

Comparison of Gene Expression Profiles of Conjunctival Cell Lines With Primary Cultured Conjunctival Epithelial Cells and Human Conjunctival Tissue

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Human conjunctival cell lines are useful tools for modeling ocular surface disease and evaluation of ocular drugs and cosmetics. However, gene expression in these cells may not be comparable to primary cultured cells, raising doubts that they could be used as a substitute. We aimed to ascertain the similarities of global gene expression between commonly used cell lines and primary cells using a microarray approach. The Affymetrix U133A chip (>22,000 genes) was used to investigate conjunctival tissue (CT), primary conjunctival epithelial cells (PCEC), two conjunctival epithelial cell lines (IOBA-NHC and ChWK), and HCEC-T, a human corneal epithelial cell line (control). Using principal component analysis, the PCEC profile was clustered more closely to conjunctival tissue than either of the two cell lines. Certain extracellular matrix genes were differentially upregulated in CT compared to PCEC, suggesting presence of fibroblasts in addition to epithelial cells in CT. Overall, 67.3% (95% CI: 66.7–67.9) of transcripts in IOBA-NHC were within 1.5-fold of the corresponding transcripts in PCEC, but only 62.2% (95% CI: 61.5–62.9) in the case of ChWK. In HCEC-T, the proportion was only 58.8% (95% CI 58.1–59.4), suggesting less resemblance to PCEC than the conjunctival epithelial cell lines. The IOBA-NHC profile was more similar to PCEC than ChWK, for all genes and genes concerned with membrane association, communication, development, and regulation of metabolism, especially protein and nucleic acid metabolism. The correlation of normalized gene expression levels was high between either the IOBA-NHC or ChWK and PCEC for genes concerned with cell defense, viral life cycle, antigen presentation, antioxidation, or ubiquitin ligation. In order to evaluate the functional significance of the altered gene expression in IOBA-NHC cells, we evaluated a few proteins important for epithelial differentiation or defense, corresponding to the transcripts for S100A9, TGM2, and TLR4. Protein levels of S100A9 and TGM2 were indeed raised, and TLR4 decreased, in IOBA-NHC compared to PCEC. Gene expression in conjunctival cell lines differs from primary cells, but the profile varies according to functional gene categories. Depending on the methodology of proposed studies, if there is limited availability of PCEC, NHC-IOBA may be more suitable than ChWK, but even then, epithelial differentiation and innate immunity functions in NHC-IOBA may differ from primary cells.

Key words: Human; Cell culture; Gene expression; Microarray; Ocular surface disease

INTRODUCTION

The use of cultured cells for the assessment of ocular surface irritation, instead of using laboratory ani-

mals, in the evaluation of cosmetics and the safety of commercial products is increasingly important from the ethical and economic points of view (20,42). Testing of human epidermal health using markers in cul-

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ured cells may be the only feasible way to rapidly screen compounds that may be allergenic (8) or pro-inflammatory (28). Apart from these considerations, culturing of cells is now an integral part of the armamentarium of techniques in ocular surface science. Cell culture as a laboratory tool for investigation of diseases has numerous advantages, including homogeneity of cell type, increased reproducibility, as well as definition and control of environmental/experimental parameters. Cell cultures (primary cells from native tissues and cell lines) have been used to study conjunctival pathology including dry eye (10,18,41), infection (44,48), inflammation (13,35), radiation (4), and drug toxicity (7,29). They are also useful for assessment of drug delivery (19,22) and understanding basic processes such as apoptosis (25) and ocular surface immunology (15,23,37).

In particular, ocular surface epithelial cell culture experiments may use primary cultured cells (55) or cell lines, including the Wong-Kilbourne derivative of the Chang cells (ChWK) (5,51) and the more recent University Institute of Applied Ophthalmobiology-Normal Human Conjunctiva (IOBA-NHC) cells (16). Primary cell cultures have the advantages of biological similarity to native tissue either normal or diseased (17), whereas in cell lines there are advantages of greater homogeneity, increased accessibility, and easy propagation, permitting more rapid, high throughput experiments. Although morphological and immunocytochemistry of ChWK (12) and IOBA-NHC (16) cells have been used to compare these cell lines to primary cultured conjunctival epithelial cells, the gene expression patterns of these cells have not been compared using an unbiased global gene expression approach. Here, we show, using a global gene analysis of these cell lines, primary conjunctival epithelial cells and native conjunctival tissue using a gene microarray approach, that ChWK and IOBA-NHC cells are suitable for modeling only selected biological functions of primary conjunctival epithelial cells.

MATERIALS AND METHODS

Cells, Materials, and Subjects

Human conjunctival tissue was obtained from the Pterygium Aetiology and Conjunctival Evaluation (PACE) study (52). This prospective and ongoing study involved patients undergoing surgical excision for pterygium. At the time of surgery, a small portion of the conjunctival patch (approximately 1.3 mm) was biopsied from an area of the superior bulbar conjunctiva uninvolved with the disease. This was rapidly frozen in liquid nitrogen after removal and stored at -150°C . The study was approved by the Institu-

tional Review Board of the Singapore Eye Research Institute. All procedures complied with the Tenets of the Declaration of Helsinki for human research. Written informed consent was obtained from all patients prior to participation in the study.

The culturing of primary conjunctival epithelial cells has previously been described (1). Briefly, human cadaveric conjunctival tissues were obtained from the Singapore Eye Bank and used for the isolation and culture of conjunctival epithelial cells within 16 h of death. All donors were males aged from 51 to 68 years of age with an average of 58 years. Four samples of human primary conjunctival epithelial cells (PCEC), passages P0–P2 were cultured. IOBA-NHC cells were obtained from Margarita Calonge and Yolanda Diebold, IOBA, University of Valladolid, Valladolid, Spain. The Wong and Kilbourne derivative of the Chang cells (ChWK) were obtained from the American Type Culture Collection (ATCC CCL-20.2, clone 1-5c-4).

Culture Conditions

Culturing of cell lines was performed as previously described (16). Briefly, cells were cultured in DMEM/F12 supplemented with 1 $\mu\text{g}/\text{ml}$ bovine pancreas insulin, 2 ng/ml mouse epidermal growth factor, 0.1 $\mu\text{g}/\text{ml}$ cholera toxin, 5 $\mu\text{g}/\text{ml}$ streptomycin, 2.5 $\mu\text{g}/\text{ml}$ amphotericin B, and 10% fetal bovine serum. Medium was changed every 2–3 days, and cell growth was assessed daily by phase-contrast microscopy.

