

Serum-Induced Differentiation of Human Meibomian Gland Epithelial Cells

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PURPOSE. We hypothesize that culturing immortalized human meibomian gland epithelial cells in serum-containing medium will induce their differentiation. The purpose of this investigation was to begin to test our hypothesis, and explore the impact of serum on gene expression and lipid accumulation in human meibomian gland epithelial cells.

METHODS. Immortalized and primary human meibomian gland epithelial cells were cultured in the presence or absence of serum. Cells were evaluated for lysosome and lipid accumulation, polar and neutral lipid profiles, and gene expression.

RESULTS. Our results support our hypothesis that serum stimulates the differentiation of human meibomian gland epithelial cells. This serum-induced effect is associated with a significant increase in the expression of genes linked to cell differentiation, epithelium development, the endoplasmic reticulum, Golgi apparatus, vesicles, and lysosomes, and a significant decrease in gene activity related to the cell cycle, mitochondria, ribosomes, and translation. These cellular responses are accompanied by an accumulation of lipids within lysosomes, as well as alterations in the fatty acid content of polar and nonpolar lipids. Of particular importance, our results show that the molecular and biochemical changes of immortalized human meibomian gland epithelial cells during differentiation are analogous to those of primary cells.

CONCLUSIONS. Overall, our findings indicate that immortalized human meibomian gland epithelial cells may serve as an ideal preclinical model to identify factors that control cellular differentiation in the meibomian gland.

Keywords: meibomian gland, epithelial cells, differentiation, gene expression, lipids

The differentiation of meibomian gland epithelial cells is critically important for the health and well-being of the ocular surface. These cells produce meibum, a lipid-rich mixture that is released by the gland at the eyelid margins. Meibum spreads onto the tear film and serves to stabilize this film, prevent its evaporation, and promote visual acuity.^{1,2} Interference with this lipid-related production and secretion, such as occurs in meibomian gland dysfunction (MGD), leads to tear film instability and evaporation¹⁻³ and is the major cause of dry eye disease throughout the world.^{1,4}

Unfortunately, despite the importance of meibomian gland epithelial cells, almost nothing is known about the control of their differentiation. Insofar as we know today, meibomian gland epithelial cell differentiation in vivo, as with other sebaceous gland epithelial cells, begins with small, undifferentiated cells located in the acinar periphery. These cells contain large numbers of free ribosomes and mitochondria, and a poorly developed smooth endoplasmic reticulum (SER) and Golgi apparatus. As cells mature and start their migration toward the lateral ductules, the SER and Golgi become more prominent, lysosomes are generated, and lipids begin to

accumulate. Ultimately, cells terminally differentiate, a process associated with a dramatic increase in volume, a profusion of lipid-filled vesicles, and nuclear pyknosis. Cells then undergo holocrine secretion, which involves cellular autophagy, apoptosis, disintegration, and release of lipid-laden contents into the ductules.^{1,5-9}

A preclinical model of this cellular differentiation process in vitro would be ideal. Such a model not only would permit the discovery of agents that regulate this cellular maturation, but also allow the development of new pharmaceutical approaches for the treatment of MGD.

We hypothesize that immortalized human meibomian gland epithelial cells can serve as this preclinical model in vitro. We further hypothesize that exposure of these cells to serum will induce differentiation and provide the opportunity to identify factors that control this process. The purpose of this investigation was to begin to test our hypotheses, and explore the impact of serum on gene expression and lipid accumulation in human meibomian gland epithelial cells.

METHODS

Meibomian Gland Epithelial Cell Culture

Procedures

Primary and immortalized human meibomian gland epithelial cells^{10,11} were cultured in keratinocyte serum-free medium (SFM) containing 5 ng/mL epidermal growth factor (EGF) and 50 µg/mL bovine pituitary extract (BPE). After reaching 70% to 90% confluence, cells were placed in SFM or serum-containing medium (10% fetal bovine serum in equal volumes of Dulbecco's modified Eagle's medium and Ham's F12 with 10 ng/mL EGF) for varying intervals. Media and serum were purchased from Invitrogen-Gibco (Grand Island, NY, USA). The use of human tissues was approved by the Institutional Review Boards of the Schepens Eye Research Institute and Massachusetts Eye and Ear and adhered to the tenets of the Declaration of Helsinki.

Lysosome and Neutral Lipid Staining

After designated time intervals, immortalized human meibomian gland epithelial cells were cultured for 30 minutes in the presence of LysoTracker Red DND-99 (Invitrogen-Gibco) to label lysosomes.¹² Cells then were fixed in paraformaldehyde, processed for staining with LipidTOX green neutral lipid stain (Invitrogen-Gibco) and 4',6-diamidino-2-phenylindole (DAPI) blue nuclear stain (Invitrogen-Gibco), and mounted with ProLong Gold antifade, as described previously.¹¹ Cells were imaged for LysoTracker (excitation/emission, 577/590 nm) and LipidTox (excitation/emission, 495/505 nm) staining with a Nikon Eclipse E800 (Nikon Instruments, Melville, NY, USA).

Cellular Lipid Analyses

Following the designated culture period, primary and immortalized human meibomian gland epithelial cells were treated with 0.25% trypsin and EDTA (Invitrogen-Gibco), washed with PBS twice, then centrifuged. Cell pellets were stored at -80°C until thawing for lipid analyses. Lipids were extracted by the method of Folch et al.¹³

For neutral lipid (i.e., cholesterol, cholesterol esters, wax esters) analysis, a lipid aliquot (100 µg) was further extracted with a Bond Elut NH2 Solid Phase Extraction 50 mg cartridge (Agilent Technologies, Columbia, MD, USA). In brief, the aliquot was dried, resuspended in 1:1 hexane:isopropanol and applied to the conditioned cartridge. The flow-through was collected, dried, resuspended in mobile phase A, and injected with internal standards into a LUNA C18(2) 100Å 150 × 1.00 mm HPLC column (Phenomenex, Torrance, CA, USA) using a Surveyor Autosampler and LC-pump (Thermo Fisher Scientific, Waltham, MA, USA), and analyzed in triplicate. Elution of the C18 column was performed with (A) 95% methanol, 5% 100 mM ammonium formate, pH 4.75 and (B) 95% isopropanol, 5% 100 mM ammonium formate, pH 4.75. The flow rate was set at 50 µL/min and the gradient was: 0 minutes 50% B, 5 minutes 50% B, 20 minutes 100% B, 95 minutes 100% B, 96 minutes 0% B with a 24-minute re-equilibration time at each run's end. Full scan spectra from a mass spectrometer (MS) were obtained by using a positive ion electrospray MS and a Orbitrap Velos Pro (Thermo Fisher Scientific) at 60,000 resolution. Neutral lipid standards used in these analyses included 1-heptadecanoyl-glycerol (17:0 MG) > 99%; 1,2-dilauroyl-sn-glycerol (12:0/12:0 DG) > 99%; 1,3-ditetradecanoyl-2-(9Z-hexadecenoyl)-glycerol (14:0/16:1/14:0 TG) > 99%; cholest-5-en-3β-yl pentadecanoate (15:0 cholesterol ester) > 99%; and 1-oleoyl-N-heptadecanoyl-D-erythro-sphingosine (17:0/d18:1 Ceramide) > 99% (Avanti Lipids, Alabaster, AL, USA); cholesterol-D7 > 98% (Cambridge

Isotope Lab, Andover, MA, USA); and oleyl laurate wax ester (Nu-Check-Prep, Inc., Elysian, MA, USA). The neutral lipids standards were mixed to 0.4 ng/µL triglycerides, 1 ng/µL diglyceride/cholesterol ester/wax ester, 2 ng/µL monoglyceride/ceramide, and 20 ng/µL cholesterol, and then diluted 1:1 with the sample extract. Statistical analysis of cholesterol levels was performed with Student's unpaired *t*-test.

