Effect of Growth Factors on the Proliferation and Gene Expression of Human Meibomian Gland Epithelial Cells

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Purpose. We hypothesize that growth factors, including epidermal growth factor (EGF) and bovine pituitary extract (BPE), induce proliferation, but not differentiation (e.g., lipid accumulation), of human meibomian gland epithelial cells. We also hypothesize that these actions involve a significant upregulation of genes linked to cell cycle processes, and a significant downregulation of genes associated with differentiation. Our objective was to test these hypotheses.

Methods. Immortalized human meibomian gland and conjunctival epithelial cells were cultured for varying time periods in the presence or absence of EGF, BPE, EGF + BPE, or serum, followed by cell counting, neutral lipid staining, or RNA isolation for molecular biological procedures.

Results. Our studies show that growth factors stimulate a significant, time-dependent proliferation of human meibomian gland epithelial cells. These effects are associated with a significant upregulation of genes linked to cell cycle, DNA replication, ribosomes, and translation, and a significant decrease in those related to cell differentiation, tissue development, lipid metabolic processes, and peroxisome proliferator-activated receptor signaling. Serum-induced differentiation, but not growth factor-related proliferation, elicits a pronounced lipid accumulation in human meibomian gland epithelial cells. This lipogenic response is unique, and is not duplicated by human conjunctival epithelial cells.

Conclusions. Our results demonstrate that EGF and BPE stimulate human meibomian gland epithelial cells to proliferate. Further, our findings show that action is associated with an upregulation of cell cycle and translation ontologies, and a downregulation of genetic pathways linked to differentiation and lipid biosynthesis.

Keywords: meibomian gland, growth factors, gene expression

Meibomian gland secretion is critically dependent upon the active proliferation of glandular epithelial cells.[1] Once generated, these sebaceous-like cells undergo a maturation process toward terminal differentiation, lipid production, and holocrine secretion. Such secretion involves the death and disintegration of fully mature, lipid-rich epithelial cells, their release into glandular ductules, and ultimately, delivery to the ocular surface.[1,2] Given this continual loss of cells, stimulation of epithelial cell proliferation is extremely important and promotes not only meibocyte replenishment, but also the production of meibum.

The stimuli that induce proliferation of human meibomian gland epithelial cells are unknown. We hypothesize that epidermal growth factor (EGF), as well as pituitary hormones and growth factors (i.e., in the components of bovine pituitary extract [BPE]) will induce such proliferation, but not differentiation (e.g., lipid accumulation). We also hypothesize that these actions will involve a significant upregulation of genes linked to cell cycle processes, and a significant downregulation of genes associated with differentiation.

In support of our hypotheses, researchers have shown that EGF is a strong mitogen that stimulates the clonal growth of rabbit meibomian gland epithelial cells,[3] enhances the mitotic activity of hamster sebocytes,[4,5] and promotes the proliferation, but not differentiation, of human amnion epithelial cells by regulating cell cycle genes.[6] Similarly, several pituitary hormones increase sebaceous gland epithelial cell growth,[2] and BPE augments the proliferation of human mammary, thymic, ureteral, and conjunctival epithelial cells.[7–10] Scientists also have reported that EGF and BPE increase the proliferation of normal and immortalized keratinocytes, whereas factors, such as serum, induce their terminal differentiation.[11] In addition, hamster sebocytes exposed to EGF do not accumulate intracellular lipids.[5]

The purpose of our study was to test our hypotheses with human meibomian gland epithelial cells. Toward that end, we examined the effects of EGF, BPE, and both factors in combination, on the proliferation, lipid accumulation, and gene expression of these cells in vitro.

METHODS

Meibomian Gland Epithelial Cell Culture Procedures

Immortalized human meibomian gland epithelial cells, recently generated in our laboratory,[12] were cultured in 6-well plates (BD Falcon, San Jose, CA) or 10 cm culture dishes (Corning Incorporated, Corning, NY) at 37°C in 5% CO_2 in one of the following media: MCDB 153 medium (MCDB; Sigma-Aldrich, St. Louis, MO), which was developed originally for the serum-free growth of specific cell types; serum-free keratinocyte basal medium (SFM; Invitrogen-Gibco, Grand Island, NY); SFM with 5 ng/mL EGF (Invitrogen-Gibco); SFM with 50 μg/mL BPE (Invitrogen-Gibco); SFM with EGF + BPE; or serum-containing medium, which consisted of an equal volume of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (Invitrogen-Gibco), supplemented with 10% fetal bovine serum (Invitrogen-Gibco) and 10 ng/mL EGF. After varying time intervals, total cells were counted with a hemocytometer under a phase-contrast microscope (Nikon Eclipse TS100; Nikon, Avon, MA).

For statistical evaluation of cell counts, ANOVA and Bonferroni multiple comparisons tests were performed with Prism 5 (GraphPad Software, Inc., La Jolla, CA).

LipidTox Staining

Immortalized human meibomian gland and conjunctival epithelial cells (gift of Ilene K. Gipson, Boston, MA) were cultured on glass coverslips (Electron Microscopy Sciences, Hatfield, PA) placed in 6-well plates in SFM supplemented with 5 ng/mL EGF and either 50 μg/mL BPE (meibomian cells) or 25 μg/mL BPE (conjunctival cells). When cells reached approximately 75% confluence, medium was changed to SFM, SFM supplemented with 50 μg/mL BPE and 5 ng/mL EGF, or serum-containing medium. Media were replaced every two to three days for seven days, when cells were washed, then fixed in 4% paraformaldehyde for 30 minutes. Following additional washes, coverslips were exposed to LipidTOX Green neutral lipid stain (Invitrogen, Grand Island, NY) in a humid chamber for 30 minutes. Coverslips were mounted on slides with ProLong Gold antifade reagent with DAPI (Invitrogen) and allowed to dry overnight before imaging with a Nikon Eclipse E800 (Nikon Instruments, Melville, NY).

