·Basic Research·

In vitro tissue engineering of lamellar cornea using human amniotic epithelial cells and rabbit cornea stroma

Xiao-Yong Liu[1], Jian Chen[1], Qing Zhou[1], Jing Wu[2], Xiao-Ling Zhang[1], Li Wang[1], Xiao-Yan Qin[1]

Foundation item: National Natural Science Foundation of China (No.30872808; No. 81100637)

[1]Department of Ophthalmology, the First Affiliated Hospital of Jinan University, Guangzhou 510630, Guangdong Province, China
[2]Laboratory of Ophthalmology, School of Medicine, Jinan University, Guangzhou 510632, Guangdong Province, China
Correspondence to: Jian Chen. Department of Ophthalmology, the First Affiliated Hospital of Jinan University, Guangzhou 510630, Guangdong Province, China. drchenj@163.com

Received: 2013-01-29 Accepted: 2013-06-27

Abstract

• **AIM: To reconstruct the lamellar cornea using human amniotic epithelial (HAE) cells and rabbit cornea stroma *in vitro* using tissue engineering technology.**

• **METHODS: Human amnia taken from uncomplicated caesarean sections were digested by collagenase to obtain HAE cells, and the cells were cultured to proliferate. Rabbit corneal epithelial cells were removed by n-heptanol to make lamellar matrix sheets. The second passage of HAE cells were cultured on the corneal stroma sheets for 1 or 2 days, then transferred to an air-liquid interface environment to culture for 2 weeks. Tissue engineered lamellar cornea (TELC) morphology was observed by Hematoxylin-eosin (HE) staining; its ultrastructure was observed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM); corneal epithelial cell-specific keratin 3 and keratin 12 were detected with immunofluorescence microscopy.**

• **RESULTS: HAE cells grew on the rabbit corneal stroma, forming a monolayer after 1-2 days. About 4-5 layers of epithelial cells developed after 2 weeks of air-liquid interface cultivation, a result similar to normal corneal epithelium. Rabbit corneal stromal cells were significantly reduced after one week, then almost completely disappeared after 2 weeks. TEM showed desmosomes between the epithelial cells; hemidesmosomes formed between the epithelial cells and the basement membrane. SEM revealed that the HAE cells which grew on the lamellar cornea had abundant microvilli. The tissue-engineered cornea expressed keratin 3 and keratin 12, as detected by immunofluorescence assay.**

• **CONCLUSION: Functional tissue-engineered lamellar corneal grafts can be constructed *in vitro* using HAE cells and rabbit corneal stroma.**

• **KEYWORDS:** amniotic epithelial cells; cornea; tissue engineering; keratin
DOI:10.3980/j.issn.2222-3959.2013.04.03

Liu XY, Chen J, Zhou Q, Wu J, Zhang XL, Wang L, Qin XY. *In vitro* tissue engineering of lamellar cornea using human amniotic epithelial cells and rabbit cornea stroma. *Int J Ophthalmol* 2013;6(4):425-429

INTRODUCTION

Today, corneal transplant surgery is still the only effective treatment for corneal blindness. However, in many regions of the world, especially in some Asian countries, traditional, cultural and religious objections block the supply of donor corneas. According to incomplete statistics, despite more than 200-300 million corneal blindness patients in China, only about 4 000-5 000 cases annually receive corneal allografts [1]. Most patients with corneal blindness are therefore deprived of effective treatment for lack of corneal donors. Even those few patients who do receive traditional corneal transplant surgery suffer with such burdens of transplant surgery as postoperative immune rejection and long-term use of anti-rejection drugs.

Human amniotic epithelial (HAE) cells are formed from amnioblasts, separated from the epiblast at about the 8th day after fertilization. Recent studies suggest that HAE cells have some of the characteristics of embryonic stem cells and can separate into three germ layers of different types of cells [2]. Because HAE cells do not express HLA-A, B, C and DR antigen, they cause almost no immune response [3], making them ideal for broad application in cell replacement therapy.

Tissue engineering corneal technology offers hope to patients without access to donor corneas. While enormous difficulties still block the engineering of full-thickness tissue corneas, artificial lamellar corneas may substitute the keratoplasty donor. We investigated the possibility of reconstructing tissue engineered lamellar cornea (TELC) using HAE cells and rabbit cornea lamella sheet *in vitro*.