For polar lipid analysis, a lipid aliquot (10 µg) and an internal standard mixture were injected into a LUNA 3 µ Silica(2) 100Å 150 × 1.00 mm column (Phenomenex) using a Surveyor Autosampler and LC-pump (Thermo Fisher Scientific), and analyzed by light chromatography/mass spectrometry (LC/MS) in triplicate and LC/MS²/MS³ in singlicate. Elution of the Silica column was carried out with (A) 58:40:02, isopropanol:hexane:100 mM ammonium formate, pH 4.75 and (B) 50:40:10, isopropanol:hexane:100 mM ammonium formate, pH 4.75. The flow rate was 50 µL/min and the gradient was: 0 minutes 0% B, 15 minutes 0% B, 40 minutes 100% B, 50 minutes 100% B, 51 minutes 0% B, with a 40-minute re-equilibration time at the end of each run. Full scan MS spectra were obtained using negative ion electrospray MS and a LTQ Orbitrap Velos (Thermo Fisher Scientific) at 60,000 resolution. The data-dependent MS² and MS³ scans were acquired on the most intense fragment ion of the top 5 intense ions in the full MS spectra. This process was used to facilitate the identification of the fatty acid composition of individual polar lipid molecular species. The chromatographic elution time for each lipid group was determined by observing the elution of the internal standard and the highest molecular weight species. The extracted ion current of each lipid molecular species was processed with SIEVE software (Thermo Fisher Scientific), and the lipid identity was determined by the retention time and exact mass (5 parts per million [ppm]). Identities were verified by using the fragment ions and the extracted ion chromatogram profiles. The phospholipid standards purchased from Avanti Lipids included: dimyristoyl phosphocholine (14:0/14:0 PC) > 99%; dimyristoyl phosphoethanolamine (14:0/14:0 PE) > 99%; dimyristoyl phosphoserine (14:0/14:0 PS) > 99%; dimyristoyl phosphoglycerol (14:0/14:0 PG) > 99%; dimyristoyl phosphate (14:0/14:0 PA) > 99%; dilauroyl sphingosylphosphocholine (d18:1/12:0 SM) > 99%; 1-myristoyl phosphate (14:0 LPA) > 99%; 1-heptadecenoyl phosphoserine (14:0 LPS) > 99%; 1-myristoyl phosphoethanolamine (14:0 LPE) > 99%; and 1-myristoyl phosphoglycerol (14:0 LPG) > 99%. The polar lipids standards were mixed to 0.1 ng/µL of PG, 1 ng/µL of PE, 2 ng/µL of LPG/LPE/PS/LPS/LPA/SM, and 20 ng/µL of PA/PC.

For all lipid groups except phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin, the ion detected was the molecular species minus one proton. For the excepted species, the ion detected was the molecular ion minus methane plus acetate. The average intensities of all ions were obtained from four replicate measurements.

Microarray Procedures

Total RNA was isolated from cells, analyzed for integrity and processed for the measurement of mRNA levels at Asuragen (Austin, TX, USA), as previously published.¹⁴ Our experiments used Illumina HumanHT-12 v3 and v4 Expression BeadChips (Illumina, San Diego, CA, USA). Data were acquired with Illumina BeadStudio software, and used background subtraction and cubic spline normalization. Standardized hybridization intensity values were adjusted by adding a constant, so that the lowest intensity value for any sample was equal to 16.¹⁵

Normalized data were evaluated without log transformation and statistical analyses were performed with Student's *t*-test (2-tailed, unpaired). These analyses were conducted with

TABLE 1. Influence of Serum on Gene Expression in Primary and Immortalized Human Meibomian Gland Epithelial Cells

Group	Genes ↑	Genes ↓	Total Genes
Undiff vs. serum			
Primary	2125	2358	4483
Immortalized	2025	2787	4812
SFM vs. serum			
Primary	1368	1814	3182
Immortalized	1602	1187	2789

Data were evaluated without log transformation. The expression of listed genes was significantly ($P < 0.05$) up- (↑) or (↓) downregulated by serum exposure, compared to the undifferentiated (“Undiff”) or SFM control groups.

GeneSifter software (Geospiza, Seattle, WA, USA), which also yielded gene ontology, KEGG pathway, and z-score (zsc) reports organized according to the guidelines of the Gene Ontology Consortium (available in the public domain at <http://www.geneontology.org/GO>).¹⁶ Gene comparisons between groups were facilitated by using the GeneSifter intersector program (Geospiza; available in the public domain at www.genesifter.net). All data from the Illumina BeadChips may be obtained and downloaded from the National Center for Biotechnology Information’s Gene Expression Omnibus (available in the public domain at <http://www.ncbi.nlm.nih.gov/geo>) via series accession numbers GSE18099 and GSE 37089.

Real-Time PCR Procedures

The differential expression of selected genes was verified by using quantitative real-time PCR (qPCR) methods. The cDNAs were generated by using SuperScript III Reverse Transcriptase (Invitrogen-Gibco) and random hexamer primers (Invitrogen-Gibco). The qPCR reactions were carried out in triplicate with TaqMan Gene Assays (Applied Biosystems, Inc., Foster City, CA,

USA), TaqMan-specific primers and probes for lipocalin 2 (Hs.204238), claudin 1 (Hs.439060), acetyl-coenzyme A acetyltransferase 2 (Hs.571037), ornithine decarboxylase 1 (Hs.467701), and GAPDH (4326317E). Differential gene expression was calculated by following the Comparative Ct method, as described in Applied Biosystems User Bulletin 2 (updated 2001).

RESULTS

Effect of Serum on Gene Expression in Human Meibomian Gland Epithelial Cells

To determine whether serum upregulates genes associated with cellular differentiation, we cultured primary and immortalized human meibomian gland epithelial cells in SFM for 5 days (“undifferentiated” cells), another 2 days to 70% to 80% confluence, followed by an additional 14 days in either SFM or serum-containing medium. Cells then were processed for analyses with Illumina BeadChips and Geospiza software.

