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Dihydrotestosterone suppression of proinflammatory gene expression in human meibomian gland epithelial cells

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Abstract

Purpose.—We discovered that dihydrotestosterone (DHT) decreases the ability of lipopolysaccharide, a bacterial toxin, to stimulate the secretion of leukotriene B4, a potent proinflammatory mediator, by immortalized human meibomian gland epithelial cells (IHMGECs). We hypothesize that this hormone action reflects an androgen suppression of proinflammatory gene activity in these cells. Our goal was to test this hypothesis. For comparison, we also examined whether DHT treatment elicits the same effect in immortalized human corneal (IHC) and conjuntival (IHConj) ECs.

Methods.—Differentiated cells were cultured in media containing vehicle or 10 nM DHT. Cells (n = 3 wells/treatment group) were then processed for RNA isolation and the analysis of gene expression by using Illumina BeadChips, background subtraction, cubic spline normalization and Geospiza software.

Results.—Our results demonstrate that DHT significantly suppressed the expression of numerous immune-related genes in HMGECs, such as those associated with antigen processing and presentation, innate and adaptive immune responses, chemotaxis, and cytokine production. DHT also enhanced the expression of genes for defensin β1, IL-1 receptor antagonist, and the anti-inflammatory serine peptidase inhibitor, Kazal type 5. In contrast, DHT had no effect on proinflammatory gene expression in HCECs, and significantly increased 33 gene ontologies linked to the immune system in HConjECs.

Conclusions.—Our findings support our hypothesis that androgens suppress proinflammatory gene expression in IHMGECs. This hormone effect may contribute to the typical absence of inflammation within the human meibomian gland.

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Disclosure/Conflict of Interest

The authors have no conflict of interest to report.

Introduction

A remarkable property of the human meibomian gland (HMG) is its apparent resistance to inflammation.[1,2] This does not mean that the HMG cannot become inflamed, such as in a single blocked gland with a chalazion. But rather that the HMG appears to resist inflammatory processes. For example, there is no evidence of overt inflammation within the glandular tissue in obstructive MG dysfunction (MGD), which affects multiple glands and may lead to conjunctival inflammation (i.e. posterior blepharitis).[1–5] Moreover, although exposure to the bacterial lipopolysaccharide (LPS) and ligand binding protein (LBP) increases the secretion of the chemoattractant leukotriene B4 (LTB4),[6] this endotoxin complex does not elicit the upregulation of proinflammatory response gene ontologies in HMG epithelial cells (ECs).[7] This lack of HMGEC response is in striking contrast to those of human corneal (HC) and conjunctival (HConj) ECs. In these cells LPS+LBP stimulates a significant up-regulation of genes associated with inflammatory and immune responses, including those related to chemokine and Toll-like receptor signaling pathways, cytokine-cytokine receptor interactions, and chemotaxis.[7]

We hypothesize that the transcription of innate anti-inflammatory genes contributes to this resistance to inflammation within the HMG, and that the generation of these transcripts is, at least in part, stimulated by androgens. In support of this hypothesis, we have found that the most highly expressed gene in the HMG encodes for the anti-inflammatory protein, leukocyte-associated immunoglobulin-like receptor-1.[8] Further, we have identified HMGEC genes that are upregulated during differentiation and are associated with the suppression of immune cell activation, pro-inflammatory cytokine production and inflammation.[9] We have also discovered that androgen administration attenuates the secretion of LTB4 by HMGECs,[6] decreases the expression of immune-related genes in proliferating HMGECs,[10] and reduces the transcription of immune-response genes in male and female mouse meibomian glands.[11,12]

The purpose of our present investigation was to continue to test our hypothesis, and determine whether androgen exposure decreases the expression of proinflammatory genes in differentiated HMGECs. For comparison, we also examined whether androgen treatment elicits the same effect in HCECs and HConjECs.