Microarray Analysis

Microarray chips, related protocols, and equipment for the processing of these chips were from Affymetrix Inc. (Santa Clara, CA). The human genome U133A GeneChip[®] consisting of more than 22,000 probe sets was used for this study. RNA was extracted as described in the manufacturer's manual. cRNA generated from 5 μg of total RNA was prepared as described in the sample preparation protocol. In order to obtain sufficient starting RNA from conjunctival tissues, each sample of RNA consists of pooled RNA from four patients' tissues. Washing and staining of arrays were performed with a GeneChip[®] Fluidics Station 450 and scanning was performed with the GeneChip[®] Scanner 3000.

Statistical analysis was performed using the GeneSpring GX 7.3 platform (Agilent Technology, Redwood City, CA). All data underwent probe level Robust Multi-Array Average (RMA) algorithm normalization as well as chip level normalization (30). Correlation analysis was performed as follows. One sample of primary conjunctival epithelial cells was

used as reference, and correlation of global gene expression data was performed against each of the four samples of ChWK and each of the five samples of IOBA-NHC cell. As a control, five samples of a less related human corneal epithelial cell line (HCEC-T) were used.

The raw gene expression data (CEL files) for the A549 cell line were downloaded from the NCBI GEO datasets GSM 47472, GSM47468, and GSM 47462 and processed subsequently as for other data as described.

The Pearson correlation coefficient was calculated, as well as the mean, SD, median, minimum, and maximum of the coefficients. The unrelated samples *t*-test was used to compare the correlation coefficients for the ChWK/PCEC and IOBA-NHC/PCEC associations, with statistical significance set at the level of $\alpha = 0.05$.

Fold change analysis was used to compare the mean expression levels of various transcripts in the cell lines compared to the primary conjunctival epithelial cells as a reference. The percentage of genes showing a 1.5-fold change (increase or decrease) relative to PCEC was calculated and tabulated, with the 95% confidence intervals of the proportions.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE8633.

Western Blot

Western blots were performed as previously published (49). Briefly, total cell lysates for PCEC or IOBA-NHC (40 μ g) were loaded on SDS-PAGE, transferred to nitrocellulose membrane, and blotted with primary antibodies against S100A9 (mouse monoclonal 1C10, Abnova, Walnut, CA), TGM2 (rabbit polyclonal Ab421, Abcam, Cambridge, MA), TLR4 (rabbit polyclonal, H-80, Santa Cruz, CA) at a dilution of 1:500. All primary antibody incubation was performed overnight at 4°C and blotted with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA) at 1:5000 dilution.

Survey of Literature

The NCBI Pubmed database was searched for articles containing "IOBA" or Chang conjunctiva cells (combination of these words or ChWK) in their abstracts. The results were manually curated to exclude review articles and to determine the predominant experimental strategy and outcome. Forty-four articles were found using the "Chang" cell line, so our analysis was restricted to articles after 2000 and for articles available on the Pubmed up to April 30, 2007. The

correlation analyses and the fold change analyses were repeated with Gene Ontology categories representing subsets of genes implicated in these published articles.

RESULTS

Analysis of Conjunctival Tissue and Primary Cultured Conjunctival Epithelial Cells

Global gene expression profiling, using principal component analysis, shows that PCEC have a pattern more similar to normal conjunctival tissue (CT) compared to either one of the conjunctival epithelial cell lines (Fig. 1). In Figure 1, the PCEC samples are spatially closer in the 3D scatter diagram to the tissue samples than the cell lines. In addition, a clear plane could be seen separating the cell lines from PCEC and the conjunctival tissue samples. Nevertheless, one would expect differences between the gene expression in CT and PCEC because the conjunctival biopsy will inevitably include some subepithelial stromal tissue together with the conjunctival epithelium, which may include fibroblasts, vascular endothelial cells, and blood cells. In order to evaluate the differences between PCEC and conjunctival tissue, we examined the possible upregulation of genes related to production of extracellular matrix in the conjunctival tissue compared to PCEC. We found that collagen types (1, 3, 6, and 18) were significantly upregulated ($p < 0.05$) by more than two times in conjunctival tissue compared to PCEC (Table 1). The same was also observed for other genes containing "extracellular matrix" within the Gene Ontology (GO) descriptions (Table 1).

We also found that three of the genes containing "blood" as a search term in the GO description were significantly upregulated ($p < 0.05$) by at least two-fold in conjunctival tissue compared to PCEC. These were the genes coding for interleukin 20 receptor alpha subunit (NM_014432), phospholipid scramblase 4 (NM_020353), and ectonucleoside triphosphate diphosphohydrolase 1 (NM_001776). These transcripts were upregulated in conjunctival tissue compared to PCEC by 5.4-, 4.6-, and 2.0-fold, respectively.

Comparison of Conjunctival Epithelial Cell Lines With Primary Cultured Conjunctival Epithelial Cells

In order to compare epithelial cells from cell lines with a similarly homogenous sample of epithelial cells, the PCEC was used as a reference in the evaluation of the cell lines.

Using a fold change analysis method, the genes in IOBA-NHC and ChWK cells were stratified into two

