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CULTIVATION OF LIMBAL STEM CELLS USING AMNIOTIC MEMBRANE, BOTH INTACT AND DENUDED

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ABSTRACT

Introduction: The sense of seeing is perhaps the most crucial. More than 80% of the information we get from the outside world comes to us through visual sources.

Aim of the study: The main aim of the study is to cultivation of limbal stem cells using amniotic membrane, both intact and denuded.

Material and method: The methodology section describes the steps taken to prepare, preserve, and use human amniotic membrane for the cultivation of limbal stem cells.

Conclusion: It is concluded that the as a result, when limb epithelial cells are cultivated on an intact AM, the basal layer of the cultured cells express ABCG2, whereas Connexin-43 and keratins 3 and 12 are expressed at modest levels.

INTRODUCTION

Lifelong corneal epithelial renewal is dependent on limbal stem cells (LSCs). The limbal stem cells make up a small percentage of the limbal epithelium and are thought to reside in the area just under the basal epithelium. 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. Many studies on human normal and keratoconus corneas have shown clusters (heterogeneity) of cells that have the potential to be limbal stem/progenitor cells due to their unique properties. Mice and rabbits were also employed as models in the single-cell studies. Comparable to the findings reported from human tissues, LSC diversity was found in mice and rabbits. The "inner" and "outer" populations of LSCs were identified by the study's authors.

Concepts of Limbal Stem Cells

The iris, pupil, and anterior chamber are located beneath the cornea, the transparent front surface of the eye (Figure 1.2A). The white, opaque sclera surrounds the structures that make up the anterior chamber, and the tissues come together at the limbus.

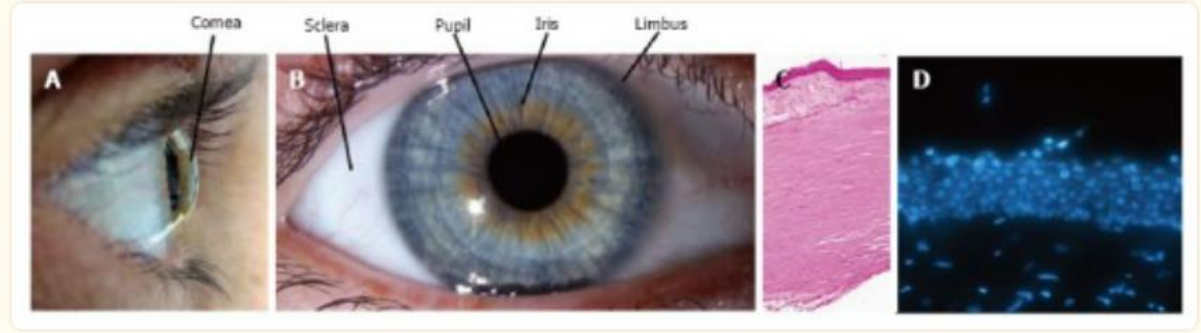

Figure 1.1 *The Eye's Anatomy.*

MATERIAL AND METHOD

Limbal Stem Cell Cultivation Using Intact and Denuded Amniotic Membrane

1. **Corneal limbal cells are cultured on both the intact and denuded amniotic membrane.**

Human Amniotic Membrane Preparation

The methodology section describes the steps taken to prepare, preserve, and use human amniotic membrane for the cultivation of limbal stem cells.

Limbal Biopsy preparation

The biopsy was handled in accordance with the steps described in the methodology section. Twenty limbal biopsies in total were taken from the cadaveric donor eyes.

Human Limbus culture on Amniotic membrane

According to the methodology, the limbal explants were positioned on the human amniotic membrane that was both intact and denuded. The explants were then covered with DMEM and F12 that had been reconstituted with growth factors.

2. **Proliferation study by Brdu labelling**

All samples were fixed in triplicate in cold methanol at 40C for 10 minutes before being subjected to the same BrdU immunofluorescent staining procedure as before. Brdu labelling indicators were evaluated in accordance with the methodology's instructions.