Material and Methods

Cell cultures

Immortalized HMGECs,[13] HCECs (from Dr. James Jester, Irvine, CA)[14]and HConjECs (from Dr. Ilene Gipson, Boston, MA)[15] were cultured, as previously described.[6,7] Briefly, HMGEC were cultured in keratinocyte serum free medium (KSFM) supplemented with 50 μg/ml bovine pituitary extract (BPE), 5 ng/ml epidermal growth factor (EGF), penicillin (100 IU/ml), and streptomycin (100 μg/ml). HCEC and HConjECs were cultured in KSFM supplemented with BPE (25 μg/ml), EGF, penicillin and streptomycin. Cells were kept in 75 cm^2 flasks and then plated for studies in 6-well culture dishes (Corning, Lowell, MA). Once cells reached confluence, their numbers ranged from approximately 4 to 5×10^5 cells/well. The actual counts depended upon the cell type. All cell culture reagents were obtained from

Invitrogen Corp. (Carlsbad, CA), except for Dulbecco's modified Eagle's medium (DMEM)/F12, which was purchased from Mediatech, Inc. (Manassas, VA).

After achieving confluence, cells (n = 3 wells/cell type/treatment) were rinsed with PBS and then placed in a differentiation medium containing DMEM/F12, 10% charcoal/dextran-treated fetal bovine serum (FBS; Invitrogen), 10 ng/ml EGF, penicillin and streptomycin in the presence or absence of ethanol or DHT (10 nM; Steraloids, Wilton, NH) for 2 days. Following this time period, cells were incubated with or without androgen (10 nM) for 6 more hours in serum-free DMEM/F12 containing 1% bovine serum albumin (Mediatech, Inc., Manassas, VA), as previously reported[6] We used 10 nM DHT because this is the physiological concentration of DHT within tissues *in vivo*, and should occupy > 90% of cellular androgen receptors.[16]

RNA extraction and gene microarray analysis

Cellular RNA samples were processed for microarray analyses, as described.[17] In brief, total RNA was purified using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), by following the manufacturer's instructions. The RNA concentrations and 260/280 nm ratios were obtained by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA integrity was examined by using a RNA Nano 6000 Series II Chip with a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). The RNA samples were then additionally processed by Asuragen (Austin, TX) for quantitation of mRNA levels using microarray expression analyses (HumanHT-12 v.4 Expression BeadChips; Illumina, San Diego, CA), as outlined[6,8]

Data that were non-log-transformed, background subtracted and cubic spline normalized were analyzed with commercial software (GeneSifter.net; Geospiza, Seattle, WA). This comprehensive program also produced Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways', gene ontology and z-score reports. Standardized hybridization intensities were all adjusted by adding a constant, such that the lowest intensity value for a sample equaled 16.[18,19] Illumina data were analyzed with Student's t-test (two-tailed, unpaired). All data are accessible through the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) via series accession number GSE111496.

Results

Effect of DHT treatment on proinflammatory gene expression in HMGECs

To determine whether androgen treatment suppresses proinflammatory gene expression in differentiated HMGECs, we exposed cells (n = 3 wells/treatment) to vehicle or DHT and then processed cell samples for Illumina BeadChip and Geospiza software analyses.

Our results show that androgen exposure induced a significant influence on the expression of over 2,000 genes in HMGECs (Table 1). The most striking effect was the DHT suppression of numerous immune-related gene ontologies in HMGECs, such as those associated with innate and adaptive immune responses, chemokine activity and receptor binding, and cytokine production (Table 2). For example, DHT decreased the expression of 67 immune response genes (e.g. IL-1β and IL-8), including those involved with antigen processing and presentation, chemotaxis, T cell mediated immunity, and the production, binding, activity

and signaling of cytokines and/or chemokines (Table 3). In contrast, DHT enhanced the expression of genes for defensin β1 (1.6-fold; $p < 0.0005$), IL-1 receptor antagonist (1.4-fold; $p < 0.0005$), and the anti-inflammatory serine peptidase inhibitor, Kazal type 5 (1.3-fold; $p < 0.05$) in IHMGECs.

In addition to these anti-inflammatory effects, DHT exerted a significant impact on many nonimmune ontologies. These included an upregulation of numerous genes associated with lipid dynamics and differentiation/autophagic processes, and a downregulation of a multitude of genes related to the cell cycle and cell proliferation (Table 4). DHT also significantly influenced assorted KEGG pathways, increasing those linked to lysosomes and mammalian target of rapamycin (mTOR) signaling, and decreasing those mediating cell cycle, chemokine signaling and antigen processing and presentation (Table 4).

Influence of DHT on proinflammatory gene expression in HCECs and HConjECs

To examine whether androgen exposure also decreases proinflammatory gene expression in differentiated HCECs and HConjECs, we treated these cells (n = 3 wells/treatment) with vehicle or DHT and then processed the samples for molecular biological analyses.

