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Conditional reprogrammed human limbal epithelial cells represent a novel in vitro cell model for drug responses

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ABSTRACT

In this study, we established human limbal epithelial cells (LECs) from normal limbal tissues by using Conditional Reprogramming (CR) technology (refer to CR-LEC cells in this study). We have successfully established CR-LEC cell strains from three human donors (3 out of 3), and normal rabbits (2 out of 2) and pig (1 out of 1) as well. CR-LEC cells sustained a continuous and stable proliferation status with a normal karyotype, normal response to DNA damage, well-defined structured spheres in matrigel 3D culture. Responses of CR-LEC cells to IFN α2b, Ganciclovir and 5-Fluorouracil were different, suggesting that these drugs had different toxicities to these cells as expected. More important, there was no significant difference of responses to drugs between early and late passages of CR-LEC cells ($p>0.05$), indicating CR-LEC cells can serve a stable normal human cell model for toxicity assessment. Toxicity tests with monolayer cultures of CR-LEC cells were measured by staining the F-actin and Dsg-1 expression. Toxicity of three drugs at LD50 concentration resulted in a gradually increased destruction of monolayer, which is, in accordance with the irritation grade of three drugs on human cornea epithelium. Therefore, CR-LEC cells provide a novel and reliable in vitro physiological cell model for corneal toxicity assessment.

1. Introduction

Eye drops and eye ointment are commonly used in clinical trials of eye diseases. Corneal epithelial cells are the primary targets exposed to the potential toxic effects of the applied drugs. To assess drug toxicity, several human corneal cell models have been developed, including immortalized human cornea epithelial cell lines and primary corneal epithelial cells [1,2].

Immortalized human cornea epithelial cell lines are generated by transduction of viral genes (e.g., HPV16-E6/E7, recombinant retrovirus, recombinant SV-40-adenovirus) or hTERT [3,4]. However, the abnormal levels of proteases result in p53 and pRb signal pathways blocked and oncogenicity in the immortalized cells [5,6]. These viral or cellular gene-immortalized corneal epithelial cells do not faithfully represent the physiological status in vitro and in vivo. Due to difficulty of establishment of initial cultures, there are still few cell lines available for the field in decades.

Primary human corneal epithelial cells under several conditions have also been used in drug toxicity assessment [7]. However, the short lifespan of these primary corneal epithelial cells limits their use in vitro [8]. Limbal epithelial cells (LECs) are the progenitor cells of corneal epithelium. LECs proliferate, differentiate and centripentally migrate to ensure corneal epithelium regeneration and integrity during homeostasis and injuries [9]. It has been reported that eyedrops induce limbal epithelial cell deficiency in a patient receiving 5-Fluorouracil in glaucoma surgeries [10]. Long-term use of preservative-containing topical drugs can be a potential risk for many corneal epithelium disorders due to the cellular toxicity [11]. Antibiotics delay stem cell regeneration and cause distortions in chromatin structure in vitro [12]. The severe effect of drug toxicity on limbal epithelial cells may eventually lead to blindness.

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Therefore, human limbal epithelial cells could be considered as the most relevant physiological system for corneal toxicity assessment.

Conditional reprogramming (CR) technique allows to establish long term cultures of normal epithelial cells without transduction of exogenous genes by using irradiated mouse fibroblast cells (feeder) and ROCK inhibitor [5,13]. Compared to all approaches discussed above, the CR method is more robust and sufficient to establish primary cultures and long term expansion [5,13]. These CR epithelial cells may meet the need for developing a human normal corneal epithelial cell model with long lifespan and physiological function for toxicity assessment.

In this study, we established primary human normal limbal epithelial cells (LEC) by CR technique. The CR-LEC cells possess the normal genetic and cellular characteristics in vitro, and express the corneal epithelial specific markers and retain the lineage of originated limbal tissue. Evaluations of its potential applications to drug toxicity were performed by exposing to three types of ocular topical drugs. CR-LEC cells provide a novel and reliable in vitro human limbal epithelial cells-based model for drug toxicity assessment.

2. Materials and methods

2.1. Cell isolation

Normal human limbal tissues (1mmx2mm) were separated carefully from the temporal side of the upper conjunctival flap used for transplanting in the pterygium surgery. Uncontaminated limbal epithelium was minced and dispersed into single cells by digestion with collagenase (Stem Cell Technoligies Inc, Vancouver, BC, Canada) plus trypsin. Primary limbal epithelial cells were co-cultured using primary epithelial culture basic medium (PECBM, ImmorTech, Shenzhen, China) with feeder cells or cultured in primary epithelial culture medium (PECM, ImmorTech, Shenzhen, China) without feeder cells at 37 °C with 5% CO_2. The cell growth curve was plotted as accumulated population doublings versus time (days). The research followed the tenets of the Declaration of Helsink. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study. The research was approved by the Institutional review boards at Shenzhen Eye Hospital and Wuhan University Shenzhen Institute.