Our results showed that serum clearly stimulates the activity of genes related to cell differentiation. As demonstrated in Table 1, serum exposure significantly ($P < 0.05$) altered the expression of over 2750 genes in primary and immortalized human meibomian gland epithelial cells. This effect was found irrespective of whether serum-associated responses were compared to those of undifferentiated or SFM-treated cells. The magnitude of serum’s influence was quite remarkable. For example, serum exposure induced 19- to 456-fold increases in the activity of genes encoding retinoic acid receptor responder 1, chemokine (C-C motif) ligand 28 (CCL28), insulin-like growth factor binding protein 3, matrix metalloproteinase 12, phospholipase A2, and prominin (Table 2). Many of these genes are associated with cell differentiation. Serum also enhanced by up to 5.9-fold the gene expression of transforming growth factor β 2, increased the mRNA levels of netrin 4 and collagen, type V, α 2 by 2.5- to 21.1-fold, and induced the appearance of polymeric immunoglobulin receptor mRNA

TABLE 2. Effect of Serum on Gene Expression in Primary and Immortalized Human Meibomian Gland Epithelial Cells

Accession #	Gene	Ratio	Ontology
Upregulation			
NM_000478	<i>Alkaline phosphatase</i>	24.7, 11.9, 12.3, 11.9	Anatomical structure morphogenesis
NM_019846	<i>chemokine (C-C motif) ligand 28</i>	15.7, 19.1, 18.9, 17.6	Chemotaxis
NM_000598	<i>Insulin-like growth factor binding protein 3</i>	54.2, 33.8, 41.3, 20.8	Regulation of cell growth
NM_002426	<i>Matrix metalloproteinase 12</i>	50.1, 45.6, 43.0, 54.8	Proteolysis
NM_007069	<i>Phospholipase A2, group XVI</i>	55.4, 17.9, 24.3, 9.0	Lipid catabolic process
NM_006017	<i>Prominin 1</i>	32.4, 31.9, 32.4, 31.9	Cell differentiation
NM_206963	<i>Retinoic acid receptor responder (tazarotene induced) 1</i>	456.0, 207.0, 30.6, 99.5	Negative regulation of cell proliferation
NM_019554	<i>S100 calcium binding protein A4</i>	24.1, 20.8, 12.3, 9.8	Epithelial to mesenchymal transition
NM_003357	<i>Secretoglobin, family 1A, member 1</i>	31.2, 18.3, 29.3, 17.7	Negative regulation of transcription from RNA polymerase II promoter
NM_030754	<i>Serum amyloid A2</i>	140.9, 32.2, 7.2, 43.6	Acute phase response
NM_003186	<i>Transgelin</i>	62.0, 107.5, 8.1, 11.9	Anatomical structure development
Downregulation			
NM_001218	<i>Carbonic anhydrase XII</i>	11.9, 9.8, 8.8, 14.4	1-Carbon metabolic process
NM_002775	<i>HtrA serine peptidase 1</i>	5.7, 6.5, 4.9, 7.5	Negative regulation of TGF β receptor signaling pathway

Relative ratios were calculated by comparing the degree of gene expression in primary and immortalized meibomian gland epithelial cells treated with serum, compared to the undifferentiated or SFM control groups. The ratios, left to right, are from the following comparisons: primary cells, serum versus undifferentiated; immortalized cells, serum versus undifferentiated; primary cells, serum versus SFM; immortalized cells, serum versus SFM.

TABLE 3. Effect of Serum Exposure on Cellular Component Ontologies in Primary and Immortalized Human Meibomian Gland Epithelial Cells

Ontology	Undifferentiated vs. Serum				SFM vs. Serum			
	Pri Genes	Pri z-Score	Imm Genes	Imm z-Score	Pri Genes	Pri z-Score	Imm Genes	Imm z-Score
Upregulation								
Cytoplasmic membrane-bounded vesicle	97	3.14	89	2.99	68	3.2	81	4.07
Cytoplasmic vesicle	105	3.5	101	3.94	72	3.29	89	4.64
Endomembrane system	213	5.4	183	3.99	161	6.65	176	6.57
Endoplasmic reticulum	144	4.14	120	2.6	105	4.69	132	6.7
Endoplasmic reticulum membrane	90	3.41	81	3.03	71	4.69	81	5.17
Endoplasmic reticulum part	101	3.55	86	2.51	82	5.27	95	6.02
Golgi apparatus	162	7.25	143	6.26	107	5.9	128	7.26
Golgi apparatus part	106	6.71	88	5.04	68	5.1	84	6.65
Golgi cisterna	16	2.96	15	2.95	13	3.5	16	4.32
Golgi cisterna membrane	13	2.51	11	2	10	2.73	13	3.72
Golgi lumen	6	2.48	6	2.72	4	2.04	5	2.56
Golgi membrane	84	5.56	73	4.65	61	5.56	72	6.53
Golgi stack	24	4.29	20	3.41	18	4.31	21	4.87
Lysosome	65	7.66	54	6.11	31	3.24	30	2.31
Lytic vacuole	65	7.66	54	6.11	31	3.24	30	2.31
Membrane-bounded vesicle	100	3.32	92	3.2	70	3.34	84	4.33
Vacuole	69	8.26	69	7.73	38	3.88	34	2.25
Vesicle	111	3.88	106	4.23	76	3.59	94	5.02
Downregulation								
Cytosolic large ribosomal subunit	28	12.9	22	8.67	29	15.54	15	9.42
Cytosolic small ribosomal subunit	29	14.19	20	8.2	32	18.23	13	8.44
Large ribosomal subunit	20	10.75	17	7.96	13	7.55	5	3
Mitochondrial envelope	84	5.93	84	4.31	69	5.78	37	2.8
Mitochondrial large ribosomal subunit	11	7.27	9	5.06	7	4.94	4	3.29
Mitochondrial nucleoid	15	6.37	11	3.52	7	2.62	5	2.38
Mitochondrial part	149	10.63	151	8.66	112	8.65	51	2.84
Mitochondrion	265	12.63	292	12.05	202	10.51	102	4.34
Organellar large ribosomal subunit	11	7.27	9	5.06	7	4.94	4	3.29
Ribonucleoprotein complex	220	24.93	201	19.29	163	20.3	60	6.66
Ribosome	113	22.93	93	16.03	98	22.68	35	8.33

Designated ontologies were chosen after analyses of nontransformed data. A z-score is a statistical rating of the relative expression of gene ontologies, and indicates how much a given ontology is over (positive)- or under (negative)-represented in a specified gene list. In other words, a z-score is a normalized difference using the expected value and standard deviation of the number of genes.¹⁷ Positive z-scores reflect gene ontology terms with a greater number of genes meeting the criterion than is expected by chance, whereas negative z-scores indicate gene ontology terms with a lower number of genes meeting the criterion than expected by chance. A z-score close to zero indicates that the number of genes meeting the criterion approximates the anticipated number.¹⁷ In this table, z-scores with values > 2.0 are reported for selected ontologies with ≥4 genes. Genes, number of genes up- or downregulated in primary (Pri) and immortalized (Imm) human meibomian gland epithelial cells after treatment with serum; z-score, significant score for the up- and downregulated genes in the serum-treated cells.

(pIgR; data not shown). Especially noteworthy was our finding that these molecular responses were very similar between primary and immortalized human meibomian gland epithelial cells (Tables 2–7). Indeed, serum elicited the same responses (i.e., significant up- or downregulation compared to “undifferentiated”) in over 2500 genes in both primary and immortalized human meibomian gland epithelial cells.