Our findings demonstrate that DHT exposure caused a significant change in the expression of more than a thousand genes in both HCECs and HConjECs and (Table 1). Notably, of the 240 biological process gene ontologies (≥ 5 genes/ontology) significantly altered by DHT in HCECs, none were proinflammatory and only one was immune-related. More specifically, DHT increased the expression of 17 genes in the immune system development ontology (z score [zsc] = 2.32). In contrast, the 14 biological process gene ontologies with the highest z scores following DHT treatment of HCECs, as well as 90% of the top 31 such ontologies, were all related to upregulation of the cell cycle (Table 5)

DHT significantly influenced the expression of 338 biological process gene ontologies (≥ 5 genes/ontology) in HConjECs. Thirty-three of these ontologies were associated with the immune system and all were upregulated by DHT (Table 6). Of interest, only five of the immune ontologies (e.g. cytokine production, regulation of defense response) increased by DHT in HConjECs were the same as those decreased by DHT in HMGECs (Table 2). DHT also had an effect on the expression of several cell cycle ontologies in HConjECs. DHT upregulated two (i.e. cell division, 15 genes increased [↑], zsc = 2.01; regulation of DNA replication, 5 genes ↑, zsc = 2.75) and downregulated four (regulation of cell cycle process, 17 genes decreased [↓], zsc = 2.62; cell cycle checkpoint, 13 genes ↓, zsc = 2.91; regulation of cell cycle arrest, 13 genes ↓, zsc = 2.75; G2/M transition, 5 genes ↓, zsc = 4.94) of these ontologies.

Discussion

The purpose of this study was two-fold. First, to test our hypothesis that DHT suppresses proinflammatory gene expression in IHMGECs. Second, to determine whether DHT elicits analogous effects in IHCECs and IHConjECs. Our results demonstrate that DHT significantly decreased the expression of numerous immune-related genes in IHMGECs, including those related to antigen processing and presentation, innate and adaptive immune

responses, chemotaxis, and cytokine production. DHT also enhanced the expression of genes encoding anti-inflammatory proteins in IHMGECs. In contrast, DHT had no influence on proinflammatory gene expression in IHCECs, and significantly enhanced the activity of genes linked to the immune system in IHConjECs. Overall, our data support our hypothesis that androgens reduce proinflammatory gene expression in IHMGECs. This hormone action, which does not occur in IHCECs and IHConjECs, may contribute to the typical lack of inflammation within the HMG.

Although we hypothesized, we also anticipated, that DHT would suppress proinflammatory gene expression in IHMGECs for a number of reasons. Androgens have long been known to exert a significant influence on the development, expression and activity of immunity in both health and disease.[20–23] For example, androgens have been shown to control the maturation, proliferation, migration, activation, and/or function of B cell precursors, T cells, monocytes, macrophages and neutrophils; and to regulate the synthesis, secretion and/or expression of antibodies, cytokines, adhesion molecules and autoantigens.[22,24,25] In general, the result of these androgen activities appears most often to be protective and immunosuppressive.[20,22,23,25] An example is this hormone's impact on the autoimmune lacrimal gland in Sjögren syndrome, where we have discovered that androgens cause a dramatic suppression of the inflammation in, and a significant increase in the functional activity of, this tissue.[26–30]

The anti-inflammatory effect in IHMGECs is in stark contrast to that observed in sebocytes, in which androgens have been reported to increase the production of pro-inflammatory cytokines.[31] This differential response, though, is not surprising, given that considerable differences appear to exist in the control mechanisms between different types of sebaceous glands.[2,32] Further, as we have previously reported, androgen action in sebaceous glands is significantly influenced by steroidogenic enzymes. The activity of these enzymes may vary according to tissue location in the body, cellular position within a pilosebaceous unit, as well as microenvironmental factors.[2]

In addition to its anti-inflammatory impact, DHT upregulated numerous genes associated with cellular differentiation and lipid dynamics, and downregulated many genes related to the cell cycle and cell proliferation. We also anticipated these effects, because androgens stimulate the differentiation and lipogenesis of the MG.[2,23,33] These actions are likely mediated through binding to androgen receptors (ARs) in HMGECs,[34,35] because androgen effects appear to depend on the presence of functional ARs.[36,37]