2.2. STR analysis

Cellular genome DNA of human limbal epithelial cells (LECs) was isolated with the kit (Tiangen, Beijing, China). Short tandem repeat (STR) analysis (DNA fingerprinting) was performed by commercial kit (Powerplex21 system; Promega Corporation, Madison, WI). Co-amplification and three-color detection of 22 loci (21 STR loci and a couple of X-chromosome-specific Amelogenin loci) were recognized. The PCR amplification was achieved on the basis of the manufacture's recommended protocol with the ABI 3100 genetic analyzer (Thermo Fisher). Data analysis and allele size determination were performed using Genotyper and PowerTyper16 Macro Software (Applied Biosystems).

2.3. Karyotype analysis

Exponentially growing CR-LECs were treated with 0.2 μg/ml Colchicines (C9754, MERCK) for 3 h. Cells were then collected and continued with hypotonic treatment and fixation. Metaphase spreads were prepared and stained to observe chromosome. Twenty metaphase spreads were analyzed and photographed under the microscope.

2.4. In vitro three-dimensional differentiation

Single-cell suspensions of CR-LEC cells were embedded in matrigel (BD Biosciences) at 1×10^4 cells per 100 μl gel in a 4-well chamber. Three-dimensional structures were formed after 9 days in a differentiation medium (keratinocyte growth medium, Life Technologies Corporation, California, USA) containing 5% matrigel. Then the morphogenesis assays were performed as previously described[15, 16].

2.5. Hematoxylin and Eeosin (H&E) staining

Normal limbal tissue was fixed in 4% paraformaldehyde, dehydrated through a stepwise series of ethanol organic solutions and paraffin-embedded. Paraffin blocks were then successively sectioned in 5 μm thickness and mounted on the glass slides. The paraffin-embedded sections were stained with Hematoxylin and Eeosin(H&E) (Zhongshan Golden Bridge Company, Beijing, China). Morphological observation of H&E-stained sections were photographed under the EVOS visual imaging microscope (Life Technologies Corp Bothell, WA, USA).

2.6. DAB staining (amplifer polymer)

The DAB staining was performed using acommercial kit called DAB Detection Kit (EliVision Super DAB, Maixin Biotech Company, Fuzhou, China). After paraffin sections of limbal tissue solvent-dewaxing and treatment with microwave antigen retrieval, endogenic peroxidase blocker were added at room temperature for 10 min and the primary antibodies mouse anti-p63 (1:100, ab735, Abcam), mouse anti-Cytokeratin 19 (1:100, ,ab7754, Abcam), mouse anti-Cytokeratin 3/12 (1:100, ab68260, Abcam) were respectively incubated on the slides at room temperature for an hour. The slides were then added the reaction-amplified reagent for 20 min and conjugated with high-sensibility enzyme conjugated lgG polymer. Reactants were visualized with the fresh-prepared DAB chromogenic solutions for 3–5 min. Hematoxylin somatic cell staining reagent was used to counterstain nuclei for 8 min. Glass slides were finally mounted with neutral balsam and visualized under Leica DM4000B fluorescence microscope.

2.7. Immunofluorescence assay

CR-LEC Cells were fixed in 4% (w/v) paraformaldehyde and permeabilized with 0.5% Triton X-100. Primary antibodies (1:100, mouse anti-p63, ab735, Abcam; 1:100, mouse anti-Cytokeratin 19, ab7754, Abcam; 1:100, mouse anti-Cytokeratin 3/12, ab68260, Abcam) were used according to manufacturer's protocols. DAPI (0.5ug/ml, D3571, Thermo Fisher) was used to stain the nucleus. Then the fluorescence was detected by Leica DM4000B fluorescence microscope.

2.8. Taqman assay and Q-PCR

hTERT mRNA expression in CR-LEC cells was assayed by quantitative real-time PCR. Total RNA was extracted with TRIzol® reagent (Invitrogen, USA) according to the manufacturer's specifications. The real-time PCR procedure was set, and hTERT mRNA was measured using the methods and primers that have been previously published[16]. The primers are p63 Forward 5′-GAA ACG TAC AGG CAA CAG CA-3′, Reverse 5′-GCT GCT GAG GGT TGA TAA GC-3'; cytokeratin 19 Forward 5′-TGA GTG ACA TGC GAA GCC AAT-3′, Reverse 5′-ACC TCC CGG TTC AAT TCT TCA-3'; cytokeratin 12 Forward 5′-TGG TCA TGT TGG TCT TTG TAA C-3′, Reverse 5′-ACT TCT CTC TAT GCT CTT GAC A-3'.

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