RESULTS

1. Culture Results

Cultivation of corneal limbal biopsy on the intact and denuded membrane:

20 limbal biopsies in total were taken over the course of the six-month period from November 2005 to April 2006. The limbal biopsies were taken from donors between the ages of 8 and 85 who had been enucleated within 6 to 8 hours of passing away. They were taken in the Transport medium. All 20 samples were used for the culture over the intact and denuded membrane (DMEM with 3% FBS and the antibiotic mixture, Penicillin, Streptomycin, and amphotericin).

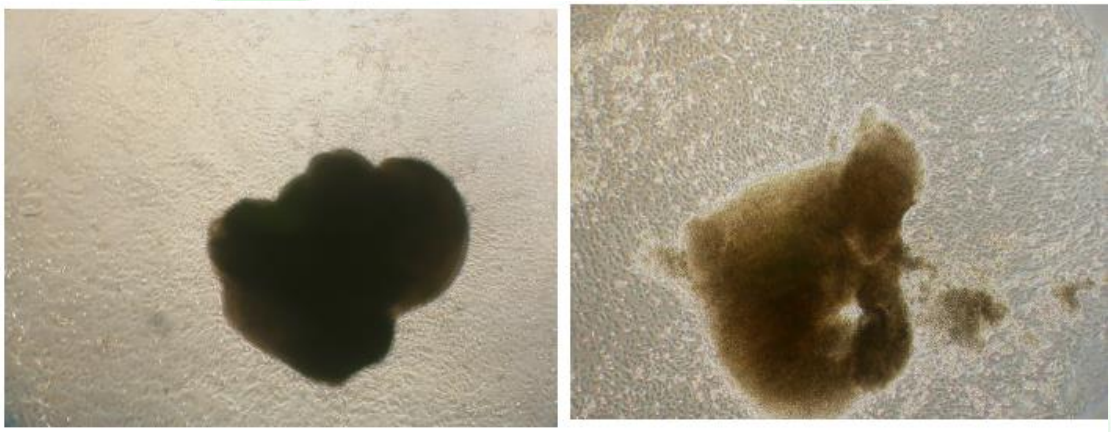

Figure 3.1 *Growth of limbal epithelial cells on the intact and denuded Amniotic membrane*

Figures 3.1A and 3.1B depict the growth on the intact and denuded amniotic membranes, respectively. Using measurement software at 20X magnification, the expanded cells appear as a monolayer of small, uniform cells with a nucleus to cytoplasmic ratio of roughly 1:1. After 12 days of culture, the cells grown over the denuded AM grew noticeably faster than those grown over the intact AM and were nearly confluent. When compared to the intact AM, the growth rate of the cells cultured over it was faster. ($p < 0.05$).

2. Immunohistochemistry results:

Using immunohistochemistry, the expression of ABCG2 and p63.:

The cultures exhibited stratification after 3 to 4 weeks of culture on the intact and denuded membrane. On the cells cultured over the intact membrane, only a small number of basal cells stained the cytoplasm positively for ABCG2. And for the p63 staining, cells cultured over both intact and denuded AM showed strong nuclear staining. (Figure 3.3)

3. RT-PCR

Results from semiquantitative RT-PCR demonstrate that cells grown on intact and denuded AM exhibit different markers' expression at the end of the 21st day of culture, including p63, ABCG2, Connexin 43, and K3/K12. ΔNp63 is more strongly expressed on cells.