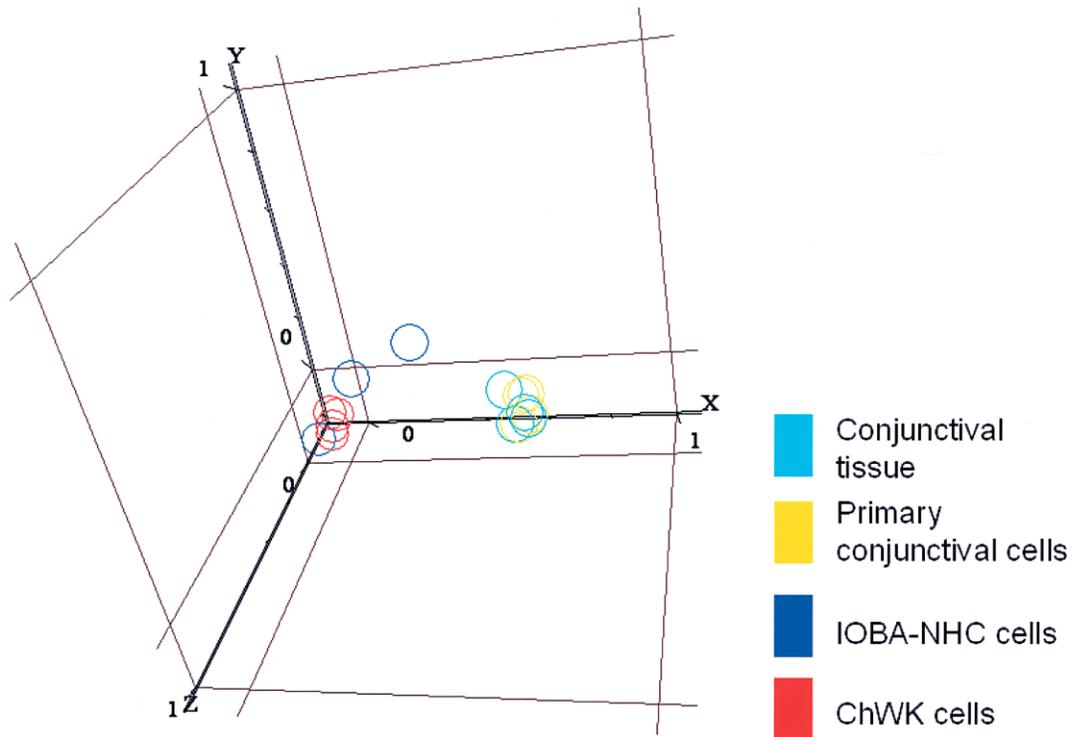


Figure 1. Global gene expression profiling in conjunctival tissue and conjunctival epithelial cells. Three-dimensional scatter plot showing the first three principal components in the x , y , and z axes, respectively. Principal component analysis (PCA) on the global gene expression data was performed for the four types of samples analyzed. Samples for different conditions are illustrated in different colors. The PCA procedure and generation of the scatter plot were performed using the Genespring GX 7.3 platform (Agilent Technology, Redwood City, CA). The option “PCA on conditions” was selected under “Tools.” The gene list was set to “All genes” and 16 experimental samples were selected, with the “mean centering and scaling” method used. On each of the axes of the scatter diagram, the data were set to “expression profile” with “linear” as the graph mode. Out of five principal components computed by the software, the first three principal components account for 31.4%, 13.5%, and 10% of the variance in the expression data, respectively, with a cumulative 54.9% for the three components.

TABLE 1
EXTRACELLULAR MATRIX GENES UPREGULATED IN CONJUNCTIVAL TISSUE
COMPARED TO PRIMARY CONJUNCTIVAL EPITHELIAL CELLS

Probe ID	Gene Symbol	Genbank Accession	Name	Fold Change
219087_at	ASPN	NM_017680	Asporin (leucine rich repeat class 1)	2.581
215016_x_at	BPAG1	BC004912	Bullous Pemphigoid antigen 1	2.715
211896_s_at	DCN	AF138302	Decorin	13.53
201893_x_at	DCN	AF138300	Decorin	8.868
211813_x_at	DCN	AF138303	Decorin	5.078
207977_s_at	DPT	NM_001937	Dermatopontin	2.217
201843_s_at	EFEMP1	NM_004105	EGF-containing tubulin-like extracellular matrix protein 1	3.185
202994_s_at	FBLN1	Z95331	Fibulin 1	2.111
202766_s_at	FBN1	NM_000138	Fibrillin 1	2.254
201744_s_at	LUM	NM_002345	Lumican	7.242
202310_s_at	COL1A1	K01228	Collagen Type I	2.473
202403_s_at	COL1A2	AA788711	Collagen Type I	6.012
202404_s_at	COL1A2	NM_000089	Collagen Type I	4.725
215076_s_at	COL3A1	AU144167	Collagen Type III	4.815
201852_x_at	COL3A1	AI813758	Collagen Type III	4.649
213428_s_at	COL6A1	AA292373	Collagen Type VI	2.542
201438_at	COL6A3	NM_004369	Collagen Type VI	2.132
209082_s_at	COL18A1	AF018081	Collagen Type VIII	2.603

categories: within 1.5-fold of the level of expression in the primary cells or outside this level of expression. The percentages of the genes that were within 1.5-fold are shown in two columns in bold in Table 2, corresponding to the two types of cell lines.

The main finding (first row Table 2) was that in IOBA-NHC cells 67.3% (95% CI 66.7–67.9) of the genes were within 1.5-fold of the expression level in PCEC, whereas in ChWK, the corresponding proportion was less, at 62.2% (95% CI 61.5–62.9). The difference in proportion was statistically significant ($p < 0.05$).

Due to biological variation, one would not expect 100% of genes to be within 1.5-fold even when comparing different samples/batches of the same cell type such as PCEC. When comparing one sample of PCEC with the other samples of PCEC, 79% (95% CI 78.5–79.6) of genes were expressed within a 1.5-fold (Table 3, first row, right-hand column). In this regard, neither IOBA-NHC nor ChWK was completely ideal as a substitute for PCEC, because 67.3% and 62.2% were each significantly lower than 79%, respectively

(Table 4, indicated by asterisks in first row, columns 4 and 5 from left).

However, the proportions (67.3% and 62.2%) are both significantly greater than 58.8% (Table 3, bold percentage in first row), confirming that the two conjunctival cell lines (IOBA-NHC and ChWK, respectively) resemble PCEC more than the less-related human corneal epithelial cell line (HCEC-T). The statistical significance of these relationships is indicated by asterisks in the first row, columns 2 and 3 in Table 4.

