EXTENDED REPORT

The expression of native and cultured human retinal pigment epithelial cells grown in different culture conditions

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Aim: To determine the transcriptional proximity of retinal pigment epithelium (RPE) cells grown under different culture conditions and native RPE.
Methods: ARPE-19 cells were grown under five conditions in 10% CO_2: ''subconfluent'' in DMEM/F12 + 10% FBS, ''confluent'' in serum and serum withdrawn, and ''differentiated'' for 2.5 months in serum and serum withdrawn medium. Native RPE was laser microdissected. Total RNA was extracted, reverse transcribed, and radiolabelled probes were hybridised to an array containing 5353 genes. Arrays were evaluated by hierarchical cluster analysis and significance analysis of microarrays.
Results: 78% of genes were expressed by native RPE while 45.3–47.7% were expressed by ARPE-19 cells, depending on culture condition. While the most abundant genes were expressed by native and cultured cells, significant differences in low abundance genes were seen. Hierarchical cluster analysis showed that confluent and differentiated, serum withdrawn cultures clustered closest to native RPE, and that serum segregated cultured cells from native RPE. The number of differentially expressed genes and their function, and profile of expressed and unexpressed genes, demonstrate differences between native and cultured cells.
Conclusions: While ARPE-19 cells have significant value for studying RPE behaviour, investigators must be aware of how culture conditions can influence the mRNA phenotype of the cell.

The retinal pigment epithelial (RPE) cell line ARPE-19, displays significant functional differentiation that mimics native RPE.[1] It provides a dependable source of cultured RPE cells for study. At the time of writing, 121 PubMed publications have utilised this cell line. The transcriptional profile of RPE cultures including ARPE-19 cells, and its proximity to native RPE, is however, unknown.

Recently, we found transcriptional differences among ARPE-19 cells grown on different matrices.[2] Unexpectedly, ARPE-19 cells grown on plastic displayed the closest phenotype to native RPE. While obvious that the transcriptome will be influenced by culture conditions, few studies are available to articulate these differences. We hypothesised that culture conditions could be optimised so ARPE-19 cells could simulate the native RPE mRNA phenotype. To accomplish this goal, we laser microdissected native RPE and compared its global transcriptional profile with ARPE-19 cells grown under different culture conditions.

METHODS

Cell culture

ARPE-19 cells were seeded at 10 000/cm^2 (''subconfluent'' or ''SS'') for 3 days or 100 000/cm^2 in T-75 cm^2 flasks and grown in Dulbecco's Modified Eagle medium/nutrient mixture F12 (DMEM/F12; BioWhittaker Inc, Walkersville, MD, USA) + 10% fetal bovine serum (FBS; UBI Upstate, Lake Placid, NY, USA) at 37°C in 10% CO_2. ''Confluent'' (CS) and ''confluent, serum withdrawn'' (CSW) cultures were grown for 7 days, and replaced with fresh medium containing serum or 1% bovine serum albumin (BSA), respectively for 3 more days. ''Differentiated'' (DS) and ''differentiated serum withdrawn'' (DSW) cells were grown for 2.5 months, and then in serum or 1%BSA for 3 days.

Tissue preparation

Ten eyes (45–95 years old) with a death enucleation time within 6 hours and from donors on life support systems for less than 24 hours were used since premorbid conditions have the greatest influence on RNA degradation[3] (table 1). Donors were free of ocular disease, systemic inflammatory disease, and diabetes mellitus. A macular calotte was dissected and cryoprotected as previously described.[4 5] Cryosections (7 μm) were stained with haematoxylin and eosin Y (Fisher Scientific, Inc) before microdis'section.

Laser capture microdissection

RPE cells were removed with an Arcturus PixCell II (Arcturus Engineering, Inc Mountain View, CA, USA) as previously described.[5] After dissection, the transfer cap was inspected for contaminating tissue before being placed in denaturing buffer.

RNA extraction

Total RNA was extracted using the RNeasy Mini-kit (Qiagen Inc, Valencia, CA, USA) and treated with DNase I (Qiagen, Inc) according to the manufacturer's recommendations. RNA quality was assessed by GAPDH expression using RT-qPCR with primers designed at the 5′ end of gene, and intact 28S and 18S ribosomal RNA bands of an RPE sample by gel electrophoresis.

Probe synthesis

Total RNA from 5000 cells was reverse transcribed with 50 μCi[33P]dCTP and 50 μCi[33P]dATP with 0.5 μg oligo-dT according to our modified method of Sgroi *et al*.[2 6] A second strand was synthesised with 50 μCi[33P]dCTP, 50 μCi[33P]dATP, 500 ng random hexamers, and 20U Klenow fragment (Gibco BRL). Probes were purified with a Bio-Spin 6 column (BioRad Laboratories, Hercules, CA, USA).

Microarray analysis

Labelled, double stranded cDNA was hybridised to the cDNA GeneFilter Human Microarray Release I (5353 genes;

Abbreviations: BSA, bovine serum albumin; CS, confluent serum; CSW, confluent, serum withdrawn; DMEM, Dulbecco's Modified Eagle medium; DS, differentiated serum; DSW, differentiated serum withdrawn; FBS, fetal bovine serum; FDR, false discovery rate; RPE, retinal pigment epithelium

Table 1 Donor eyes used for transcriptional analysis

Donor	Age (years)	Sex	Race	D-E* (hours)	Cause of death
Microarray analysis					
1	60	M	W	4:00	Myocardial infarction
2	80	M	W	3:35	Respiratory failure
3	45	M	W	4:45	CVA†
4	74	F	W	3:35	Lung cancer
5	95	F	W	3:05	Leukaemia
Real time RT-PCR					
6	83	M	W	3:10	Myasthenia gravis
7	82	M	W	3:00	Melanoma
8	84	M	W	5:00	CHF‡
9	51	F	W	4:45	Lung cancer
10	57	F	W	5:45	Ovarian cancer

*D-E, death to enucleation time; † CVA, cerebral vascular attack; ‡ CHF, congestive heart failure.

