UNIVERSITY OF CALIFORNIA, SAN DIEGO

An understanding of gene regulation in corneal epithelium pathogenesis

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by

Jin Zhu

Committee in charge:

Professor Liangfang Zhang, Chair
Professor Shaochen Chen
Professor Kang Zhang

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The Thesis of Jin Zhu is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

An understanding of gene regulation in corneal epithelium pathogenesis

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Master of Science in Nanoengineering

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Professor Liangfang Zhang, Chair

The cornea is consisted with continuous and smooth epithelial cells in the center and limbal stem cells in the edge. Their transparency plays important role in maintaining our vision. Any deficiency in cornea epithelial cells (CECs) or limbal stem cells (LSCs) will lead to transparency lost and blindness. In our study, we successfully developed a protocol for cell culture to expand LSCs from human donors and animal tissues. Enabling us to generate a homogenous cell population to study key factors involved in LSCs fate determination and CEC differentiation. Through the genome-wide gene expression analysis, we found WNT7A and PAX6 were highly involved in LSCs and CECs, which

gave us a insight of these two genes regulation for limbal stem cell fate determination.

1. Introduction

The cornea is a group of cell layers in the outer part of our eyeball system. It is highly transparent and smooth. It is very easy to be damaged by external injury both chemically or physically. Any deficiency in cornea epithelial cells or limbal stem cells will lead to convert the cells into a non-transparent status, which looks like skin epithelial cell. Corneal and skin epithelium both shares a lot of similarities and differences. The morphology of them both looks like stratified epithelium. They both maintain by their stem cells which highly express p63 (transcription factor 63) and keratin 5/keratin 14 in the basal cell layer in the limbus and epidermis[1-3]. But during skin epithelial stem cells differentiation, they move upwards and the keratin 5/keratin 14 are replaced by skin-specific keratin 1/ keratin 10. For LSCs, in limbus area, the specific marker was subjected by K19, but after they differentiate into the central cornea, K5/K14 are replaced by corneal-specific K3/K12. The transparency maintains by the CECs is essential for vision. The pathological change of CECs into skin like epithelial cells, will lead to the morphological change and convert the keratin expression. For the abnormal cornea epithelium tissue, K3/K12 are replaced by K1/K10, which will cause the loss of transparency and blindness[4], but the mechanism is sill unknown.

In order to study the mechanism of LSCs fate determination and differentiation, we successfully developed a cell culture protocol to isolate and culture LSCs from human donor and animal tissue. We use p63 and K19 with to identify our isolated and cultured

LSCs, the mitotic marker Ki67 was highly expressed in the cells which indicating the cells is going through an active proliferating process. Following this protocol, we also successfully developed a protocol for a three-dimensional CEC sphere complex after we seed the single LSCs into the reduced growth factor matrigel. The CEC-specific markers K3/K12 was highly expressed in these differentiated cells. We also use the same protocol to isolate and culture the SECs from human eyelids honor tissues. Hair follicles were removed by tweezers under microscope. SESCs specific markers p63 and K5 were highly expressed in our cultured cells. And followed by the three-dimensional cell culture protocol, the differentiated SECs also express the skin-specific marker K1/K10 as expected.

Between these specific marker gene in LSCs and SESCs, we don't know how is the regulation mechanism in cell fate determination. In order to find the key factor among these genes, we demonstrated a genome-wide gene expression analysis. We found two genes *WNT7A* and *PAX6* were highly expressed in LSCs and CECs, which may give us an insight for further study these two genes in LSCs cell fate determination. At last, we identified *WNT7A* and *PAX6* were both highly expressed in our *in vitro* conditions but no observation in SECs.

