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Influence of sex on gene expression in human corneal epithelial cells

Tomo Suzuki,[1] Stephen M. Richards,[1] Shaohui Liu,[1] Roderick V. Jensen,[2] David A. Sullivan[1]

[1]Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, MA; [2]Department of Biological Sciences, Virginia Tech, Blacksburg, VA

Purpose: Sex-associated differences have been identified in the anatomy, physiology and pathophysiology of the human cornea. We hypothesize that many of these differences are due to fundamental variations in gene expression. Our objective in this study was to determine whether such differences exist in human corneal epithelial cells both in vivo and in vitro.Methods: Human corneal epithelial cells were isolated from the corneoscleral rims of male and female donors. Cells were processed either directly for RNA extraction, or first cultured in phenol red-free keratinocyte serum-free media. The RNA samples were examined for differentially expressed mRNAs by using of CodeLink Bioarrays and Affymetrix GeneChips. Data were analyzed with GeneSifter.Net software.Results: Our results demonstrate that sex significantly influences the expression of over 600 genes in human corneal epithelial cells in vivo. These genes are involved in a broad spectrum of biological processes, molecular functions and cellular components, such as metabolic processes, DNA replication, cell migration, RNA binding, oxidoreductase activity and nucleoli. We also identified significant, sex-related effects on gene expression in human corneal epithelial cells in vitro. However, with few exceptions (e.g. X- and Y-linked genes), these sex-related differences in gene expression in vitro were typically different than those in vivo.Conclusions: Our findings support our hypothesis that sex-related differences exist in the gene expression of human corneal epithelial cells. Variations in gene expression may contribute to sex-related differences in the prevalence of certain corneal diseases.

For almost five decades it has been recognized that sex exerts a significant influence on the anatomy, physiology and pathophysiology of the cornea. Thus, investigators have identified significant, sex-related differences in the diameter, curvature, thickness, sensitivity and wetting time of the cornea, the mitotic rate of corneal epithelial cells, the density of corneal endothelial cells, as well as the survival rate of corneal grafts [1-15]. Researchers have also reported significant, sex-associated variations in the prevalence of Salzmann's nodular corneal degeneration, against-the-rule astigmatism, keratoconus, viral keratopathy, pseudophakic bullous keratopathy, aphakic bullous keratopathy, interstitial keratitis, and Fuchs' dystrophy [14,16,17], as well as in the response to LASIK surgery [18].

In addition to these observations, scientists have discovered that sex-specific differences in the cornea may also occur during the menstrual cycle, pregnancy and menopause. These alterations include changes in the thickness, hydration, curvature and sensitivity of the cornea, incidence of central corneal endothelial pigmentation, foreign body sensation, contact lens tolerance and visual acuity [19-30].

We hypothesize that many of these differences are due to fundamental, sex-associated variations in gene expression. Our objective in this study was to determine whether such differences exist in human corneal epithelial cells both in vivo and in vitro.

Correspondence to: David A. Sullivan, Ph.D., Schepens Eye Research Institute, 20 Staniford Street, Boston, MA, 02114; Phone: (617) 912-0287; FAX: (617) 912-0101; email: david.sullivan@schepens.harvard.edu

METHODS

Human corneal epithelial cell isolation and culture procedures: Corneal epithelial cells were isolated from the corneoscleral rims of human donors. These tissues were obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA), as well as from physicians at the Massachusetts Eye & Ear Infirmary (MEEI) after corneal transplant surgery. All tissues were de-identified prior to our use, according to Health Insurance Portability and Accountability Act of 1996 (HIPAA) regulations. Corneal epithelial cells were either processed directly for RNA extraction, or first cultured in vitro. For direct processing, epithelial cells were scraped off the rims of male (n=3; 34, 44, and 52 years old) and female (n=3; 31, 44, and 50 years old) donors with a crescent knife, collected into TRIzol (Invitrogen, Carlsbad, CA) and stored at -80 °C until RNA extraction.

For cell culture, the rims (n=2/sex; males=56 and 60 years old; females=42 and 53 years old) were rinsed with Dulbecco's phosphate buffered saline (PBS) without $Ca2^{+}$ or $Mg2^{+}$ (Invitrogen), and containing 20 µg/ml gentamicin (Invitrogen), for two to three min. Each rim was trimmed, and then the conjunctiva, endothelial layer, and iris remnants were removed. The residual rim was sectioned into three or four pieces. Each piece was placed with its epithelial side down onto a collagen-coated 6-well plate (Biocoat Collagen I Cellware; BD Biosciences, San Jose, CA). After a 20 to 30 min period, during which time the epithelium adhered to the plate, a drop of keratinocyte serum-free medium (KSFM; Invitrogen) was administered to the top of each tissue piece.

Tissues were incubated overnight at 37 °C under 95% humidity and 5% CO_2. The explants were then cultured in KSFM supplemented with 50 µg/ml of bovine pituitary extract and 0.005 µg/ml of human epidermal growth factor. The medium was replaced every two days. The tissue pieces were removed with sterile forceps after five to seven days of culture. When epithelial outgrowths were 70% confluent, they were split and seeded onto coated 6-well plates at 0.5×105 cells/well. Cells were cultured in KSFM without phenol red for 48 h, then removed with trypsin and processed for molecular biological procedures. We selected media without phenol red for the final cell cultures because this dye has estrogen activity [31].

Molecular biological procedures: To examine the influence of sex on human corneal epithelial cell gene expression, total RNA was first extracted by using TRIzol reagent. Samples were then exposed to RNase-free DNase (Invitrogen) and analyzed on an RNA 6000 Nano LabChip with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) to verify RNA integrity. After these steps, the RNA samples were processed using two different methods.