The proportions 67.3% appear rather close to 62.2% (Table 2, bold percentages, first row). However, these proportions are significantly different (Table 2, first row, evidenced by nonoverlapping 95% confidence intervals). To illustrate the statistical significance or otherwise of the proportions in the bold columns in Table 2, Table 4 (first column from left) is constructed with asterisks indicated whenever there is a significant difference ($p < 0.05$). IOBA-NHC was more similar to PCEC than ChWK (Table 4, asterisks in the first column) in genes concerned with

TABLE 2
COMPARISON OF GENE EXPRESSION PROFILES

	GO No.	Total No.	IOBA-NHC % 1.5× (95% CI)	ChWK % <1.5× (95% CI)
Overall		22,283	67.3 (66.7–67.9)	62.2 (61.5–62.9)
Membrane bound organelles	43,227	5,899	61.6 (60.3–62.9)	57.0 (55.7–58.3)
Nucleus	5,634	4,140	62.9 (61.4–64.4)	58.1 (56.6–59.7)
Extracellular region	5,576	1,119	70.8 (67.9–73.5)	66.5 (63.6–69.3)
Chromosome	5,694	249	53.0 (46.5–54.4)	49.0 (42.5–55.5)
Response to wounding	9,611	477	66.9 (62.3–71.1)	61.6 (57.0–66.1)
Response to stress	6,950	1,091	62.2 (59.2–65.2)	56.7 (53.7–59.8)
Response to inflammation	6,954	235	67.2 (60.6–70.2)	61.3 (54.6–67.6)
Cell communication	7,154	4,121	72.2 (70.8–73.6)	67.0 (65.5–68.4)
Transcription regulator	30,528	1,636	71.9 (69.6–74.1)	67.8 (65.4–70.1)
Transporter	5,215	1,953	66.5 (64.3–68.6)	62.4 (60.1–64.6)
Development	7,275	2,152	72.0 (70.0–73.9)	66.5 (64.5–68.6)
Differentiation	30,154	315	72.4 (66.9–77.3)	64.8 (59.1–70.1)
Motor activity	3,774	167	67.7 (59.8–74.7)	61.7 (53.7–69.1)
Cellular defense	6,968	128	69.5 (60.5–77.3)	58.6 (49.4–67.3)
Viral life cycle	16,032	43	55.8 (39.7–70.8)	55.8 (39.7–70.8)
Antigen presentation	19,882	47	63.8 (48.2–77.1)	59.6 (44.0–73.5)
Antioxidant	16,209	43	48.8 (33.3–64.5)	48.8 (33.3–64.6)
Ubiquitin ligase complex	151	33	57.6 (39.1–74.3)	45.5 (28.3–63.7)
Regulation of metabolism	19,222	1,752	69.1 (66.8–71.3)	64.6 (62.2–66.8)
Lipid metabolism	6,629	649	64.9 (61.0–68.6)	60.7 (56.7–64.5)
Protein metabolism	19,538	2,853	63.3 (61.5–65.1)	56.6 (54.8–58.5)
Carbohydrate metabolism	5,975	467	63.4 (58.7–67.8)	58.9 (54.2–63.5)
Nucleic acid metabolism	6,139	2,961	62.3 (60.5–64.1)	57.8 (56.0–59.6)
Organic acid metabolism	6,082	469	56.7 (52.0–61.3)	50.1 (45.4–54.8)

The transcript level of various classes of genes in each of the two types of conjunctival cell lines (IOBA-NHC and ChWK) was compared with the corresponding transcripts in the primary conjunctival epithelial cells using fold change analysis. Total No.: number of genes in that Gene Ontology (GO) category in the GeneChip. 1.5×: 50% increased or decreased from the level in primary cells. CI: confidence interval of the proportion.

TABLE 3
COMPARISON OF EXPRESSION PROFILE BETWEEN UNRELATED CELL LINE (HCEC-T)
AND PRIMARY CONJUNCTIVAL EPITHELIAL CELLS (PCEC)

	Total No.	HCEC-T vs. PCEC		PCEC vs. PCEC	
		% <1.5×	95% CI	% <1.5×	95% CI
Overall	22,283	58.8	58.1, 59.4	79.0	78.5, 79.6
Membrane bound organelles	5,899	56.9	55.6, 58.2	76.7	75.6, 77.8
Nucleus	4,140	56.2	54.7, 57.7	77.2	76.0, 78.5
Extracellular region	1,119	59.8	56.9, 62.7	79.3	76.8, 81.7
Chromosome	249	49.0	42.8, 55.2	73.9	67.8, 79.3
Response to wounding	477	57.0	52.6, 61.5	77.5	73.4, 81.2
Response to stress	1,091	54.1	51.1, 57.0	75.0	72.3, 77.6
Response to inflammation	235	58.7	52.4, 65.0	77.8	71.8, 82.9
Cell communication	4,121	60.7	59.2, 62.2	81.5	80.2, 82.6
Transcription regulator	1,636	59.5	57.1, 61.9	82.0	80.1, 83.9
Transporter	1,953	61.0	58.8, 63.1	80.7	78.9, 82.5
Development	2,152	60.8	58.7, 62.8	80.0	78.3, 81.7
Differentiation	315	57.5	52.0, 62.9	80.9	76.0, 85.1
Motor activity	167	53.3	45.7, 60.9	76.0	68.6, 82.2
Cell defense	128	56.3	47.7, 64.8	83.5	75.6, 89.4
Viral life cycle	43	55.8	41.0, 70.7	74.4	58.2, 86.1
Antigen presentation	47	51.1	36.8, 65.4	65.9	50.3, 78.9
Antioxidant	43	60.5	45.9, 75.1	65.1	48.7, 78.7
Ubiquitin ligase	33	51.5	34.5, 68.6	69.6	50.8, 84.9
Regulation metabolism	1,752	59.1	56.8, 61.4	80.8	78.9, 82.7
Lipid metabolism	649	57.2	53.4, 61.0	77.3	73.8, 80.5
Protein metabolism	2,853	57.6	55.7, 59.4	78.0	76.5, 79.6
Carbohydrate metabolism	467	56.5	52.0, 61.0	78.1	74.0, 81.8
Nucleic acid metabolism	2,961	56.5	54.7, 58.3	76.9	75.4, 78.4
Organic acid metabolism	469	60.3	55.9, 64.8	73.5	69.2, 77.5

membrane binding, cell communication, development, regulation of metabolism, in particular regulation of protein and nucleic acid metabolism.

Not surprisingly, the expression levels of genes in the less-related human corneal epithelial cell line (HCEC-T) were significantly less similar to PCEC than comparing PCEC to PCEC itself (Table 3, lower percentages in the left compared to right bold columns). The last column in Table 4 illustrates that this relationship was significant for all GO categories apart from genes concerned with viral life cycle, antigen presentation, antioxidant, and ubiquitin ligase. The reason for lack of statistical significance in these four categories is the relatively small number (less than 50) of genes present in each of these categories on the U133A GeneChip used in this study.

