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# Novel phototoxicity assay using human embryonic stem cell-derived retinal pigment epithelial cells

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ABSTRACT

Some chemicals are harmful in to light-exposed tissues such as skin and eyes. The 3T3 Neutral Red Uptake Phototoxicity Test has been validated and adopted by the Organization of Economic and Community Development (OECD) as a method of evaluating chemical phototoxicity using mouse 3T3 fibroblasts. However, the high rate of false positive results associated with this test eventually led to increased laboratory animal usage. Although the eye is vulnerable to light damage because of constant exposure to environmental radiation, few approaches are available to predict ocular phototoxicity in humans. Here, we propose a tier one test that identifies the potential ocular phototoxicity of chemical substances. Using a three-dimensional culture technique, human embryonic stem cells (hESCs) were differentiated to retinal pigment epithelial cell (RPE) precursors. The precursors after prolonged treatment with FBS formed a uniform hexagonal lattice of cells with well-developed tight junctions and time-dependent elevation of melanin content and RPE maturation marker levels. Hierarchical clustering of gene transcripts revealed that hESC-derived RPEs were very similar to tissue-derived adult RPEs. Interestingly, there were a high percentage of chemicals eliciting a positive response in 3T3 cells and negative in hESC-derived RPEs under the experimental conditions used in the phototoxicity test. The response to treatment of hESC-derived RPEs with these negative chemicals became positive at a higher dose of UVA irradiation; however, the biological responses to these chemicals differed between the two cells. Taken together, we conclude that hESC-derived RPEs are novel tool for future toxicological and mechanistic studies of ocular phototoxicity in humans.



## 1. Introduction

Exposure to sunlight has been shown to effect photochemical change in biological systems. The photochemical reactions of pharmaceuticals and cosmetics are a key trigger of phototoxicity in to light-exposed tissues such as skin and eyes (ICH, 2013). The 3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU-PT) has been validated and adopted by the Organization of Economic and Community Development (OECD) as its test for phototoxicity (Spielmann et al., 1994). Although the 3T3 NRU-PT test (which uses the mouse embryo fibroblast cell line 3T3) is considered to be easily performed and sensitive, it has yielded high rates of false positive results (Lynch and Wilcox, 2011). Factors affecting the performance of the 3T3 NRU-PT include its high Photo Irritancy Factor (PIF) and reduced maximum test concentration (Ceridono et al., 2012). In addition, it has been shown that human reconstructed epidermis can detect the phototoxic effects of topical or systemically applied chemicals (Lelièvre et al., 2007).

The eye, after skin, has the highest vulnerability to light damage because of its constant exposure to environmental radiation. Chronic exposure to intense light and/or age-related changes can lead to light-induced eye damage (Roberts, 2001). Although numerous studies have reported that certain chemicals induce skin phototoxicity, it remains unclear whether these chemicals also exhibit ocular phototoxicity. Though several animal tests have been developed to evaluate the ocular phototoxicity of chemicals (Shimoda et al., 1993; Adachi et al., 2015), there is still an urgent need for reliable *in vitro* human cells-based ocular phototoxicity assays to avoid errors in interpretation due to species differences, and to reduce animal use.

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Light damages the retina through thermal, mechanical, or photochemical mechanisms. Since retinal pigment epithelial cells (RPEs) are postmitotic after birth, damage to these cells can lead to early retinal degeneration and thereby to transient or permanent loss of vision (Wielgus et al., 2010). The two most commonly used *in vitro* models of human RPEs are human fetal RPEs and the RPE cell line ARPE-19. A previous study has shown that properties of fetal RPEs are similar to those of functional tissue-derived RPEs, while ARPE-19 cells resemble pathologic or aged RPEs (Ablonczy et al., 2011).

Various types of human cells that are structurally and functionally similar to tissue-derived cells have been produced from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs). We recently developed a three-dimensional culture technique to induce hESCs to retinal progenitors, which give rise to neural retina and RPEs (Nakano et al., 2012; Kuwahara et al., 2015). In addition, we demonstrated that the inhibition of glycogen synthase kinase 3 and fibroblast growth factor receptor could induce the transition from retinal progenitors to RPE fate (Kuwahara et al., 2015). Furthermore, we have recently demonstrated hESC-derived neural retinal tissue has a potential to differentiate into mature photoreceptors with outer segments in primate models (Shirai et al., 2016). These findings allowed us to prepare large amounts of highly pure retinal progenitors derived from hESCs. In this study, we at first optimized culture protocol to differentiate retinal progenitors to expand RPEs. We then characterized hESC-derived RPEs, and examined whether these cells could be used in the phototoxicity assay. Based on the results of the present study, we concluded that hESC-derived RPE is a novel tool for studying ocular phototoxicology and ocular phototoxicity mechanisms in humans.