Microarray Procedures

Total RNA was extracted from cells, evaluated for integrity, and processed for the determination of mRNA levels at Asuragen (Austin, TX), as described previously.[13] Our studies used Illumina HumanHT-12 v3 and v4 Expression BeadChips (Illumina, San Diego, CA). Data were obtained with Illumina BeadStudio software, and used background subtraction and cubic spline normalization. Normalized hybridization intensity values were adjusted by adding a constant, so that the lowest intensity value for any sample was equal to 16.[14]

Standardized data were analyzed without log transformation and statistical analyses were performed with Student's *t*-test (two-tailed, unpaired). These evaluations were conducted with GeneSifter software (Geospiza, Seattle, WA), which also generated 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).[15,16] Comparison of gene data between groups was facilitated by the use of the GeneSifter intersector program (Geospiza; available in the public domain at www.public.genesifter.net). All data from the Illumina BeadChips are accessible for download through 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 37,089.

Real-Time PCR Procedures

The differential expression of selected genes was confirmed by using quantitative real-time PCR (qPCR) methods. The cDNAs were transcribed by using SuperScript III Reverse Transcriptase (Invitrogen) and random hexamer primers (Invitrogen). The qPCR reactions were performed in triplicate by using TaqMan Gene Assays (Applied Biosystems, Inc., Foster City, CA), TaqMan-specific primers and probes for small proline-rich protein 3 (Hs01878180_s1), keratin 10 (Hs00166289_m1), TIMP metallopeptidase inhibitor 1 (Hs00171558_m1), laminin alpha 3 (Hs00165042_m1), 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 EGF and BPE on the Proliferation of Human Meibomian Gland Epithelial Cells

To determine whether EGF and/or BPE stimulate the proliferation of human meibomian gland epithelial cells, we conducted an extensive series of time course experiments.

Our first set of studies was performed after culturing cells (3.76×10^4cells/well; 3 wells/condition) to 20% to 30% confluence, then for an additional 1, 3, and 7 days in one of 4 different media. These included MCDB, SFM, SFM + EGF + BPE, and serum-containing media. Our results demonstrate that SFM + EGF + BPE induced a marked rise in cell proliferation (Fig. 1A). The total number of cells increased approximately 50-fold from day 1, with the largest rise occurring between days 3 and 7. The cells appeared to reach confluence by day 7. In contrast, MCDB supported minimal to no cell proliferation; rather, the total number of cells decreased by approximately 80% over the 7-day period. SFM promoted a gradual increase in the cell proliferation rate. Compared to the cell count at day 1, the total number of cells rose 2-fold, then 8-fold by days 3 and 7. Serum-containing media induced little to no proliferation. The cell count remained the same between days 1 and 7. In addition, cellular morphology appeared flat and enlarged (data not shown).

To confirm and extend these experiments, we cultured 20% to 30% confluent cells for 1, 3, 5, and 7 days in the 4 different media. As shown in Figure 1B, our findings again demonstrated that SFM + EGF + BPE stimulated an ever-increasing cell proliferation rate, and a 45-fold rise in the total cell count between days 1 and 7. MCDB did not support cell proliferation, and most cells detached from the well within 3 days of culture. SFM permitted slight, but steady, cell proliferation. The total number of cells, relative to the quantity at day 1, increased by 5.4-fold during the 7-day time course. Serum-containing media supported little or no proliferation, and the cell count remained constant from days 1 to 7.

To examine the impact of individual supplements on cellular proliferation, we cultured cells in SFM in the presence or absence of EGF, BPE, or EGF + BPE. Cells were 20% to 30% confluent on day 0, and were maintained for 1, 3, 5, 7, 10, and 14 days in culture. Our results demonstrated that cell proliferation rates increased during the time course in all media conditions (Fig. 2). The relative rates were as follows: EGF + BPE > BPE > EGF > SFM. Within 5 days of culture, total

