Contribution of 3T3 to the Culture of Cornelimbal Stem Cells[1]

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Received: 19 August 2021; Accepted: 18 October 2021; Published: 22 October 2021

ABSTRACT

Introduction: *Researchers may now examine limbal epithelial cells on an individual basis thanks to recent developments in single-cell RNA sequencing and single-cell quantitative real-time PCR.*

Aim of the study: *the main aim of the study is to Contribution Of 3T3 To The Culture Of Cornelimbal Stem Cells*

Material and method: *Human amniotic membrane was prepared in accordance with the methodology section's protocol*

Conclusion: *The limbal phenotypic cells could be preserved by the limbal epithelial cells when they were cultivated on the denuded AM in the presence of a 3T3 feeder layer.*

INTRODUCTION

Limbal Stem Cell Conundrums

Although much of the evidence is indirect, it is sufficient to conclude that stem cells do indeed reside in the limbus. The final piece of the puzzle will be revealed once a stem cell marker is discovered that provides unambiguous information about stem cell functionality.

Principles of Corneal Epithelial Regeneration

Without a doubt, the most specialised surface on the body is the corneal surface. It is constantly mending, much like the surface of the body. The corneal epithelium's top layer constantly loses cells to desquamation and must be replenished by cell growth. Experimental evidence of the upward mobility of previously labelled basal cells suggests that it may be caused by the pressure on proliferation inside the basal cell layer. After experimental corneal epithelial injury, it was shown that corneal epithelial cells moved horizontally from the periphery to the centre.

Numerous findings suggest that human corneas also exhibit the centripetal movement. The limbal epithelium is excluded from corneal erosions, which first heal centripetally. Second, when the sutures are removed from corneal grafts, tiny subepithelial cysts that form between the sutures shift towards the centre. Third, specular microscopy revealed the centripetal migration of epithelial cells under physiological circumstances.

LITERATURE REVIEW

Behaegel, Joséphine & Dhubhghaill (2017) Cultivating limbal stem cells outside the body, or ex vivo, is a promising approach to treating limbal stem cell deficiency. Clinical trial results have been widely reported since the technique's introduction in 1997, but little is known about the potential health risks associated with production and transplantation. There is a risk of introducing toxic or infectious agents into culture procedures due to the use of

[1] *How to cite the article:* Pohekar A.S., Gupta N., Contribution of 3T3 to the Culture of Cornelimbal Stem Cells, *IJPPS*, Oct-Dec 2021, Vol 5, Issue 4, 16-21

animal and/or human-derived products, which may be contaminated with known or unknown additives. The dangers stem from differences in customs and procedures at various institutions worldwide.

Selver, Ozlem & Yagci, Ayse & Egrilmez (2017) The cornea, the outermost layer of the eye, must be clear for vision to work normally. When this area is injured or destroyed, it leads to a lack of limb stem cells. When limbal stem cells die off, the conjunctival epithelium may start to colonise the cornea. Many methods for cultivating limbal epithelial cells have been developed since then. Methods to improve stem cell viability are still being investigated. Cultures of limbal cells are typically grown in an explant culture format. The transplanted growth is subsequently surgically attached to the recipient eye (cultivated limbal epithelial cells). Regulations around cell-based therapy are constantly changing, and as a result, the transplantation of produced limbal epithelial cells, grown in xenobiotic-free environments, is becoming increasingly popular in Turkey and elsewhere.

Haagdorens, Michel & Behaegel, Joséphine & Rozema (2017) FDOCT was utilised to take pictures of the upper and lower limbus of the 50 healthy subjects' eyes. Matlab was utilised for picture analysis and processing. Using fluorescent labelling, we were able to prove that stem cells exist in these clusters. There were around 7.4 palisades per millimetre based on the average interpalisade epithelial rete peg width (ERP) and the average palisade ridge width (PR), both of which were on the order of 72 m. Iris colour was shown to have a substantial effect on PR, ERP, and PD, whereas age had a significant effect on PD and PD (p0.05). The limbus may be safely and successfully imaged with in vivo OCT, a technique that can also be used to see the Vogt palisades. Matlab's image processing tools allowed researchers to count and determine the density of voluminous ciliary palisades of Vogt in scanned images. This technique has the potential to enhance the accuracy of limbal biopsies taken before to transplantation.