SUBJECTS AND METHODS

Subjects Human amnia from uncomplicated caesarean sections were obtained from the First Affiliated Hospital of

Jinan University with the approval of the institutional review board. Informed consent was obtained from each patient. New Zealand white rabbits weighing 2.5-3.0kg were purchased from the Experimental Animal Center of Guangdong Province, China. The hospital review board approved all care and treatment protocols related to the animals.

Methods The amnion layer was mechanically peeled off the chorion and washed several times with phosphate buffer saline (PBS, pH 7.4) containing antibiotics (100Ku/L penicillin, 0.1g/L streptomycin). The amnion was subsequently cut into small pieces about 1mm×1mm. To release the HAE cells, the amnion membrane was incubated at 37℃ with 0.2% collagenase II (Invitrogen, Carlsbad, CA, USA) for 2h. The upper liquid was discarded after 1 000r/min centrifugation for 5min and the remaining tissue was digested with a solution containing 0.25% trypsin and 0.02% EDTA (1:1, v:v) (Amresco, Solon, OH, USA) at room temperature for 10min. Digestion was terminated with a serum containing Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Carlsbad, CA, USA). The cells were collected and washed three times with PBS. Viability of the HAE cells was determined by exclusion of trypan blue dye and cells were counted with a hemocytometer. HAE cells were resuspended to a density of 1×10^5mL with DMEM supplemented with 10% superior grade fetal bovine serum (Gibco, Carlsbad, CA, USA) and 10ng/mL epidermal growth factor (PeproTech GmbH, Hamburg, Germany). About 2mL worth of solution of HAE cells was cultured in T25 culture flasks at 37℃ with 95% humidity and 50mL/L CO_2. Culture medium was changed every other day. The morphology and growth status of the HAE cells were observed with an inverted microscope (Leica Camera AG, Solms, Germany). Cells were passaged with 1:2 splits when the solution reached 80%-90% confluence, at about 7-10 days.

Rabbit corneal stroma lamella After rabbits were anesthetized by intramuscular injection, the bulbar conjunctiva and Tenon capsule were removed under sterile conditions. Cotton sheet soaked in n-heptanol were placed over the corneas for 30s; then the corneal epithelial cells were wiped off with a swab. The corneas were observed under a dissecting microscope to ensure that no residual corneal epithelial cells remained. Corneas were then washed 3 times with PBS. Lamellar stroma sheets about 1/2 the thickness of the cornea and 7.5mm in diameter were obtained using a corneal trephine. The sheets were attached to the bottom of sterile 96-well culture plates. Three random corneal lamellar stroma sheets were checked by scanning electron microscope (SEM) for any remaining epithelial cells.

Reconstruction of TELC In the experimental group, about 5×10^4 second generation HAE cells were planted on each lamella sheet. After submerged culture for 2 days, the sheets were transferred to the air-liquid interface culture on Millipore-CM insert dishes with 0.4μm pore size (Millipore, Temecula, CA, USA) for 2 weeks. Culture medium was changed every other day. The control group had all the same conditions as the experimental group, except that no HAE cells were seeded on the rabbit corneal stroma sheets.

Histological characterization of TELC The growth status of HAE cells on the corneal stroma was observed daily with the inverted microscope. The histological morphology of the reconstructed TELC was examined with paraffin section and hematoxylin-eosin (HE) staining. The surface ultrastructure of the reconstructed TELC was examined with a Philips ESEM-30 SEM (Philips, Amsterdam, Netherlands). The inner ultrastructure of TELC was examined with a Philips TECANAI-10 transmission electron microscope (TEM; Philips, Amsterdam, Netherlands).

Immunohistochemistry assay The reconstructed TELC were frozen into sections and treated with 0.25% Triton X-100 at room temperature for 10min. Endogenous peroxidase was blocked with 3% H_2O_2 at room temperature for 10min. Then, the sections were blocked with 5% bovine calf serum (Invitrogen, Carlsbad, CA, USA) containing D-Hanks solution at 37℃ for 30min. Each frozen section was incubated with polyclonal antibodies of goat anti-human keratin 3 and keratin 12, (Chemicon, Temecula, CA, USA) at 4℃ overnight according to manufacturer's instructions. Then, the sections were incubated with fluorescent isothiocyanate (FITC) -conjugated rabbit anti-goat IgG antibody (Biosynthesis Biotechnology, Beijing, China) at 37℃ for 1h. Omission of primary antibodies was used as controls. The specimens were analyzed with a Zeiss LSM 510 laser scanning confocal microscope (LSCM; Carl Zeiss AG, Oberkochen, Germany). Signals were collected through the dual-channel, first passage excitation wavelength of 488nm and emission wavelength of 515nm-525nm, and the second channel excitation wavelength of 405nm and emission wavelength of 420nm-470nm.