The first method to evaluate gene expression involved the use of CodeLink Uniset Human 20K I Bioarrays (Amersham Biosciences/GE Healthcare, Piscataway, NJ), which target 21,108 transcripts and 19,881 well-annotated human genes. The RNA samples were hybridized according to reported techniques [32]. In brief, cDNA was synthesized from RNA (2 µg) with a CodeLink Expression Assay Reagent Kit (Amersham, Piscataway, NJ) and purified with a QIAquick purification kit (Qiagen, Valencia, CA). After sample drying, cRNA was produced with a CodeLink Expression Assay Reagent Kit (Amersham), recovered with an RNeasy kit (Qiagen) and quantified with a UV spectrophotometer. Fragmented, biotin-labeled cRNA was then incubated and shaken (300 rpm shaker) for 18 h on a CodeLink Bioarray at 37 °C. Following this time period, the Bioarray was washed, exposed to streptavidin-Alexa 647, and scanned using ScanArray Express software and a ScanArray Express HT scanner (Packard BioScience, Meriden, CT) with the laser set at 635 nm, laser power at 100%, and photomultiplier tube voltage at 60%. Scanned image files were evaluated using CodeLink image and data analysis software (Amersham), which yielded both raw and normalized hybridization signal intensities for each array spot. The spot intensities (~20,000) on the microarray image were normalized to a median of one. Standardized data, with signal intensities greater than 0.50, were analyzed with GeneSifter.Net software (VizX Labs LLC, Seattle, WA). This comprehensive program also generated gene ontology and z-score reports. These ontologies included biological processes, molecular functions and cellular components, and were organized according to the guidelines of the Gene Ontology Consortium [33].

The second method to assess, and to verify, gene expression involved the use of Affymetrix U133A 2.0 GeneChips (Affymetrix Inc., Santa Clara, CA), which target 18,400 transcripts and 14,500 genes. The Affymetrix and CodeLink platforms identify 12,697 and 13,604 unique Entrez Gene genes, respectively. Over 80% of the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) genes are common to both platforms. The Affymetrix procedure utilized the same fragmented, biotin-labeled cRNA samples that had been prepared for CodeLink Bioarrays. The cRNA was hybridized to GeneChips according to the manufacturer's protocol. Hybridized GeneChips were then scanned with an Affymetrix Model 700 Scanner and expression data files were generated from array images using Affymetrix Microarray Suite 4.0 software. GeneChip data were normalized by selecting the default scaling in Affymetrix GeneChip Operating Software, which produces a trimmed mean intensity of 500 for each GeneChip microarray. Standardized data with a quality value of 1.0 were then examined with GeneSifter software.

CodeLink and Affymetrix gene expression data were analyzed with and without log transformation and statistical evaluation of these data was performed with Student's t test (two-tailed, unpaired). Data from each platform were also compared using the GeneSifter intersector program. The data from the individual Bioarrays (n = 10) and GeneChips (n= 6) are accessible for download through the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) via series accession number (GSE14621).

Real Time PCR procedures: The differential expression of selected genes was verified by using quantitative real-time PCR (qPCR) procedures. Human corneal epithelial cells from male (n=3; 40, 62, and 79 years old) and female (n=4; 71, 73, 79, and 83 years old) donors were obtained from NDRI and MEEI, and RNA was extracted using either Trizol or RNAqueous Kits (Ambion, Austin, TX). The RNA samples were evaluated with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and a BioAnalyzer. The cDNAs were transcribed by utilizing SuperScript III Reverse Transcriptase (Invitrogen) and random hexamer primers (Invitrogen). Duplex reactions in triplicate were then performed by using TaqMan Gene Assays (Applied Biosystems, Inc., Foster City, CA) and TaqMan-specific probes for X (inactive)-specific transcript (Hs00300535_s1), jumonji, AT rich interactive domain 1D (Hs00190491), carcinoembryonic antigen-related cell adhesion molecule 6 (Hs00366002), schwannomin interacting protein 1 (Hs00205829), guanine nucleotide binding protein, β14 (Hs00388871), GTP-binding protein 10 (putative; Hs00414912) and β-actin endogenous control (4326315E). Differential gene expression was calculated according to the ΔΔCt method outline in Applied Biosystems User Bulletin two (updated in 2001).

RESULTS

Influence of sex on overall gene expression in human corneal epithelial cells: Sex has a significant effect on gene expression

Table 1. Influence of sex on gene expression in human corneal epithelial cells.

	Genes M>F	Genes F>M	Total genes
CodeLink			
No transformation	216	367	583
Log transformation	219	393	612
Total	238	423	661
Affymetrix			
No transformation	307	118	425
Log transformation	282	114	396
Total	329	129	458

Data were analyzed with and without log transformation. The number of common and non-overlapping genes between analytical categories was determined, and then the total numbers were calculated. The expression of listed genes was significantly ($p<0.05$) influenced by sex. Abbreviations in the table are M = male and F = female.

in human corneal epithelial cells. Analysis of CodeLink data showed that sex influenced the expression of 661 genes, with 423 of these genes more highly expressed in females and 238 in males (Table 1). Similarly, evaluation of Affymetrix data demonstrated significant, sex-related differences in the expression of 458 genes. However, with this platform, the majority of genes were more highly expressed in males, as compared to females (Table 1).

The reason for this apparent discrepancy appears to be due, in large part, to differences in the lists of genes identified as differentially expressed between the array platforms. In our studies, 13,440 CodeLink genes and 11,026 Affymetrix genes were above threshold sensitivity in their respective microarrays. However, many of these genes were not the same on each platform. Analysis of the Entrez Gene identifications of above threshold genes showed that 7,525 genes were identical between the platforms. Yet, 5,915 CodeLink genes and 3,501 Affymetrix genes did not have counterparts expressed above threshold on the other platform. And, if another gene identifier, such as Gene ID was used, then even greater differences in gene expression existed between platforms.

In effect, although the gene populations on the CodeLink and Affymetrix arrays had many similarities, they also had many dissimilarities. These variations could account for why 43% of the genes showing significant, sex-related differences on the CodeLink Bioarray were unique to this platform, and had no corresponding transcripts on the Affymetrix array (Table 2). Similarly, 22% of the significant Affymetrix genes were unique, and not present in the above threshold CodeLink genes (Table 2).