Yin, Jia & Jurkunas, Ula (2017) The limbus is home to adult somatic stem cells known as corneal epithelial stem cells. These cells are responsible for replenishing the cornea's clear epithelium. When there aren't enough healthy limbal stem cells or if the ones that are are damaged, a condition known as limbal stem cell deficiency (LSCD) might set in. Chemical and thermal ocular surface burns are more likely to develop LSCD, a major contributor to corneal scarring. There are many reported protocols for transplanting limbal stem cells (LSCT).

MATERIAL AND METHOD

Effect of 3T3 in Culturing of Corneal Limbal Stem Cells

1. **Preparation of Human Amniotic Membrane:**

Human amniotic membrane was prepared in accordance with the methodology section's protocol.

2. **Preparation of 3T3 fibroblasts:**

“Confluent murine 3T3 fibroblasts from the National Centre for Cell Sciences (NCCS, Pune) were plated at a density of 2.2 x 104 cells/cm2 onto cell culture dishes after being treated with 4 g/mL mitomycin C (MMC) for two hours at 37°C and 5% CO2. Following plating, these feeder cells were employed 4 to 24 hours later.”

3. **Limbal Biopsy preparation:**

A 2 mm3 corneal limbal biopsy from a cadaveric donor eye was taken and transported to the cell biology lab for further processing in accordance with the methodology section's protocol. The sample was taken in DMEM with 3% FCS and antibiotics.

4. **Cultivation of limbal explants on denuded AM with and without 3T3 feeder layer**

Human limb explants cultured with and without the feeder layer on the deep epithelized amniotic membrane:

AM was attached to the culture insert as previously mentioned, with the basement membrane facing up. Every two to three days, the medium was changed. The cultures were cultured for around 3 to 4 weeks until confluence was reached. The cultures were implanted in an OCT compound and immediately frozen for immunohistochemistry after three weeks.

5. Immunostaining:

The frozen section was immunostained using the p63 antibody at a dilution of 1: 100 and ABCG2 at a dilution of 1: 75, respectively.

6. Western Blot analysis:

The expression of ABCG2 and p63 by cultured limbal limbal epithelial cells on denuded membrane and denuded membrane with 3T3 feeder layer was verified using western blot. To get enough cells for protein extraction, we started and sampled two cultures from each donor. After denuded AM with 3T3 (n=3) and denuded AM cultures of limbal epithelium (n=3) reached a growth plateau, the cultured cells over the membrane were scraped with a cell scraper and collected in lysis buffer (Proprep protein extraction Kit, CA, USA), and protein was extracted in accordance with the methodology section's protocol.

RESULTS

Effect of 3T3 in Culturing of Corneal Limbal Stem Cells

Growing corneal limbal explants on denuded AM while the 3T3 feeder layer is present:

20 limbal biopsies in total were taken over the course of six months, from December 2005 to May 2006. Within two hours of the donor's passing, the tissues were collected. The donor's age ranged from 8 to 90. The growth of the cells on the denuded AM and with the 3T3 feeder layer is depicted in Figure 4.1. By the end of day 3, the explant cells had begun to grow, and by the end of day 21, they had nearly reached confluence.

Cultivation of corneal limbal explants on denuded AM in the absence of the 3T3 feeder layer:

Up until the cultures' confluence in 3–4 weeks, the outgrowth rate of the 10 cultures was documented and measured each time the culture medium was switched. (No data shown) By the end of the 21st day, the cells cultured over the denuded AM and denuded AM with 3T3 had almost reached confluent growth.

Cultured corneal limbal explants on denuded AM were shown to express ABCG2 and p63 by immunohistochemistry and western blotting in the presence of the 3T3 feeder layer:

At the conclusion of three weeks, immunohistochemistry was performed on the cultured cells. P63 and ABCG2 are both expressed by the cells cultured over the denuded AM in the presence of 3T3. Only a few basal cells displayed both markers' expression. (Fig. 4.2).