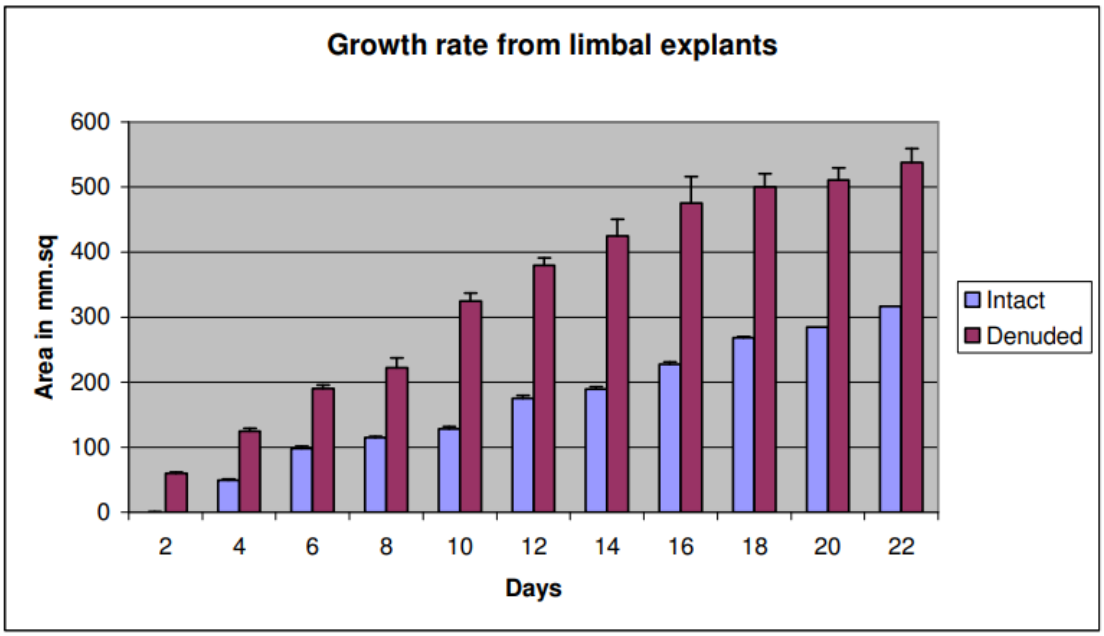

Figure 3.2: *Differential proliferation of amniotic membrane limbal epithelial cells between the whole membrane and its removed regions*

Both types of cells grew to confluence after around 3 weeks. The rates of cell expansion cultivated over intact and denuded AMs are compared in this figure.

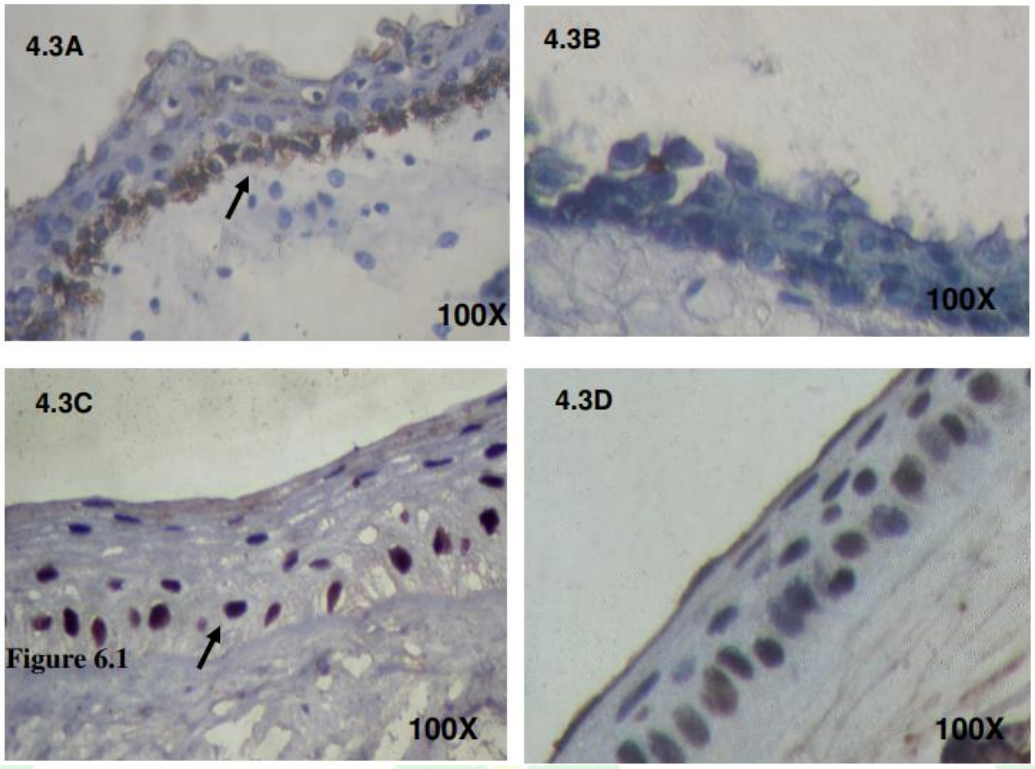

Figure 3.3

Figure 3.3 is an immunohistochemistry image of harvested limbal epithelial cells after three weeks of culture on an intact and denuded membrane. Cells in 4.3B with a denuded membrane lacked expression. 4.3C p63 expression in cells grown over intact tissue. The brown nuclei are indicative of positive cells. Connexin 43 was expressed negatively in cells cultured over an intact amniotic membrane, but positively in cells cultured over a denuded amniotic membrane.