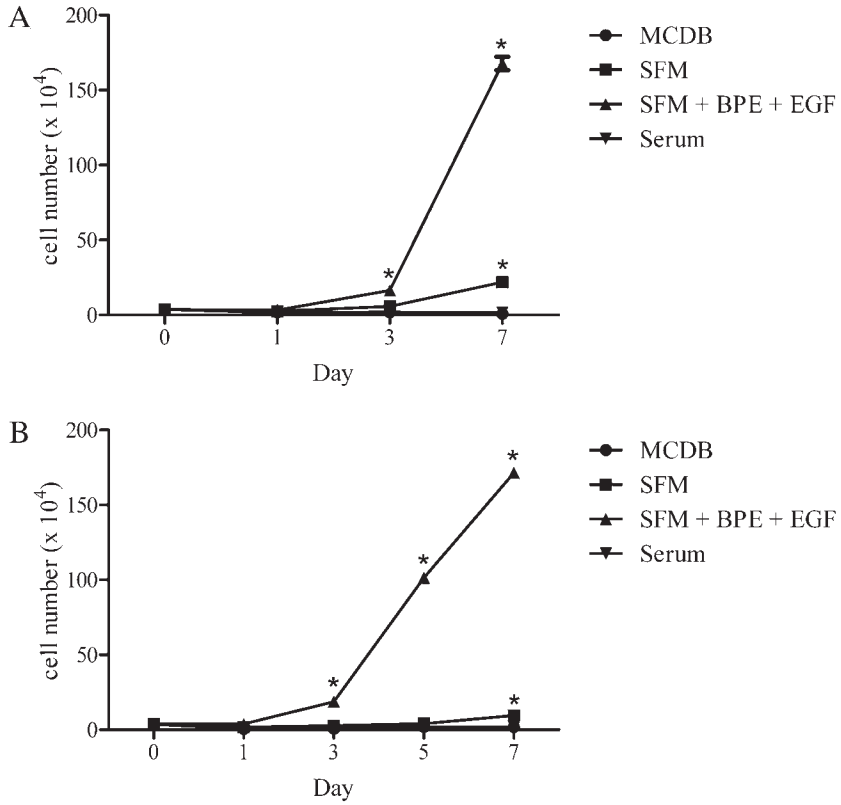

Figure 1. Effect of EGF + BPE, serum, and media on the proliferation of human meibomian gland epithelial cells. Cells ($n = 3$ wells/condition) at passages 44 (**A**) and 49 (**B**) were cultured as described in the text. Values equal the mean ± SE. *Significantly ($P < 0.0001$) greater than values of all other conditions on that day.

cell counts had increased 2.9-, 3.3-, 12.8-, and 18.2-fold in SFM, SFM + EGF, SFM + BPE, and SFM + EGF + BPE media, respectively. By day 5 of culture, cells cultured in the SFM + EGF + BPE media appeared to be 90% to 95% confluent. After 14 days of culture, total cell counts had risen 25.0-, 36.8-, 43.6-, and 60.7-fold in SFM, SFM + EGF, SFM + BPE, and SFM + EGF + BPE media, respectively. These findings indicated that cell proliferation may continue after cells reach confluence. However, the rate of proliferation appeared to decline after confluence was achieved.

The rapidity and magnitude of the proliferative response to EGF and BPE were influenced by the cell passage number. As illustrated in Figure 3A, exposure of passage 50 human meibomian gland epithelial cells to EGF + BPE led to 1.7-, 4.3-, and 62.8-fold increases in cell number by 1, 3, and 7 days after treatment, respectively. By day 7, these cells were completely confluent and had begun to stratify. In contrast, earlier passage cells required more time to reach log phase growth. As shown in Figure 3B, the number of passage 16 cells increased 1.06-, 1.4-, and 13.7-fold within 1, 3, and 7 days after culture in EGF and BPE. At day 7, cells were approximately 90% confluent. Of particular note, these earlier passage cells did not proliferate in SFM, and most cells died and detached from the plates within 3 days of culture (Fig. 3A).

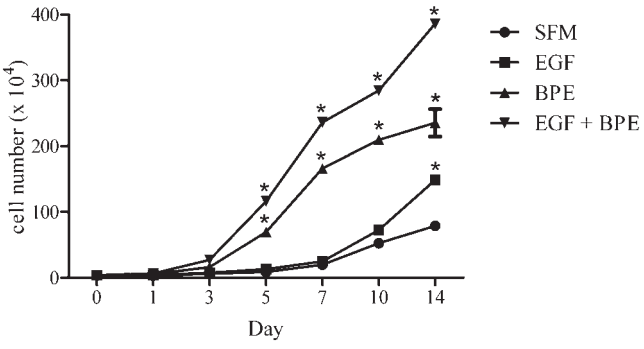

Figure 2. Influence of EGF, BPE, and EGF + BPE on the proliferation of human meibomian gland epithelial cells. Cells at passage 50 were cultured as explained in the text. Values represent the mean ± SE. *Significantly ($P < 0.0001$) greater than SFM control.

Influence of EGF and BPE on Gene Expression in Human Meibomian Gland Epithelial Cells

To examine whether growth factors upregulate genes linked to cell cycle processes, and downregulate genes associated with differentiation, we treated human meibomian gland epithelial cells with EGF and/or BPE for varying periods of time and then processed the cells for analyses with Illumina BeadChips and bioinformatic software.

In the first set of experiments, we cultured subconfluent, passage 16, human meibomian gland epithelial cells for 2 days in SFM ($n = 3.76 \times 10^4$cells/well), followed by an additional 5 days in media containing EGF, BPE, or EGF + BPE ($n = 3$ wells/condition). Our findings demonstrate that growth factor-induced proliferation is associated with a significant ($P < 0.05$) change in the expression of thousands of genes (Table 1). As compared to the basal state, EGF, BPE, and their combination stimulated a significant increase in the expression of genes associated with the cell cycle, cell proliferation, DNA replication, ribosomes, and translation (Table 2). Conversely, these growth factors caused a significant decrease in ontolo-

