi^2 = 34.027, P < 0.005$). Up to 55% of P1 cells in explant culture were passaged over P10 and were stably subcultured through at least 25 passages. However, cells cultured in suspension culture never achieved confluence in P2 (Figure 2).

CEF of P1 in cell-suspension culture and explant culture was 1.14% ±0.81% and 2.82% ±0.62%, respectively. There were statistical significance between them ($n = 5, P = 0.02$). CEF of P20 in explant culture was 30.2% ±5.84%. There were statistical significance between P1 and P20 of explant culture ($n = 5, P = 0.001$).

Immunostaining images showed expression of progenitor markers p63 and K19 in cell-suspension culture P1 and explant culture P1 and P20, and the expression of p63 and K19 in explant culture P20 was stronger. In P1 of both cell-suspension culture and explant culture, K12 was expressed. However, there was no K12 expressed in P20 of explant culture (Figure 3).

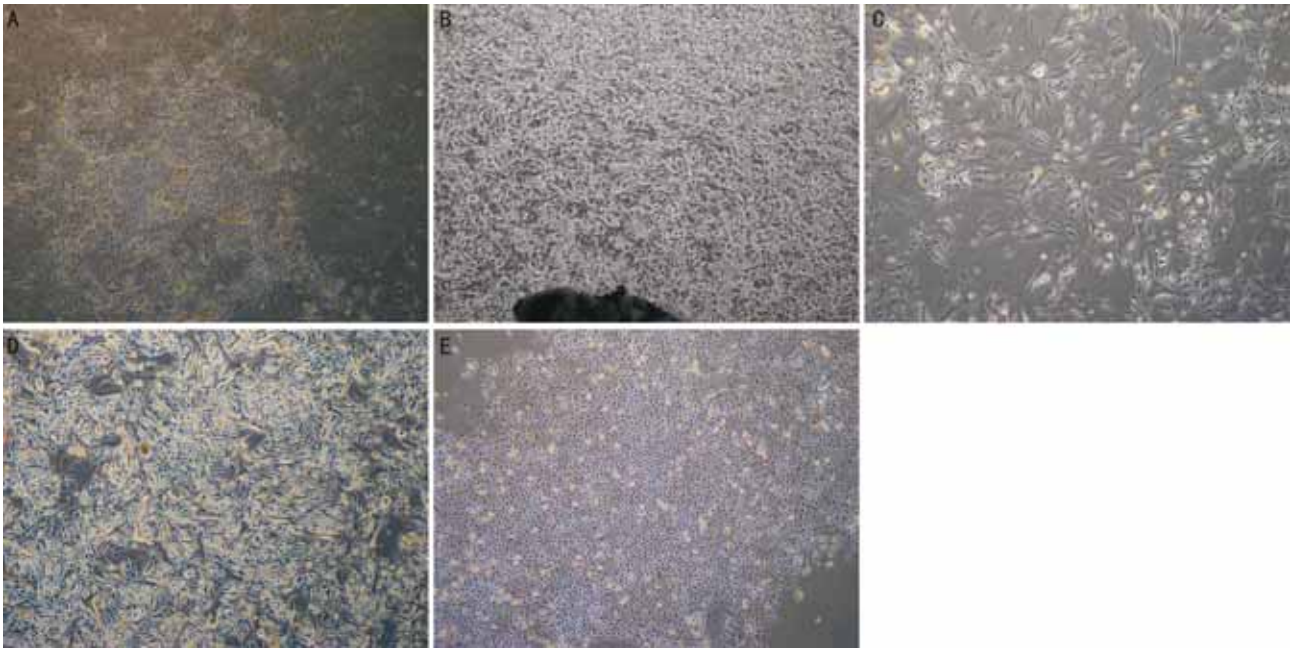

Figure 1 Mouse of MCEC in culture (×100) A: Cell-suspension culture P0; B: Explant culture P0; C: Cell-suspension culture P1; D: Explant culture P1; E: Explant culture P20.