## 2. Materials and methods

### 2.1. Test chemicals

Bithionol, chlorpromazine, 8-methoxypsoralen, 6-methylcoumarin, rose bengal, chlorohexidine, dichlorophene, piroxicam, L-ascorbic acid, and penicillin G were purchased from Sigma-Aldrich (St. Louis, MO); ciprofloxacin hydrochloride, sodium diclofenac, and sodium lauryl sulfate were from Wako Pure Chemical (Osaka, Japan), and musk ambrette was from Tokyo Chemical Industry (Tokyo, Japan). The chemicals were dissolved in appropriate solvents, dimethyl sulfoxide, ethanol, or distilled water. The purity of all chemicals tested was more than 98.0%.

### 2.2. Generation of hESC-derived RPEs

hESC (KhES-1), which were established by Drs. Nakatsuji, N. and Suemori, H. (Kyoto University), were used following the hESC research guidelines of the Japanese government, and the protocol of this study was approved by the ethics committees of Sumitomo Chemical Co., Ltd. Undifferentiated hESCs were maintained and differentiated into retinal progenitors as previously described (Kuwahara et al., 2015). Briefly, undifferentiated hESCs on the mouse embryo fibroblast feeder cells were maintained in serum-free medium, and differentiated into retinal progenitors using the serum-free floating culture of embryoid body-like aggregates with quick aggregation (SFEBq) method in the presence of bone morphogenetic protein-4.

To induce RPEs, retinal progenitors at twenty-one days after differentiation were dissociated with accutase (Innovative Cell Technologies, San Diego, CA), and then plated as single cells onto plates coated with Synthemax (Corning, Corning, NY) and cultured in N2B27 medium (DMEM/F12 + GlutaMAX (Thermo Fischer Scientific, Waltham, MA), neurobasal medium (Thermo Fischer Scientific), 0.5xB27 supplement (Thermo Fischer Scientific), 0.5xN2 supplement (WAKO Pure Chemical), 0.1 mM β-mercaptoethanol and 0.2 mM L-glutamine) supplemented with 50 ng/ml Activin A. Four weeks later, hESC-derived RPE precursors were cryopreserved in CELLBANKER freezing medium (Nippon Zenyaku Kogyo, Fukushima, Japan) before use. Cryopreserved hESC-derived RPE precursors were thawed and seeded in N2B27 medium onto Synthemax-coated plates until the cells formed confluent monolayers, and then the spent N2B27 medium was changed to DMEM/F12 supplemented with 10% fetal bovine serum (FBS) for RPEs maturation.

### 2.3. DNA microarray analysis

The comprehensive gene expression analyses were performed using the GeneChip system as previously described (Higashi et al., 2014). Briefly, total RNA was extracted using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany), and cDNA for *in vitro* transcription was prepared. For primary cultured RPEs, total RNA was purchased from ScienCell Research Laboratories (Carlsbad, CA). Fragmented *in vitro* transcripts were hybridized overnight onto Human Genome U133 plus 2.0 microarrays, stained, washed, and scanned with an Affymetrix GeneChip Scanner 3000 7G. The obtained image files were analyzed with the Affymetrix data suite system. Hierarchical clustering analysis of 144 RPEs-specific genes was performed with GeneSpring GX 7.3.1 software (Agilent Technologies, Santa Clara, CA) using the gene expression microarray data from the National Center for Biotechnology Information Gene Expression Omnibus (accession nos. GSM466534, GSM248214, and GSE18811 for ARPE-19, dermal fibroblasts, and tissue-derived RPEs, respectively).

### 2.4. Real time RT-PCR assay

Expression of human *BEST1* was quantified using a TaqMan probe and TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA). The relative expression level was normalized to that of *GAPDH* gene in the same RNA preparation.

### 2.5. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min at 4 °C. After blocking, the cells were incubated with rat anti-ZO-1 antibody (Thermo Fisher Scientific). Localization of antigen was visualized using Alexa Flour 488 anti-rabbit IgG (Molecular Probes, Eugene, OR). Cryosections of hESC-derived RPEs at D7 on Transwell cell culture inserts (Corning) were stained with Hoechst 33258.

### 2.6. Melanin content

hESC-derived RPEs were dissociated with accutase, and the optical density of the cell pellet after dissolution in 0.5 M NaOH was measured at 450 nm. Melanin and protein contents were determined using purified melanin from *Sepia officinalis* (Sigma-Aldrich) and bovine serum albumin as a standard, respectively.

### 2.7. Phototoxicity test

The addition of 1000-fold excess of chemicals in appropriate solvents to Earle's balanced salt solution produced the desired final concentration. The hESC-derived RPEs and mouse 3T3 fibroblasts were treated in triplicate with several doses of chemicals for 1 h prior UVA exposure. Two plates were then exposed to UVA (5 or 20 J/cm$^2$) in the presence of chemicals using a solar simulator SXL-2500V2 (320–800 nm, Seric, Tokyo, Japan); one of the plates was kept in the dark by covering it with aluminum foil. To reduce the cytotoxicity of chemicals, the cells were washed and incubated in