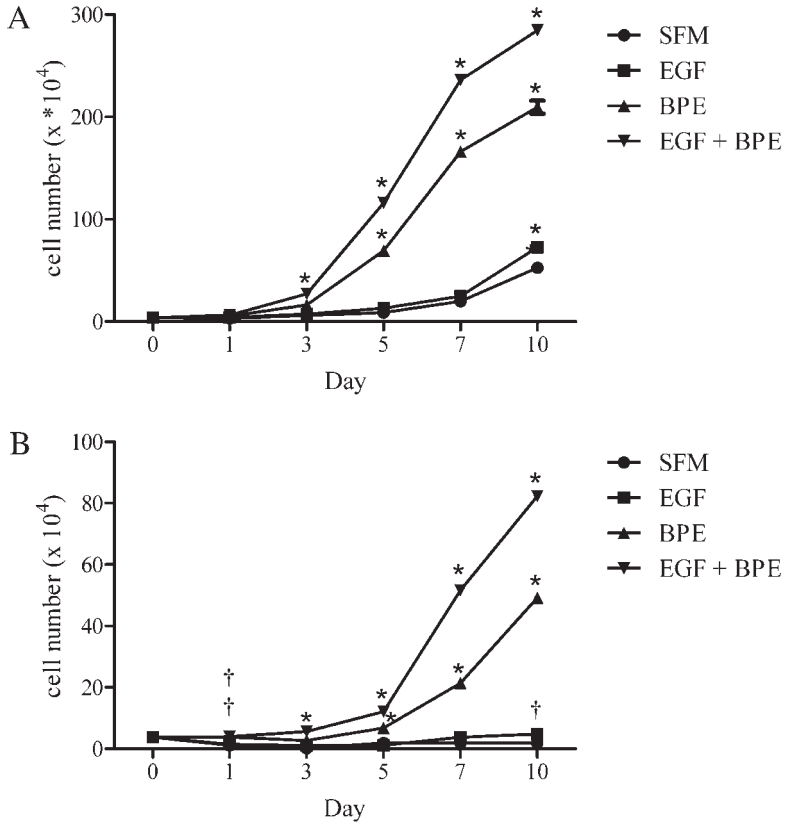

Figure 3. Impact of EGF, BPE, and EGF + BPE on the proliferation of human meibomian gland epithelial cells. Cells ($n = 3$ wells/condition) at passages 50 (**A**) and 16 (**B**) were cultured as described in the text. Values equal the mean ± SE. Significantly (*$P < 0.0001$ and †$P < 0.01$) greater than SFM control. The *double †* in (**B**) indicates that BPE and EGF + BPE conditions were significantly different than the SFM control.

gies related to cell differentiation, keratinization, tissue development, and fatty acid and lipid metabolic processes (Table 3).

These modulatory effects were duplicated in a second series of experiments, which involved the treatment of passage 24 human meibomian gland epithelial cells ($n = 3$ wells/condition) under proliferating (i.e., EGF + BPE) or differentiating (i.e., serum) conditions for 4 days. The combined growth factors significantly ($P < 0.05$) increased ontologies associated with cell cycle processes (zsc = 17.1), cell proliferation (zsc = 3.6), DNA replication (zsc = 11.2), ribosome biogenesis (zsc = 8.6), and translation (zsc = 6.9), and reduced those linked to epithelial cell differentiation (zsc = 5.8), tissue development (zsc = 3.0), lipid metabolic processes (zsc = 3.1), and lipid modification (zsc = 4.5).

The influence of growth factors on human meibomian gland epithelial cells also was shown by the analysis of KEGG pathways. As demonstrated in Table 4, EGF, BPE, and EGF + BPE promoted the activity of cell cycle, DNA replication, and ribosome pathways, and decreased processes related to fatty acid metabolism and peroxisome proliferator–activated receptor (PPAR) signaling (e.g., PPARδ and retinoid X receptor α [RXRα]). Of interest, the growth factors also suppressed pathways associated with acid secretion and lysosomes.

Table 1. Effect of EGF and/or BPE on Gene Expression in Human Meibomian Gland Epithelial Cells

Group	Genes ↑	Genes ↓	Total Genes
EGF	1711	1709	3420
BPE	1941	1803	3744
EGF + BPE	1622	1670	3292

Data were evaluated without log transformation. The expression of listed genes was significantly ($P < 0.05$) up- (↑) or downregulated (↓) by growth factor exposure, compared to the basal SFM control group.

Examples of cell cycle and translation genes upregulated by EGF + BPE treatment of human meibomian gland epithelial cells are listed in Table 5. An illustration of keratin- and lipid-related genes downregulated by combined growth factor exposure is shown in Table 6.

To confirm in part the Illumina BeadChip results, selected genes were analyzed by qPCR. This experimental approach verified the regulatory effects of EGF, BPE, and EGF + BPE on laminin, α3; keratin 10; small proline-rich protein 3; and TIMP metallopeptidase inhibitor 1 (Table 7).

The influence of EGF and BPE on human meibomian gland epithelial cells was not completely the same. In fact, BPE significantly ($P < 0.05$) increased the expression of 1088 genes compared to EGF. In turn, EGF significantly ($P < 0.05$) upregulated the activity of 918 genes relative to BPE. Major differences occurred between these growth factors in the extent of their impact on ontologies and KEGG pathways. For example, BPE induced far greater effects on cell cycle processes (zsc = 14.1) and DNA replication (zsc = 8.5), whereas EGF exerted a stronger influence on ribosomes (zsc = 11.3) and translation (zsc = 11.9).

Impact of EGF + BPE on Lipid Accumulation in Human Meibomian Gland Epithelial Cells

To test our hypothesis that growth factors do not promote differentiated cell functions, such as lipid accumulation, we induced human meibomian gland epithelial cells to proliferate

Table 2. Growth Factor Upregulation of Cell Cycle and Translational Process Ontologies