RESULTS

Biological Characteristics of HAE Cells More than half of the HAE cells adhered to the bottom of the culture plates in primary culture after 24h; those attached cells were round with good refraction. HAE cells grew rapidly after 3 days; that is, the cells entered the exponential growth phase. They fused together at about 5-7 days, then covered the flasks after about 10 days. Cells were flat, irregular polygons with abundant cytoplasm and round or oval nuclei (Figure 1A). The second passage HAE cells attached onto the lamella after 4 hours and fused together at 1-2 days (Figure 1B).

Histological Characterization of TELC Conventional HE staining of tissue sections showed that HAE cells formed a continuous single layer of epithelial cells at 2 days, with a

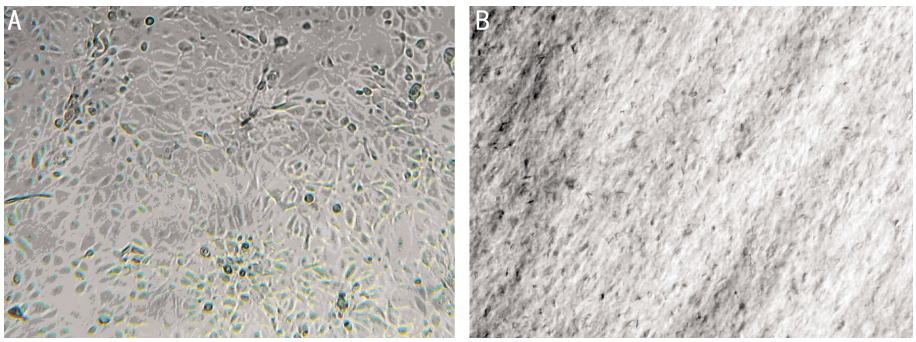

Figure 1 HAE cells were cultured in the flask and on the rabbit corneal stroma sheets A: Primary cultured HAE cells; B: HAE cells were seeded on the rabbit corneal stroma sheets (inverted microscope, ×100).

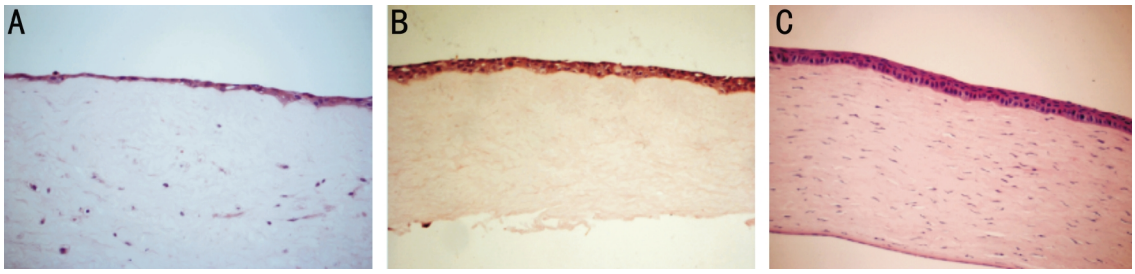

Figure 2 HE staining of the TELC and normal rabbit cornea A: HAE cells, cultured for 2 days, formed a monolayer on the corneal stroma while rabbit stromal cells were scattered throughout the corneal stroma sheet; B: HAE cells formed a stratified epithelium on the corneal stroma sheet; C: Normal rabbit cornea (HE×200).

number of scattered stromal cells in the corneal stroma (Figure 2A). After two weeks in the air-liquid interface culture, they formed a stratified epithelium in the inserted dishes. At the same time, almost all the rabbit stromal cells in the corneal stroma disappeared (Figures 2B). The reconstructed TELC *in vitro* therefore consisted of rabbit corneal stroma lamella and multi-layer epithelial cells formed from the HAE cells. A total of 4-5 layers of epithelial cells were continuously attached to the corneal stroma, which showed a cuboid or columnar-to-flat appearance similar to normal corneal epithelium and with similar polarity (Figure 2C). HAE cells were cultured at an air-liquid interface to reconstruct TELC for two weeks (Figure 3). Blank control lamella showed no epithelial cells growing on the lamella.