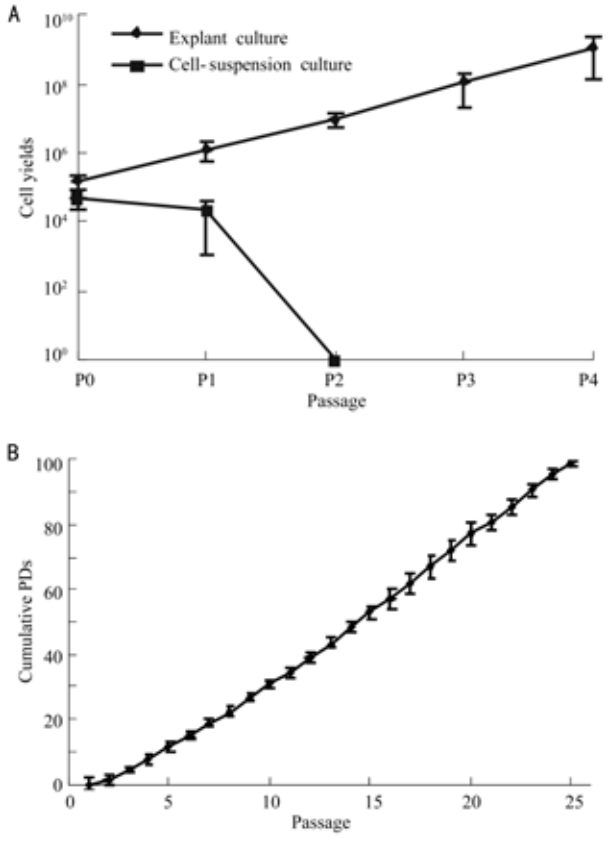

Figure 2 Mouse MCEC culture A: Cell yields of cell-suspension culture and explant culture; B: Cumulative PD of explant culture.

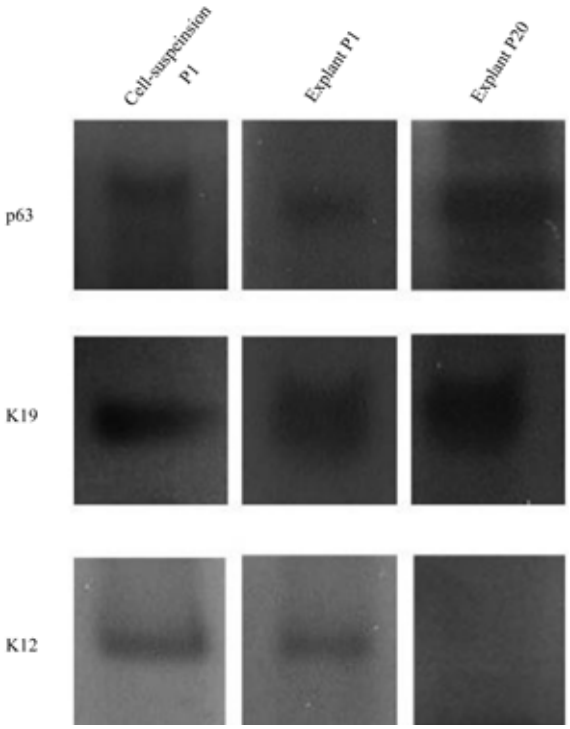

Figure 3 Western blotting.

DISCUSSION

Mice are used in many different types of studies for several reasons: they are well-suited for genetic manipulations such as genomic sequence analyses; different strains with different characteristics are readily available, and many transgenic (Tg) and knockout strains have been created and are commercially available. Furthermore, *in vitro* approaches with cultured mouse cells allow the investigation of tissue or cell specific properties. However, culture of MCEC from a single mouse has been notoriously difficult to establish. In order to find an optimum culture condition, we compared cell-suspension and explant culture of mouse corneal epithelial cells in this paper.

There are several approaches for the primary culture of corneal epithelial cells *in vitro* which include the cell-suspension culture and explant culture techniques [2]. Each approach has its own advantages. While cell-suspension culture can decrease fibroblasts contamination from the stroma of explants

to some extent, it requires a large amount of tissue. Since the mouse cornea is small, it is difficult to obtain sufficient cells from one single mouse by cell-suspension. Moreover, during the process of isolating cells from tissue into single cells, some of the cells including epithelial progenitor cells were damaged by enzyme and pipetting. Therefore, cells of P0 which could attach and subculture to P1 were low.

In explant culture, more MCEC were preserved and there weren't almost stem cells lost. The study of Li *et al*[8] found during *ex vivo* expansion, some limbal epithelial progenitor cells indeed migrated from the explant surface. In our study, we also found the CEF in P1 of explant culture was higher than cell-suspension culture and P20 was higher than P1 in explant culture. Moreover, using the explant culture techniques, we have cultured the cells though more than 25 passages with a high proliferative capacity without signs of undergoing replicative senescence. Cells in P20 had similar morphology, characterized by homogenous small cell size with typical cobblestone appearance, which suggests the cells maintain progenitor cell state [9]. The expression of p63 [10] and K19 [11] was localized to progenitor cells with high proliferative capacity, including both LSC and TAC at present, while the K12 is expressed by all corneal epithelial cells and limbal suprabasal epithelial cells, except the limbal basal cells and is the marker of differentiation [12, 13]. In our study, western blotting showed cells were positive for p63 and K19 and negative for K12 in P20 of explant culture, which indicates that the cells may be the equivalent to limbal epithelial progenitor cells, including stem cells.

Although a main problems during explant culture is contamination by fibroblasts [2, 14], by using keratinocyte growth medium and by handling the explant gently when removing the tissue within 10 days after initiating the culture ensured that fibroblast outgrowth was minimal. In conclusion, in mouse corneal epithelial cell culture the explant culture is a preferable option.