Ontology	EGF Genes ↑	EGF Z-Score	BPE Genes ↑	BPE Z-Score	EGF + BPE Genes ↑	EGF + BPE Z-Score
Cell cycle						
Cell cycle	147	5.9	231	12.9	167	8.8
Cell cycle arrest	54	5.5	75	8.4	60	7.1
Cell cycle checkpoint	41	5.9	65	10.6	51	8.8
Cell cycle phase	95	5.1	169	13.4	123	9.6
Cell cycle process	112	5.3	190	13.0	138	9.2
Cell division	52	3.9	92	9.9	70	7.7
Cell growth			38	2.2	33	2.3
Cell proliferation	143	5.1	155	4.5	131	4.3
Chromosome			98	7.8	70	4.9
Chromosome, centromeric region			42	8.6	29	5.7
Condensed chromosome	19	2.4	46	9.8	30	6.0
Condensed chromosome kinetochore	11	2.2	27	8.3	18	5.4
Condensed chromosome, centromeric region	12	2.4	29	8.6	20	5.9
DNA replication	34	3.4	57	7.7	44	6.1
G1/S transition checkpoint	12	2.3	14	2.6	12	2.5
G1/S transition of mitotic cell cycle	31	4.8	43	7.1	35	6.2
G2/M transition of mitotic cell cycle	11	0.5	25	4.4	20	3.7
Interphase	51	4.7	80	9.1	63	7.5
Interphase of mitotic cell cycle	50	4.7	77	8.8	61	7.3
Kinetochore			29	7.9	20	5.3
M phase	56	3.3	112	11.4	79	7.8
M phase of mitotic cell cycle	43	3.3	93	11.8	65	8.1
M/G1 transition of mitotic cell cycle	14	3.1	26	7.2	19	5.4
Mitosis	42	3.3	90	11.6	64	8.2
Mitotic cell cycle	92	5.7	163	14.0	120	10.3
Mitotic cell cycle checkpoint	23	4.1	34	6.8	28	6.0
Mitotic cell cycle G1/S transition checkpoint	12	2.3	14	2.6	12	2.5
Mitotic cell cycle G1/S transition DNA damage checkpoint	11	2.4	13	2.7	10	2.1
Mitotic metaphase/anaphase transition	11	3.5	16	5.4	13	4.7
Mitotic prometaphase			32	9.0	23	6.7
mRNA binding	19	5.4	19	4.8	14	3.6
Positive regulation of cell cycle			16	3.6	12	2.7
Positive regulation of cell cycle process	12	1.2	20	3.4	15	2.5
Positive regulation of cell proliferation	59	3.4	63	2.9		
Regulation of cell cycle	87	6.1	121	9.6	93	7.5
Regulation of cell cycle arrest	42	5.8	65	10.2	51	8.4
Regulation of cell cycle process	51	4.9	86	10.5	63	7.7
Regulation of cell proliferation	105	4.1	108	2.9	93	3.0
Regulation of mitosis	15	3.5	21	5.2	18	4.9
Regulation of mitotic cell cycle	34	3.1	54	6.6	40	4.8
Regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	14	3.2	20	5.0	14	3.4
RNA binding	147	11.6	149	10.2	143	11.8
S phase			38	7.6	28	5.7
S phase of mitotic cell cycle	23	4.2	35	7.1	26	5.4
Translation						
Ribonucleoprotein complex	116	13.4	126	12.9	120	13.6
Ribosome	69	15.2	52	9.3	56	11.9
Regulation of translation	25	3.7			26	4.2
Structural constituent of ribosome	67	16.4	47	9.3	55	13.3
Translation	114	14.7	86	8.1	103	13.2
Translation factor activity, nucleic acid binding	22	6.3	16	3.3	19	5.3
Translation initiation factor activity	15	5.8	9	2.2	13	5.0
Translational elongation	54	16.4	36	8.9	47	14.3
Translational initiation	19	6.0	12	2.4	16	4.9
Translational termination	50	16.2	33	8.7	42	13.5

Specific ontologies were selected 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 designated gene list. In other words, a Z-score is a normalized difference using the expected value and standard deviation of the number of genes.[16] Positive Z-scores represent gene ontology terms with a higher 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 near zero indicates that the number of genes meeting the criterion approximates the anticipated number.[16] In this table, Z-scores with values > 2.0 are reported for selected ontologies with ≥ 14 genes. Genes ↑, number of genes upregulated in human meibomian gland epithelial cells after treatment with EGF, BPE, or EGF + BPE; Z-score, specific score for the upregulated genes in the growth factor-exposed cells.

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Table 3. Growth Factor Downregulation of Cell Differentiation and Lipid Synthesis Ontologies

Ontology	EGF Genes ↓	EGF Z-Score	BPE Genes ↓	BPE Z-Score	EGF + BPE Genes ↓	EGF + BPE Z-Score
Cell differentiation						
Cell differentiation			205	2.46	194	2.58
Cornified envelope	16	11.83	16	11.53	16	12.01
Epidermal cell differentiation			27	7.41	27	7.83
Epidermis development	50	8.29	47	7.16	44	6.91
Epithelial cell differentiation	41	6.3	40	5.7	39	5.92
Epithelium development	55	4.32	55	3.94	53	4.09
Keratinization	13	5.68	12	4.89	13	5.76
Keratinocyte differentiation	26	8.48	25	7.76	25	8.18
Tissue development	110	4.75	108	3.95	107	4.58
Lipid synthesis						
Cellular lipid catabolic process	20	3.51	18	2.61	17	2.58
Cellular lipid metabolic process	84	4.41	88	4.51	84	4.6
Cholesterol biosynthetic process			12	4.68	12	4.95
Cholesterol metabolic process	15	2.27	17	2.77	15	2.35
Fatty acid β-oxidation	14	5.56	12	4.28	11	4.01
Fatty acid biosynthetic process			20	3.32		
Fatty acid catabolic process	14	4.46	12	3.31	11	3.09
Fatty acid metabolic process	38	4.05	37	3.5	31	2.53
Fatty acid oxidation	18	4.94	16	3.89	13	2.92
Glycerolipid biosynthetic process	19	3.17	18	2.61	18	2.92
Glycerophospholipid biosynthetic process	14	3.26	12	2.25	12	2.49
Lipid biosynthetic process	55	3.79	56	3.59	53	3.58
Lipid catabolic process	27	2.36				
Lipid metabolic process	121	5.13	124	4.91	116	4.74
Lipid modification	21	3.89	21	3.65	18	2.96
Lipid oxidation	18	4.94	16	3.89	13	2.92
Phospholipid biosynthetic process	19	3.21			17	2.61
Phospholipid metabolic process	25	2.28				

