CHAPTER III

MATERIALS AND METHODS

1. Isolation and Cultivation of Limbal Epithelial Cells

Human limbal tissues were received from Department of Ophthalmology, Thai Red Cross after corneal removal for transplantation. The tissue was washed in Mg^{2+} and Ca^{2+} free phosphate buffer saline (1X PBS) containing 1% Penicillin-Streptomycin (GIBCO) and 2.5 mg/L amphotericin B. After carefully removal of excessive sclera and conjunctiva, the limbal ring was incubated in dispase II (1.2 IU/mL in 1X PBS) at 37°C under humidified 5% CO_2 for 1 hour. After that, limbal ring was rinsed with Dulbecco's modified Eagle's medium (DMEM):Ham's F12 (1:1), supplemented with 10% fetal bovine serum (Hyclone), the loosened limbal tissue was cut into 2x2 mm^2. Then the cut limbal explants were put on the center of the 2 $\mu g/cm^2$ laminin coated 6-well plate and cultured at 37°C with 5% CO_2 and humidified atmosphere in limbal complete media: 1:1 mixture of Dulbecco modified Eagle medium (DMEM) and Ham F-12 containing 10% fetal bovine serum USA grade, 1% Penicillin-Streptomycin, 2.5 mg/L amphotericin B, 1% L-glutamine, 0.5 mg/L Hydrocortisone, 2.5 mg/ml NaHCO3, 10 ng/mL epidermal growth factor (EGF), (R&D) and 5 mg/L Insulin (Invitrogen). Culture Media was changed every 1-2 days. After limbal epithelial outgrowth from limbal explants on laminin for 5 days, limbal epithtlial cells were dissociated into single cells by using 0.05%Trypsin-EDTA (invitrogen). Numbers of cells were counted under an inverted microscope (Nikon, Japan) before sub-cultivation for colony formation and further clonal analysis.

2. Isolation and Cultivation of Limbal Stromal Cells

After the limbal ring was incubated in dispase II as described previously, loosened epithelial sheets were removed with a cell scraper. The remaining stromal tissue was cut into 2x2 mm^2 and cultured on fibronectin coated plate at 37°C under humidified 5% CO2 in limbal complete media. Medium was changed every 2 days. Upon reaching 70% to 80% confluence, the outgrowing limbal fibroblasts from limbal stromal

tissue were sub-cultured to the next passage on fibronectin for serial propagation and further analysis.

3. Preparation of 3T3 Fibroblasts

3T3 cell lines were purchased from American Type Culture Collection (ATCC). 3T3 were cultured in DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin (GIBCO) and incubated at 37°C in humidified incubator with 5% CO2 in air. Media were changed every 2-3 days and passage by trypsinization when they reached 70-80% confluent. For colony forming, confluent 3T3 fibroblasts were treated with 4 µg/mL mitomycin C (MMC) in culture madia and incubated for 2 hours at 37°C under 5% CO_2. Then cells were trypsinised and plated into cell culture dishes at 2.2 x 10^4 cells/cm^2.

4. Preparation of BMP and BMP antagonist (Noggin) overexpression in 3T3

BMP4 and Noggin plasmid were purchased from ORIGENE. Construction of BMP4 and Noggin expression vector was generated by ligation of coding sequence fragments cut with *EcoRI* and *XbaI* restriction enzyme into pcDNA3.1/myc-HIS B. In order to generate 3T3-BMP4 and 3T3-Noggin, 3T3 cells were transfected with each expression vector using FuGENE® HD Transfection Reagent (ROCHE) according to manufacturer's instruction. Next day, media was changed to completed media and cultured for another one day. Then cells were trypsinized and seeded on 10 cm dish in completed media supplemented with 600 µg/mL geneticin, only transfected-3T3 cells could grow in Geneticin containing media. Geneticin resistance colonies were selected and expanded as 3T3-BMP4 and 3T3-Noggin. 3T3-BMP4 and 3T3-Noggin selected clones were tested for gene expression levels by Semi-quantitative RT-PCR and Western blot before use as feeder cells.

5. Colony Forming Assay

Single cells were seeded at density of 300-500 cells into multi-well plates or culture dishes containing MMC-treated 3T3. For protein treatments such as TGF-β1 (R&D systems), TGFβ-inhibitor SB431542 (Stemgent), BMP2 and Noggin (R&D systems) were added at the first day of seeding. After 7-10 days of cultivation, limbal colonies were identified and photographed under an inverted microscope (Nikon, Japan), their diameters were measured by Axiovision Program, Fluorescence Microscope (Carl Zeiss, Oberkochen, Germany). Colonies on culture dish were fixed 12 days later in 4% paraformaldehyde and stained with rhodamine B for colonogenic capacity determination. One colony contained at least 30 cells. Colonies were counted under an inverted microscope and determined the colony-forming Efficiency (%CFE).

$$\%CFE = \frac{\text{number of colonies}}{\text{number of cells seeded}} \times 100\%.$$

6. RNA Extraction

Limbal colonies in each culture system were picked at day 10 and lysed by using TRI reagent (Molecular Research Center; MRC). The lysate was homogenized by pipetting and incubated at room temperature for 5 minutes, 0.1 mL of BCP (Molecular Research Center; MRC) was added to the lysate and shaked vigorously for 15 seconds. The resulting mixture was stored at room temperature for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes at 4°C. After that, the colorless upper aqueous phase (RNA containing solution) was transfered to a fresh tube. RNA was precipitated by mixing with 0.5 mL isopropanol and stored at -20°C overnight. Next day, sample was centrifuged at 12,000 g for 8 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol and subsequent centrifugation at 7,500 g for 5 minutes at 4°C. The ethanol was removed and the RNA pellet was air-dry for 5-10 minutes. RNA was dissolved in the RNA storage solution (Ambion). RNA concentration was measured by Nanodrop spectrophotometer (Thermo Scientific, U.S.A.).