Invitrogen, Inc, Huntsville, AL, USA) using the manufacturer's protocol. This array contains an insert DNA from a sequence verified IMAGE/LLNL clone using the 3′ end of the gene. Arrays were exposed for 3 days to a high density phosphorimager screen (BioRad Laboratories) and scanned at 50 μm resolution in a phosphorimager (FX Pro-Plus, BioRad Laboratories). The data appear on Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) using platform number GPL1488, and accession numbers GSM32029-GSM32048. Since Lee *et al* demonstrated that at least three replicate experiments are necessary for reliable microarray results,[7] for each condition, at least three independent experiments were performed.

Image and statistical analysis

Signal intensity for each gene was quantified and normalised to 75% of the average signal intensity of the entire array by Pathways 3 software (Invitrogen, Inc). An individual gene was ''expressed'' if the signal intensity was ⩾1.4-fold above background[8] in at least two of three, and three of five experiments for cultured and native RPE, respectively.

Gene expression signals were scaled according to the method of Tusher *et al*.[2 9] A reference set was generated using the average expression from all arrays. Each hybridisation signal was compared with the reference signal in a cube root scatter plot, and intensity values for each gene were corrected with a ''scaling factor.''

Hierarchical cluster analysis was performed with average linkage clustering by Cluster, and visualised with TreeView.[10] Significance analysis of microarrays (SAM; version 1.12) determined differential gene expression between conditions.[11]

Real time RT-PCR

Total RNA (100 ng) was reverse transcribed with Sensiscript (Qiagen, Inc) as previously described.[2] Primer sequences were designed to span consecutive exons using Primer 3 (Whitehead Institute/MIT, Cambridge, MA, USA). Sequences were verified using NCBI Unigene (table 2). First strand cDNA was amplified using the LightCycler (Roche Diagnostics, Nutley, NJ, USA) in a final volume of 20 μl containing SYBR Green PCR Master Mix (10 μl; Qiagen, Inc, USA), primers (0.5 μM each), and 2 μl DNA in 2.5 mM $MgCl_2$ The standard curve for the gene of interest consisted of PCR products ($1–10^{-6}$ pg). PCR products were checked by melting point analysis and quantified using the second derivate maximum values calculated by the Light-Cycler analysis software. Expression was normalised to acidic ribosomal phosphoprotein expression.[12] The Student's *t* test was used to compare the differential gene expression between conditions.

RESULTS

Expression profile of native and cultured RPE cells

Figure 1A shows healthy macular RPE cells with cuboidal columnar epithelial morphology and normal Bruch's membrane. Figure 1B shows the cryosection after microdissection, and figure 1C shows the microdissected cells adherent to the transfer cap. Figure 2 demonstrates spindly appearing ''SS'' cells (fig 2A) and regular ''CS'' cells (Fig 2B). ''DS'' cells had cobblestone morphology with melanin pigment (fig 2C).

Scatter plots of microarray analysis for pairwise comparison of native RPE from the five donors, and cultured cells showed reasonable reproducibility (data not shown; $R^2 = 0.829–0.995$ for all comparisons). Using our expression criterion, the number of genes expressed by native RPE was 78% (4177 genes) of the array. The number of genes expressed by ARPE-19 cells ranged from 45.3% (CS), 45.4% (DS), 46.5% (SS), 47.2% (CSW), to 47.7% (DSW).

Table 2 Real time RT-PCR primers and conditions

Gene name	GenBank Acc No		Sequence	Location	Size (BP)	Cycles	Tm (℃)
SEC13-like 1 (*S cerevisiae*)	AA496784	F	CGTGTGTTCATTTGGACCTG	868–1103	236	45	55
		R	CCCTCTGTCACTGATGCTGA				
Topoisomerase (DNA) II α170 kDa	AA504348	F	TCCTGCCAAAACCAAGAATC	4494–4666	173	45	55
		R	GTACAGATTTTGCCCGAGGA				
ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	AA427472	F	TGCCCTGCACTACATAGCAC	3326–3514	189	45	55
		R	GGGGAAGATCTCAGGGTCTC				
Oxidase (cytochrome c) assembly 1-like	AA598582	F	GTCGAATCAGAGAGGCCAAG	722–948	227	45	55
		R	GAGATCCTGGAACCACCAGA				
START domain containing 4, sterol regulated	H11369	F	ACCGCTCAAGGGGTTATTCT	979–1158	180	45	55
		R	CCAACACTTTGGGAGGCTAA				
Acidic ribosomal phosphoprotein PO	M17885	F	CGACCTGGAAGTCCAACTAC	93–201	109	45	53
		R	ATCTGCTGCATCTGCTTG				