2. Methods and Materials

LSCs isolation and cell culture

The human cornea was isolated from human eye bank donation. Limbus regions were taken from the cornea edge. The cornea epithelial tissues should be removed as much as possible. The limbus tissue was washed in cold PBS with 1% Pen/Strep and cut into small piece. For this step, the smaller piece you can get, the better results you would obtain. The single stem cells could be isolated by a two-steps digestion. The first step is to use 0.2% collagenase IV to digest at 37℃ for 2 hours. The second step followed by a 15 minutes 0.25% trypsin-EDTA at 37℃, then centrifuge at 1000 rpm for 5 minutes to spin down the cells. The last step is to seed the cells on a plastic cell culture plate coated with a reduced growth factor Matrigel (354230, BD Biosciences).

We used the same protocol for skin epithelial stem cell (SESCs). The skin eyelid tissue was obtained from human donor and rabbit skin. Cell culture medium formula was shown as follows: DMEM/F12 and DMEM (1:1) with 1% Pen/Strep, 10% fetal bovine serum, 10 ng/ml EGF, 5 ug/ml insulin, 0.4 ug/M 3,3',5-triiodo-L-thyronine.

In order to obtain the differentiated CECs, we demonstrated the three-dimensional sphere structure. The single stem cell were mixed with Matrigel. Cell concentration is 2*104 cells per 50 ul/gel. Then we used differentiation medium to directional differentiate the stem cells. CnT-30 was used for limbal stem cell differentiation and CnT-02 was used for skin stem cell differentiation. (Cellntec)

In order to detect the expressed gene product proteins, we use immunofluorescence and laser confocal microscopy to observer the protein staining. We first treated the cell with 4% paraformaldehyde for 20 min, and then treated with Triton X-100-PBS for 5 min twice. The blocking process was followed by a PBS solution with 5% FBS. The primary antibody should be treated for 4℃ overnight. After followed by three times PBS washing, the specimen was incubated with secondary antibody. We use DAPI for staining, then followed by immunofluorescence microcopy observation.

3. Results

As Figure 1 shown, both corneal and skin epitheliums share some similarities. They both express p63 in the stem cell layer. (Figure 1, a and b right panel) and K5/K14 markers (Figure 2, a and b). But they also have many differences. The cornea-specific marker K3/K12 replaced the K3/K14 after the LSCs differentiated and moved to the central cornea. (Figure 1, c). After SESCs differentiated and moved upward, the skin-specific marker K1/K10 replaced the initial K3/K12. (Figure, 2 c) The abnormal CECs convert to a skin-like epithelial cells and the skin specific marker K1/K10 were started to express. (Figure, 1 d)

After we followed our protocol, we successfully developed our LSCs and SESCs culture population. (Figure 3, a and c, with positive stem cell marker p63 K5/K19 and Ki67). The three-dimensional differentiation structure was performed as Figure 3 b and d shown. The cornea-specific markers K3/K12 were observed in the CECs sphere and the skin-specific marker K1/K10 were observed in the SECs sphere. We also demonstrated a genome-wide gene expressing analysis and found the WNT7A and PAX6 were highly expressed in LSCs and CECs compare to SESCs. (Figure 3 e, *WNT7A* 8.8x in LSC and 12.3x in CEC, *PAX6* 4.5x in LSC and 6x in CEC). Further more, we tested these two genes expression in LSCs and CECs. (Figure 3, f left and middle left panels were limbus and skin region, middle right was the cultured LSCs, the right panel is the three-dimensional CEC spheres.). The results suggest that these two genes were highly

expressed in LSCs and CECs, which may play an important role in LSCs fate determination and differentiation.

4. Discussion

The successful protocol for LSCs isolation and culture helped us to enabling an approach for studying limbal stem cell fate determination. Based on this progress, the discovery of WNT7A and PAX6 play important role in gene regulation of LSCs fate is essential for further study. Our further study indicated WANT7A and PAX6 are responsible for maintaining cornea cell fate. By knocking down WNT7A and PAX6 in LSCs did not change the morphological properties but the K3/K12 marker are significantly decreased after the LSCs differentiated into CECs sphere. Meanwhile, the K1/K10 became more active. Further more, we tested the engineered expression of PAX6 was be able to convert SESCs into LSC-like cell be the evidence of K3/K12 expression increased and K1/k10 decreased.