To explore the gene expression profile of another unrelated cell line (bronchial epithelial cell A549) in place of HCEC-T, we repeated the above analyses and showed this in Tables S2 (available at <http://www.seri.com.sg/publications/others/Table S2.doc>) and S3 (available at <http://www.seri.com.sg/publications/others/Table S3.doc>). In general, the same conclusions can be drawn as in Tables 3 and 4.

We also use a different method to compare the gene expression profiles in IOBA-NHC or ChWK

cells with PCEC (Table 5). This method used the correlation of paired expression data between either IOBA-NHC or ChWK against PCEC. The information provided by this analysis is different from that in Tables 2 and 3. A large percentage of genes expressed within 1.5-fold could still be observed with low correlation and vice versa.

The correlation analysis was useful for GO categories with relatively few genes (30–50 each). The correlation of gene expression levels, using PCEC as a reference was generally high ($r > 0.70$) for either IOBA-NHC or ChWK cells in the categories described as “viral life cycle,” “antigen presentation,” “antioxidant,” and “ubiquitin ligase” (Table 5). However, correlation varied in different gene categories; for example, the genes involved in the inflammatory response were poorly correlated to PCEC in either of the cell lines.

In order to shed some light on the genes that were expressed differently between a conjunctival cell line and PCEC, we tabulated the list of 55 genes that demonstrated a change of at least 1.5-fold in IOBA-NHC cells compared to primary conjunctival cells (Table S1: available at <http://www.seri.com.sg/publications/others/Table S1.xls>). For these genes, the exact number of fold of up- or downregulation is il-

TABLE 4
COMPARISONS BETWEEN CELL LINES AND ASPECTS OF REPRODUCIBILITY

	IOBA vs. ChWK	IOBA vs. HCEC-T	ChWK vs. HCEC-T	IOBA vs. PCEC	ChWK vs. PCEC	HCEC-T vs. PCEC
Overall	*	*	*	*	*	*
Membrane bound organelles	*	*		*	*	*
Nucleus		*		*	*	*
Extracellular region		*	*	*	*	*
Chromosome				*	*	*
Response to wounding		*		*	*	*
Response to stress		*		*	*	*
Response to inflammation		*		*	*	*
Cell communication	*	*	*	*	*	*
Transcription regulator		*	*	*	*	*
Transporter		*		*	*	*
Development	*	*	*	*	*	*
Differentiation		*			*	*
Motor activity		*				*
Cell defense					*	*
Viral life cycle						
Antigen presentation						
Antioxidant						
Ubiquitin ligase						
Regulation metabolism	*	*	*	*	*	*
Lipid metabolism		*		*	*	*
Protein metabolism	*	*		*	*	*
Carbohydrate metabolism				*	*	*
Nucleic acid metabolism	*	*		*	*	*
Organic acid metabolism				*	*	*

Note: Cell type A versus cell type B does not refer to direct comparison between cell type A and cell type B (refer to text for explanation). IOBA: IOBA-NHC cell line, ChWK: Chang cell line WK derivative, PCEC: primary conjunctival epithelial cell, HCEC-T: unrelated corneal epithelial cell line.
**p* < 0.05.

lustrated in the table. Some examples include the S100A8 (NM_002964) and S100A9 (NM_002965), which are important inflammatory markers that can be noninvasively monitored in human tears, and interleukin-1 α (M15329), an important proinflammatory cytokine.

In Table 5, genes concerned with cellular defense and protein metabolism, relatively high correlation coefficients (>0.55) were obtained for IOBA-NHC or ChWK against PCEC, which agreed with the relatively high percentages in the corresponding rows in Table 2.

In order to evaluate the functional relevance of the transcript changes, we performed Western blots to relatively quantify the levels of three proteins that are expressed in the PCEC and IOBA-NHC cells corresponding to the transcripts evaluated in the microarray (Fig. 2). S100A9 was selected for this purpose because of its importance in differentiation and regulation of calcium-associated stress signal transduction (21), and its transcript levels were greatly increased in IOBA-NHC (Table S1: available at <http://www.seri.com.sg/publications/others/Table S1.xls>) compared

to PCEC cells. The same was also true for transglutaminase (TGM)-2, which was expressed at 3.1-, 2.6-, 2.7-, and 1.1-fold higher in the IOBA-NHC cells, compared to the PCEC for the probesets 201042_at, 211003_x_at, 211573_x_at, and 216183_at, respectively. TGM2 is an important molecule involved in wound healing, apoptosis, and regulation of wound healing, oncogenesis and tumor metastasis, and expressed in the eye (39,45). On the other hand, the Toll-like receptor (TLR)-4 expression was lower in the IOBA-NHC cells compared to PCEC (Table S1: available at <http://www.seri.com.sg/publications/others/Table S1.xls>). TLR-4 is an important pathogen recognition receptor molecule in the innate defense of the eye (36). Figure 2 shows that the protein level changes in the three proteins were consistent with differences in transcript levels, confirming the functional significance of our study.

DISCUSSION

PCEC resemble the native conjunctival tissue more than any of the cell lines. This is not surprising, con-

TABLE 5
COMPARISON OF GENE EXPRESSION BETWEEN CONJUNCTIVAL CELL LINES AND
PRIMARY CONJUNCTIVAL EPITHELIAL CELLS USING CORRELATION