Specific ontologies were selected after evaluation of nontransformed data. Z-scores with values > 2.0 are reported for selected ontologies with ≥14 genes. Genes ↓, number of genes downregulated in human meibomian gland epithelial cells after treatment with EGF, BPE, or EGF + BPE; Z-score, specific score for the downregulated genes in the growth factor-exposed cells.

with EGF + BPE, or to differentiate with serum. In addition, to demonstrate the specificity of the lipid-related response of meibomian (i.e., sebaceous-like) cells, we exposed human conjunctival epithelial cells to the same conditions. Following 7 days of treatment, cells were fixed and stained with LipidTox (green) and DAPI (red).

As shown in Figure 4, EGF + BPE exposure had no apparent effect on cellular lipid levels compared to those of vehicle-treated cells. In contrast, serum treatment elicited a pronounced lipid accumulation in human meibomian gland epithelial cells. This lipogenic response is unique, and is not duplicated in human conjunctival epithelial cells (Fig. 4).

Discussion

Our studies showed that EGF and BPE, alone or in combination, stimulated a significant, time-dependent proliferation of human meibomian gland epithelial cells. These effects were associated with a significant upregulation of genes linked to

Table 4. Growth Factor Influence on KEGG Pathways in Human Meibomian Gland Epithelial Cells

Ontology	EGF Genes	EGF Z-Score	BPE Genes	BPE Z-Score	EGF + BPE Genes	EGF + BPE Z-Score
Upregulation						
Cell cycle	27	4.5	38	7.1	32	6.4
DNA replication	11	4.3	17	7.3	14	6.4
Ribosome	53	15.8	35	8.7	43	12.9
Downregulation						
Collecting duct acid secretion	6	2.4	9	4.5	8	3.9
Fatty acid metabolism	11	3.8	10	3.4	10	3.5
Lysosome	20	2.7	23	3.8	18	2.4
PPAR signaling pathway	14	3.1	14	3.2	12	2.5

Pathways were selected after analyses of nontransformed data. Analogous results were found in the experiments comparing cellular responses to EGF + BPE, compared to serum. In those studies, KEGG pathways for cell cycle (zsc = 8.3), DNA replication (zsc = 8.7), and ribosome (zsc = 3.7) all were upregulated, and those for collecting duct acid secretion (zsc = 3.4), fatty acid metabolism (zsc = 2.4), and lysosome (zsc = 5.8) all were downregulated.

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Table 5. EGF Plus BPE Upregulation of Selected Cell Cycle- and Translation-Related Genes in Human Meibomian Gland Epithelial Cells

Accession #	Gene	Ratio	*P* Value
Cell cycle			
NM_015714	G0/G1switch 2	16.11	0.0206
NM_001255	Cell division cycle 20 homolog	11.94	0.0000
NM_001067	Topoisomerase (DNA) IIα	10.96	0.0028
NM_181803	Ubiquitin-conjugating enzyme E2C	10.18	0.0007
NM_004701	Cyclin B2	8.38	0.0052
NM_005915	Minichromosome maintenance complex component 6	7.37	0.0000
NM_182776	Minichromosome maintenance complex component 7	6.54	0.0001
NM_030928	Chromatin licensing and DNA replication factor 1	6.39	0.0026
NM_002916	Replication factor C (activator 1) 4	5.01	0.0012
NM_002388	Minichromosome maintenance complex component 3	4.96	0.0004
NM_001237	Cyclin A2	3.56	0.0130
NM_004354	Cyclin G2	3.49	0.0027
NM_002689	Polymerase (DNA directed), α2 (70kD subunit)	2.73	0.0052
NM_001760	Cyclin D3	2.43	0.0011
NM_053056	Cyclin D1	2.34	0.0000
NM_199246	Cyclin G1	2.3	0.0156
NM_057735	Cyclin E2	2.07	0.0449
NM_182851	Cyclin B1 interacting protein 1	1.99	0.0011
NM_001759	Cyclin D2	1.43	0.0023
NM_001259	Cyclin-dependent kinase 6	1.34	0.0420
NM_000075	Cyclin-dependent kinase 4	1.31	0.0401
Translation			
NM_138957	Mitogen-activated protein kinase 1	3.61	0.0199
NM_003246	Thrombospondin 1	3.28	0.0006
NM_002759	Eukaryotic translation initiation factor 2-alpha kinase 2	2.65	0.0154
NM_004280	Eukaryotic translation elongation factor 1 epsilon 1	2.16	0.0266
NM_001402	Eukaryotic translation elongation factor 1 alpha 1	1.81	0.0002
NM_001568	Eukaryotic translation initiation factor 3, subunit E	1.81	0.0006

Relative ratios were calculated by comparing the degree of gene expression in meibomian gland epithelial cells exposed to EGF and BPE for 5 days, compared to that of the basal state.