The influence of serum on genes associated with cell differentiation also was quite evident from zsc analyses of biological process, molecular function, and cellular component ontologies in human meibomian gland cells. Compared to undifferentiated or SFM controls, serum induced a significant increase in ontologies related to the endoplasmic reticulum, Golgi apparatus, lysosomes, vesicles, growth factor activity, cell differentiation, epithelium development, and vesicle-mediated transport (Tables 3–5). Conversely, serum also caused a significant decrease in the expression of genes linked mitochondria, ribosomes, cell cycle, mitosis and translation (Tables 3–5). Examples of gene activities in the tissue

development ontology that were significantly altered by serum are shown in Table 6.

These differentiative effects were duplicated in another series of experiments, which involved the exposure of immortalized human meibomian gland epithelial cells ($n = 3$ wells/condition) to serum-containing or serum-free media for 4 days. Serum treatment significantly ($P < 0.05$) increased ontologies and pathways linked to cytoplasmic vesicles (zsc = 4.7), epithelium development (zsc = 3.3), Golgi apparatus (zsc = 8.4), insulin signaling pathway (zsc = 2.1), lysosomes (zsc = 7.2), vesicle-mediated transport (zsc = 7.0), and autophagy (zsc = 4.0), and decreased those associated with cell cycle (zsc = 16.2), focal adhesion (zsc = 3.6), mitosis (zsc = 15.0), mitochondrion (zsc = 10.0), ribosomes (zsc = 7.1), and translational elongation (zsc = 4.2).

The differentiating impact of serum on primary and immortalized human meibomian gland epithelial cells also was demonstrated by the analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. As shown in Table 7,

TABLE 4. Influence of Serum on Biological Process Ontologies in Primary and Immortalized Human Meibomian Gland Epithelial Cells

Ontology	Undifferentiated vs. Serum				SFM vs. Serum			
	Pri Genes	Pri z-Score	Imm Genes	Imm z-Score	Pri Genes	Pri z-Score	Imm Genes	Imm z-Score
Upregulation								
Anatomical structure development	349	2.64	334	3.35	256	4.28	291	4.4
Apoptosis	195	6	184	6.13	132	5.21	159	6.43
Apoptotic mitochondrial changes	11	3.68	9	2.89	10	4.69	8	2.97
Cell adhesion	117	4.49	96	2.78	97	6.52	103	5.88
Cell death	209	5.85	199	6.17	142	5.16	166	5.88
Cell differentiation	237	2.25	223	2.49	162	2.38	195	3.38
Cell migration	87	4.13	76	3.31	62	4.11	71	4.4
Cell morphogenesis	86	2.81	75	2.07	62	3.11	73	3.64
Cellular response to stimulus	500	4.25	446	3.07	337	3.99	387	4.31
Epidermis development	42	4.72	46	6.23	28	3.86	27	2.82
Epithelium development	65	4.3	61	4.28	46	4.09	49	3.67
Induction of apoptosis	60	3.99	59	4.47	36	2.44	54	5.16
Induction of programmed cell death	60	3.95	59	4.43	37	2.62	54	5.11
Organ morphogenesis	76	2.24	75	2.84	53	2.29	72	4.24
Programmed cell death	196	5.99	185	6.12	134	5.36	160	6.45
Proteolysis	132	5.22	130	5.98	87	4.18	108	5.59
Signal transduction	430	4.31	391	3.71	294	4.3	328	3.95
Signaling	453	3.21	412	2.65	314	3.77	347	3.11
Tissue development	136	5.27	128	5.33	98	5.39	111	5.61
Vesicle-mediated transport	115	4.36	103	3.76	77	3.67	80	2.79
Wound healing	84	3.67	71	2.55	59	3.56	75	5.01
Downregulation								
Cell cycle	209	7.95	327	15.98	124	2.54	84	2.34
Cell cycle checkpoint	56	7.04	77	9.92	28	2.25	22	2.85
Cell cycle phase	154	9.09	248	17.46	79	2.22	54	2.11
Cell cycle process	168	8	275	16.69	95	2.37	64	2.12
Cell division	79	6.01	139	13.58	47	2.47	37	3.32
Gene expression	505	9.8	555	8.19	412	9.61	226	3.75
M phase	92	6.38	172	15.94	52	2.12	37	2.26
M phase of mitotic cell cycle	78	7.33	143	16.41	40	2.2	29	2.39
Mitosis	76	7.31	139	16.26	39	2.23	29	2.59
Mitotic cell cycle	151	10.08	233	17.53	76	2.72	50	2.17
NcRNA metabolic process	93	12.6	96	11.27	71	10.55	32	4.49
NcRNA processing	66	10.51	68	9.37	54	9.73	25	4.45
Nuclear division	76	7.31	139	16.26	39	2.23	29	2.59
Nuclear export	39	9.3	30	5.31	30	7.88	14	3.76
Ribonucleoprotein complex assembly	33	8.13	30	6.03	19	4.44	10	2.38
Ribonucleoprotein complex biogenesis	91	15.81	80	11.5	70	13.43	27	4.84
Ribonucleoprotein complex subunit organization	34	8.06	31	5.97	20	4.52	11	2.63
Ribonucleotide metabolic process	18	4.82	17	3.63	58	3.28	36	2.17
Ribosome biogenesis	69	15.54	60	11.38	61	15.71	20	4.84
RNA processing	172	13.95	180	12.43	126	10.82	52	3.11
rRNA metabolic process	47	11.72	44	9.39	41	11.7	17	4.98
rRNA processing	46	11.88	43	9.53	40	11.8	17	5.24
Sterol biosynthetic process	19	6.39	19	5.52	9	2.52	10	4.56
Translation	180	21.45	157	15.28	151	20.43	67	9.34
Translational elongation	75	19.97	55	12.1	77	23.76	32	11.05
Translational initiation	27	7.6	26	6.21	23	7.41	12	4.27
Translational termination	68	19.2	52	12.31	74	24.37	31	11.52

Specific ontologies were selected after analyses of nontransformed data. Data are reported for designated ontologies with ≥ 8 genes.

serum enhanced the activity of lysosome, phagosome and p53 signaling pathways, and decreased those associated with cell cycle and ribosomes.

To verify in part the Illumina BeadChip results, selected genes were analyzed by qPCR. This experimental approach confirmed the regulatory effects of serum on *lipocalin 2*, *claudin 1*, *ornithine decarboxylase 1*, and *acetyl-coenzyme A acetyltransferase 2* (Table 8).