Sex-related impact on specific gene expression and gene ontologies in human corneal epithelial cells in vivo: As anticipated, sex has a significant ($p<0.05$) influence on the expression of X and Y chromosome-linked genes in human corneal epithelial cells (Table 3). However, sex also exerts a significant impact on many other genes. As shown in Table 4, Table 5, and Table 6, the activity of numerous genes, such as those encoding phosphoserine phosphatase, NF-kB2, neuritin 1, vasoactive intestinal peptide receptor 1, GalNac-T6 and notch homolog 4 was significantly greater in corneal epithelial cell from males. In contrast, the transcription of many other genes, such as cyclin D1, transglutaminase 1, carcinoembryonic antigen-related cell adhesion molecule 6, purinergic receptor P2X, ligand gated ion channel, 3, and β2 microglobulin was significantly higher in corneal epithelial cells from females.

The influence of sex on gene expression in human corneal epithelial cells involved a broad spectrum of biological processes, molecular functions and cellular components. For example, sex altered the expression of many genes (e.g. 100 genes/category) involved in activities such as molecular processes, biological regulation and catalysis (Table 7). In addition, sex had a considerable effect on the occurrence of specific gene ontologies. Thus, as demonstrated by z-score analysis, sex had a significant impact on the relative expression of genes related to metabolic processes, DNA replication, cell migration, RNA binding, oxidoreductase activity, nucleoli and other ontologies (Table 8 and Table 9). Analysis of Affymetrix data also revealed that male corneal epithelial cells, as compared to those of females, had a significant increase in the transcription of genes (M=4↑; F=1↓) associated with the androgen receptor signaling pathway (z score=3.95), and of genes (M=7↑; F=1↓) related to T cell activation (z score=2.80).

It is important to note that the nature of the sex-associated influence on gene ontologies was not identical on the CodeLink and Affymetrix platforms (Table 6, Table 7, and Table 8). This finding was most likely due, as noted above, to the large differences in gene expression between the array platforms. Some molecular function and cellular component results were similar with both arrays (Table 10). However, almost none of the genes within the ontologies were the same, which again reflects the differences between the platform gene populations.

Table 2. Significant, sex-related differences in gene expression: Comparisons between CodeLink and Affymetrix arrays.

	Genes M>F	Genes F>M	Total genes
CodeLink			
Number of genes with significant differences in expression	216	367	583
Number of genes with same results on Affymetrix	22	20	42
Number of genes changed in same direction on Affymetrix	82	133	215
Number of genes changed in opposite direction on Affymetrix	10	58	68
Number of genes with opposite results on Affymetrix	0	4	4
Number of unique genes, not expressed by Affymetrix	102	152	254
Affymetrix			
Number of genes with significant differences in expression	307	118	425
Number of genes with same results on CodeLink	22	20	42
Number of genes changed in same direction on CodeLink	141	69	210
Number of genes changed in opposite direction on CodeLink	66	8	74
Number of genes with opposite results on CodeLink	4	0	4
Number of unique genes, not expressed by CodeLink	74	21	95

Data were analyzed without log transformation. The phrase "Number of genes with same (or opposite) results" means that the findings were significant ($p<0.05$) on both platforms. The term "Number of genes changed in same (or opposite) direction" means that results were significant on one platform, but not on the other. The phrase "same direction" was also used for a gene demonstrating significant up- or down-regulation on one platform and a corresponding, but not significant, alteration in at least one gene transcript on the other array (note: some genes had several transcripts). Genes labeled as "unique" were not expressed at above threshold levels on the other array platform.

Table 3. Sex-related expression of X and Y chromosome genes in human corneal epithelial cells.

Entrez gene identification	Gene	CL ratio	Affy ratio	CL p value	Affy p value	Ontology
Male>Female						
Y chromosome						
6192	Ribosomal protein S4	314.1	1289.3	<0.0000	<0.0001	translation
8287	Ubiquitin specific peptidase 9	54.4	110.0	<0.0241	<0.0103	ubiquitin cycle
8284	Jumonji, AT rich interactive domain 1D	33.7	64.8	<0.0000	<0.0004	chromatin modification
9086	Eukaryotic translation initiation factor 1A	12.4	27.5	<0.0039	<0.0023	translational initiation
8653	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3	4.8	197.3	<0.0139	<0.0002	nucleotide binding
Female>Male						
X chromosome						
7503	X (inactive)-specific transcript	248.7	373.7	<0.0003	<0.0136	inactivation of X chromosome
1964	Eukaryotic translation initiation factor 1A, X-linked	1.8	1.5	<0.0479	<0.0177	translational initiation
7403	Ubiquitously transcribed tetratricopeptide repeat	1.7	1.7	<0.0085	<0.0001	binding

Relative ratios were calculated by comparing the degree of gene expression in corneal epithelial cells from men and women. Abbreviations in the table are CL = CodeLink; Affy = Affymetrix.

Analogous observations were made when analyzing the effect of sex on KEGG pathways in human corneal epithelial cells. CodeLink and Affymetrix data showed that pathways for purine and pyrimidine metabolism were both upregulated (i.e. z scores >2.0) in males, as compared to females, but a number of the genes were platform-specific.

To confirm in part the CodeLink and Affymetrix results, selected genes were analyzed by qPCR. This experimental approach confirmed the sex-related differences in the expression of X (inactive)-specific transcript (F>M; up to 973 fold), jumonji, AT rich interactive domain 1D (M>F; infinitely greater, because this mRNA was not detected in female qPCR samples) and carcinoembryonic antigen-related cell adhesion molecule 6 (F>M; up to 30 fold). The transcript levels of schwannomin interacting protein 1, guanine nucleotide binding protein, β14 and GTP-binding protein 10 (putative) were too low (i.e. average thresholds typically

Table 4. Gene expression in human corneal epithelial cells: Significant, sex-related differences identified by both CodeLink and Affymetrix arrays.