It was of particular interest that the DHT effects on immune, lipid, cell cycle and mTOR signaling genes in differentiated IHMGECs were similar to those induced by DHT in proliferating IHMGECs.[10] Our finding was surprising, given that gene expression in proliferating versus differentiating IHMGECs is typically quite different.[38] As we recently discovered, significant differences exist in the expression of more than 9,200 genes in proliferating versus differentiating IHMGECs.[38]

Our results demonstrate a very potent DHT influence on IHMGEC gene expression, and this finding is quite different than that recently published by other investigators.[39] These

researchers reported that DHT had minimal or no effect on specific gene expression, as well as on the morphology, proliferation, metabolic activity, and lipid accumulation of IHMGECs. Our apparently 'opposite' results can be explained by differences in experimental design. For our differentiation experiments, we used charcoal- and dextran-treated FBS. The charcoal and dextran pretreatment removes all free steroids, a procedure that has been used for over 45 years in endocrine studies.[40,41] In contrast, the differentiation studies of Schröder et al. involved culturing IHMGECs in 10% FBS.[39] Such FBS, without charcoal-dextran stripping, contains testosterone, at levels that can be converted to 10 nM DHT within cells that harbor steroidogenic enzymes.[16] This concentration of DHT results in the occupation of > 90% of the intracellular androgen receptors.[16] The human meibomian gland, in turn, which is made up solely of epithelial cells, contains all these steroidogenic enzymes.[42] Thus, the Schröder et al. "control" IHMGECs were cultured in androgen-containing FBS, and essentially exposed to 10 nM DHT. The Schröder "DHT" treated cells were treated with another 10 nM DHT. Given that the first 10 nM DHT would occupy almost all of the androgen receptors, it would be anticipated that there would be no differences found between the responses of "control" and "DHT" treated IHMGECs. That is what Schröder et al. reported.[39] In effect, Schröder et al. compared the results of DHT-treated control cells versus DHT-treated cells. Our data are based upon results from IHMGECs cultured in the absence (controls), or presence (treated), of 10 nM DHT, an experimental design that would permit the identification of an androgen influence.

The DHT-induced suppression of proinflammatory genes in IHMGECs was not duplicated in IHCECs or IHConjECs. Rather, DHT exerted no influence on proinflammatory gene expression in IHCECs, and significantly increased the activity of genes related to the immune system in IHConjECs. These observations are similar to our previous findings when we compared the DHT effects on the gene activity of proliferating IHMGECs versus proliferating IHConjECs,[10] as well as on the protein synthesis of the lacrimal gland versus other mucosal tissues.[10,43,44] In brief, the nature of androgen influence is most often cell- and tissue-specific.[10,43,44]

The most striking effect of DHT on IHCECs was its upregulation of a tremendous number of cell cycle genes. This proliferative response may help to explain why androgen administration has been demonstrated to stimulate mitosis and repair defects in the cornea[45–47] In contrast, the most marked effect of DHT on differentiated IHConjECs was an upregulation of immune response genes. Thus, DHT increased the expression of 33 immune-related gene ontologies (e.g. cytokine production, regulation of defense response) in IHConjECs, and the majority of these were different than those suppressed by DHT in IHMGECs. DHT also modulated several cell cycle ontologies in IHConjECs, with the majority being downregulated.

There are several limitations in our research. First, we did not perform RT-PCR confirmations of selected BeadChip data. Our reason was that for RT-PCR verification of Illumina results we try to select genes with intensities ≥ 500, at least in one group, and a mean difference between groups ≥ 2.0. We find it very difficult to detect genes with lower Illumina intensities and lower comparative ratios with RT-PCR. In our current study, there were only three genes that met our intensity and ratio criteria in the IHMGECs (i.e. S100

calcium binding protein A7, TAF15 RNA polymerase II and aspartic peptidase, retroviral-like 1), one gene in the HCECs (heat shock 10kDa protein 1) and one gene in the IHConjECs (activating transcription factor 3). However, we have confirmed DHT's influence on three of these genes in earlier experiments, which involved IHMGECs cultured in serum-free media (Table 7). The effects of DHT (i.e. significantly up- or down-regulated) were the same as in the current study.