Influence of Serum on Neutral and Polar Lipid Expression in Human Meibomian Gland Epithelial Cells

To examine whether serum-induced differentiation is paralleled by changes in the lipid profile of primary and immortalized human meibomian gland epithelial cells, we

TABLE 5. Impact of Serum on Molecular Function Ontologies in Primary and Immortalized Human Meibomian Gland Epithelial Cells

Ontology	Undifferentiated vs. Serum				SFM vs. Serum			
	Pri Genes	Pri z-Score	Imm Genes	Imm z-Score	Pri Genes	Pri z-Score	Imm Genes	Imm z-Score
Upregulation								
Cysteine-type endopeptidase activity	13	2.74	12	2.57	9	2.46	13	3.9
Cytoskeletal protein binding	70	2.76	62	2.13	58	4.49	56	2.99
Enzyme binding	94	2.92	93	3.49	68	3.42	72	2.75
Growth factor activity	23	3.8	17	2.19	23	6.15	21	2.78
Peptidase activity	78	3.38	84	4.91	54	3.29	68	4.5
Downregulation								
DNA-dependent ATPase activity	16	3.38	25	5.94	12	2.73	8	2.17
Electron carrier activity	31	3.28	31	2.29	24	2.8	16	2.21
GTP binding	60	3.67	64	2.92	49	3.61	29	2
GTPase activity	44	4.76	41	2.95	29	2.81	23	3.32
Ligase activity	64	2.46	83	3.77	57	3.36	37	2.43
mRNA binding	25	6.13	24	4.84	21	5.96	9	2.28
Nucleic acid binding	437	7.33	492	6.55	351	7.1	211	3.51
Nucleotide binding	345	8.25	389	7.76	250	5.57	150	2.67
Purine nucleotide binding	257	4.97	310	5.94	191	3.46	126	2.52
Purine ribonucleoside triphosphate binding	253	5.06	309	6.32	187	3.44	126	2.8
Purine ribonucleotide binding	255	4.93	309	6	189	3.38	126	2.6
Ribonucleotide binding	255	4.93	309	6	189	3.38	126	2.6
RNA binding	251	19.9	223	13.73	198	17.62	89	7.18
Structural constituent of ribosome	109	24.09	86	16.09	99	25.05	38	10.23
Structure-specific DNA binding	36	3.92	44	4.63	32	4.46	18	2.47
Transferase activity, transferring alkyl or aryl (other than methyl) groups	12	3.17	12	2.54	13	4.65	7	2.67
Translation factor activity, nucleic acid binding	33	8.51	32	7.05	25	7.12	12	3.46

Designated ontologies were chosen after analyses of nontransformed data. Data are reported for selected ontologies with ≥ 7 genes.

analyzed the neutral and polar lipid content in cells after the 2-week culture in SFM or serum-containing medium.

Our results showed that serum exerts quantitative and qualitative alterations in neutral and polar lipid patterns of human meibomian gland epithelial cells. In addition, the nature of the serum-related response was similar in primary and immortalized cells. Serum exposure was associated with a significant ($P < 0.05$, 1-tail) 34% increase in cholesterol levels (primary [P] + immortalized [I] SFM = 189.1 ± 24.5 ng, P + I serum = 253.4 ± 39.9 ng). Similarly, serum treatment led up to 34-fold differences in the amount of various fatty acids in wax and cholesterol esters (Table 9).

The response to serum-induced differentiation appeared to be most pronounced in cellular phospholipids. As shown in Table 10, primary and immortalized human meibomian gland epithelial cells contained many different phospholipids, including phosphatidylcholine, sphingomyelin, lysophosphatidylethanolamine, lysophosphatidylserine, phosphatidylserine, phosphatidylethanolamine, lysophosphatidylglycerol, lysophosphatidic acid, phosphatidic acid, phosphatidylglycerol, lysophosphatidylcholine, lysophosphatidylinositol, and phosphatidylinositol. Phosphatidylcholine was expressed at the highest level (i.e., 47%–57% of total measured phospholipids). Culture of cells in serum resulted in numerous alterations in the type and amount of fatty acids in most of the phospholipid species. Two very consistent changes were the often dramatically increased levels (e.g., up to 109-fold greater) of polyunsaturated fatty acids, as well as arachidonic acid, in differentiated cells (Table 11).

All our neutral and polar lipid raw data are shown in the Supplementary Tables.

The exposure of human meibomian gland epithelial cells to serum, as we previously discovered,¹¹ was associated with the

acquisition of lipid-containing vesicles. As demonstrated in the Figure, much of this lipid accumulation appeared to occur within lysosomes.

DISCUSSION

Our results supported our hypothesis that serum stimulates the differentiation of human meibomian gland epithelial cells in vitro. This serum-induced effect is associated with a significant increase in the expression of genes linked to cell differentiation, epithelium development, the endoplasmic reticulum, Golgi apparatus, vesicles, and lysosomes, and a significant decrease in gene activity related to the cell cycle, mitochondria, ribosomes, and translation. These cellular responses are accompanied by an accumulation of lipid-containing organelles (e.g., lysosomes), as well as alterations in the fatty acid content of neutral and polar lipids. Of particular importance, our results showed that the molecular and biochemical changes of immortalized human meibomian gland epithelial cells during differentiation are similar to those of primary cells. Overall, our findings indicated that immortalized human meibomian gland epithelial cells may serve as an ideal preclinical model to identify factors that control cell differentiation in vitro.

Our hypothesis that exposure of human meibomian gland epithelial cells to serum would induce differentiation, compared to proliferation, was prompted by several considerations. First, serum has been shown to promote the differentiation of other epithelial cell types, including those from human foreskin, mammary, bronchial, and corneal tissues.^{18–23} This developmental process is associated with an upregulation of genes related to cell differentiation and tissue development, and a downregulation of genes linked to cell

TABLE 6. Serum-Induced Alteration of Genes Related to Tissue Development in Primary and Immortalized Human Meibomian Gland Epithelial Cells