	GO No.	IOBA (<i>n</i> = 5) vs. PCEC			ChWK (<i>n</i> = 4) vs. PCEC		
		Median <i>r</i>	Min	Max	Median <i>r</i>	Min	Max
Overall		0.322	0.319	0.326	0.312	0.310	0.315
Membrane bound organelles	43,227	0.551	0.542	0.557	0.548	0.545	0.553
Nucleus	5,634	0.51	0.495	0.518	0.510	0.507	0.516
Extracellular region	5,576	0.11	0.096	0.114	0.080	0.076	0.094
Chromosome	5,694	0.618	0.613	0.638	0.640	0.613	0.666
Response to wounding	9,611	0.157	0.14	0.175	0.128	0.121	0.139
Response to stress	6,950	0.225	0.221	0.227	0.207	0.198	0.211
Response to inflammation	6,954	-0.0115	-0.031	0.003	-0.040	-0.048	-0.035
Cell communication	7,154	0.348	0.335	0.354	0.345	0.323	0.355
Transcription regulator	30,528	0.441	0.411	0.47	0.442	0.436	0.448
Transporter	5,215	0.373	0.363	0.38	0.369	0.35	0.381
Development	7,275	0.208	0.2	0.221	0.188	0.18	0.195
Differentiation	30,154	0.208	0.2	0.221	0.188	0.18	0.195
Motor activity	3,774	0.66	0.589	0.794	0.654	0.603	0.717
Cell defense	6,968	0.785	0.774	0.809	0.782	0.761	0.805
Viral life cycle	16,032	0.776	0.723	0.811	0.810	0.788	0.822
Antigen presentation	19,882	0.776	0.724	0.784	0.775	0.764	0.804
Antioxidant	16,209	0.738	0.682	0.753	0.736	0.727	0.739
Ubiquitin ligase	151	0.769	0.719	0.798	0.771	0.701	0.816
Regulation metabolism	19,222	0.462	0.430	0.477	0.442	0.431	0.448
Lipid metabolism	6,629	0.334	0.312	0.35	0.400	0.393	0.420
Protein metabolism	19,538	0.586	0.572	0.595	0.562	0.556	0.567
Carbohydrate metabolism	5,975	0.482	0.446	0.505	0.502	0.474	0.571
Nucleic acid metabolism	6,139	0.547	0.543	0.563	0.548	0.541	0.551
Organic acid metabolism	6,082	0.443	0.423	0.471	0.414	0.396	0.432

Table values show Pearson correlation coefficients (*r*) where 1.0 = perfect correlation, 0.0 = no correlation, -1.0 = negative correlation.

sidering that the ChWK is artificially (51), whereas the IOBA-NHC cells spontaneously (16), immortalized. The IOBA-NHC cells appear to resemble PCEC more than the ChWK cells, especially for the genes involved in cell communication and development. Although the PCEC expectedly show a greater resemblance to the conjunctival tissue than any cell line, the gene expression profile was still distinct from the tissue as other cell types/components may be present in the tissue but not the primary cells.

A high level of genes in cellular defense showed similarities in expression profile between IOBA-NHC and primary cells, validating the use of IOBA-NHC in the previous studies in this field (Table 6). As possible differences in the gene expression data between cell lines and primary epithelial cells may be dependent on gene function category, we highlighted the methodology of some studies employing cell lines in Table 6. It is interesting that a large number of such studies deal with microbial infection and, indeed, the correlation of the gene expression levels between IOBA and PCEC was relatively high ($r > 0.70$) in the categories of cellular defense and viral life cycle-related genes. When there were disparities in the gene

expression levels between NHC-IOBA and PCEC in important entities such as S100A9, TGM2, and TLR4, which play important roles in the biology of conjunctiva epithelium, the transcript level differences were mirrored by protein level differences, suggesting that our approach is valid and that even in the areas of differentiation and innate immunity, definite differences in molecular processes do exist between NHC-IOBA and PCEC.

A SV40 immortalized human corneal epithelial cell line, as described previously, and that expressed cornea-specific, 64-kDa cytokeratin in addition to five major insoluble proteins, was used as a control because this cell line has no conjunctival epithelial cell origin, although it is still an ocular surface epithelial cell type (2).

Not surprisingly, the gene expression profile for HCEC-T was significantly different from PCEC, and from IOBA-NHC cells. Some gene categories in HCEC-T (nuclear genes and differentiation), however, showed no significant difference in expression from ChWK cells, suggesting that some features of ChWK gene expression profile resembles the HCEC-T, perhaps related to the method of immortalization.

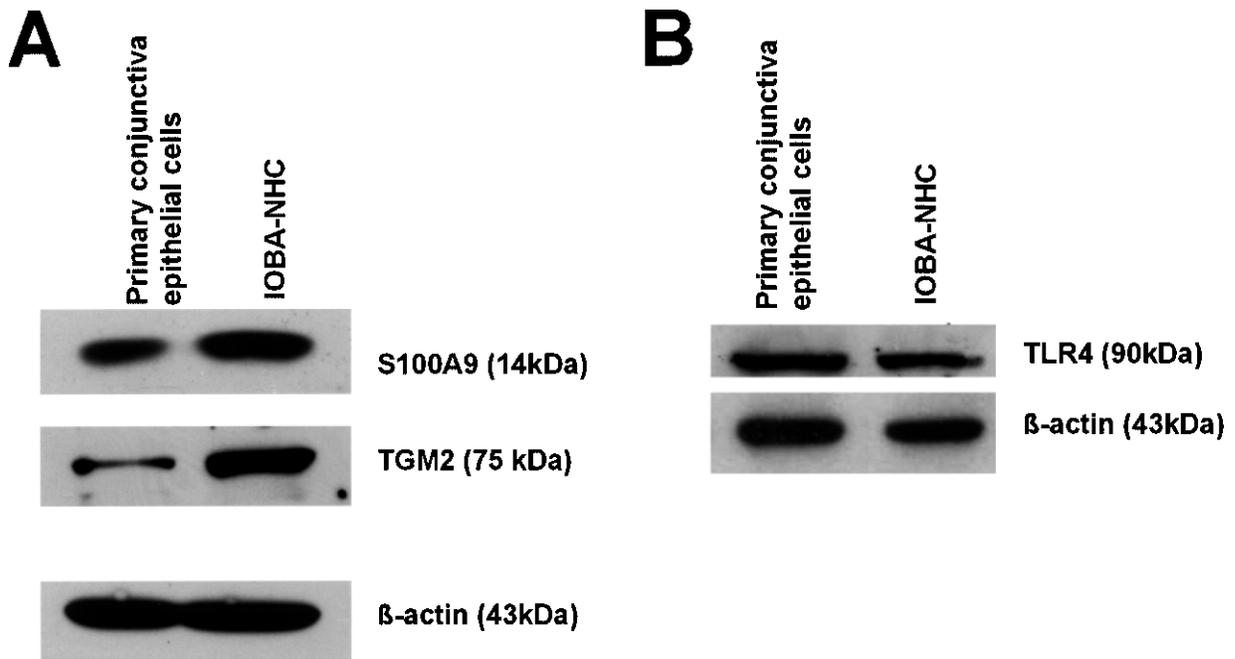


Figure 2. Protein levels in primary cultured conjunctival epithelial cells compared to the IOBA-NHC. Western blots performed on total cell lysate showing that evaluated proteins are higher in the IOBA-NHC (A) or lower in the IOBA-NHC (B) compared to primary conjunctival epithelial cells.