Table 6. EGF Plus BPE Downregulation of Selected Keratin- and Lipid-Related Genes in Human Meibomian Gland Epithelial Cells

Accession #	Gene	Ratio	*P* Value
Keratin			
NM_173086	Keratin 6C	251.79	0.0000
NM_207392	Keratinocyte differentiation-associated protein	175.04	0.0001
NM_005987	Small proline-rich protein 1A	166.23	0.0000
NM_001024209	Small proline-rich protein 2E	63.06	0.0001
NM_003125	Small proline-rich protein 1B	62.37	0.0000
NM_005988	Small proline-rich protein 2A	56.75	0.0000
NM_005557	Keratin 16	52.72	0.0000
NM_001014291	Small proline-rich protein 2G	51.78	0.0000
NM_182507	Keratin 80	51.01	0.0002
NM_002964	S100 calcium binding protein A8	10.86	0.0001
NM_002965	S100 calcium binding protein A9	8.72	0.0000
Lipid			
NM_004354	Cyclin G2	3.49	0.0027
NM_001444	Fatty acid binding protein 5	37.75	0.0026
NM_018404	ArfGAP with dual PH domains 2	25.36	0.0001
NM_001031615	Aldehyde dehydrogenase 3 family, member B2	21.55	0.0001
NM_004364	CCAAT/enhancer binding protein (C/EBP), α	20.52	0.0001
NM_004605	Sulfotransferase family, cytosolic, 2B, member 1	17.83	0.0001
NM_001955	Endothelin 1	10.72	0.0002
NM_025153	ATPase, class V, type 10B	10.02	0.0000
NM_152443	Retinol dehydrogenase 12 (all-trans/9-cis/11-cis)	9.35	0.0007
NM_002899	Retinol binding protein 1, cellular	9.26	0.0000

Relative ratios were calculated by comparing the degree of gene expression in meibomian gland epithelial cells treated with EGF and BPE for 5 days, compared to that of the basal state.

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Table 7. Confirmation of Selected BeadChip Gene Expression Results

Gene	EGF Array	EGF qPCR	BPE Array	BPE qPCR	EGF + BPE Array	EGF + BPE qPCR
Upregulation						
LAMA3	11.2	8.2	9.6	5.7	10.3	4.9
TIMP1	11.7	13.6	4.8	4.9	8.4	6.9
Downregulation						
KRT10	10.6	10.8	10.2	13.7	12.4	16.3
SPRR3	14.0	10.5	1.6	3.0	22.9	11.4

The expression of selected genes that were shown to be up- or downregulated by growth factors in human meibomian gland epithelial cells by using Illumina BeadChips ("Array"), were reexamined with qPCR procedures. LAMA3, Laminin, α3; TIMP1, TIMP metallopeptidase inhibitor 1; KRT10, keratin 10; SPRR3, small proline-rich protein 3.

cell cycle, DNA replication, ribosomes, and translation, and a significant decrease in those related to cell differentiation, tissue development, lipid metabolic processes, and PPAR signaling. Serum-induced differentiation, but not growth factor-related proliferation, elicits a pronounced lipid accumulation in human meibomian gland epithelial cells. This lipogenic response is unique, and is not duplicated by human conjunctival epithelial cells. Overall, our results supported our hypothesis that growth factors stimulate human meibomian gland epithelial cells to proliferate through processes involving gene regulation.

Our finding that EGF and BPE increased the proliferation, but not the differentiation, of human meibomian gland epithelial cells is consistent with the responses of other cell types to these growth factors.[2–11] EGF + BPE induced the highest rate of proliferation, followed by BPE alone, then EGF alone. In contrast, MCDB and serum-containing media supported minimal to no proliferation. SFM permitted slight growth in late-, but not early-passage cells. Of particular interest, the rapidity and magnitude of the proliferative response to EGF and BPE was influenced by the cell passage number. Late-passage (i.e., 50) human meibomian gland epithelial cells proliferated more quickly than early-passage (i.e., 16) cells, despite being exposed to the same culture conditions. This difference may reflect a positive selection of faster-growing cells,[17,18] as well as the development of an altered karyotype,[12] with increasing passage number. We found previously that these human meibomian gland epithelial cells express slight changes in karyotype after passage 34.[12] For this reason, we performed our microarray analyses on early-passage cells, which feature a normal karyotype.[12]

The growth factor stimulation of human meibomian gland epithelial cell proliferation was associated with a significant upregulation of genes linked to DNA replication, cell cycle, ribosomes, and translation. Stimulated genes related to DNA replication included DNA directed polymerase, minichromosome maintenance complex components, replication factor C, and replication proteins. EGF and BPE also increased the expression of genes for cyclin B2 and D1-3, cyclin-dependent kinase (CDK) 4 and 6, and E2F transcription factor, and downregulated those genes encoding CDK inhibitors. Collectively, these genes are the major molecular components that drive cell cycle progression. Cyclin is a regulatory subunit that modulates the activation state of the catalytic subunit CDK in the cyclin-CDK complex.[19] Cyclin B is involved in the control of G2/M transition and mitosis.[19] Cyclin Ds are proto-oncogenic components of the retinoblastoma pathway, and are able to form complexes with Cdk2, Cdk4, Cdk5, and Cdk6.[20] Indeed, the accumulation of cyclin D1-Cdk4/6 complexes is of great importance for cell cycle progression.[21]