Ultrastructure of the TELC TEM showed that the multilayer epithelial cells on the corneal stroma had rich organelles, oval nuclei and clear nuclear membranes. Desmosome connections were found among the cells (Figure 4A), as well as hemidesmosome connections between the cells and the basement membrane (Figure 4B).

No epithelial cells were found on the denuded stroma lamellae, but SEM detected cord-like bundles of collagen fibers (Figure 5A). Cells on the corneal stroma of TELC displayed polygonal, irregular shapes, with SEM showing growth among the cells and rich microvilli on the cell surface (Figure 5B).

Immunohistochemical Characterization of TELC TELC stained positive for the corneal epithelium-specific keratin 3 and keratin 12, as detected by immunohistofluorescence, which showed green fluorescence with DAPI-stained blue nuclei (Figure 6). The control group was negative.

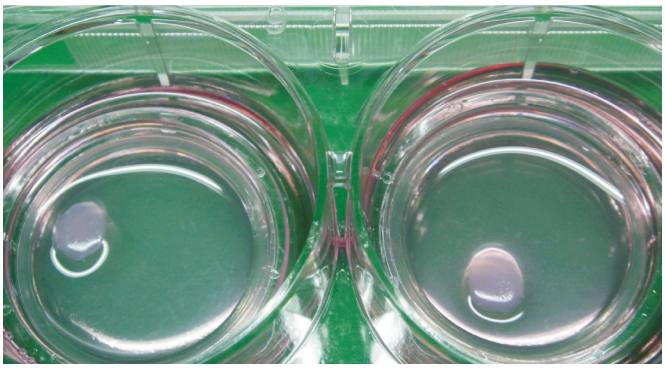

Figure 3 HAE cells were cultured on rabbit corneal stroma in the inverted dishes to establish an air-liquid interface culture.

DISCUSSION

Obtaining sufficient corneal donors remains problematic in many countries, especially in China. As a result, many ophthalmologists have turned to tissue engineering corneas. The key to tissue engineering corneas is using the proper combination of seed cells and carrier material. Scientists had already found that limbal stem cells, oral mucosa epithelial cells, dental pulp stem cells and skin stem cells may be good candidates for tissue engineering corneal cells. Corneal stem cells located in the limbal basal epithelium with little differentiation, a long life cycle, high proliferative potential and self-renewal capacity, making them an obvious source of corneal epithelial cells. Because of the limited number of stem cells available and the importance to the cornea, it is difficult to obtain a sufficient number of autologous limbal stem cells. In addition, limbal stem cell culture requires

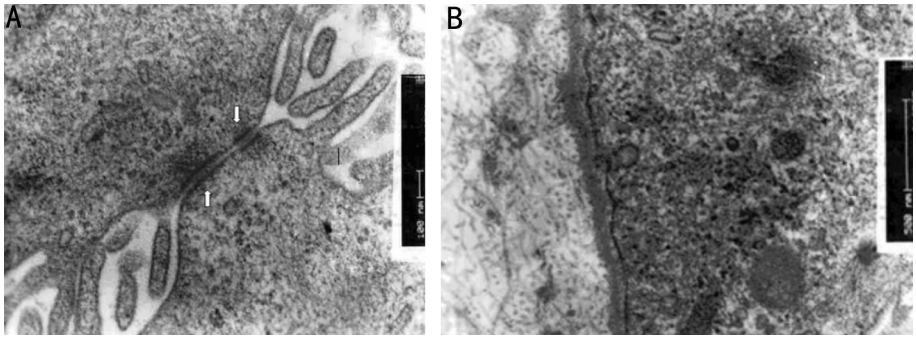

Figure 4 The inner ultrastructure of the TELC A: Desmosomes (arrows) formed between the epithelial cells on the TELC (TEM, bar = 100nm); B: Hemidesmosomes formed between the epithelial cells and the basement membrane (TEM, bar =500nm).