7. Complementary DNA (cDNA) Synthesis

Isolated RNA was reverse transcribed by using RevertAid™ H Minus M-MuLV (Fermentus) according to the manufacturer's instruction. Briefly, 1 µg of total RNA in 11 µL of DNase/Rnase free water was incubated with 1 µL of 0.5 µg/µL Oligo dT at 70°C for 5 minutes and incubated on ice. Then, 7 µL of mastermix containing 4 µL of 5X reaction buffer, 2 µL of 10mM dNTP and 1 µL of RiboLock™ RNase Inhibitor (20 u/µL) was added into RNA template tube and incubated at 37°C for 5 minutes. Finally, 1 µL of RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 u/µl) was added into reaction tube and cDNA was transcribed at 42°C for 60 minutes. The reaction was terminated by incubating at 70°C for 5 minutes. Complementary DNA was kept at -20°C until used for measuring gene expression by real-time PCR.

8. Semi-quantitative RT-PCR Analysis

Transcript levels were assessed by PCR using specific primers for human BMP4 (Appendix A), GAPDH was used for normalization. Thermal cycling conditions consisted of the following: denaturation at 94°C for 3 minutes, followed by 35 cycles each at 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds and a final extension at 72°C for 1 minute to terminate reactions. Ethidium bromide–stained agarose gels were visualized with Gel Documentation System (Bio-Rad, U.S.A.).

9. Quantitative Real-Time PCR Analysis

The mRNA levels were measured by an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Singapore) in a final volume of 25 µL. Each reaction was performed using reagents from Maxima SYBR (Fermentus), with 0.3 µM of primer, and 2 µL of cDNA. Amplification consisted of 40 cycles of denaturation at 94°C for 10 seconds, annealing at 50°C - 60°C, depending on the primer pair, for 30 seconds, and extension at 72°C for 40 seconds. Appendix A. shows the sequence of primer pairs (purchased from Operon, Hawthorne, NY USA). After the processes were completed, the real-time PCR results were automatically reported by Applied Biosystme software version 7500 and analyzed by relative quantification method (comparative Ct method).

10. Western Blot

Nuclear and cytoplasmic proteins were extracted from 3T3 and 3T3-Noggin feeder cells by using Mammalian Protein Extraction Reagent (M-PER®) (PIERCE, Thermo) according to the manufacturer's instruction. Total protein concentration was measured by spectrofluorometer (Thermo Labsystems, U.S.A.). Protein 30 μg was electrophoresed on 12% SDS-polyacrylamode gel at 150 Volt for 60 minutes and transferred to nitrocellulose at 150 Volt for 60 minutes. To detect Noggin protein, nitrocellulose was blocked with 5% non-fat dry milk in PBS for 1 hour at room temperature with constant agitation, and incubated with goat anti-noggin antibody (Santa cruz), diluted in 5% non-fat dry milk in TTBS (0.2% Tween 20 in Tris buffer saline) (1:200) at 4°C for overnight. Next day, the nitrocellulose was washed with TTBS for 5 min three times and incubated with donkey anti-goat HRP conjugated in 5% non-fat dry milk in TTBS (1:2000) for 60 min at room temperature with constant agitation. Finally, the nitrocellulose was visualized by chemiluminescence. β-actin protein level was used as a control for equal protein loading. For β-actin protein detection, total protein was electrophoresed on 12% SDS-polyacrylamode gel at 150 V for 60 min and transferred to nitrocellulose at 150 Volt for 60 min. After blocking with 5% non-fat dry milk in PBS, nitrocellulose membrane was incubated with mouse anti-mouse β-actin diluted in 5% non-fat dry milk in TTBS (1:500). Subsequently, the nitrocellulose was incubated with goat anti-mouse HRP conjugated IgG in 5% non-fat dry milk in TBST and visualized by chemiluminescence.

11. Immunofluorescence Staining

The immunofluorescence staining was performed to evaluate the expression and the location of different molecular markers that have been proposed to identified LESCs or differentiated cells. In brief, limbal colonies on each 3T3 feeder system plate were fixed with 4% formaldehyde for 20 minutes at room temperature and then permeabilized with 1X PBS supplemented with 0.3% Triton X-100 for 15 minutes, and blocked in blocking buffer (10% goat serum and 0.3% Triton X-100 in PBS) for 30 minutes at room temperature. After each step, cells were washed twice with PBS for 5 minutes to

decrease nonspecific antibody interaction. Primary antibodies were diluted in diluents (5% goat serum, 0.3% Triton X-100 [Sigma] in PBS) and incubated overnight at 4°C in humidified conditions. Cells were washed with PBST (PBS + 0.3% TritonX-100) supplemented with 0.1% BSA three times for 5 minutes. Secondary antibodies, Alexa Flour 488 (green)/546 (red) conjugated goats anti mouse antibody (Molecular Probes) or goat anti rabbit antibody (Molecular Probes), were also diluted in diluents at dilution 1:200 and incubated for 1 hour at room temperature in dark. For nuclear staining, 0.1 µg/mL DAPI (Molecular Probes) was used and incubated for 5 minutes at room temperature. Fluorescence images were obtained using Fluorescence microscope (Carl Zeiss, Germany) and Confocal microscope (Nikon, Japan). Antibodies: rabbit anti-human ΔNp63α (Cell Signaling) and goat anti-human noggin 1:50 (Santa Cruz).

12. Statistical Analysis

Statistical analysis was performed using the SPSS software (version 13). Statistical significance was assayed by Student's t-test in normal distribution data. The results were considered significant if $p < 0.05$.