Accession #	Gene	Ratio	Ontology
Upregulation			
NM_000598	<i>Insulin-like growth factor binding protein 3</i>	54.2, 33.8, 41.3, 20.8	Regulation of cell growth
NM_005046	<i>Kallikrein-related peptidase 7</i>	52.5, 80.9, 4.5, 2.9	Epidermis development
NM_001878	<i>Cellular retinoic acid binding protein 2</i>	44.8, 20.9, 2.0, 3.8	Epidermis development
NM_000900	<i>Matrix Gla protein</i>	39.2, 3.5, 33.2, 4.8	Protein complex assembly
NM_001323	<i>Cystatin E/M</i>	31.3, 59.2, 3.2, 5.6	Epidermis development
NM_002167	<i>Inhibitor of DNA binding 3</i>	21.5, 28.5, 3.3, 5.5	Negative regulation of transcription
NM_021229	<i>Netrin 4</i>	21.1, 4.2, 6.3, 3.7	Neuron remodeling
NM_172037	<i>Retinol dehydrogenase 10</i>	18.0, 5.2, 8.4, 3.3	Organ morphogenesis
NM_019554	<i>S100 calcium binding protein A4</i>	17.5, 20.8, 8.4, 3.5	Cell differentiation
NM_002166	<i>Inhibitor of DNA binding 2</i>	14.9, 36.8, 5.3, 3.1	Negative regulation of transcription
NM_004936	<i>Cyclin-dependent kinase inhibitor 2B</i>	14.4, 15.9, 5.0, 3.7	Regulation of cyclin-dependent protein kinase activity
NM_001901	<i>Connective tissue growth factor</i>	14.0, 9.2, 4.6, 10.5	Angiogenesis
NM_001453	<i>Forkhead box C1</i>	11.4, 8.8, 3.2, 3.1	Blood vessel development
NM_000393	<i>Collagen, type V, α2</i>	7.6, 3.9, 2.5, 8.8	Axon guidance
NM_001955	<i>Endothelin 1</i>	6.1, 4.1, 2.0, 4.1	Prostaglandin biosynthetic process
NM_001018004	<i>Tropomyosin 1α</i>	4.6, 8.4, 2.1, 6.0	Cellular component movement
NM_000693	<i>Aldehyde dehydrogenase 1 family, member A3</i>	2.9, 2.5, 3.1, 3.5	Retinoic acid biosynthetic process
NM_000362	<i>TIMP metalloproteinase inhibitor 3</i>	2.2, 4.6, 2.8, 2.7	Central nervous system development
NM_001039348	<i>EGF-containing fibulin-like extracellular matrix protein 1</i>	2.1, 2.4, 1.9, 12.6	Epidermal growth factor receptor signaling pathway
NM_000963	<i>Prostaglandin-endoperoxide synthase 2</i>	2.0, 7.2, 5.6, 13.7	Prostaglandin biosynthetic process
Downregulation			
NM_003068	<i>Snail homolog 2</i>	5.6, 4.2, 3.1, 2.2	Negative regulation of transcription
NM_005213	<i>Cystatin A</i>	5.2, 3.2, 4.3, 16.9	Negative regulation of peptidase activity
NM_000575	<i>Interleukin 1α</i>	4.5, 3.6, 2.2, 2.6	Inflammatory response
NM_033260	<i>Forkhead box Q1</i>	4.2, 5.2, 2.5, 3.7	DNA fragmentation involved in apoptotic nuclear change
NM_006142	<i>Stratifin</i>	4.1, 2.3, 2.4, 2.0	Regulation of cyclin-dependent protein kinase activity
NM_000067	<i>Carbonic anhydrase II</i>	4.0, 2.6, 13.3, 7.3	1-Carbon metabolic process
NM_004429	<i>Epbriin-B1</i>	4.0, 2.9, 2.2, 2.2	Neural crest cell migration

Relative ratios were calculated by comparing the degree of gene expression in primary and immortalized meibomian gland epithelial cells exposed to serum, compared to the undifferentiated or SFM control groups. The ratios, left to right, are from the following comparisons: primary cells, serum versus undifferentiated; immortalized cells, serum versus undifferentiated; primary cells, serum versus SFM; immortalized cells, serum versus SFM.

cycle and cell division.^{24,25} Second, serum is known to reduce or inhibit the proliferation of many types of epithelial cells, including those from the trachea, bronchus, mammary gland, urothelium, conjunctiva, and oral mucosa.^{18-20,26-28} We also recently have found that serum decreases the proliferation of

primary and immortalized human meibomian gland epithelial cells, compared to that of cells cultured in SFM.^{10,11} Third, we have discovered that serum treatment increases the accumulation of neutral lipids in immortalized human meibomian gland epithelial cells.^{10,11} This response is characteristic of

TABLE 7. Effect of Serum on KEGG Pathways in Primary and Immortalized Human Meibomian Gland Epithelial Cells

Ontology	Undifferentiated vs. Serum				SFM vs. Serum			
	Pri Genes	Pri z-Score	Imm Genes	Imm z-Score	Pri Genes	Pri z-Score	Imm Genes	Imm z-Score
Upregulation								
Lysosome	35	6.5	31	5.96	21	4.29	17	2.29
p53 signaling pathway	18	4.15	19	5.05	12	3.26	16	4.51
Phagosome	37	5.54	31	4.57	25	4.45	26	4.02
Downregulation								
Aminoacyl-tRNA biosynthesis	12	3.1	17	4.96	11	3.58	7	2.82
Cell cycle	36	5.3	51	8.51	24	3.43	21	4.83
Ribosome	69	18.22	50	11.32	74	23.08	31	11.08
RNA transport	51	7.58	57	8.19	40	6.71	17	2.45
Steroid biosynthesis	10	5.04	10	4.66	6	3.05	6	4.43

Selected ontologies were chosen after analyses of nontransformed data. Data are reported for designated ontologies with ≥ 6 genes.

TABLE 8. Confirmation of Selected BeadChip Gene Expression Results

Gene	Cell	SFM BeadChip	SFM qPCR	Serum BeadChip	Serum qPCR
Upregulation					
<i>LCN2</i>	Primary	6.2	7.5	72.0	161.9
<i>LCN2</i>	Imm	22.6	34.0	21.7	40.3
<i>CLDN1</i>	Primary	15.1	13.9	10.2	11.5
<i>CLDN1</i>	Imm	8.1	9.0	8.6	9.8
Downregulation					
<i>ODC1</i>	Primary	7.8	12.1	21.9	19.7
<i>ODC1</i>	Imm	4.5	5.9	6.9	14.6
<i>ACAT2</i>	Primary	10.3	15.4	12.4	17.4
<i>ACAT2</i>	Imm	4.3	3.4	7.1	9.5

The expression of selected genes, that were up- or downregulated by serum exposure in human meibomian gland epithelial cells according to Illumina BeadChip analyses, were reevaluated with qPCR procedures. Numbers represent the relative increase or decrease of gene expression, compared to control values. *LCN2*, lipocalin 2; *CLDN1*, claudin 1; *ODC1*, ornithine decarboxylase 1; *ACAT2*, acetyl-coenzyme A acetyltransferase 2.

differentiation in other sebaceous gland epithelial cells,^{11,29,30} as well as in adipocytes.^{31,32} Of interest, we observed in the present study that lipid accumulation in differentiated meibomian gland epithelial cells appears to occur, at least in part, in lysosomes.

The serum-induced differentiation of human meibomian gland epithelial cells is associated not only with an increased expression of tissue development genes, but also those associated with anti-inflammatory and antibacterial activities. For example, serum upregulates the gene encoding secretoglobin, family 1A, member 1 (i.e., uteroglobin), which suppresses inflammation,³⁵ and downregulates the gene for interleukin 1 α , a proinflammatory cytokine. In addition, serum enhances the expression of genes for phospholipase A2, group XVI, and CCL28. Phospholipase A2 kills gram-positive bacteria and is a key bactericide in human tears.³⁴ Also, CCL28 has antimicrobial activity against gram-positive and gram-negative bacteria, and *Candida albicans*.³⁵ These transcripts, if translated, could contribute to the absence of inflammation and bacterial invasion within the meibomian gland in obstructive MGD.^{14,36-38}

It also is of interest that CCL28 attracts IgA-positive cells to mucosal tissues.³⁹ Given that serum stimulates *pIgR* gene expression in primary human meibomian gland epithelial cells, it is possible that these differentiated cells could have a role in transporting polymeric IgA antibodies to the ocular surface and protecting against microbial infection. In support of this concept, we previously have identified IgA α chain in human meibomian gland secretions,⁴⁰ and others have shown that sebaceous glands can secrete IgA.⁴¹

A particularly intriguing finding was the observation that serum upregulates the expression of genes encoding netrin 4 and collagen, type V, α 2. These proteins are involved with neuron remodeling and axon guidance, and, if translated and secreted in vivo during cellular differentiation, could possibly influence meibomian gland innervation. The meibomian gland is the only sebaceous gland that is heavily innervated,⁵ and human meibomian gland epithelial cells are responsive to neurotransmitters.⁴² However, essentially nothing is known about the control of meibomian gland innervation in vivo.