4. Western Blot

At the conclusion of the 21-day incubation period, proteins were extracted from the cells cultured over the intact and denuded membrane. After three weeks, the cells harvested from the intact membrane expressed ABCG2, whereas the cells on the denuded membrane did not. Both showed expression of p63 (clone 4A4).

5. Labeling index for Brdu in cells grown on intact and removed membranes

Label-retaining stem cells that cycle slowly or are quiescent during mitosis may be found using thymidine or BrdU labelling. Thymidine, an endogenous DNA base, may be replaced with the analogue BrdU to specifically identify just dividing

cells. After being labeled, slow cycling cells should keep it for a much longer time than other, more mitotically active cells, who will lose it through repeated mitosis.

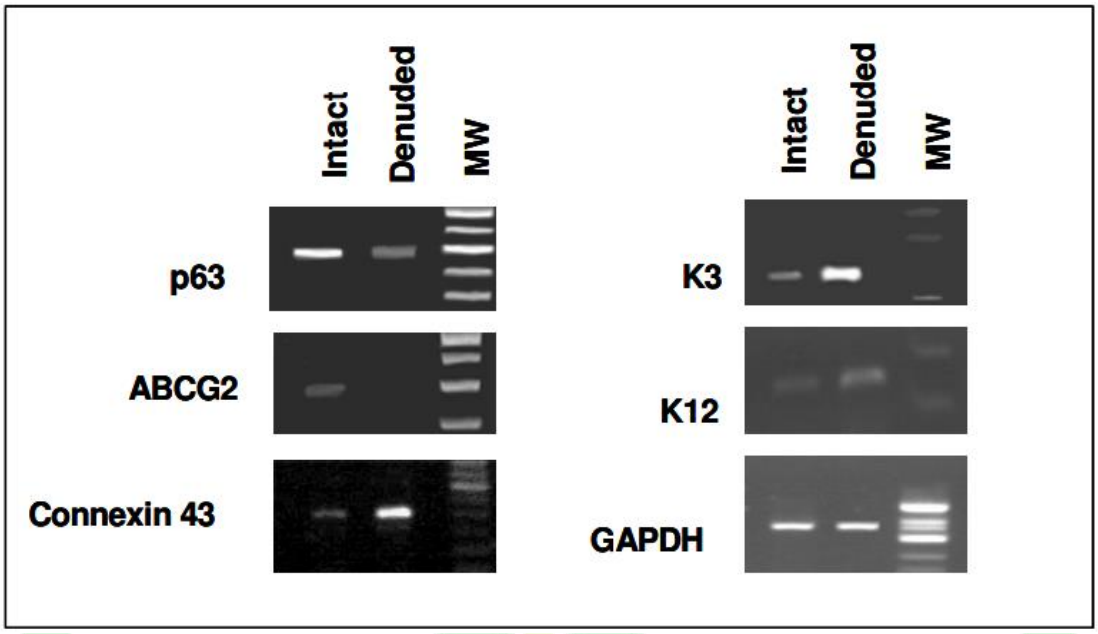

Figure 3.4A *shows the semiquantitative RT-PCR electrophoretogram. In the figure, cells cultured over intact and denuded membranes are shown to express p63, ABCG2, connexin-43, Keratin 3 and K12. The internal control is GAPDH. X Hinf I digest, Molecular Weight*

CONCLUSION

As a result, when limb epithelial cells are cultivated on an intact AM, the basal layer of the cultured cells express ABCG2, whereas Connexin-43 and keratins 3 and 12 are expressed at modest levels. The cultivated cells also maintained the label for an additional 21 days, mimicking the in vivo phenotype of stem cells incorporating human limb basal epithelial cells. Devitalized amniotic membrane combined with epithelium replicates the stromal niche seen in vivo to preserve stem cell properties.

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