We should also note that we have consistently found that differences in gene expression in the MG are typically modest (i.e. ≤ 2.0-fold), irrespective of the comparison (e.g. hormone versus placebo, male versus female, etc). For example, we have found that over 96.5% of the sexually dimorphic genes in the BALB/c mouse MGs display modest differences in gene expression.[48] Such differences, though, are far greater than observed in somatic tissues, the vast majority of which are very modest (i.e. ≤ 1.2-fold). As we have previously reported,[48] this is true for 71.4% of sexually dimorphic genes in adipose tissue, 81.3% in liver, 82.5% in muscle and 94.4% in brain.[49] These differences are biologically valid.[50]

A second limitation in our research is that our data reflect mRNA levels, and we have yet to determine whether these transcripts are translated into functional proteins. A third limitation is that the IHMGECs, IHCECs and IHConjECs all originated from male tissues.[51] Because equivalent cell lines from female tissues do not exist, we could not evaluate the effects of androgens on immortalized "female" cells. It may be that the vast majority of genes regulated by androgens in cells from male and female meibomian glands, for example, are the same, given our findings in mice.[52] However, it is also known that there may be distinct, sex-related differences in cellular receptor and signaling pathways, as well as in cellular responses to pharmaceuticals and stress.[53–55] Indeed, the understanding that sex-specific cellular characteristics mediate sex-specific responses to a variety of stimuli has led cardiovascular researchers to propose sex-specific treatments for cardiovascular disease.[56] The reality is that cells have a "sex," and cells from males and females can behave differently.[57] Future research will have to address whether sex-related differences occur in the behavior of human ocular surface and adnexal cells to androgen exposure.

Lastly, given the apparent property of the HMG to resist inflammation,[1,2] might this ability be related to the glandular location? More specifically, given that the MGs are embedded in the tarsus of the eyelids, is it possible that the MGs are poorly accessible to the adaptive immune system with its cell populations (e.g. macrophages, lymphocytes, plasma cells, neutrophils)? Studies would indicate that MGs are not so hidden from the immune system. For example, investigators have reported extensive periglandular inflammation in eyelids of patients with Sjögren syndrome.[58] And others have reported that hyperreflective, polymorphous cellular structures are present outside HMG acini in patients with MGD.[59] Qazi et al. also suggest that these structures may be immune cells, and that some of them may also be found in intraepithelial locations and within conjunctival ducts (i.e. presumably MG).[59] As an additional consideration, proinflammatory cytokines in the tear film may promote hyperkeratinization of the MG terminal duct epithelium and thereby facilitate the development of obstructive MGD.[1,2,60,61]

Overall, our results support our hypothesis that androgens decrease proinflammatory gene expression in IHMGECs. This hormone action, which is not reproduced in IHCECs or IHConjECs, may contribute to the typical absence of overt inflammation within the HMG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

DHT influence on gene expression in differentiated IHMGECs, IHCECs and IHConjECs

Epithelial cells	Genes ↑	Genes ↓	Total genes
Meibomian gland	1,130	1,158	2,288
Cornea	555	555	1,110
Conjunctiva	516	622	1,138

Data were evaluated without log transformation. The expression of listed genes was significantly ($p < 0.05$) up (↑)- or down (↓)-regulated by DHT treatment.

Table 2.

DHT down-regulation of immune-related gene ontologies in HMGECs

Ontology	Genes ↓	Z-score
Chemokine receptor binding	10	4.45
Positive regulation of adaptive immune response	8	4.16
Positive regulation of positive chemotaxis	5	4.11
Response to cytokine stimulus	33	3.98
Positive regulation of T cell mediated cytotoxicity	4	3.96
Regulation of positive chemotaxis	5	3.94
I-κB kinase/NF-κB cascade	22	3.94
T cell mediated immunity	7	3.72
Regulation of T cell mediated cytotoxicity	4	3.56
Chemokine activity	8	3.56
Regulation of adaptive immune response	10	3.51
Regulation of I-κB kinase/NF-κB cascade	17	3.47
Regulation of transforming growth factor- β production	4	3.39
Transforming growth factor-β production	4	3.39
Positive regulation of leukocyte mediated immunity	7	3.37
Positive regulation of lymphocyte mediated immunity	7	3.37
Cellular response to type I interferon	10	3.18
Type I interferon-mediated signaling pathway	10	3.18
Response to type I interferon	10	3.13
T cell mediated cytotoxicity	4	3.08
Regulation of immune effector process	17	3.06
Regulation of adaptive immune response based on somatic recombination of immune receptors built from	9	3.02
Response to interferon-γ	11	2.97
Antigen processing and presentation of peptide antigen via MHC class I	4	2.94
Regulation of chemotaxis	9	2.91
Regulation of cytokine production	25	2.87
Negative regulation of immune effector process	5	2.84
Cytokine receptor binding	19	2.69
Cytokine production	26	2.56
Chemotaxis	42	2.52
Innate immune response	33	2.47
Cellular response to cytokine stimulus	21	2.41
Positive regulation of leukocyte mediated cytotoxicity	4	2.37
Positive regulation of immune effector process	9	2.34
Regulation of defense response	26	2.27
Regulation of interleukin-2 production	5	2.25
Positive regulation of chemotaxis	6	2.21
Positive regulation of cytokine production	13	2.18
Regulation of lymphocyte mediated immunity	7	2.16