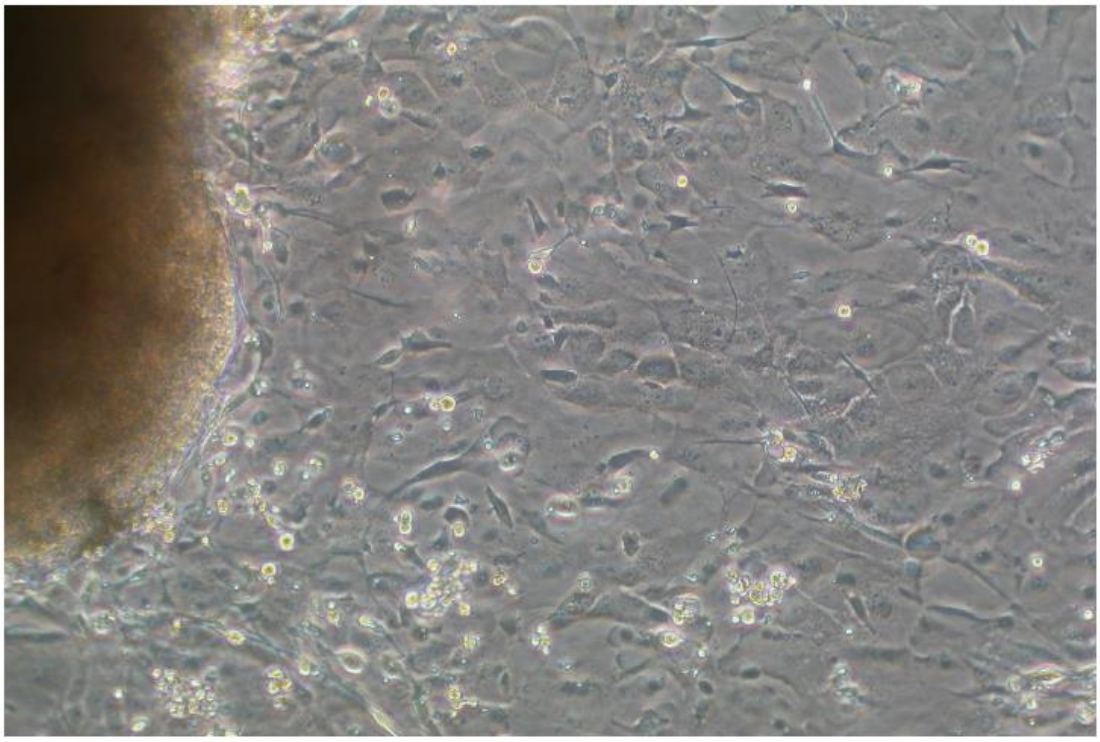

Figure 4.1 *Growth of the cells in the presence of 3T3 feeder layer*

The growth of the limbal epithelial cells derived from limbal explants cultured in the presence of the 3T3 feeder layer is shown in this figure. The culture was photographed after two weeks of incubation.

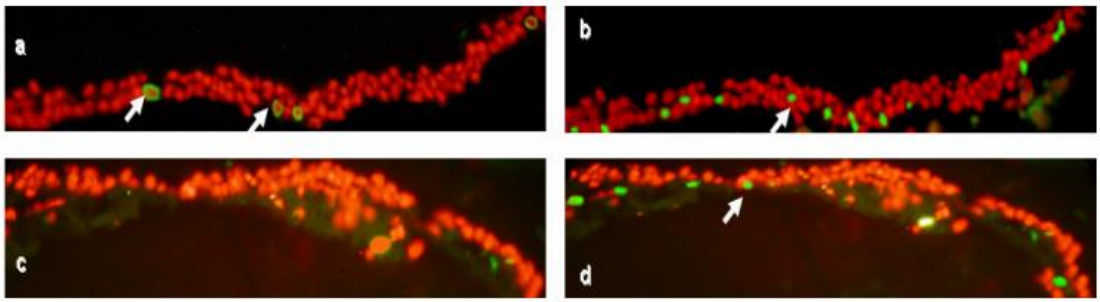

Figure 4.2

The immunofluorescence image of p63 and ABCG2 on the cultured over the denuded AM with and without 3T3 feeder layer is shown in this figure at the conclusion of three weeks. Figures 4.2a and 4.2c show ABCG2 expression. Cells a and b were cultured on the denuded AM with the 3T3 feeder layer present, while cells c and d were grown on the denuded AM without the 3T3 feeder layer. On the cells cultured in the presence of the 3T3 feeder layer, only a small number of basal cells express ABCG2. C - cells exhibit negative expression when cultured over a denuded layer without a 3T3 feeder layer. B - depicts the cells cultured over the denuded AM +3T3 feeder layer as having positive p63 expression. d - Positive expression on 3T3 feeder layer-free denuded AM. (Mag – 20X)

Western blot confirmed that this marker was expressed. The cells harvested at the end of three weeks had a weakly positive expression of both p63 and ABCG2, according to Western blot results. (Fig. 4.3).