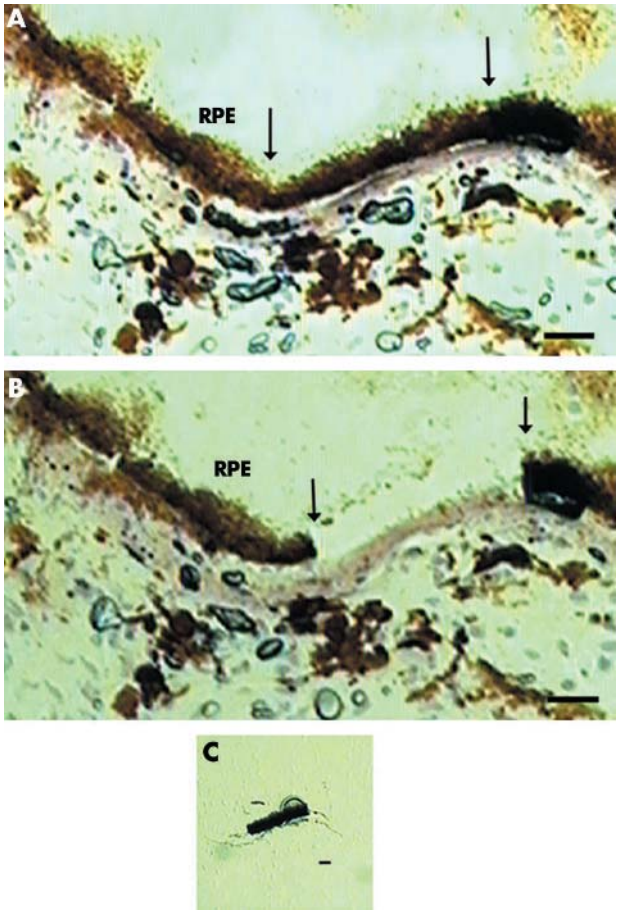

Figure 1 Photomicrograph of macular RPE/Bruch's membrane with normal morphology before (A) and after (B) laser capture microdissection, as described in the methods. Arrow indicates area of laser dissection. Five laser spots dissected five RPE cells, as seen on the transfer cap (C). Bar = 10 μm.

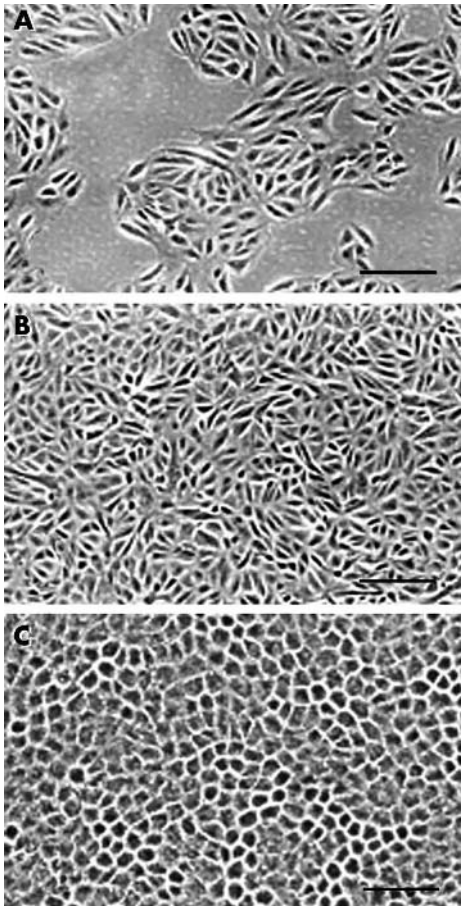

Figure 2 Phase contrast micrograph of ARPE-19 cells grown under various conditions, as outlined in the methods. (A) Subconfluent cells grown in DMEM/F12 + 10% FBS; (B) confluent cells grown in DMEM/F12 + 10% FBS; and (C) differentiated cells grown for 2.5 months in DMEM/F12+10% FBS. Bar = 50 μm.

Table 3 shows the 50 most abundant genes from native RPE cells, of which 48 were also the 50 most abundantly expressed genes by ARPE-19 cells, regardless of culture condition (each separately analysed). While some genes illustrate the multiple functions of the RPE such as melanin biosynthesis (D-dopachrome tautomerase) or antioxidant function (Selenoprotein T), most genes have general cellular function such as protein processing (14%), cytoskeleton (8%), cell cycle (8%), and differentiation (6%). Two class unpaired SAM using a twofold expression differential showed no differentially expressed genes with any false discovery rate (FDR).

The majority of the 50 lowest abundance genes expressed by native RPE cells have function related to transcription factors (16%), metabolism (6%), or are unknown (48%). However, a great number of genes had low expression. To further assess the proximity of low abundance genes between native and cultured RPE, 807 genes expressed by native RPE were identified by doubling the arbitrary expression units of the lowest 50 genes. The number of genes expressed by different culture conditions was low: SS (239 genes; 29.6%), CSW (241 genes; 29.8%), CS (223 genes; 27.6%), DSW (263 genes, 32.4%), DS (226 genes; 28.0%).

Cluster analysis of native and cultured RPE

Unsupervised hierarchical cluster analysis showed that CSW or DSW cells clustered closest to native cells and were segregated from SS, CS, and DS cells (fig 3A). Since RPE cells undergo apoptosis and morphological deterioration with ageing, we identified 713 genes involved with differentiation or cell cycle/apoptosis. Supervised cluster analysis was similar to unsupervised cluster analysis (fig 3B).

Differential expression of native and cultured RPE cells

The expression profiles of CSW and SS cells were used to assess their proximity to native RPE since they were similar and dissimilar, respectively, to native RPE by cluster analysis. Firstly, CSW and SS cultures expressed only 60.5% and 59.6% of the genes, respectively, that were expressed by native RPE. Of the differentially expressed genes identified by SAM (FDR 7%) between native RPE and SS (n = 592 genes), and native RPE and CSW (n = 318 genes), 250 differentially expressed genes were in common, or 42% of SS and 78% of CSW genes, respectively. Of these differentially expressed genes, 36% had no reported function while 7.2% were related to cell cycle/apoptosis, 4.4% to cell structure, 5.6% to metabolism, 7.6% to protein processing, 8.4% to signal transduction, and 6.8% to transcriptional regulation.