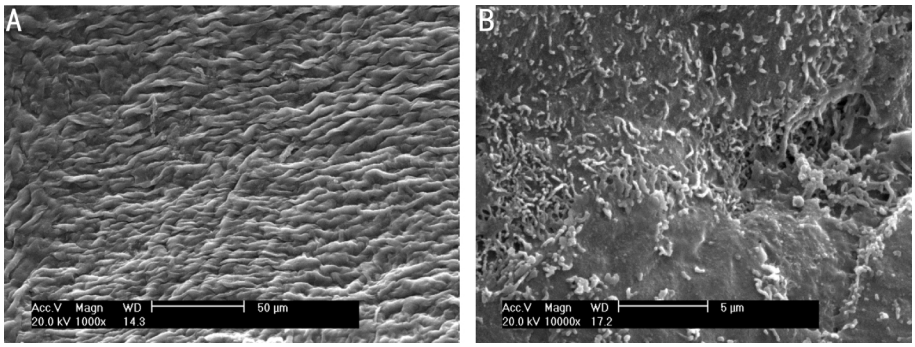

Figure 5 The surface ultrastructure of the denuded stroma lamellae and the TELC A: Bundles of collagen fibers, but not epithelial cells, were found on the denuded stroma lamella sheets (SEM×1 000, bar=50μm); B: The surface of HAE cells, with rich microvilli (SEM× 10 000, bar =5μm).

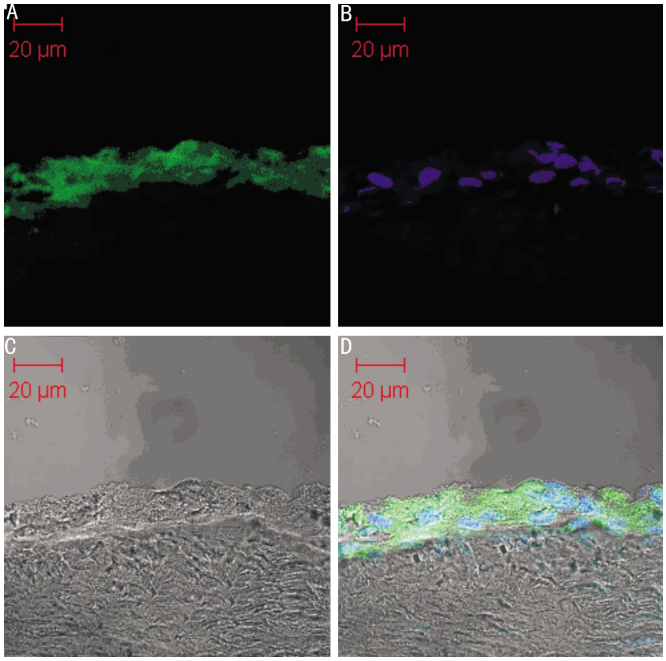

Figure 6 After culture for 2 weeks, epithelial cells cultured on the rabbit corneal stroma expressed keratin 3 and keratin 12, as detected by immunofluorescence (laser confocal microscopy, bar=20μm) A: Keratin 3 and keratin 12 in the HAE cells plasma; B: DAPI labeled HAE cells nuclei; C: TELC under the LSCM without laser emission; D: Stacked pictures of A to C.

sophisticated, expensive technology to accomplish. Once the cell culture fails *in vitro*, both patients and doctors suffer keen disappointment, in addition to time lost. Other types of seed cells have their own technical drawbacks. Therefore, the need is pressing to find a more ideal type of seed cell by which to construct tissue-engineered cornea.

Parmar *et al* [4] reported that he cultured HAE cells which seeded onto collagen corneal shields for 7 days when transplanted using a collagen shield supported by a bandage contact lens. In this way, he successfully treated three eyes with persistent corneal epithelial defects. HAE cells come from the epiblast as soon as 8 days after fertilization and before gastrulation, so they are likely to retain the plasticity of pregastrulation embryo cells. HAE cells express surface markers normally present on embryonic stem cells and the pluripotent stem cell-specific transcription factors octamer-binding protein 4 and nanog[2].

HAE are immunologically privileged cells that do not express human leukocyte antigen markers under normal conditions, making them ideal candidates for transplantation [3,5]. In different microenvironments and with different growth factors, HAE cells have been made to differentiate into liver cells, glial cells, neurons, hair follicles and skin epithelial cells[6-9].

The different ingredients of the tissue microenvironment are closely related to cell differentiation and development. Corneal stroma, the important microenvironment of corneal epithelial cells, can induce non-cornea cells on the corneal surface to transdifferentiate into corneal epithelial cells [10].