We discovered that the process of differentiation is associated with significant alterations in the amount of cholesterol, as well as the fatty acid profiles of wax esters, cholesterol esters and phospholipids, in both primary and immortalized human meibomian gland epithelial cells. That such a response can occur is not surprising, given that meibomian glands contain the transcripts necessary for all the key lipogenic enzymes involved in cholesterol and fatty acid synthesis.^{43,44} Further, the expression of these enzymatic mRNAs is known to be significantly increased by factors (e.g., androgens) that promote differentiated functions in meibomian gland epithelial cells.⁴³⁻⁴⁶

It is possible that the quantitative and qualitative changes in fatty acid levels during cellular differentiation might be influenced by the relative activity of several enzymes. These include: phospholipase A2, group XVI, which catalyzes the release of fatty acids from phosphatidylcholine and phosphatidylethanolamine³³; secretoglobin, family 1A, member 1, which may inhibit phospholipase A2³³; and prostaglandin-endoperoxide synthase 1 and 2, also known as cyclooxygenase (COX) 1 and 2, which convert free arachidonic acid after its release from membrane phospholipids by phospholipase A2 to prostaglandin H2.³³ The gene expression for these enzymes is significantly altered during serum-stimulated differentiation of human meibomian gland epithelial cells.

TABLE 9. Influence of Serum on Wax and Cholesterol Ester Fatty Acid Expression in Primary and Immortalized Human Meibomian Gland Epithelial Cells

Fatty Acid	Serum-Free Media		Serum-Containing Media	
	Primary Cells	Immortalized Cells	Primary Cells	Immortalized Cells
Wax ester				
33:1	587.0, 476.6	707.2, 897.2	166.7, 115.8	291.8, 236.1
41:3	58.0, 41.9	24.3, 25.2	162.9, 92.4	78.7, 121.5
42:2	56.4, 84.2	37.7, 67.2	234.5, 205.0	291.4, 292.7
Cholesterol ester				
20:3	89.0, 216.2	166.2, 176.8	854.7, 1023.9	818.1, 1216.9
20:4*	70.4, 364.5*	220.6, 470.3*	744.7, 1152.0*	887.9, 1192.5*
22:4	1.0, 8.5	6.8, 5.7	148.0, 173.7	154.8, 279.6

Primary and immortalized human meibomian gland epithelial cells ($n = 2$ samples/condition) were cultured serum-free or serum-containing media and then processed for the isolation of lipids and the analysis of fatty acids in wax and cholesterol esters. The lipid weights (mg) of each sample were: primary cells, serum-free = 2.6 and 1.5; immortalized cells, serum-free = 2.4 and 3.4; primary cells, serum-containing = 2.8 and 1.8; immortalized cells, serum-containing = 2.7 and 3.2. The numbers in the "Fatty Acid" column, such as 33:1, take the form C:D, where C is the number of carbon atoms in the fatty acids (i.e., 33) and D is the number of double bonds (i.e., 1). The numbers in the cell columns represent the amount (pg) of designated wax and cholesterol ester fatty acids in each sample/condition.

* The fatty acid (20:4) reflects arachidonic acid.

TABLE 10. Phospholipid Expression in Human Primary and Immortalized Meibomian Gland Epithelial Cells In Vitro

Phospholipid	Serum-Free Media		Serum-Containing Media	
	Primary Cells	Immortalized Cells	Primary Cells	Immortalized Cells
Phosphatidylcholine	57.00, 52.35	49.43, 43.93	55.97, 50.63	47.04, 44.97
Sphingomyelin	19.93, 16.91	20.55, 20.47	13.49, 15.45	18.47, 17.95
Lysophosphatidylethanolamine	11.24, 13.16	11.98, 15.48	14.13, 15.30	14.77, 16.89
Lysophosphatidylserine	3.70, 5.01	5.61, 7.03	6.45, 6.76	7.24, 7.97
Phosphatidylserine	2.39, 4.69	4.17, 4.53	4.05, 5.27	5.47, 4.91
Phosphatidylethanolamine	2.47, 3.63	3.29, 3.05	2.59, 3.60	3.02, 2.68
Lysophosphatidylglycerol	1.55, 2.26	2.46, 2.55	1.94, 1.29	1.51, 2.67
Lysophosphatidic acid	1.27, 1.17	1.41, 1.46	0.91, 1.12	1.57, 1.22
Phosphatidic acid	0.41, 0.70	0.97, 1.31	0.41, 0.51	0.84, 0.63
Phosphatidylglycerol	0.05, 0.13	0.13, 0.19	0.08, 0.07	0.07, 0.10

Primary and immortalized human meibomian gland epithelial cells ($n = 2$ samples/condition) were cultured serum-free or containing media and then processed for the analysis of phospholipids. The numbers represent the percentage expression of a given phospholipid in the total phospholipid amount of each sample/condition. The total amount was determined by calculating and adding the picogram quantities of phospholipids shown in this table. Lysophosphatidylcholine, lysophosphatidylinositol, and phosphatidylinositol also were identified in the cellular samples. However, without internal standards for these species, their picogram amounts could not be calculated.

TABLE 11. Effect of Serum Exposure on Phospholipid Fatty Acid Content in Human Primary and Immortalized Meibomian Gland Epithelial Cells

Phospholipid	Serum-Free Media		Serum-Containing Media	
	Primary Cells	Immortalized Cells	Primary Cells	Immortalized Cells
Phosphatidylcholine				
38:2	2480, 2976	2798, 3794	1,795, 828.2	1,687, 1,168
38:4*	567.2, 1206*	699.4, 647.2*	14,642, 9,083*	5,157, 5,304*
38:5	277.4, 629.3	338.3, 292.5	10,131, 6,107	4,206, 4,535
38:6	97.2, 156.1	66.0, 59.9	2,565, 1,674	1,169, 1,115
Lysophosphatidylethanolamine				
20:4*	2155, 5363*	2192, 3504*	19,861, 12,622*	11,603, 15,859*
22:2	204.2, 386.6	364.0, 1091	75.6, 45.7	104.5, 77.5
22:4	312.6, 811.0	585.6, 1457	3,154, 2,254	2,220, 3,016
22:6	456.0, 1080	479.1, 916.8	21,845, 16,015	12,697, 14,728
Lysophosphatidylserine				
22:5	193.3, 521.9	240.6, 481.3	2,080, 1,383	1,449, 1,585
22:6	75.8, 222.9	103.6, 198.4	3,872, 2,435	2,111, 2,756
Phosphatidylserine				
40:6	56.7, 235.0	107.5, 160.8	2,836, 2,301	1,866, 1,897
40:7	17.8, 51.3	66.7, 25.0	221.3, 213.1	164.3, 131.4
42:9	0.4, 1.8	0.5, 0.8	118.8, 104.9	81.9, 79.6
Phosphatidylethanolamine				
38:2	312.8, 569.5	440.2, 618.0	167.0, 142.5	203.1, 118.4
38:4*	330.0, 975.4*	365.8, 404.6*	2,970, 2,792*	1,810, 1,753*
38:6	30.7, 73.2	26.1, 26.9	551.7, 571.7	329.5, 321.0
40:7	17.0, 49.9	35.3, 35.3	925.7, 898.9	685.5, 616.5
Lysophosphatidylglycerol				
20:2	212.4, 823.6	585.1, 973.0	3,608, 1,574	1,550, 3,348
20:4*	39.2, 131.8*	119.3, 155.1*	642.5, 277.9*	232.3, 569.1*
22:5	21.8, 85.0	24.7, 39.6	530.4, 205.6	232.2, 490.5
22:6	19.3, 75.2	18.8, 37.2	2,261, 1,029	970.4, 2,117
Phosphatidylglycerol				
40:7	0.6, 4.8	1.7, 3.9	117.1, 53.6	51.7, 97.0