Secondly, of 1175 genes unexpressed by native RPE, 324 (27.6%) of these genes were expressed by CSW cells. The function of genes expressed by CSW cells include signal transduction (7.7%), cell cycle/apoptosis (7.7%), transcriptional regulation (7.1%), protein processing (6.5%), intracellular transport (4%), cell adhesion (3.3%), cytoskeleton (1.5%), and unknown function (27.8%). Of genes not

Table 3 50 Most abundant genes expressed by native macular RPE cells

Gene name	GenBank Acc No	Signal (AU)*	Biological function
Cut-like 1, CCAAT displacement protein (*Drosophila*)	AA284408	49.2	Development
cDNA FLJ34046 fis, clone FCBBF2007610	W15465	44.7	Unknown
Solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	N49856	37.7	Neurotransmitter
Nephronophthisis 3 (adolescent)	H93118	36.3	Unknown
Junctional adhesion molecule 2	R68464	36.2	Cell adhesion
Prefoldin 4	AA253430	35.9	Protein folding
EST	R32754	34.1	Unknown
Selenoprotein T	R78516	33.5	Antioxidant
Ubiquitin conjugating enzyme E2I (UBC9 homologue, yeast)	AA487197	33.1	Protein degradation
Biogenesis of lysosome related organelles complex-1, subunit 1	H94857	32.2	Unknown
CDC28 protein kinase regulatory subunit 1B	AA459292	31.4	Cell cycle
D-dopachrome tautomerase mRNA, complete cds	AA292995	31.0	Unknown
Zinc finger protein 258	AA280676	29.4	Development
Endothelin converting enzyme 1	AA279429	28.6	Cell-cell signalling
Multiple endocrine neoplasia I	AA261796	27.6	Transcription regulation
CDC28 protein kinase regulatory subunit 2	AA397813	27.0	Cell cycle
Interleukin 24	AA281635	26.5	Immune response, apoptosis
EST	N92646	26.0	Unknown
Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	AA448184	24.9	electron transport
Profilin 1	AA521431	23.5	Cell structure
BCL2 related protein A1	AA459263	23.1	Apoptosis inhibition
EST	R76499	23.1	Unknown
Kinesin family member 5B	AA046690	21.9	Organelle transport
Rho GTPase activating protein 1†	AA443506	21.5	Cell structure
Adaptor related protein complex 1, γ1 subunit	W07300	21.5	Protein sorting
NudE nuclear distribution gene E homologue-like 1 (A. nidulans)	R94775	21.1	Unknown
EST	H93842	20.8	Unknown
Sarcolipin	AA196465	20.7	Intracellular transport
Myeloid cell nuclear differentiation antigen	N29376	20.7	Transcription regulation
PDGFA associated protein 1†	AA490300	20.6	Cell cycle
Guanine nucleotide binding protein (G protein), α inhibiting activity polypeptide 3	AA490256	20.3	Unknown
Chitinase 3-like 1 (cartilage glycoprotein-39)	AA434115	20.2	Metabolism
EST	H93906	20.1	Unknown
Chromosome 14 open reading frame 2	T90621	20.1	Unknown
Ataxin 2 related protein	AA029963	19.9	Unknown
Zinc finger protein 258	AA280677	19.9	Development
DnaJ (Hsp40) homolog, subfamily B, member 1	AA481758	19.8	Protein folding
Vesicle docking protein p115	AA504342	19.5	Vesicle docking during exocytosis
Secretory carrier membrane protein 2	R32802	19.4	Protein transport
Cas-Br-M (murine) ecotropic retroviral transforming sequence	N94234	19.4	Cell growth
EST	T60223	19.3	Unknown
Chromosome X open reading frame 12	AA455272	18.8	Unknown
EST	R69566	18.8	Unknown
Decay accelerating factor for complement (CD55, Cromer blood group system)	R09561	18.6	Complement pathway
CTF8, chromosome transmission fidelity factor 8 homologue (*S cerevisiae*)	N57731	18.5	Unknown
EST	R25153	18.5	Unknown
Villin 2 (ezrin)	AA411440	18.5	Cell structure
Ankyrin repeat domain 1 (cardiac muscle)	AA488072	18.5	Defence response
Cytoskeleton associated protein 1	AA504554	18.5	Cell structure
EST	W44701	18.3	Unknown

*AU, arbitrary units.
†Indicate genes not in the top 50 of cultured RPE cells (any condition).

expressed by native RPE, 349 genes (29.7%) were expressed by SS cells. The most common function of genes expressed by SS cells included cell cycle/apoptosis (9.7%), protein processing (7.7%), signal transduction (6.9%), transcription regulation (6.3%), small molecule transport (4.9%), cell adhesion (4.3%), immune response (3.4%), RNA processing (2.3%), and unknown function (28.9%).

The number of differentially expressed genes between native and ARPE-19 cells was used as a third assessment of transcriptional proximity. An FDR<10% is a reliable indicator of statistical precision for studies using human tissue.[13] Using two class unpaired SAM analysis between native RPE and CSW (FDR 7.2%) or SS cells (FDR 7.4%), 317 and 591 differentially expressed genes respectively, were identified.