REFERENCES

1 Miyashita H, Higa K, Kato N, Kawakita T, Yoshida S, Tsubota K, Shimmura S. Hypoxia enhances the expansion of human limbal epithelial progenitor cells *in vitro*. *Invest Ophthalmol Vis Sci* 2007;48(8):3586-3593

2 Zhang X, Sun H, Tang X, Ji J, Li X, Sun J, Ma Z, Yuan J, Han ZC. Comparison of cell-suspension and explant culture of rabbit limbal epithelial cells. *Exp Eye Res* 2005;80(2):227-233

3 Hazlett L, Masinick S, Mezger B, Barrett R, Kurpakus M, Garrett M. Ultrastructural, immunohistological and biochemical characterization of cultured mouse corneal epithelial cells. *Ophthalmic Res* 1996;28(1):50-56

4 Kawakita T, Espana EM, He H, Yeh LK, Liu CY, Tseng SC. Calcium-induced abnormal epidermal-like differentiation in cultures of mouse corneal-limbal epithelial cells. *Invest Ophthalmol Vis Sci* 2004;45(10):3507-3512

5 Ma X, Shimmura S, Miyashita H, Yoshida S, Kubota M, Kawakita T, Tsubota K. Long-term culture and growth kinetics of murine corneal epithelial cells expanded from single corneas. *Invest Ophthalmol Vis Sci* 2009;50(6):2716-2721

6 Kawakita T, Shimmura S, Hornia A, Higa K, Tseng SC. Stratified epithelial sheets engineered from a single adult murine corneal/limbal progenitor cell. *J Cell Mol Med* 2008;12(4):1303-1316

7 Ma XL, Kong J, Liu HQ, Zhao JY, Zhao JS. Comparative study of four culture methods to engineer murine corneal epithelial sheet. *Guoji Yanke Zazhi* 2008;8(9):1743-1746

8 Li W, Hayashida Y, He H, Kuo CL, Tseng SC. The fate of limbal epithelial progenitor cells during explant culture on intact amniotic membrane. *Invest Ophthalmol Vis Sci* 2007;48(2):605-613.

9 Romano AC, Espana EM, Yoo SH, Budak MT, Wolosin JM, Tseng SC. Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. *Invest Ophthalmol Vis Sci* 2003;44(4):5125-5129

10 Hernandez Galindo EE, Theiss C, Steuhl KP, Meller D. Gap junctional communication in microinjected human limbal and peripheral corneal epithelial cells cultured on intact amniotic membrane. *Exp Eye Res* 2003;76(3):303-314

11 Yoshida S, Shimmura S, Kawakita T, Miyashita H, Den S, Shimazaki J, Tsubota K. Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci* 2006;47(11):4780-4786

12 Tanifuji-Terai N, Terai K, Hayashi Y, Chikama T, Kao WW. Expression of keratin 12 and maturation of corneal epithelium during development and postnatal growth. *Invest Ophthalmol Vis Sci* 2006;47(2):545-551

13 Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res* 2005;81(3):247-264

14 Liu S, Li J, Wang C, Tan D, Beuerman R. Human limbal progenitor cell characteristics are maintained in tissue culture. *Ann Acad Med Singapore* 2006;35(2):80-86

小鼠角膜上皮细胞消化培养法和组织块培养法的比较研究

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摘要

目的:比较小鼠角膜上皮细胞消化培养法和组织块培养法。

方法:分别使用消化培养法和组织块培养法培养小鼠角膜上皮细胞。比较两种方法中小鼠角膜上皮细胞的克隆形成率(CFE)和群体倍增(PD)。通过 Western blotting 方法检测 p63、角蛋白 19 以及角蛋白 12 的表达。

结果:其中 80% 组织块培养法的原代培养可成功传代,而仅 12% 的消化培养法原代培养可成功传代;两者比较有显著性差异($P < 0.05$)。传代培养中,组织块培养法中 55% 的第一代(P1)细胞可以传代超过 P10 并继续稳定传代至少可传至 P25。而消化培养法传代至 P2 即不能融合。在 P1,组织块培养法细胞的 CFE 高于消化培养法($P = 0.02$);而组织块培养法 P20 细胞的 CFE 又显著高于其 P1 细胞($P = 0.001$)。免疫荧光染色显示消化培养法的 P1 细胞和组织块培养法的 P1,P20 细胞均表达 p63 和 K19。K12 仅在消化培养法的 P1 细胞和组织块培养法的 P1 中表达,而组织块培养法的 P20 细胞中,K12 阴性表达。

结论:小鼠角膜上皮细胞的培养,组织块培养法优于消化培养法。

关键词:角膜;上皮;细胞培养;组织块法;消化法