Entrez gene identification	Gene	CL Ratio	Affy Ratio	CL p value	Affy p value	Ontology
Male>Female						
5168	Ectonucleotide pyrophosphatase/ phosphodiesterase 2	2.4	3.6	<0.0089	<0.0314	cell motility
5923	Ras protein-specific guanine nucleotide-releasing factor 1	2.0	2.5	<0.0342	<0.0215	regulation of Rho protein signal transduction
26577	Procollagen C-endopeptidase enhancer 2	1.7	2.1	<0.0133	<0.0383	protein binding
11113	Citron (rho-interacting, serine/threonine kinase 21)	1.6	1.8	<0.0232	<0.0062	cell cycle
7298	Thymidylate synthetase	1.5	1.6	<0.0439	<0.0472	dTMP biosynthetic process
51313	Chromosome 4 open reading frame 18	1.4	3.3	<0.0460	<0.0279	Golgi membrane
8991	Selenium binding protein 1	1.4	1.4	<0.0489	<0.0202	protein transport
Female>Male						
1048	Carcinoembryonic antigen-related cell adhesion molecule 5	7.9	3.0	<0.0001	<0.0202	plasma membrane
6590	Secretory leukocyte peptidase inhibitor	2.9	2.6	<0.0300	<0.0337	serine-type endopeptidase inhibitor activity
7051	Transglutaminase 1	2.2	2.1	<0.0220	<0.0344	protein modification process
11001	Solute carrier family 27 (fatty acid transporter), member 2	2.1	1.7	<0.0121	<0.0462	very-long-chain fatty acid metabolic process
66002	Cytochrome P450, family 4, subfamily F, polypeptide 12	2.1	2.4	<0.0224	<0.0239	electron transport
831	Calpastatin	1.3	1.1	<0.0444	<0.0370	calpain inhibitor activity
8202	Nuclear receptor coactivator 3	1.2	1.3	<0.0296	<0.0032	signal transduction

Data were analyzed with and without transformation. Abbreviations in the table are CL = CodeLink and Affy = Affymetrix

Table 5. Gene expression in human corneal epithelial cells: Analogous, sex-related differences identified with CodeLink and Affymetrix arrays.

Entrez gene identification	Gene	Ratio	p value	Ontology
CodeLink				
Male>Female				
4953	Ornithine decarboxylase 1	3.2 (1.5)	<0.0493	polyamine biosynthetic process
4217	Mitogen-activated protein kinase kinase kinase 5	2.5 (1.4)	<0.0443	MAPKKK cascade
7433	Vasoactive intestinal peptide receptor 1	2.3 (1.4)	<0.0365	signal transduction
4035	Low density lipoprotein-related protein 1 (α2-macroglobulin receptor)	2.1 (1.4)	<0.0246	lipid metabolic process
64699	Transmembrane protease, serine 3	2.1 (1.5)	<0.0263	proteolysis
Female>Male				
11343	Monoglyceride lipase	4.8 (2.6)	<0.0281	lipid metabolic process
2952	Glutathione S-transferase theta 1	4.6 (2.0)	<0.0227	response to stress
23659	Lysophospholipase 3 (lysosomal phospholipase A2)	3.5 (1.2)	<0.0274	lipid metabolic process
10461	C-mer proto-oncogene tyrosine kinase	2.4 (1.3)	<0.0238	protein amino acid phosphorylation
567	β_2 microglobulin	1.7 (1.3)	<0.0422	antigen processing and presentation of peptide antigen via MHC class I
Affymetrix				
Male>Female				
79644	Steroid 5α-reductase 2-like	3.4 (1.6)	<0.0437	lipid metabolic process
51299	Neuritin 1	3.1 (2.2)	<0.0387	plasma membrane
5099	Protocadherin 7	2.9 (1.5)	<0.0052	cell adhesion
23705	Cell adhesion molecule 1	1.9 (1.6)	<0.0205	apoptosis
2852	G protein-coupled estrogen receptor 1	1.8 (2.0)	<0.0466	signal transduction
Female>Male				
8000	Prostate stem cell antigen	3.7 (2.4)	<0.0180	plasma membrane
2152	Coagulation factor III (thromboplastin, tissue factor)	2.3 (2.6)	<0.0316	immune response
10748	Killer cell lectin-like receptor subfamily A, member 1	1.8 (1.3)	<0.0069	signal transduction
3708	Inositol 1,4,5-triphosphate receptor, type 1	1.8 (17.8)	<0.0225	ion transport
6484	ST3 βgalactoside α2,3-sialyltransferase 4	1.7 (1.3)	<0.0157	protein amino acid glycosylation

Significant, sex-related differences in gene expression were identified with either CodeLink or Affymetrix arrays. The other array showed similar, but not significant, directional changes in gene expression. The extent of these changes on the corresponding array are shown in parentheses.

Table 6. Gene expression in human corneal epithelial cells: Significant, sex-related differences identified uniquely with either CodeLink or Affymetrix arrays.

Entrez gene identification	Gene	Ratio	p value	Ontology
CodeLink				
Male>Female				
4016	Lysyl oxidase-like 1	3.23	<0.0326	electron transport
11226	GalNAc-T6	2.19	<0.0082	protein amino acid O-linked glycosylation
4855	Notch homolog 4	2.04	<0.0070	cell fate determination
10686	Claudin 16	1.67	<0.0180	ion transport
*	Integrin subunit α-2 gene	1.65	<0.0347	cell adhesion
Female > Male				
23762	Oxysterol binding protein 2	3.31	<0.0095	lipid transport
85865	GTP-binding protein 10 (putative)	2.76	<0.0095	ribosome biogenesis and assembly
5024	Purinergic receptor P2X, ligand-gated ion channel, 3	2.65	<0.0127	ion transport
5307	Paired-like homeodomain 1	2.24	<0.0363	regulation of transcription, DNA-dependent
10630	Podoplanin	1.88	<0.0332	cell morphogenesis
Affymetrix				
Male>Female				
29970	Schwannomin interacting protein 1	2.64	<0.0321	protein binding
23531	Monocyte to macrophage differentiation-associated	2.07	<0.0038	cytolysis
5723	Phosphoserine phosphatase	2.05	<0.0383	L-serine biosynthetic process
4791	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	1.91	<0.0141	regulation of transcription, DNA-dependent
26227	Phosphoglycerate dehydrogenase	1.75	<0.0088	L-serine biosynthetic process
Female>Male				
9630	Guanine nucleotide binding protein, α14	2.62	<0.0028	signal transduction
595	Cyclin D1	1.7	<0.0090	G1/S transition of mitotic cell cycle
8379	Mitotic arrest deficient-like 1	1.64	<0.0010	mitotic metaphase
3669	Interferon stimulated exonuclease	1.48	<0.0268	DNA catabolic process, exonucleolytic
10801	Septin 9	1.46	<0.0388	carbohydrate metabolic process