The function of differentially expressed genes between native RPE and individual culture conditions was a fourth factor used to evaluate the similarity of expression profiles. The functional annotation of the 317 differentially expressed genes between native RPE and CSW cells included cell cycle/apoptosis (10.7%), protein processing (6.9%), signal transduction (5.7%) and transcription factors (5.7%), metabolism (6.3%), and unknown (42.7%). Analysis using highly stringent conditions (FDR 1.5%) showed that 48 genes were underexpressed and five genes were overexpressed by native RPE compared to CSW cells (table 4). Of this gene set, 21% were related to protein processing.

The biological function of the 591 differentially expressed genes between native and SS cells included cell cycle/apoptosis (9.6%), protein processing (8.8%), metabolism (8.3%), signal transduction (7.4%), transcriptional regulation (5.9%), cytoskeleton (4.7%), cell adhesion (3.4%), and unknown (35.4%). Analysis using highly stringent conditions (FDR 1.4%) identified 41 genes that were underexpressed and 5 genes that were overexpressed by native RPE compared to SS cells (table 5). Again, protein processing genes comprised 17% of differentially expressed genes.

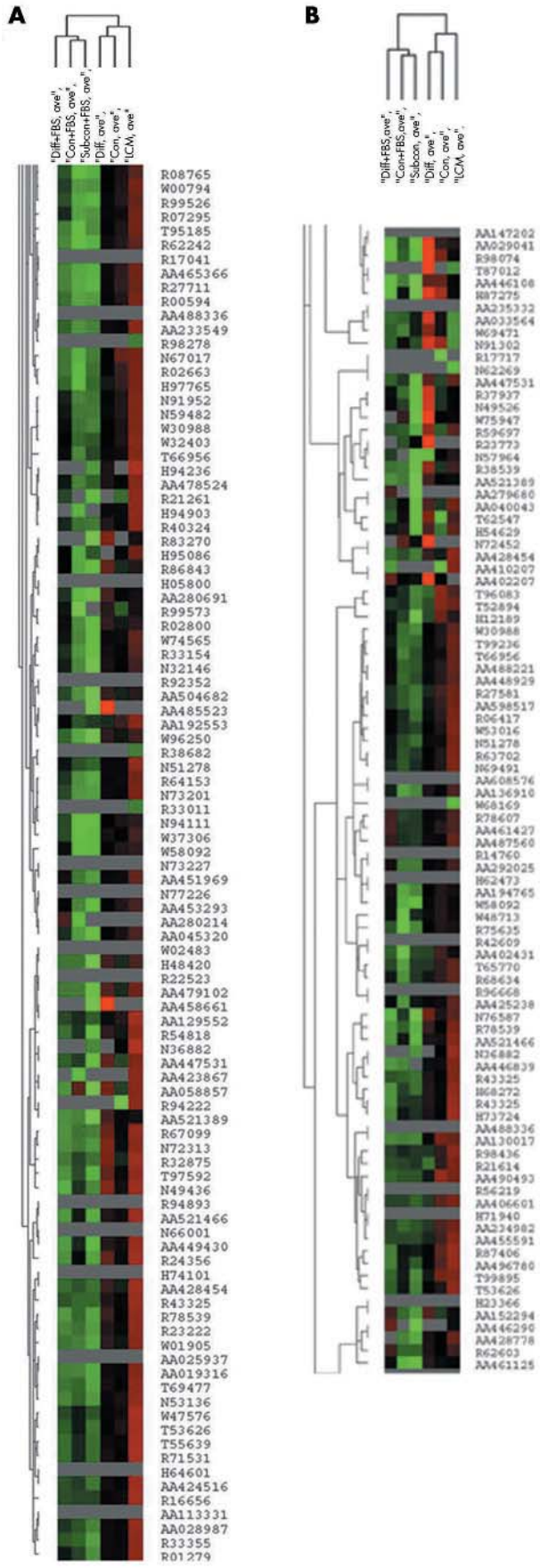

Figure 3 A cluster node of laser capture microdissected RPE cells and ARPE-19 cells grown under five culture conditions. The similar dendrite length indicates relative similarity between conditions. (A) Unsupervised cluster analysis; (B) supervised cluster analysis. Red is $\log_2$ intensity value<1, green is $\log_2$ intensity value>1 after transformation, black is $\log_2$ intensity value$=1$, and grey indicates not expressed. ''Diff+FBS'' is differentiated ARPE-19 cells grown in serum; ''Native'' is laser captured RPE cells; ''Subcon + FBS'' is subconfluent ARPE-19 grown in serum; ''Con+FBS'' is confluent ARPE-19 cells grown in serum; ''Con'' is confluent serum withdrawn ARPE-19 cells; and ''Diff'' is differentiated serum withdrawn ARPE-19 cells. Individual genes are designated by ACC number.

Serum influences the expression of cultured RPE cells

Since serum divided the culture conditions, and native RPE clustered with serum withdrawn conditions, two class unpaired SAM analysis (FDR 9.9%) between serum and serum withdrawn conditions identified 317 differentially expressed genes. Cells that were grown with serum removed had 207 genes overexpressed and 110 genes underexpressed compared to cells grown in serum. The biological function of differentially expressed genes included protein processing (11.7%), signal transduction (11.7%), cell cycle (5.4%), metabolism (5.4%), transcription factors (4.7%), cell adhesion (2.8%), cell structure (1.8%), and unknown (38.8%). The actual differential expression was small, ranging from 15–30%. To evaluate the influence of genes with larger differential expression, SAM analysis with more stringent FDR (4.6%) and a twofold differential expression threshold was performed (table 6). Three apoptosis, and three differentiation genes were identified.