This discovery gave us a new strategy for corneal disease treatment. By transplantation of the engineered SESCs with PAX6 gene, the non-transparent cornea may be able to convert back to the transparent status, which give us a hope for the potential therapeutic treatment.

5. Acknowledgements

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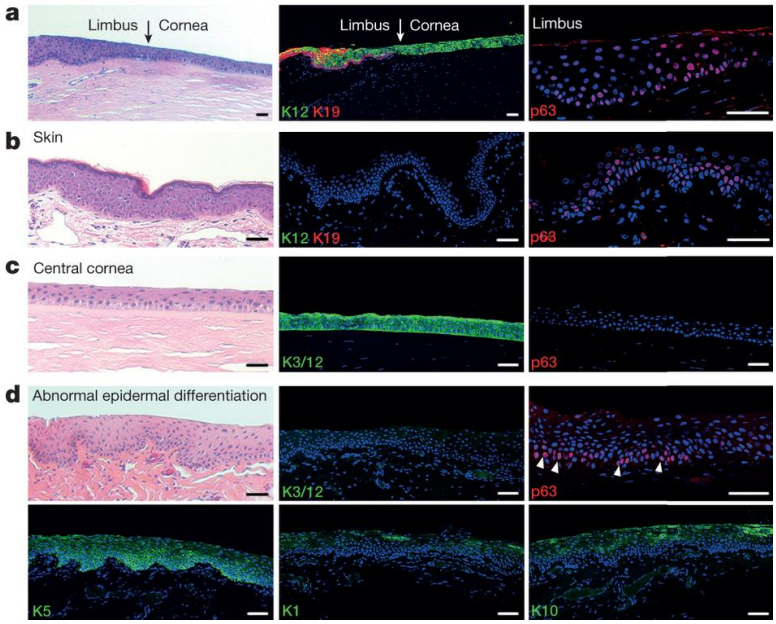