Genes expressed on the Codelink Bioarray were not present at above threshold sensitivity in the Affymetrix array. Similary, genes expressed on the Affymetrix array were not present at above threshold sensitivity on the CodeLink Bioarray. Abbreviations in the table are GalNAc-T6 = UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase. The asterisk indicates Gene accession number = AF113511 .

Table 7. Influence of sex on the expression of human corneal genes related to biological processes, molecular functions and cellular components.

Ontologies	Array	Total genes	Male>Female	Female>Male
Biological process ontologies				
cellular process	CL	346	121	225
	Affy	267	195	72
metabolic process	CL	244	83	161
	Affy	207	143	64
biological regulation	CL	186	60	126
	Affy	160	119	41
Molecular Function ontologies				
binding	CL	342	127	215
	Affy	279	204	75
catalytic activity	CL	171	70	101
	Affy	128	90	38
Cellular component ontologies				
cell	CL	396	148	248
	Affy	311	221	90
organelle	CL	266	99	167
	Affy	224	154	70

All genes displayed significant ($p<0.05$) differences in sex-related expression. Results are shown for selected ontologies containing at least 100 genes on both array platforms. Abbreviations in the table are CL = CodeLink; Affy = Affymetrix.

exceeded 31 cycles) to reliably quantitate with qPCR procedures.

Sex-related impact on specific gene expression and gene ontologies in human corneal epithelial cells in vitro: To determine whether sex-related differences in gene expression are maintained in cultured human corneal epithelial cells, cells were cultured as described in the Methods and then processed for molecular biological procedures and analysis with CodeLink Bioarrays.

Our results show that sex-associated differences exist in the expression of 437 genes, with 220 genes more highly expressed in females and 217 in males. These genes are linked to X and Y chromosomes (Table 11), as well as to autosomes that encode such proteins as small proline-rich protein 3, defensin β1, lipocalin 2 and Sjögren syndrome nuclear autoantigen 1 (Table 12). The nature of these sex differences encompassed genes involved in cell growth, wound response, tyrosine kinase signaling and chromatin modification (Table 13). The majority of these genes were different than those identified in the non-cultured corneal epithelial cells.

If data from cultured and noncultured human corneal epithelial cells were combined, then significant, sex-related differences were identified in 255 genes (M>F=84; F>M=171). These genes included those encoding retinol dehydrogenase 8, retinoid X receptor α, α 1,4 galactosyltransferase and the estrogen receptor 1 (Table 14).

DISCUSSION

The present study demonstrates that sex has a significant influence on the expression of over 600 genes in human corneal epithelial cells in vivo. These genes are associated with a broad array of biological processes, molecular functions and cellular components, including such activities as metabolic processes, DNA replication, cell migration, RNA binding, oxidoreductase activity and nucleoli. These results support our hypothesis that fundamental variations in gene expression may contribute to the sex-associated differences in the anatomy, physiology and pathophysiology of the human cornea.

However, the precise nature of these sex-related differences in gene expression, as identified with the CodeLink Bioarrays and Affymetrix GeneChips, varied depending upon the microarray platform. Originally, we had chosen to run CodeLink Bioarrays to evaluate the influence of sex on human corneal epithelial cell gene expression, and to confirm possible significant differences by using a separate platform, the Affymetrix GeneChip. We found, though, that there were tremendous differences in gene populations between the array platforms, such that over 5,900 CodeLink genes and more than 3,500 Affymetrix genes had no counterparts expressed above threshold on the other platform. Indeed, 43% of the genes showing significant, sex-related differences on the CodeLink Bioarray were unique to this platform, and had no corresponding transcripts on the

Table 8. Effect of sex on the expression of gene ontologies in human corneal epithelial cells, as shown with CodeLink Bioarrays.

Ontology	M Genes ↑	F Genes ↑	M z-score	F z-score
Biological process				
hexose metabolic process	6	4	3.5	0.67
DNA replication	6	5	2.47	0.45
regulation of cellular metabolic process	16	48	-2.03	0.52
RNA metabolic process	16	53	-2.4	0.74
monocarboxylic acid metabolic process	5	10	1.42	2.4
fatty acid metabolic process	4	8	1.38	2.31
Molecular function				
actin binding	9	4	3.52	-0.62
calcium ion binding	16	11	2.48	-1.31
iron ion binding	7	6	2.48	0.37
oxidoreductase activity	14	16	2.41	0.65
transcription factor activity	3	15	-2.05	-0.28
ligase activity	7	13	1.6	2.3
Cellular component				
nucleolus	5	7	2.22	2.21
intracellular	107	193	0.08	2.87
nucleus	42	95	-0.88	2.64
mitochondrial part	6	17	0.04	2.48
extracellular region	19	17	0.38	-2.28
integral to membrane	48	58	0.26	-2.4

A z-score is a statistical measure of the relative expression of gene ontologies, and shows how much each ontology is over- or under-represented in a gene list. More specifically, the z-score is a standardized difference using the expected value and standard deviation of the number of genes meeting the criterion of a gene ontology term under a hypergeometric distribution [89]. Positive z scores indicate gene ontology terms with a greater number of genes meeting the criterion than is expected by chance, whereas negative z scores reflect gene ontology terms with fewer genes meeting the criterion than expected by chance. A z score near zero suggests that the number of genes meeting the criterion approximates the expected number [89]. Selected z-scores with values >2.0 or less than <-2.0 are reported for ontologies with ≥10 genes. Data were analyzed without transformation. In the table, the terms are: M Genes ↑ - number of genes up-regulated in human corneal epithelial cells of males (M), as compared to those of females (F); F Genes ↑ - number of genes up-regulated in human corneal epithelial cells of females, as compared to those of males; z-score - specific score for the up-regulated genes in the male and female cells.