Real time RT-qPCR validation

Real time RT-qPCR was performed on cells grown under identical culture conditions, but separate experiments from the microarray analysis, and native RPE from five different globes to expand the differential expression pattern beyond the eyes used for microarray experiments. Since the purpose of this investigation was to evaluate global gene expression changes, genes were randomly selected. Table 7 shows that all five genes had similar expression patterns as observed on the arrays.

DISCUSSION

We showed previously that confluent ARPE-19 cells grown on different matrices had different transcriptional profiles from native RPE, and that cells grown on plastic had the closest transcriptome to native RPE.[2] To improve the transcriptional proximity of ARPE-19 cells grown on plastic to native RPE, we varied the culture conditions and evaluated global expression trends, a more informative benchmark than individual gene expression. With this end point, CSW and DSW cells were most similar to native RPE. While each culture condition preserved the expression of the most abundant genes, significant transcriptional differences exist between native and cultured cells including the number of genes on the array that were expressed and not expressed, the number and function of differentially expressed genes, and the number of expressed low abundance genes. Our results suggest that the global expression profiles can be improved by varying the culture conditions, but significant widespread differences remain between cultured and native RPE.

With ageing, the RPE undergoes apoptosis and morphological deterioration. Supervised cluster analysis of 713 genes related to differentiation or cell cycle/apoptosis was similar to unsupervised analysis, further suggesting that CSW or DSW cells are most similar to native RPE. Further analysis of the transcriptome of CSW cells however, showed significant differences from native RPE including the number of genes

Table 4 Differential gene expression between confluent, serum withdrawn cultured and native RPE cells sorted by SAM (FDR 1.5%). Genes are sorted by biological function

Gene name	GenBank Acc No	Score (d)	Fold change*	Biological function
48 Genes overexpressed by confluent serum withdrawn RPE				
Lectin, galactoside binding, soluble, 9 (galectin 9)	AA434102	4.6899019	NA†	Cell adhesion
Cyclin dependent kinase 4	AA486312	4.0367285	NA	Cell cycle
Pospholipase A2 receptor 1, 180 kDa	R91516	3.9299213	NA	Cell cycle
Integrin β1 binding protein 1	AA456882	3.8089025	NA	Cell-matrix adhesion
Integrin, α2 (CD49B, α2 subunit of VLA-2 receptor)	AA463610	3.7711619	NA	Cell-matrix adhesion
Actin, β	R44290	4.0403841	NA	Cytoskeleton
Caldesmon 1	AA076063	3.7303184	1.2	Cytoskeleton; muscle contraction
Oxidase (cytochrome c) assembly 1-like	AA598582	3.4692439	10.5	Electron transport
Tumour suppressor candidate 3	N66008	5.2150083	NA	Electron transport
Cytochrome c oxidase subunit VIc	AA456931	4.3479318	NA	Electron transport
Reproduction 8	AA465570	5.736559	NA	Fertilisation
Sialyltransferase 1 (β galactoside α-2,6-sialyltransferase)	AA598652	4.0207485	NA	Immune response
Epstein-Barr virus induced gene 3	AA425028	3.4711393	NA	Immune response
Tumour necrosis factor receptor superfamily, member 5	H98636	3.3610776	NA	Immune response; apoptosis
Fucosyltransferase 4 (α(1,3) fucosyltransferase, myeloid specific)	R28447	3.8948373	NA	Metabolism; carbohydrate metabolism
Histidine ammonia-lyase	W86776	4.2938508	NA	Metabolism; histidine catabolism
Epoxide hydrolase 2, cytoplasmic	R73525	5.1584747	NA	Metabolism; xenobiotic metabolism
Protein phosphatase 1, catalytic subunit, α isoform	AA443982	3.7866788	NA	Metabolism; glycogen metabolism
CAZ associated structural protein	R52873	3.3870227	NA	Protein degradation
Ubiquitin protein ligase E3C	AA284599	4.808889	NA	Protein degradation
Ubiquitin conjugating enzyme E2D 3 (UBC4/5 homologue, yeast)	AA017199	4.1638422	NA	Protein degradation
Proteasome regulatory particle subunit p44S10	AA424807	3.8497784	NA	Protein degradation
HECT type E3 ubiquitin ligase	R87212	3.2685684	NA	Protein degradation
ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	AA427472	16.602038	2.8	Protein degradation; acidification of organelles
Protein-O-mannosyltransferase 1	R13777	3.9364101	NA	Protein modification
Aspartylglucosaminidase	R42153	3.4922915	NA	Protein modification
Adaptor related protein complex 3, β 2 subunit	H11692	4.1620878	NA	Protein transport
SEC13-like 1 (S. cerevisiae)	AA496784	4.1282042	NA	Protein transport
α-2-macroglobulin	H06516	3.6385137	NA	Protein transport
RAE1 RNA export 1 homolog (*S pombe*)	AA504128	3.4039961	NA	RNA transport
Protein tyrosine phosphatase, non-receptor type 1	R06605	3.5456862	NA	Signal transduction
Zinc finger protein 593	AA033532	3.3121002	NA	Transcription regulation
Signal transducer and activator of transcription 3 interacting protein 1	N77731	3.2713342	NA	Transcription regulation
Zinc finger protein 161	AA232647	6.8052672	1.5	Transcription regulation; cellular defense
Topoisomerase (DNA) II alpha 170 kDa	AA504348	45.484875	17.4	Transcription regulation; DNA topology
Synaptoporin	H98620	4.1875559	NA	Transport
ATP binding cassette, subfamily B (MDR/TAP), member 10	R83875	3.3779001	NA	Transport
EST	H66616	5.2260079	NA	Unknown
HIV-1 rev binding protein 2	W52273	4.6580643	2.6	Unknown
EST	R73909	4.2685967	NA	Unknown
EST	AA411554	4.1332	1.5	Unknown
EST	R02609	3.9193518	1.5	Unknown
Cisplatin resistance associated	W77812	3.8728312	NA	Unknown
Reticulocalbin 1, EF-hand calcium binding domain	AA457719	3.3784786	NA	Unknown
Family with sequence similarity 13, member A1	N51424	3.3669014	NA	Unknown
Transcribed sequence with weak similarity to protein ref:NP_060219.1 (*H sapiens*) hypothetical protein FLJ20294 (*H sapiens*)	R51835	3.3217265	10.3	Unknown
Similar to non-histone chromosomal protein HMG-14 (high mobility group nucleosome binding domain 1) (LOC400452), mRNA	R53889	3.315474	NA	Unknown
EST	W19429	3.2800179	NA	Unknown
5 genes underexpressed by confluent serum withdrawn RPE				
Phosphofructokinase, platelet	R38433	−5.491407	−2.0	Metabolism; glycolysis
Mannosyl (α-1,6-)-glycoprotein β-1,2-N-acetylglucosaminyltransferase	AA485653	−3.719321	−2.0	Metabolism; oligosaccaride synthesis
START domain containing 4, sterol regulated	H11369	−3.790357	−2.6	Metabolism; cholesterol transporter
Snail homologue 2 (*Drosophila*)	H57309	−3.733474	−6.3	Transcription regulation
CDNA FLJ42496 fis, clone BRACE2035003	AA410207	−4.944185	−12.2	Unknown