Comparison With Previously Published Data

There were no previous data on the global gene expression comparison between PCEC and cell lines. However, the differential expression of a selected set of proteins or markers has been previously evaluated. For example, after stimulation by IFN- γ and TNF- α , ChWK cells had less upregulation of inflammatory genes when compared to PCEC (12). ChWK cells were also cytokeratin-4 negative, whereas PCEC demonstrated obvious expression of this well-known conjunctival epithelial differentiation marker (12). Immortalized rabbit lacrimal acinar cells were shown to express higher levels of proteins such as vimentin, compared to primary lacrimal acinar cells (46), but this study only used immunolabeling of proteins and did not address transcript levels. A glial cell line has been compared to oligodendrocytes, but only with respect to myelin-associated glycoproteins and glycolipids (54). As these studies are focused on an arbitrary set of markers they cannot address the relative differences in different functional or biological subsets. In the nonocular literature, global gene profiling has been performed for cancer-derived cell lines compared to primary chondrocytes (27), normal leukocytes (50), or Schwann cells (34), while one study compared cancer cell lines to clinical cancer specimens (38). These studies primarily address the differences in diseased cells compared to normal cells, not

the differences between a nontransfected primary cell equivalent with its natural counterpart.

The reasons for the ChWK and IOBA-NHC cells to retain potential for continuous proliferation after repeated passaging are not completely known. The ChWK cells may have been contaminated by HeLa cells (33). The IOBA-NHC, though not contaminated by Langerhans cells, endothelial cells, or fibroblasts, may have been exposed to an unknown oncogenic stimulus, because it is heteroploid, with a near triploid chromosome number (65+/-4) and no Y chromosome despite the original source of a male human donor (16). The reasons for the immortalization are likely to explain the deviation of the gene expression of these cells from PCEC.

Strengths and Limitations

Because this is a microarray experiment with a global human gene array, we were unbiased in the selection of genes to compare. We accept there is some arbitrary selection of the panel of specific genes in some analyses. However, we have selected these categories based on the type of studies that had been conducted using these specific conjunctival cell lines in the literature.

The main limitation to this article is the restriction of the analysis to gene expression data and limiting the functional evaluation to only three proteins ex-

TABLE 6
PREVIOUS STUDIES EMPLOYING IOBA-NHC AND ChWK CELL LINES

Study	Purpose	Biological Process
IOBA-NHC		
Buron 2006 (4)	UV and drug toxicity	Apoptosis
Corrales 2001 (7)	Drug toxicity	Cell viability
Diebold 1998 (15)	Cytokine stimulation	Immunity
Diebold 2003 (16)	Cytokine stimulation	Morphology, proliferation
Diebold 2007 (19)	Drug delivery	Nanoparticle penetration
Enriquez de Salamanca 2005 (23)	Cytokine stimulation	Membrane receptor expression
Enriquez de Salamanca 2006 (22)	Drug delivery, toxicity	Drug uptake, cell viability
Narayanan 2006 (41)	Cytokine stimulation	Cytokine expression
Talreja 2005 (48)	LPS stimulation	TLR signaling
ChWK (only 21/44 shown)		
Garweg 2006 (26)	Drug toxicity	Cell proliferation
Gallyas 2006 (25)	Viral cytopathy	Apoptosis
Chen 2006 (6)	Cytokine stimulation	Signaling, Immunity
Buron 2006 (4)	As above	
Talreja 2005 (48)	As above	
Guenoun 2005 (29)	Drug toxicity	Antioxidant, free radicals
De Saint Jean 2004 (9)	Cytokine stimulation	Differentiation
Zhan 2003 (55)	Cytokine stimulation	Immunity
Papa 2003 (44)	Drug toxicity	Cell proliferation
Jendrossek 2003 (31)	Bacterial infection	Apoptosis, immunity
Sinniah 2002 (47)	Toxicology	Cell morphology
Fillon 2002 (24)	Bacterial infection	Apoptosis
Debbasch 2002 (13)	Drug toxicity	Cell viability, free radicals
Wu 2001 (53)	Viral infection	Membrane proteins
Ochiai 2001 (43)	Drug toxicity	Growth factor expression
Jendrossek 2001 (32)	Bacterial infection	Signaling, apoptosis
Debbasch 2001 (12)	Drug toxicity	Apoptosis, free radicals
Debbasch 2001 (14)	Drug toxicity	Apoptosis, free radicals
Blom 2001 (3)	Bacterial infection	Immunity
Minor 2000 (40)	Bacterial infection	Carbohydrate metabolism
De Saint Jean 2000 (11)	Cytokine stimulation	Apoptosis

pressed in the IOBA-NHC and PCEC. Our data would have been more valuable if it can be supplemented by proteomics profiling and other functional assays.

We only use IOBA-NHC and ChWK cells from a single batch. It is theoretically possible that, over the years, some mutations and changes of characteristics may have occurred after the propagation of these cells. Therefore, there may be some limitations to the extrapolation of our data to other IOBA-NHC and ChWK cells.

Potential Applications

Researchers should exercise caution in the selection of conjunctival cell lines in their research. Figure 3 provides some guidelines for the selection of study samples in research related to the ocular surface. For example, data on the general response of cells in IOBA-NHC and ChWK cells to inflammation may not be applicable to native conjunctival tissue. However, given the limited availability of human tissue and PCEC, specific questions and cellular mecha-

nisms related to inflammation, such as characteristics of cell communication and defense may still be properly addressed in these cells. In such cases, particular caution must be exercised when studying transcript levels of certain markers of inflammation such as S100A8 in cell lines, because these could vary tremendously from the corresponding primary cultured cells.

In nonocular research, the vast majority of the scientific literature that utilized cell lines did not include a parallel assessment of the induced changes in equivalent native cells, which may not be available. The findings in these studies may or may not apply to human disease processes, depending on the area of biology involved, and should be addressed in their specific context.