Ontology	Genes ↓	Z-score
Defense response	62	2.11
Immune system process	90	2.1
Adaptive immune response	13	2.09
Regulation of type I interferon production	6	2.09
Negative regulation of defense response	6	2.04
Immune response	61	2.03

Biological process and molecular function immune ontologies (≥ 5 genes/ontology) were identified following the analysis of non-transformed Illumina data. All of the immune ontologies listed in this Table were downregulated in HMGECs following DHT treatment. A z-score is a statistical rating of the relative expression of genes, and demonstrates how greatly they are over- or under-represented in a specific gene list.[19] Positive z- scores reflect a greater number of genes meeting the criterion than is anticipated by chance, and values > 2.0 are significant. Terms: Genes ↓ - number of genes down-regulated in a given ontology; z-score - specific score for the down-regulated genes in a given onotology.

Table 3.

DHT downregulation of immune-related gene expression in HMGECs

Accession #	Gene	Ratio	p value	Ontology
NM_004591	Chemokine (C-C motif) ligand 20	2.05	0.0042	chemotaxis
NM_002993	Chemokine (C-X-C motif) ligand 6	1.99	0.0367	chemotaxis
NM_001511	Chemokine (C-X-C motif) ligand 1	1.99	0.0028	chemotaxis
NM_000584	Interleukin 8	1.93	0.0019	angiogenesis
NM_001565	Chemokine (C-X-C motif) ligand 10	1.67	0.0013	chemotaxis
NM_002994	Chemokine (C-X-C motif) ligand 5	1.61	0.0002	chemotaxis
NM_002994	Chemokine (C-X-C motif) ligand 5	1.54	0.0159	chemotaxis
NM_022873	Interferon, alpha-inducible protein 6	1.39	0.0049	immune response
NM_006290	Tumor necrosis factor, alpha-induced protein 3	1.39	0.0304	B-1 B cell homeostasis
NM_000576	Interleukin 1, beta	1.33	0.0017	chronic inflammatory response to antigenic stimulus
NM_006332	Interferon, gamma-inducible protein 30	1.29	0.0293	cytokine-mediated signaling pathway
AB074172	Interleukin 6 signal transducer	1.29	0.0060	positive regulation of acute inflammatory response
NM_006147	Interferon regulatory factor 6	1.28	0.0065	cytokine-mediated signaling pathway
NM_021649	Toll-like receptor adaptor molecule 2	1.28	0.0074	toll-like receptor signaling pathway
NM_001004196	CD200 molecule	1.27	0.0246	regulation of immune response
NM_138806	CD200 receptor 1	1.26	0.0239	regulation of immune response
NM_003921	B-cell CLL/lymphoma 10	1.26	0.0112	toll-like receptor signaling pathway
NM_003238	Transforming growth factor, β2	1.25	0.0109	immune effector process
NM_002089	Chemokine (C-X-C motif) ligand 2	1.19	0.0425	chemotaxis
NM_022059	Chemokine (C-X-C motif) ligand 16	1.13	0.0199	chemotaxis
NM_004048	Beta-2-microglobulin	1.1	0.0073	positive regulation of T cell mediated cytotoxicity
NM_002198	Interferon regulatory factor 1	1.09	0.0206	cytokine-mediated signaling pathway

Accession numbers are the sequence identities of gene fragments expressed on the Illumina BeadChips. These sequences are listed in the nucleotide database of the National Center for Biotechnology Information (NCBI). Relative ratios were calculated by comparing the degree of gene expression in HMGECs after vehicle and DHT treatment. Ratios were determined by using non-transformed data.