Affymetrix GeneChip. Similarly, over 20% of the significant Affymetrix genes were unique to this platform. Given these differences in gene populations, it is not surprising that the lists of sex-associated differentially expressed genes and gene ontologies were not identical on the CodeLink and Affymetrix platforms.

A question, then, is whether these platform-specific data have any biological meaning. The answer, based upon recent studies, is yes. A number of investigations have found that significant differences exist between CodeLink and Affymetrix platforms in their ability to detect differential gene expression [34-36]. These studies have also reported little agreement between these platforms concerning the lists of the differentially expressed genes [34-37]. Even if exactly the same sequences and genes are compared, there is only 60 to 70% overlap in CodeLink and Affymetrix data [38]. This low concordance in gene identification appears to be due to intrinsic differences in platform design, including variations in probe length and content, deposition technology, labeling approaches, hybridizing protocols, image segmentation, signal detection, background correction, data normalization and data mining [34-36,38], combined with the intrinsic instability of lists of significantly changed genes based on p-value cut-offs [39]. The result is that CodeLink and Affymetrix arrays, both of which have proven reproducibility and accuracy, seem to measure different things [36]. However, the majority of gene expression changes revealed by each of the platforms are believed to be biologically correct, and these differences cannot be attributed to technological variations [34,35]. It has also been suggested that for a more meaningful transcriptome assessment, one may have to analyze the same sample with different microarray platforms [35]. The genes contained in the intersection of the two lists can be used as reliable biomarkers, while the genes in the union can be used to identify biological pathways.

Given this information, the CodeLink and Affymetrix microarray data concerning sex-related differences in gene expression of human corneal epithelial cells are biologically relevant. However, since these arrays do not evaluate the same gene populations, the results should be different. Such

Table 9. Influence of sex on the expression of gene ontologies in human corneal epithelial cells, as shown with Affymetrix arrays.

Ontology	M Genes ↑	F Genes ↑	M z-score	F z-score
Biological process				
macromolecular complex assembly	23	11	4.36	3.92
cell motility	14	3	2.53	0.15
cell migration	9	2	2.29	0.29
response to stress	11	7	-2.02	-0.11
ribonucleoprotein complex biogenesis and assembly	5	6	0.74	4.02
metabolic process	144	63	1.58	2.97
transcription from RNA polymerase II promoter	15	10	0.75	2.54
Molecular function				
transcription activator activity	12	4	2.69	1.19
protein binding	139	51	2.49	0.8
RNA binding	20	10	2.38	2.5
transmembrane receptor activity	8	2	-2.01	-1.73
transcription coactivator activity	8	4	2.34	2.18
receptor activity	17	3	-1.54	-2.23
Cellular component				
actin cytoskeleton	11	2	3.32	0.27
nucleoplasm part	14	8	2.43	3.05
cytoplasmic membrane-bound vesicle	11	5	2.21	1.87
intracellular organelle part	69	37	1.99	3.78
Golgi membrane	9	7	1.53	3.4
extracellular region	20	4	-1.59	-2.2

Selected z-scores with values >2.0 or less than <-2.0 are listed for ontologies with ≥10 genes. Terminology and abbreviation explanations are presented in the legend to Table 8.

platform-dependent differences in experimental outcomes are thought to be prominent in biological systems where the magnitude of differences between the two samples is relatively low [35].

Our microarray analyses showed that numerous genes were expressed to a significantly greater extent in corneal epithelial cells of men, as compared to women. These included a variety of genes associated with signal transduction pathways, such as CD47 (binds thrombospondin), jagged 2 (activates Notch receptors), vasoactive intestinal peptide receptor 1 and G protein coupled estrogen receptor 1 (binds estrogen and promotes nongenomic signaling events). Males also expressed higher activities of genes promoting cell adhesion (cell adhesion molecule 1 and claudin 16), elastin deposition in the extracellular matrix (lysyl oxidase-like 1), mucin-type O-linked glycosylation (UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6), thyroid hormone inactivation (Type III iodothyronine deiodinase), lysophospholipid hydrolysis (ectonucleotide pyrophosphatase/ phosphodiesterase 2) and neurite outgrowth and arborization (neuritin 1).

Of particular interest were the increased expression in males of corneal genes encoding: a) selenium-binding protein 1, a retinal antigen that may contribute to the pathogenesis of uveitis in patients with Behcet's disease [40]; b) citron, a dual specificity protein kinase that plays an important role in the regulation of cytokinesis [41]. It is possible that activity of this protein may contribute to the greater mitotic index found in the corneal epithelium of male mice (2); c) epidermal growth factor receptor, which stimulates corneal epithelial cell proliferation and wound healing [42]. A significant increase in epidermal growth factor receptor levels are also found in peripheral tissues of males, as compared to females [43]; and d) thymidylate synthetase, an enzyme that promotes DNA synthesis and repair [41].

These latter sex-related effects are especially intriguing, given that males have a significantly higher expression of corneal epithelial cell genes associated with DNA replication and cell migration. These sex-associated influences may be due to the influence of androgens. The reason is that androgens have been reported to repair defects, promote wound healing and stimulate mitosis in the corneal epithelium, as well as to suppress angiogenesis and correct dystrophies in the cornea [2,44-47]. Indeed, a Brazilian pharmaceutical firm has marketed topical androgens to treat corneal trauma, cicatrization, erosions, ulcers and atrophy, as well as to facilitate post-operative care after corneal transplantation.