*Confluent, serum withdrawn/native RPE; †NA, undetectable in native RPE.

expressed and not expressed, and differentially expressed genes with a diversity of function. These data collectively suggest that while clustering closest to native RPE, the global transcriptional profile of CSW cells displayed multiple mRNA phenotypic differences which may influence ageing studies.

Culture conditions were segregated by serum. The function of differentially expressed genes, regardless of culture density, was quite wide ranging, which suggests that serum influences multiple cellular functions including protein processing, signal transduction, cell cycle, metabolism, and

Table 5 Differential gene expression between subconfluent cultured and native RPE cells sorted by SAM (FDR 1.4%). Genes are sorted by biological function

Gene name	GenBank Acc No	Score (d)	Fold change*	Biological function
41 Genes overexpressed by subconfluent RPE				
Collagen, type XVI, α1	R54778	6.2086958	NA†	Cell adhesion
Integrin, α2 (CD49B, α2 subunit of VLA-2 receptor	AA463610	5.9930352	NA	Cell adhesion
Thrombospondin 2	H38240	5.5952514	NA	Cell adhesion
Insulin-like growth factor binding protein 3	AA598601	6.4324886	NA	Cell cycle
Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	AA487700	5.9964532	NA	Cell cycle
v-yes-1 Yamaguchi sarcoma viral related oncogene homologue	R83836	9.0139898	NA	Cell cycle
Gap junction protein, β2, 26 kDa (connexin 26)	AA490688	5.8023088	NA	Cell-cell signaling
Actin, β	R44290	9.7128396	NA	Cytoskeleton
Caldesmon 1	AA076063	7.83121	1.4	Cytoskeleton; Muscle contraction
Reproduction 8	AA465570	6.971362	NA	Fertilisation
Tumour necrosis factor receptor superfamily, member 25	W76376	6.1518795	NA	Immune response, apoptosis
Transcription factor 12 (HTF4, helix-loop-helix transcription factors 4)	AA488497	7.1312589	NA	Immune response; development
Glutamate receptor, ionotropic, N-methyl D-aspartate 2A	H08933	5.6530117	NA	Ion transport
Fucosyltransferase 4 (α(1,3) fucosyltransferase, myeloid specific)	R28447	5.7747679	NA	Metabolism, carbohydrate
Microsomal glutathione S-transferase 1	AA495936	6.6792779	NA	Oxidative stress response
HECT type E3 ubiquitin ligase	R87212	7.9988818	NA	Protein degradation
CAZ associated structural protein	R52873	5.6053736	NA	Protein degradation
26 serine protease	H04028	7.4968725	NA	Protein degradation; protease
Elastase 3B, pancreatic	W40123	6.9857814	NA	Protein degradation; protease
Aspartylglucosaminidase	R42153	8.4918723	NA	Protein modification
SEC13-like 1 (*S cerevisiae*)	AA496784	10.424023	NA	Protein transport
Adaptor related protein complex 3, β2 subunit	H11692	5.6233762	NA	Protein transport
Peroxisome biogenesis factor 1	AA427472	16.184002	2.4	Proton transport
Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	AA425853	7.7310373	NA	RNA processing
Tyrosine kinase 2	AA482128	7.5206556	NA	Signal transduction
Inositol 1,4,5-triphosphate receptor, type 2	AA479093	7.3995941	NA	Signal transduction
Protein phosphatase 1, catalytic subunit, αisoform	AA443982	6.5780078	NA	Signal transduction
Zinc finger protein 161	AA232647	9.8923477	1.6	Transcription regulation; defense response
Topoisomerase (DNA) II β170 kDa	AA504348	39.16232	16.9	Transcription regulation; DNA topology
Synaptoporin	H98620	10.04159	NA	Transport
Guanine nucleotide binding protein (G protein), α inhibiting activity polypeptide 3	AA490256	5.6303528	1.5	Transport
ATP binding cassette, subfamily B (MDR/TAP), member 10	R83875	5.5977134	NA	Transport
Human neuropeptide Y receptor Y1 (NPYY1) mRNA, exon 2-3 and complete cds.	R19478	13.345719	NA	Unknown
Syndecan 2 (heparan sulfate proteoglycan 1, cell surface associated, fibroglycan)	H64346	9.7652404	NA	Unknown
Integral membrane protein 2C	AA034213	7.76812	NA	Unknown
Cisplatin resistance associated	W77812	7.6011771	NA	Unknown
IMAGE:363103	AA019511	7.0814745	NA	Unknown
IMAGE:327676	W23757	6.326244	NA	Unknown
IMAGE:28309	R40460	6.3181421	1.5	Unknown
IMAGE:197888	R96220	6.1341687	NA	Unknown
S-phase 2 protein	T69532	5.8673496	NA	Unknown
5 Genes Underexpressed by Subconfluent RPE				
ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	AA427472	−6.104330	−3.6	Protein degradation; Acidification of organelles
Phosphofructokinase, platelet	R38433	−6.797924	−2.4	Metabolism, glycolysis
HIV-1 rev binding protein 2	W52273	−7.691879	−12.2	Unknown
CDNA FLJ42496 fis, clone BRACE2035003	AA410207	−7.320713	NA	Unknown
Solute carrier family 43, member 3	N76193	−6.725273	−2.2	Unknown