Table 4.

DHT regulation of non-immune-related gene ontologies in HMGECs

Ontology / Pathway	DHT genes ↑	DHT genes ↓	Z-score ↑	Z-score ↓
Biological process ontologies				
Lipids				
Lipid metabolic process	89	41	**6.26**	−1.81
Cellular lipid metabolic process	57	28	**4.44**	−1.47
Phosphatidylcholine biosynthetic process	4	1	**3.66**	0.12
Steroid metabolic process	25	12	**3.55**	−0.6
Lipid biosynthetic process	36	25	**3.49**	0.43
Phosphatidylcholine metabolic process	5	1	**3.43**	−0.34
Phospholipid metabolic process	19	9	**2.99**	−0.62
Lipid transport	17	8	**2.86**	−0.58
Regulation of lipid metabolic process	14	6	**2.86**	−0.58
Glycerophospholipid biosynthetic process	9	5	**2.77**	0.41
Lipid localization	18	9	**2.68**	−0.6
Phospholipid biosynthetic process	12	8	**2.67**	0.65
Glycerolipid biosynthetic process	12	9	**2.63**	1.03
Lipid catabolic process	18	5	**2.26**	−2.09
Cellular lipid catabolic process	11	3	**2.21**	−1.41
Sphingolipid metabolic process	9	3	**2.17**	−0.93
Glycerophospholipid metabolic process	11	6	**2.12**	−0.25
Cholesterol efflux	4	1	**2.09**	−0.54
Differentiation / autophagy				
Vacuole	42	22	**6.8**	1.31
Macroautophagy	8	1	**6.18**	−0.34
Lysosome	35	19	**6.08**	1.3
Lytic vacuole	35	19	**6.08**	1.3
Autophagy	13	3	**5.76**	−0.25
Vacuole organization	11	2	**5.59**	−0.46
Epidermal cell differentiation	16	5	**5.56**	−0.01
Autophagic vacuole assembly	6	1	**5.5**	0
Keratinocyte differentiation	13	5	**4.83**	0.38
Vacuolar membrane	20	10	**4.81**	0.81
Autophagic vacuole	6	0	**4.47**	−1.16
Keratinization	8	2	**4.25**	−0.19
Epithelial cell differentiation	20	9	**3.17**	−0.71
Cell cycle				
Mitotic cell cycle	22	94	−2.02	**10**
Cell cycle phase	23	97	−2.34	**9.42**
Cell cycle process	31	110	−2.08	**9.31**
Cell cycle	51	134	−1.02	**9.28**

Ontology / Pathway	DHT genes ↑	DHT genes ↓	Z-score ↑	Z-score ↓
Regulation of cell cycle	31	77	0.36	**8.26**
Interphase	11	52	−1.54	**7.88**
Interphase of mitotic cell cycle	11	50	−1.46	**7.6**
Mitosis	12	46	−1.08	**6.89**
Nuclear division	12	46	−1.08	**6.89**
Cell proliferation	56	118	−0.52	**6.84**
M phase of mitotic cell cycle	12	46	−1.2	**6.63**
M phase	16	56	−1.51	**6.35**
G1/S transition of mitotic cell cycle	6	28	−0.91	6.19
Ribosome biogenesis	3	23	−1.39	6.14
KEGG pathways				
Lysosome	24	5	**7.28**	−0.85
Metabolic pathways	81	64	**3.69**	−0.03
mTOR signaling pathway	8	1	**3.36**	−1.21
Aldosterone-regulated sodium reabsorption	6	1	**2.71**	−0.95
RNA transport	5	25	−1.11	**5.51**
Cell cycle	5	19	−0.59	**4.51**
Chemokine signaling pathway	9	20	−0.27	**2.79**
Antigen processing and presentation	6	8	1.41	**2.14**

Biological process ontologies (≥ 5 genes/category) related to lipids, differentiation / autophagy and cell cycle, as well as assorted KEGG pathways, were selected after analyses of non-transformed data. Those ontologies and pathways with a z-score > 2.0 are highlighted in bold. Terms are analogous to those described in the legend to Table 2.

Table 5.