Fresh rabbit corneal stroma lamella was used as scaffolding for HAE cells. Corneal stroma is composed of the extracellular matrix, basement membrane and Bowman layer. The complete natural corneal stroma has growth factors which produce complex signals that regulate cell growth, proliferation and differentiation. Under physiological conditions, the corneal epithelium is exposed to the air, so the air-liquid interface plays an important role in corneal epithelial cell growth and differentiation [11]. The air-liquid interface culture simulates the corneal epithelial cell environment; in our experiment, it was seen to promote HAE cell stratification. While the lamellae floated on the surface of the medium, their 4.0μm diameter hole facilitated the exchange of material across the medium, so that the surface cells grew when exposed to the air in conditions which mimicked the normal growth environment of corneal epithelial cells. This environment helped encourage cell differentiation and maturation, as well as basement membrane formation.

HAE cells as seed cells were cultured on fresh rabbit cornea lamella carrier to construct an effective TELC *in vitro*. The experiments showed that cultured HAE cells can amplify and proliferate on the denuded corneal stroma, to form a 4- to 5-layer epithelium with desmosomes between the epithelial cells and hemidesmosomes between the epithelial cells and the basement membrane. The reconstructed tissue engineered cornea epithelium was similar to normal cornea in structure and morphology. The induced HAE cells expressed corneal epithelial cell-specific keratin 3 and keratin 12; that is, the TELC exhibited corneal-specific function. It was interesting to note that rabbit corneal stroma cells gradually disappeared during the culture period *in vitro* which coincided with the removal of corneal stromal cells to construct an acellular corneal stroma scaffolding on which to tissue engineer new cornea. Why did the corneal stromal cells disappear? It was not clear that the stromal cells gradually went into apoptosis. The mechanisms of this process have yet to be determined.

HAE cells and fresh rabbit corneal stroma can be used to construct a corneal lamella graft with similar morphology and function as human cornea. HAE cells may be a potential seed cell and rabbit corneal stroma may be a promising support material for tissue engineering of corneal reconstructions in lieu of corneal transplants when such are in short supply.

REFERENCES

1 Yuan J, Chen JQ. Strengthen the research on corneal transplantation immunology. *Ophthalmol CHN* 2009;18(6):370-373

2 Miki T, Lehmann T, Cai H, Stolz DB, Strom SC. Stem cell characteristics of amniotic epithelial cells. *Stem Cells* 2005;23(10):1549-1559

3 Akle CA, Adinolfi M, Welsh KI, Leibowitz S, McColl I. Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. *Lancet* 1981;2(8254):1003-1005

4 Parmar DN, Alizadeh H, Awwad ST, Li H, Neelam S, Bowman RW, Cavanagh HD, McCulley JP. Ocular surface restoration using non-surgical transplantation of tissue-surgical transplantation of tissue-cultured human amniotic epithelial cells. *Am J Ophthalmol* 2006;141(2):299-307

5 Adinolfi M, Akle CA, McColl I, Fensom AH, Tansley L, Connolly P, Hsi BL, Faulk WP, Travers P, Bodmer WF. Expression of HLA antigens, beta 2-microglobulin and enzymes by human amniotic epithelial cells. *Nature* 1982;295(5847):325-327

6 Takahashi N, Enosawa S, Mitani T, Lu H, Suzuki S, Amemiya H, Amano T, Sakuragawa N. Transplantation of amniotic epithelial cells into fetal rat liver by in utero manipulation. *Cell Transplant* 2002;11(5):443-449

7 Sakuragawa N, Thangavel R, Mizuguchi M, Hirasawa M, Kamo I. Expression of markers for both neuronal and glial cells in human amniotic epithelial cells. *Neurosci Lett* 1996; 209(1):9-12

8 Okawa H, Okuda O, Arai H, Sakuragawa N, Sato K. Amniotic epithelial cells transform into neuron-like cells in the ischemic brain. *Neuroreport* 2001;12(18):4003-4007

9 Fliniaux I, Viallet JP, Dhouailly D, Jahoda CA. Transformation of amnion epithelium into skin and hair follicles. *Differentiation* 2004;72 (9-10): 558-565

10 KurpakusM A, Stock EL, Jones JC. The role of the basement membrane in differential expression of keratin proteins in epithelial cells. *Dev Biol* 1992;150(2):243-255

11 Koizumi N, Cooper LJ, Fullwood NJ, Nakamura T, Inoki K, Tsuzuki M, Kinoshita S. An evaluation of cultivated corneal limbal epithelial cells. using cell-suspension culture. *Invest Ophthalmol Vis Sci* 2002;43 (7): 2114-2121