In the absence of the 3T3 feeder layer, ABCG2 and p63 were expressed by immunohistochemistry and western blot on cultured corneal limbal explants on denuded AM:

On the cells cultured over the denuded membrane, ABCG2 expression was completely absent, but after three weeks, p63 expression could be seen on the few basal cells. 4.3c and 4.3d in Figure The western blot on the cells that were harvested after three weeks confirmed this. The ABCG2 protein was totally absent, however the p63 protein was little expressed.

RT-PCR Results:

Data from RT-PCR on the expression of Np63, ABCG2, Connexin 43, and K3/K12 on cells grown over a denuded AM layer without a 3T3 feeder layer

Only cells harvested at day 8 showed any p63 or ABCG2 expression at all; cells harvested at day 21 showed none at all. Connexin-43 and Keratin 3 & 12 expression was also noticeably higher on cells cultured over the denuded AM without 3T3 feeder layer.

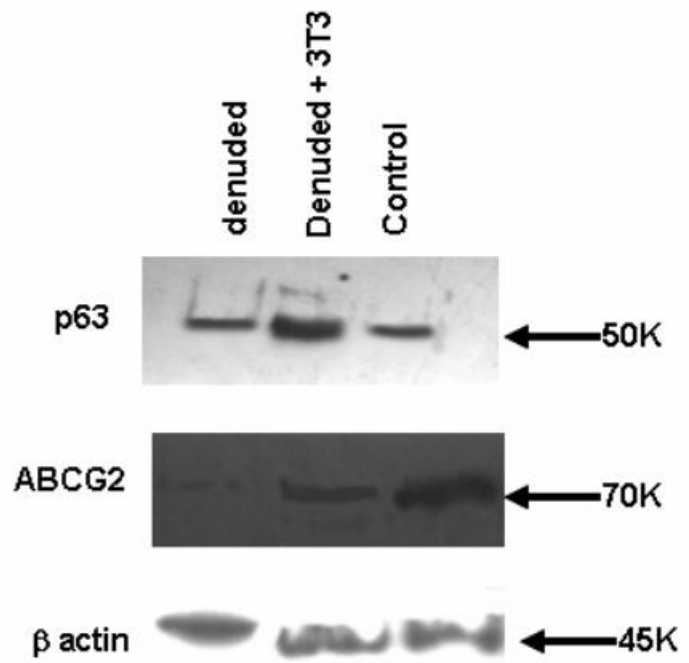

Figure 4.3 *Western blot results*

This image displays the results of a western blot on cells that were collected after 21 days and cultivated over both denuded AM and denuded AM with 3T3. Both denuded AM and denuded AM with 3T3 feeder layer showed the positive expression of p63. While ABCG2 expression is present in the cells grown with the 3T3 feeder layer, it is totally gone from the cells retrieved from the denuded membrane. Positive control lane: p63-SiHa cell lysate; control lane: ABCG2-MCF 7 cell lysate (positive control). The loading control is shown by actin.

Np63, ABCG2, Connexin 43, and K3/K12 expression data from RT-PCR on cells cultivated over the denuded AM with the 3T3 feeder layer:

p63 and ABCG2 were expressed by the cultivated cells up to 21 days of incubation. The expression of these markers associated with stem cells gradually decreased but was still present after 21 days of incubation. Connexin 43 and keratin 3&12 showed similar expression, however it was noticeably less than those cultivated just on the denuded AM.

CONCLUSION

By being grown on the denuded AM with a 3T3 feeder layer, limbal epithelial cells were able to maintain their characteristic phenotype. There is a need for more study to identify the substances secreted by these cells that aid in the preservation of their limbal phenotype.

Financial Support and Sponsorship: Nil

Conflict of Interest: None

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