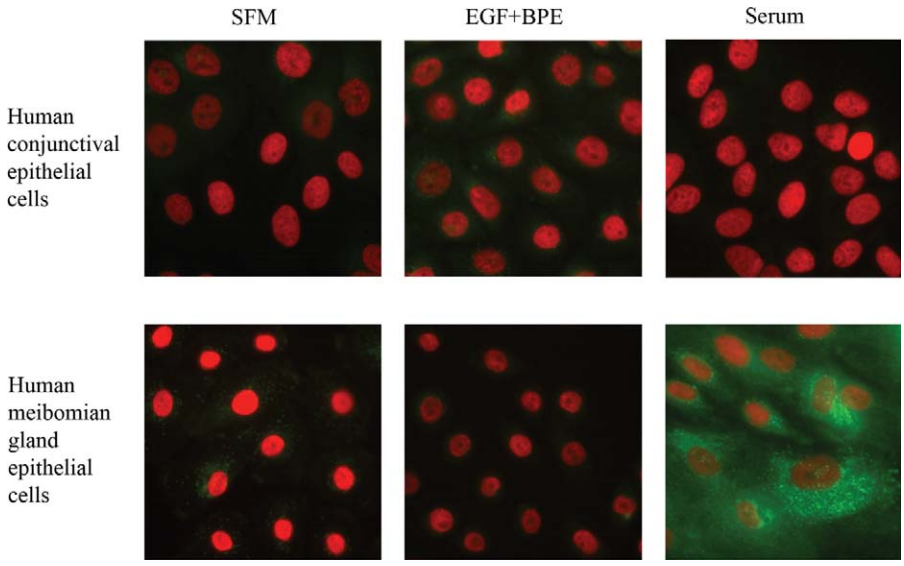

Figure 4. Effect of EGF + BPE, and serum on lipid accumulation in human meibomian gland and conjunctival epithelial cells. Cells were cultured in SFM or in media supplemented with EGF + BPE or serum. Cells were fixed and stained with LipidTox Green neutral lipid stain and DAPI (*red*). Magnification: ×400.

These complexes promote release of E2F transcription factors, which allow the transcription of genes required for the transition from G1 through the S phase.[22–24] The growth factor upregulation of ribosome- and translation-associated genes was to be expected, given that ribosome biogenesis consumes approximately 80% of the energy of a proliferating cell,[25,26] and that ribosomal protein mRNAs monopolize the translational capacity of cells.[26]

We discovered that EGF and BPE cause a significant decrease in the expression of genes related to cell differentiation and keratinization, such as small proline-rich proteins (SPRR), keratins, the S100 calcium binding proteins A8 (S100A8) and A9 (S100A9), and keratinocyte differentiation-associated protein. The SPRR are precursors of the cornified cell envelope of terminally differentiating, stratified squamous epithelia and influence the rigidity of this envelope.[27–31] The SPRR, whether alone[27] or combined with S100A8 and A9,[32] are known to promote keratinization in the conjunctiva[33,34] and elsewhere in the body.[35,36] Of particular interest, the expression of SPRR, S100A8, and S100A9 genes is increased significantly in meibomian glands of patients with meibomian gland dysfunction (MGD).[13] It is possible application of these growth factors might be able to suppress the keratinization process, which is a primary cause of MGD.[2,37]

We also found that EGF and BPE significantly reduce the expression of genes associated with lipid biosynthesis and PPAR signaling. This growth factor-induced gene effect could account for the profound lack of lipid accumulation in proliferating meibomian (this study)[12] and sebaceous[38] gland epithelial cells. As concerns PPARs, these ligand-activated nuclear factors form heterodimers with retinoid X receptors (RXR) to regulate transcription.[39–42] PPARs have been identified in mitochondria, peroxisomes, and microsomes of sebocytes, and are important modulators of multiple lipid metabolic genes.[43] There are three subclasses of PPARs: α, β/δ, and γ, each with different tissue localizations and transcriptional activities.[39,44,45] PPARδ is a potent metabolic regulator in various tissues, such as fat, skeletal muscle, and the heart. PPARδ enhances fatty acid catabolism and energy uncoupling in adipose tissue, and suppresses macrophage-derived inflammation.[46,47] PPARδ also regulates the late stages of sebocyte differentiation and stimulates sebaceous lipid synthesis.[48] In our study, we demonstrated that EGF and BPE significantly downregulate the genes encoding PPARδ and RXRα in human meibomian gland epithelial cells. These growth factor actions possibly may contribute to the suppression of lipid accumulation in these cells.

The influence of EGF and BPE on human meibomian gland epithelial cells was not identical. There were significant differences between these factors in terms of their impact on specific genes, ontologies, and KEGG pathways. For example, BPE induced far greater effects on genes related to cell cycle processes, compared to EGF. This BPE influence may reflect the fact that pituitary extracts should contain adrenocorticotrophic hormone, fibroblast growth factor, growth hormone, insulin-like growth factor 1, thyroid-stimulating hormone, α-melanocyte stimulating hormone, and transforming growth factor-α,[49,50] as well as EGF, all of which have been shown to stimulate the proliferation of sebocytes.[37] However, this stimulatory effect of BPE may be countered somewhat by β-endorphin, which also is present in the pituitary and reportedly decreases sebocyte proliferation.[51]

Our results demonstrated that serum-induced differentiation, but not growth factor-elicited proliferation, promotes a striking accumulation of lipids within human meibomian gland epithelial cells. This response, which was not found in human conjunctival epithelial cells, also may occur after a decrease in the proliferation of meibomian gland epithelial cells[12] and sebocytes.[38] These findings are consistent with earlier observations that neutral lipids accumulate in sebaceous gland epithelial cells during differentiation.[38,52]

Overall, our investigation demonstrated that EGF and BPE exert a significant effect on the proliferation and gene expression of human meibomian gland epithelial cells.

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