*Subconfluent/native RPE; †NA, undetectable in native RPE.

transcription. Native RPE clustered with cells grown in serum withdrawn medium. Since the outer blood-retinal barrier is composed of the RPE and Bruch's membrane, the RPE is probably partially shielded from serum by Bruch's membrane. Our results suggest that serum was a negative factor that separated RPE cells from native cells, and that RPE cells in vivo are not significantly exposed to serum.

Our interpretations are based on a small collection of eyes. The statistical precision of the arrays, however, enabled us to make statistically valid comparisons. While a wide age range was used to reduce age bias, our results may not be generalisable to all native RPE. We were comforted by the validation of our array results by RT-qPCR using a different set of eyes which likely reduces expression differences related to donor to donor variation. We evaluated a single RPE cell line so extrapolation to other cultured RPE cells is unknown. The use of laser microdissection and unamplified RNA for microarray analysis has limitations. While amplification bias

Table 6 Differential gene expression between cultured RPE cells grown in serum and serum withdrawn conditions sorted by SAM with an FDR of 4.6% and at least a twofold differential expression threshold

Gene name	GenBank Acc No	Score (d)	Fold change*	Biological function
13 Genes overexpressed by cells grown in serum withdrawn medium				
Myeloid cell leukaemia sequence 1 (BCL2 related)	AA488674	2.87841767	11.5	Apoptosis
Phytoceramidase, alkaline	W58013	2.60586649	2.8	Apoptosis/ceramide metabolism
Reticulocalbin 1, EF-hand calcium binding domain	AA457719	2.67862204	3.2	Calcium binding/signal transduction
Visinin-like 1	H65066	2.60870158	8.6	Calcium binding/signal transduction
Phytanoyl-CoA hydroxylase interacting protein	AA405628	3.75618502	8.3	Development/differentiation
Leucine rich repeat containing 28	R95132	2.99928312	6.9	Development/differentiation
Ephrin-B1	AA428778	2.7292518	3.2	Development/differentiation
ATP binding cassette, subfamily C (CFTR/MRP), member 13	H47929	3.63023830	6.7	Ion transport
Ubiquitin conjugating enzyme E2D 3 (UBC4/5 homologue, yeast)	AA017199	2.72914196	3.8	Protein degradation
RNA binding motif protein 4	AA456271	2.79296662	2.9	RNA processing
Chromosome 22 open reading frame 8	H94903	3.71707843	6.3	Unknown
IMAGE:3895112	T95342	2.66350001	4.7	Unknown
Mesoderm development candidate 2	AA284495	2.79383791	2.3	Unknown
2 Genes underexpressed by cells grown in serum withdrawn medium				
v-myb myeloblastosis viral oncogene homolog (avian)-like 2	AA456878	−3.6013598	−4.5	Apoptosis/transcription factor
IMAGE:295501	W23543	−3.4456702	−2.4	Unknown

*Serum withdrawn/serum conditions.

Table 7 Differential gene expression between cultured and native RPE by real time RT-PCR

Gene name	GenBank Acc No	Fold change, array*	Fold change, RT-PCR*	p Value
Subconfluent *v* native RPE				
SEC13-like 1 (*S cerevisiae*)	AA496784	0†	−12.5	0.040
ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	AA427472	−3.6	−1135	0.039
Topoisomerase (DNA) II α170 kDa	AA504348	16.9	∞‡	–
Confluent, serum withdrawn *v* native RPE				
START domain containing 4, sterol regulated	H11369	−2.5	−5.7	0.0012
Oxidase (cytochrome c) assembly 1-like	AA598582	10.5	2.71	0.0032
Topoisomerase (DNA) II α170 kDa	AA504348	17.4	∞‡	–

*Cultured RPE/native RPE; †0, undetectable in subconfluent ARPE-19 cells; ‡∞, undetectable in native RPE,

is eliminated, RNA quantity prevents a comprehensive validation even with RT-qPCR. The array utilised was not RPE specific, and contains both a relatively limited gene set and uncharacterised genes. With further study, the uncharacterised genes may provide new insights into general or RPE specific functioning. Many investigators use a wide range of culture densities, medium, and duration to study RPE function. Our results provide evidence in support of the long held opinion that culture conditions alter the cellular phenotype and validate the long held assumption that great care must be utilised when extrapolating results of gene expression experiments from cultured to native RPE cells.

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