Figure 1 Normal limbus,cornea and skin morphology and their specific marker

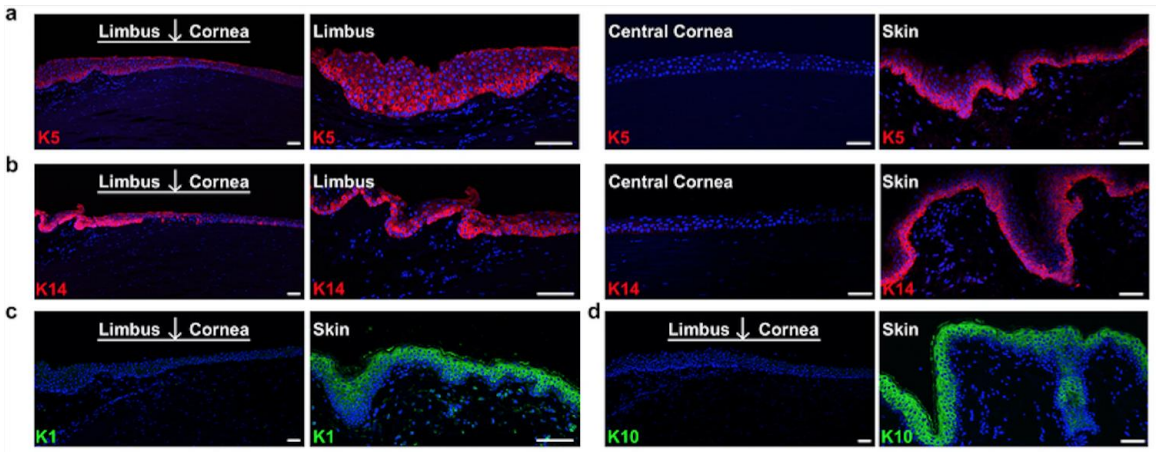

Figure 2 Early corneal and skin epithelial cell marker and their differentiation

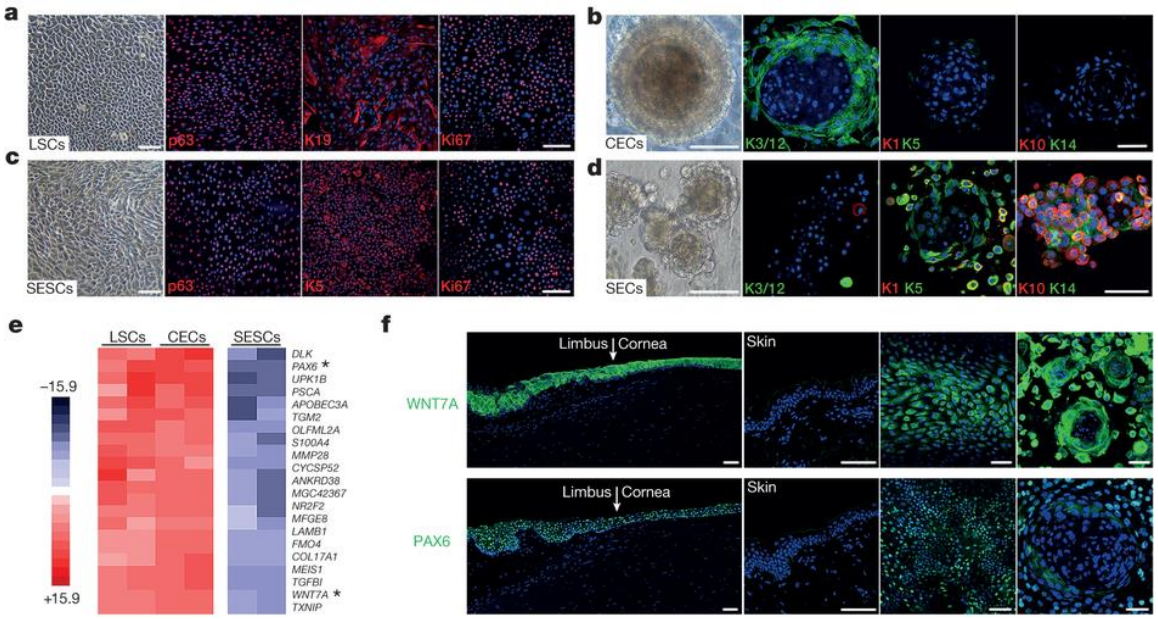

Figure 3 Stem cell isolation and culture, WNT7A and PAX6 expression in limbus and cornea

Reference

1 Pellegrini, G. Dellambra E, Golisano O, Martinelli E, Fantozzi l, Bondanza S, Ponzin D, Mckwon F, De Luca M. p63 identifies keratinocyte stem cells. Proc. Natl Acad. Sci. USA 98, 3156-3161 (2001)

2 Argyris, T.S & A.J.P. Klein-Szanto. Regeneration and the mechanism of epidermal tumor promotion. CRC Crit. Rev. Toxicol. 14, 211-258 (1985)

3 Buck, R. C. Cell migration in repair of mouse corneal epithelium. Invest. Ophthalmol. Vis. Sci. 18,767-784 (1979)

4 Davanger. M. & Evensen, A. Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature 229, 560-561(1971)

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Figure 1: Normal limbus,cornea and skin morphology and their specific marker
http://www.nature.com/nature/journal/vaop/ncurrent/fig_tab/nature13465_F1.html

Figure 2: Early corneal and skin epithelial cell marker and their differentiation
http://www.nature.com/nature/journal/vaop/ncurrent/fig_tab/nature13465_SF1.html

Figure 3: Stem cell isolation and culture, WNT7A and PAX6 expression in limbus and cornea
http://www.nature.com/nature/journal/vaop/ncurrent/fig_tab/nature13465_F2.html