In contrast, females had greater expression of many other genes, including those related to pain responses (purinergic receptor P2X, ligand gated ion channel, 3), neural signaling (γ-aminobutyric acid A receptor β3), cell cycle (cyclin D1), arachidonic acid hydroxylation (cytochrome P450, family 4, subfamily F, polypeptide 12), cysteine protease inhibition (calpastatin), prolactin regulation (paired-like homeodomain 1) and a variety of cellular processes associated with G protein signaling (GTP-binding protein 10).

Table 10. Impact of sex on the expression of gene ontologies in human corneal epithelial cells, as shown with both CodeLink and Affymetrix arrays.

Ontology	Array	M Genes ↑	F Genes ↑	M z-score	F z-score
Molecular function					
small conjugating protein ligase activity	CL	5	4	2.77	0.71
	Affy	6	2	2.35	1.09
acid-amino acid ligase activity	CL	5	4	2.5	0.49
	Affy	6	2	2.06	0.94
cytoskeletal protein binding	CL	10	6	2.76	-0.69
	Affy	13	3	2.18	0.09
transcription coactivator activity	CL	5	5	2.1	0.7
	Affy	8	4	2.34	2.18
Cellular component					
nuclear body	CL	1	8	-0.24	4.21
	Affy	4	4	1.43	3.69
nucleus	CL	42	95	-0.88	2.64
	Affy	84	37	1.75	2.11
intracellular	CL	107	193	0.08	2.87
	Affy	174	76	2.11	3.31
intracellular part	CL	103	184	0.16	2.57
	Affy	168	75	2.07	3.53
intracellular organelle	CL	85	153	0.08	2.11
	Affy	142	63	1.98	2.95
intracellular membrane-bound organelle	CL	74	139	-0.21	2.21
	Affy	127	56	1.89	2.63
membrane-bound organelle	CL	74	139	-0.22	2.2
	Affy	127	56	1.88	2.62
organelle	CL	85	153	0.07	2.1
	Affy	142	63	1.97	2.94
extracellular region	CL	19	17	0.38	-2.28
	Affy	20	4	-1.59	-2.2

Selected z-scores with values >2.0 or less than <-2.0 are reported. Terminology and abbreviation explanations are presented in the legend to Table 8. In the table, abbreviations are: CL = CodeLink; Affy = Affymetrix.

Several other sex-related differences in gene expression were quite notable. Females had a lower expression of genes encoding phosphoserine phosphatase, an enzyme that catalyzes L-serine formation. Deficiency of this protein has been linked to Williams syndrome [48], which often presents with hyperopia [49]. Women, in turn, are more likely to develop hyperopia [50,51]. Women had a higher expression of the genes encoding: a) carcinoembryonic antigen-related cell adhesion molecule 6, a protein often increased in cancer [41]; b) X (inactive)-specific transcript, which is expressed exclusively from the X inactivation center of the inactive X chromosome [41], and interestingly may be downregulated by desiccation stress [52]; and c) transglutaminase 1, enzyme that catalyzes protein cross-linking. This expression of this protein is typically increased in dry eye and corneal keratinization [53,54].

We wonder if this increased expression of transglutaminase 1 may contribute to the increased prevalence of dry eye in women [55]. We also wonder whether this heightened expression may be due to the influence of estrogens, given that these hormones are known to increase the levels of various transglutaminases in other tissues [56-58]. If so, then estrogen could potentially promote corneal abnormalities and dry eye. Consistent with this hypothesis is the finding that estrogen administration is associated with a significant increase in the signs and symptoms of dry eye [59,60]. Indeed, estrogen treatment has been linked to the induction of photophobia, blurred vision, foreign body sensation, heightened sensitivity, contact lens intolerance and variations in corneal thickness, edema and curvature [19,26, 27,29,61-65]. These effects may account for why hormone replacement therapy in postmenopausal women may reduce visual acuity [66], and why oral contraceptive use in pre-menopausal women may increase corneal hydration, sensitivity and contact lens discomfort [27,67,68], and lead to an elevated blink rate [69].

If androgens and estrogens do mediate some of the sex-related differences in gene expression in human corneal epithelial cells, then the mechanism by which sex steroids act most likely involves the local, intracrine synthesis of these hormones from adrenal sex steroid precursors and a consequent hormone association with saturable, high-affinity and steroid-specific receptors. Classically, the monomeric, activated steroid-receptor complex would then bind to a response element(s) in the regulatory region of specific target genes, dimerize with another steroid-bound complex and, in combination with appropriate co-activators, regulate gene transcription [70,71]. In support of this hypothesis, we and

Table 11. Sex-related expression of X and Y chromosome genes in human corneal epithelial cells in vitro.

Entrez gene identification	Gene	Ratio	p value	Ontology
Male>Female				
Y chromosome				
6192	Ribosomal protein S4, Y-linked 1	349.1	<0.0090	translation
8287	Ubiquitin specific peptidase 9, Y-linked (fat facets-like, Drosophila)	165.0	<0.0212	ubiquitin-dependent protein catabolic process
8653	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	27.4	<0.0117	nucleotide binding
9086	Eukaryotic translation initiation factor 1A, Y-linked	20.6	<0.0018	translational initiation
8284	Jumonji, AT rich interactive domain 1D	16.5	<0.0050	spermatogenesis
22829	Neuroligin 4, Y-linked	8.3	<0.0282	cell adhesion
84663	Chromosome Y open reading frame 15B	4.3	<0.0020	
Female>Male				
X chromosome				
7503	X (inactive)-specific transcript	348.4	<0.0000	inactivation of X chromosome
9643	Mortality factor 4 like 2	3.0	<0.0385	regulation of cell growth

Relative ratios were calculated by comparing the degree of sex-related gene expression in corneal epithelial cells that had been cultured in vitro. Data were analyzed with and without log transformation.