CONCLUSIONS

Conjunctival cell lines do not resemble native conjunctival tissue as much as primary cultured conjunc-

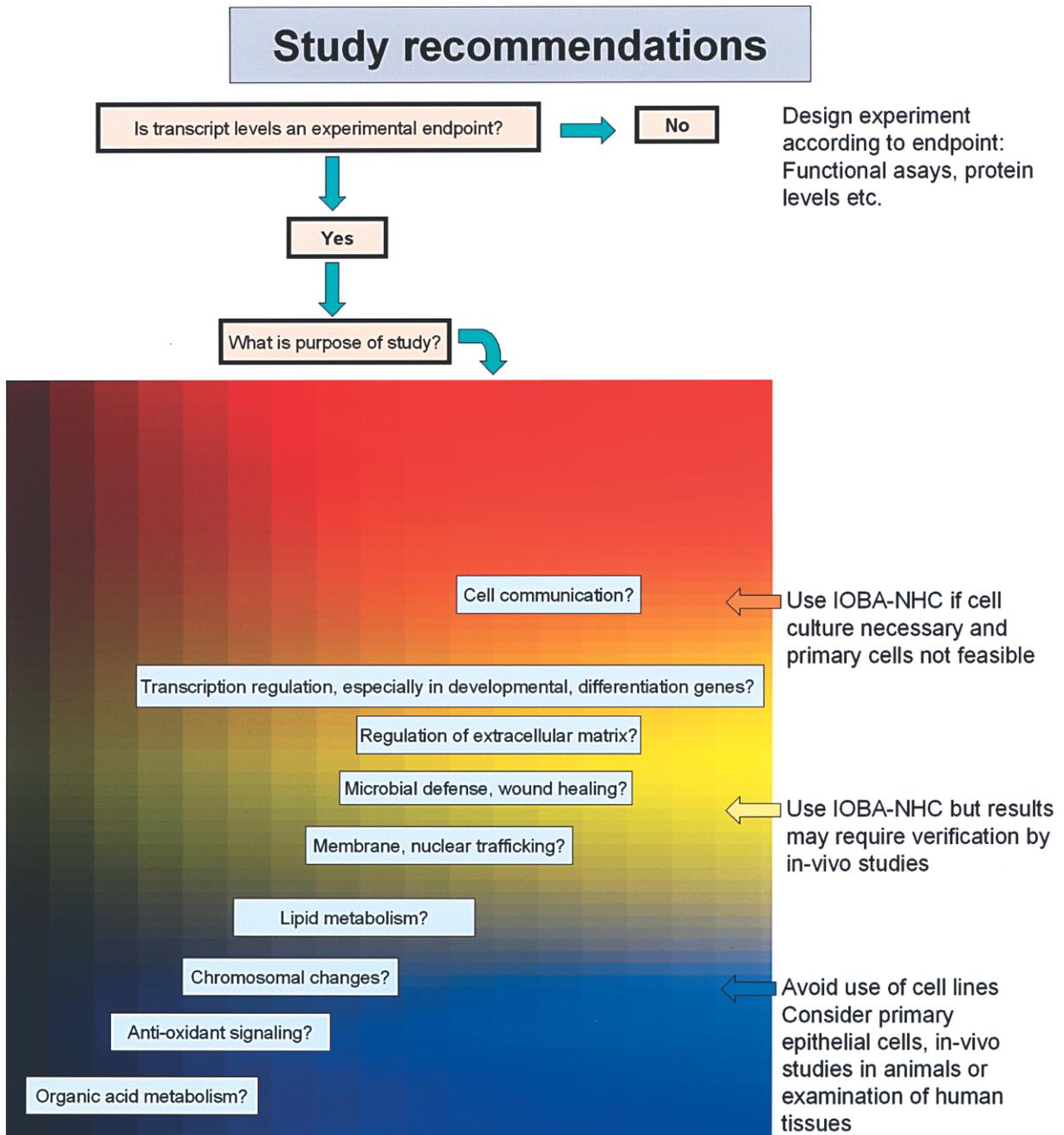


Figure 3. Selection of cells for ocular surface research. This chart summarizes how investigators can select appropriate cells in the ocular surface research in the future, based on the gene expression data in the current study.

tival epithelial cells, which also differed from native tissue in some ways. However, when laboratory and industrial settings dictate the use of conjunctival cell lines, scientists should be aware that there are some differences in the gene expression profile of IOBA-NHC and ChWK cells, and studies using one of these cell types may be more valid than the other depending on the aim and context of the experiment.

ADDITIONAL FILES

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE8633.

Table S1: Genes in the inflammatory response category and the mean number of fold of alteration of expression

in IOBA-NHC cells relative to primary conjunctival epithelial cells. Microsoft excel file (available at <http://www.seri.com.sg/publications/others/Table S1.xls>)

Table S2: Comparison of gene expression profile between bronchial epithelial cell line (A549) and PCEC. Internal epithelial conjunctival control: this refers to the comparison of one sample of PCEC against other samples of PCEC. Microsoft word file (available at <http://www.seri.com.sg/publications/others/Table S2.doc>)

Table S3: Comparisons between cell lines and aspects of reproducibility. IOBA: IOBA-NHC cell line, ChWK: Chang cell line WK derivative, PCEC: primary conjunctival epithelial cell, and A549: unrelated bronchial epithelial cell line. Microsoft word file (available at <http://www.seri.com.sg/publications/others/Table S3.doc>)

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L.T. performed the analysis for the data and drafted the manuscript. R.W.B. conceived of the study,

participated in the coordination of study, and helped to draft the manuscript. J.P.G. performed the cell cultures and RNA extraction for the study. M.E.S., M.C., and Y.D. carried out the initial experiments for the characterization of the IOBA-NHC cell line, and participated in the design of the study. All authors read and approved the final manuscript. This study was funded by Biomedical Research Council grant: BMRC 03/1/35/19/231, Singapore Eye Research Institute Grant R502/51/2006, and an unrestricted grant from Allergan, Inc. The funding bodies did not play a role in the study design, in the collection, analysis, and interpretation of data, nor in the writing of the manuscript; but BMRC has approved the manuscript for publication. We would like to thank Dr. Li Jing, Singapore Eye Research Institute, for her help in the study on TLR4.

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