DHT up-regulation of cell cycle gene ontologies in HCECs

Ontology	Genes ↑	Z-score
Pyrimidine ribonucleotide metabolic process	4	5.62
Cell cycle process	47	5.27
Mitotic cell cycle	38	5.22
Cell cycle phase	39	4.8
Cell cycle	55	4.75
Negative regulation of cell cycle process	8	4.74
Regulation of cell cycle process	22	4.69
DNA-dependent DNA replication initiation	5	4.33
Negative regulation of mitosis	5	4.33
Negative regulation of nuclear division	5	4.33
DNA-dependent DNA replication	9	4.32
Regulation of cell cycle arrest	16	4.3
Base-excision repair	5	4.25
Spindle checkpoint	5	4.25
DNA conformation change	12	4.13
Mitosis	20	4.09
Nuclear division	20	4.09
Cell cycle checkpoint	15	4.06
Cell cycle arrest	20	4
Regulation of cell cycle	30	3.95
Organelle fission	20	3.93
M phase of mitotic cell cycle	20	3.93
Pyrimidine nucleoside metabolic process	5	3.85
Mitotic cell cycle checkpoint	10	3.76
Double-strand break repair	7	3.7
M phase	24	3.61
DNA strand elongation involved in DNA replication	4	3.59
Negative regulation of cell cycle	21	3.58

Cell cycle-related ontologies (≥ 5 genes/ontology) were selected after the analysis of biological process data. All of the ontologies listed in this Table were upregulated in HCECs following DHT treatment.

Table 6.

DHT up-regulation of immune-related gene ontologies in HConjECs

Ontology	Genes ↑	Z-score
Myd88-dependent toll-like receptor signaling pathway	8	4.78
Positive regulation of defense response	13	4.75
Toll-like receptor signaling pathway	9	4.72
Pattern recognition receptor signaling pathway	9	4.56
Innate immune response-activating signal transduction	9	4.52
Activation of innate immune response	9	4.44
Positive regulation of innate immune response	10	4.18
Toll signaling pathway	7	3.88
Regulation of interleukin-6 production	5	3.79
Toll-like receptor 4 signaling pathway	7	3.76
Intracellular transport	35	3.74
Cytokine secretion	5	3.73
Interleukin-6 production	5	3.67
Toll-like receptor 1 signaling pathway	6	3.46
Toll-like receptor 2 signaling pathway	6	3.41
Regulation of defense response	16	3.33
Positive regulation of NF-κB transcription factor activity	6	3.29
Immune response-activating signal transduction	11	3.25
Immune response-regulating signaling pathway	11	3.17
Regulation of innate immune response	10	3
Positive regulation of immune system process	19	2.95
Positive regulation of immune response	14	2.87
Myd88-independent toll-like receptor signaling pathway	5	2.82
Leukocyte cell-cell adhesion	3	2.82
Cytokine production	14	2.79
Toll-like receptor 3 signaling pathway	5	2.78
Activation of immune response	11	2.48
Regulation of cytokine production	12	2.4
Positive regulation of response to stimulus	27	2.25
Positive regulation of T cell activation	7	2.18
Positive regulation of lymphocyte activation	8	2.1
Regulation of adaptive immune response based on somatic recombination of immune receptors built from	4	2.08
Regulation of adaptive immune response	4	2.04
Cytokine activity	10	2.59

Immune-related ontologies (≥ 5 genes/ontology) were identified after the analysis of biological process and molecular function ontology data. All of the ontologies listed in this Table were upregulated in HConjECs following DHT treatment.

Table 7.

Confirmation of the DHT influence on the expression of specific genes in IHMGECs, IHCECs and IHConjECs

		Current study		Previous study	
Cells	**Gene**	**Ratio**	**p value**	**Ratio**	**p value**
DHT gene ↑					
IHConjECs	Activating transcription factor 3	2.50	0.0001	5.00	0.0006
DHT genes ↓					
IHMGECs	TAF15 RNA polymerase II	2.03	0.0198	1.43	0.0003
IHCECs	Heat shock 10kDa protein 1	2.17	0.0372	1.30	0.0273

The previous study involved culturing cells in the presence or absence of 10 nm DHT in serum-free media for 3 (IHMGECs), 4 (IHConjECs), or 5 (IHCECs) days. The experimental procedures, and methods for determining gene expression, were analogous to those previously reported.10 The IHCECs were a gift from Dr. Ilene Gipson (Boston, MA). Relative ratios were calculated by comparing the degree of gene expression in cells after vehicle and DHT exposure. Abbreviations: ↑- upregulation; ↓- downregulation