Table 12. Sex-associated differences in gene expression in human corneal epithelial cells in vitro.

Entrez gene identification	Gene	Ratio	p value	Ontology
Male>Female				
3105	Major histocompatibility complex, class I, A	154.0	<0.0048	antigen processing and presentation of peptide antigen via MHC class I
6707	Small proline-rich protein 3	4.3	<0.0316	epidermis development
1672	Defensin β1	3.3	<0.000	chemotaxis
3934	Lipocalin 2 (oncogene 24p3)	3.0	<0.0193	transport
23705	Cell adhesion molecule 1	2.3	<0.0290	T cell mediated cytotoxicity
Female>Male				
*	CDNA FLJ40891 fis, clone UTERU2001110	10.0	<0.0091	
822	Capping protein (actin filament), gelsolin-like	2.9	<0.0124	protein complex assembly
284217	Laminin α1	2.1	<0.0045	multicellular organismal development
2559	Gamma-aminobutyric acid (GABA) A receptor α6	2.1	<0.0467	ion transport
8636	Sjogren syndrome nuclear autoantigen 1	1.6	<0.0148	identical protein binding

Data were analyzed with and without log transformation. The asterisk indicates gene accession number = BQ068355

others have shown that the cornea contains the enzymatic machinery necessary for the intracrine synthesis and metabolism of androgens and estrogens [72-74]. Moreover, we and others have shown that the cornea contains androgen and estrogen receptors [75-78] and that sex steroids may regulate gene expression in primary and immortalized human corneal epithelial cells [79,80] (Dr. Payal Khandelwal, personal communication).

Our current investigation also demonstrates that sex exerts a significant impact on gene expression in human corneal epithelial cells in vitro. However, with few exceptions (e.g. X- and Y-linked genes), these sex-related differences in gene expression in vitro were typically different than those in vivo. There are several possible explanations for this finding. First, the influence of sex steroids on gene expression is lost during culture. Second, the molecular biological effects of hormones from the hypothalamic-pituitary axis, which is differentially regulated by sex steroids, are also absent during cell culture. Loss of this axis' hormonal impact has been shown to underlie the striking differences in gene expression between other cell types in vivo and in vitro [81,82].

Additional explanations for the sex-related differences in gene expression in vivo and/or in vitro include the effects of Y-linked genes in males [83], and of X inactivation and associated X escapees in females. X inactivation is a chromosome-wide silencing mechanism that evolved to restore equal gene expression between males and females. However, although the process of X inactivation silences a majority of genes, 100 to 200 genes may escape this silencing and be expressed from both X chromosomes in females [83-86]. There are also a number of other hormone-independent mechanisms that may account for genes that are

Table 13. Effect of sex on the expression of gene ontologies in human corneal epithelial cells in vitro.

Ontology	M Genes ↑	F Genes ↑	M z-score	F z-score
Biological process				
regulation of cell morphogenesis	8	4	3.32	1.1
regulation of cell growth	6	4	3.27	2.02
response to wounding	9	4	2.07	-0.04
transmembrane receptor protein tyrosine kinase signaling pathway	4	6	1.36	3.14
chromatin modification	4	6	1.13	2.83
response to stimulus	28	12	1.03	-2.09
intracellular signaling cascade	17	6	0.55	-2.1
Molecular function				
carbohydrate binding	10	4	4.18	0.8
heparin binding	4	2	3.37	1.34
UDP-glycosyltransferase activity	4	1	2.62	-0.09
glycosaminoglycan binding	4	2	2.78	0.98
calcium ion binding	16	4	2.31	-1.61
phosphoprotein phosphatase activity	0	5	-1.29	2.88
anion transmembrane transporter activity	2	4	0.52	2.43
protein binding	79	82	0.59	2.16
Cellular component				
Golgi membrane	11	2	3.91	-0.86
cytoplasmic vesicle membrane	4	2	2.83	0.91
cytoplasm	78	74	2.15	1.64
cytosol	6	11	0.63	3.05
centrosome	4	4	2.06	2.09

Selected z-scores with values >2.0 or less than <-2.0 are shown for selected ontologies with ≥5 genes. Terminology and abbreviation explanations are presented in the legend to Table 8.

Table 14. Sex-related differences in gene expression in human corneal epithelial cells in vivo and in vitro.

Entrez gene identification	Gene	Ratio	p value	Ontology
Male>Female				
8793	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	1.9	<0.0066	apoptosis
1735	Deiodinase, iodothyronine, type III	1.5	<0.0154	thyroid hormone catabolic process
154091	Solute carrier family 2 (facilitated glucose transporter), member 12	1.5	<0.0085	carbohydrate transport
3931	Lecithin-cholesterol acyltransferase	1.6	<0.0460	lipid metabolic process
50700	Retinol dehydrogenase 8 (all-trans)	1.4	<0.0472	estrogen biosynthetic process
Female>Male				
26354	Guanine nucleotide binding protein-like 3 (nucleolar)	2.1	<0.0225	regulation of cell proliferation
51552	RAB14, member RAS oncogene family	1.9	<0.0459	Golgi to endosome transport
53947	α 1,4-galactosyltransferase (globotriaosylceramide synthase)	1.9	<0.0282	glycosphingolipid biosynthetic process
6256	Retinoid X receptor α	1.8	<0.0499	transcription
2099	Estrogen receptor 1	1.7	<0.0102	transcription

Data were analyzed without log transformation.

expressed in a sex-specific (i.e. exclusively in males or females) or a sex-biased (i.e. higher level in either males or females) manner [87,88]. The number of sex-biased genes appear to be considerable, although fold-differences in gene expression, at least in somatic tissues (e.g. liver, muscle, adipose tissue, brain), tend to be modest (e.g. <1.2 fold) [88].

Overall, our findings support our hypothesis that sex-related differences exist in the gene expression of human corneal epithelial cells. Further studies are required to explore how these variations in gene expression may contribute to sex-related differences in the prevalence of certain corneal diseases.

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