Primary and immortalized human meibomian gland epithelial cells ($n = 2$ samples/condition) were cultured serum-free or serum-containing media and then processed for the analysis of fatty acids in the phospholipids. The numbers in the cell columns equal the amount (pg) of selected phospholipid fatty acids in each sample/condition.

* The fatty acids represent (20:4), or may contain (38:4), arachidonic acid.

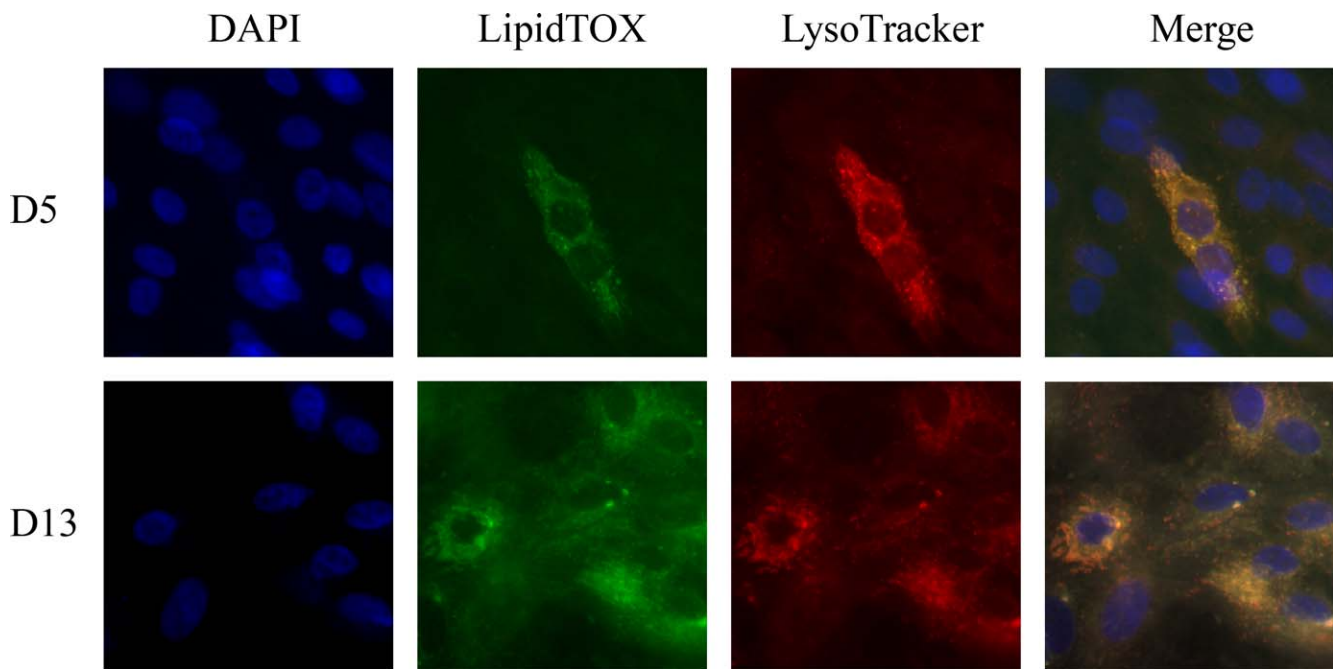


FIGURE. Lipid accumulation in immortalized human meibomian gland epithelial cells. Cells were cultured in serum for 5 or 13 days, and then processed for the identification of neutral lipids with LipidTOX, lysosomes with LysoTracker Red DND-99, and nuclei with DAPI. The figures were overlaid with Adobe Photoshop (Adobe Systems, San Jose, CA, USA) to create the “Merge” figure.

Two of the most striking changes during cellular differentiation were the increases in arachidonic acid content in, and polyunsaturation of, phospholipid fatty acids. If released by the action of phospholipase A2, arachidonic acid could serve as the precursor of many biologically active products (i.e., eicosanoids) that modulate epithelial cell growth and differentiation.^{47,48} Arachidonic acid also may be metabolized by 5-lipoxygenase, ultimately yielding leukotriene B₄ (LTB₄).⁴⁷⁻⁴⁹ The LTB₄ is secreted by human meibomian gland epithelial cells in response to bacterial toxin exposure⁵⁰ and theoretically could promote ocular surface inflammation. It remains to be determined whether these changes in arachidonic acid levels also occur *in vivo* during differentiation. It is possible that they reflect increased arachidonic acid uptake from the serum-containing media *in vitro*.⁵¹⁻⁵³ As concerns the increased polyunsaturation, it is of interest that a decreased unsaturation of nonpolar fatty acids has been associated with MGD.⁵⁴⁻⁵⁶

The human meibomian gland epithelial cell lipids detected in our study do not reflect the levels of neutral and polar lipids typically found in human meibum.^{2,57-60} The reason is that we analyzed cellular extracts, not meibum, and the lipid components of these products are not necessarily the same. To explain, the meibomian gland secretes through a holocrine process, which involves disintegration of the whole cell and secretion of the entire cell components into the lateral ductile and, ultimately, the central and terminal ducts.^{1,5-9} This would include the membranes, which are enriched in phospholipids. However, ductal cells, as in other exocrine glands, often alter the content of their luminal secretions. Such ductal activity also has been proposed for sebaceous glands.⁶¹ More specifically, Thiboutot speculated that phospholipids are recycled in sebaceous gland ducts after holocrine secretion, resulting in the delivery of predominantly neutral lipids to the skin.⁶¹ This recycling process also may occur in the meibomian gland, which would explain, for example, why meibum is depleted of phospholipids.^{2,57-59} Further research is required

to determine the precise role of ductal cells in modulating the lipid content in the human meibomian gland.

Lastly, our study demonstrated that the molecular and biochemical changes of immortalized human meibomian gland epithelial cells during differentiation are similar to those of primary cells. This observation is particularly important, given that other immortalized human ocular surface cells do not necessarily mimic their primary cell counterparts, and that their responses may not be physiologically relevant.^{62,63} Overall, our results indicated that immortalized human meibomian gland epithelial cells may serve as an ideal preclinical model to identify factors that control cell differentiation.

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