Influence of sex on gene expression in human corneal epithelial cells

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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.

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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 1 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 (KSF-M; Invitrogen) was administered to the top of each tissue piece.
Tissues were incubated overnight at 37 °C under 95% humidity and 5% CO₂. 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 RNaseasy 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 a 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 NDRRI 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 using 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), carcioembryonic 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.
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.
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 2. Significant, sex-related differences in gene expression: Comparisons between CodeLink and Affymetrix arrays. |
|---------------------------------------------------------------|
| **CodeLink**                                                 |
| Number of genes with significant differences in expression 216 |
| Number of genes with same results on Affymetrix 22            |
| Number of genes changed in same direction on Affymetrix 82    |
| Number of genes changed in opposite direction on Affymetrix 10|
| Number of genes with opposite results on Affymetrix 0        |
| Number of unique genes, not expressed by Affymetrix 102       |
| **Affymetrix**                                               |
| Number of genes with significant differences in expression 307|
| Number of genes with same results on CodeLink 22              |
| Number of genes changed in same direction on CodeLink 141     |
| Number of genes changed in opposite direction on CodeLink 66  |
| Number of genes with opposite results on CodeLink 4           |
| Number of unique genes, not expressed by CodeLink 74          |

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               |                               |          |            |            |              |                           |
| 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                |                               |          |            |            |              |                           |
| 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 tetracopeptide 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.
## 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                             |
| 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-| 2.1 (1.4) | <0.0246  | lipid metabolic process                          |
|                            | macroglobulin receptor)                        |           |          |                                                  |
| 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                        | β2 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               | Lysyl oxidase-like 1                      | 3.23   | <0.0326   | electron transport                            |
| 4016                        | GalNAc-T6                                 | 2.19   | <0.0082   | protein amino acid O-linked glycosylation    |
| 11226                       | Notch homolog 4                           | 2.04   | <0.0070   | cell fate determination                       |
| 4855                        | Claudin 16                                | 1.67   | <0.0180   | ion transport                                 |
| *                           | Integrin subunit α-2 gene                 | 1.65   | <0.0347   | cell adhesion                                 |
| Female > Male               | Oxysterol binding protein 2               | 3.31   | <0.0095   | lipid transport                               |
| 23762                       | GTP-binding protein 10 (putative)         | 2.76   | <0.0095   | ribosome biogenesis and assembly              |
| 85865                       | Purinergic receptor P2X, ligand-gated ion channel, 3 | 2.65 | <0.0127 | ion transport |
| 5024                        | Paired-like homeodomain 1                 | 2.24   | <0.0363   | regulation of transcription, DNA-dependent    |
| 5307                        | Podoplanin                                | 1.88   | <0.0332   | cell morphogenesis                            |
| **Affymetrix**              |                                           |        |           |                                               |
| Male > Female               | Schwannomin interacting protein 1         | 2.64   | <0.0321   | protein binding                               |
| 29970                       | Monocyte to macrophage differentiation-associated | 2.07 | <0.0038 | cytolysis |
| 23531                       | Phosphoserine phosphatase                 | 2.05   | <0.0383   | L-serine biosynthetic process                 |
| 5723                        | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 | 1.91 | <0.0141 | regulation of transcription, DNA-dependent |
| 4791                        | Phosphoglycerate dehydrogenase            | 1.75   | <0.0088   | L-serine biosynthetic process                 |
| Female > Male               | Guanine nucleotide binding protein, α14   | 2.62   | <0.0028   | signal transduction                           |
| 9630                        | Cyclin D1                                 | 1.7    | <0.0090   | G1/S transition of mitotic cell cycle         |
| 595                         | Mitotic arrest deficient-like 1           | 1.64   | <0.0010   | mitotic metaphase                             |
| 8379                        | Interferon stimulated exonuclease         | 1.48   | <0.0268   | DNA catabolic process, exonucleolytic         |
| 3669                        | 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. Similarly, 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.
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

| 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.
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.

### 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.
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 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.
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 premenopausal 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

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**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.
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

| Entrez gene identification | Gene | Ratio | p value | Ontology |
|----------------------------|------|-------|---------|----------|
| Male>Female Y chromosome | Ribosomal protein S4, Y-linked 1 | 349.1 | <0.009 | translation |
| 6192 | Ubiquitin specific peptidase 9, Y-linked (fat facets-like, Drosophila) | 165.0 | <0.021 | ubiquitin-dependent protein catabolic process |
| 8287 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked | 27.4 | <0.011 | nucleotide binding |
| 8653 | Eukaryotic translation initiation factor 1A, Y-linked | 20.6 | <0.001 | translational initiation |
| 9086 | Junonji, AT rich interactive domain 1D | 16.5 | <0.005 | spermatogenesis |
| 8284 | Neuroulin 4, Y-linked | 8.3 | <0.028 | cell adhesion |
| 22829 | Chromosome Y open reading frame 15B | 4.3 | <0.002 | |
| 84663 | Female>Male X chromosome | X (inactive)-specific transcript | 348.4 | <0.000 | inactivation of X chromosome |
| 7503 | Mortality factor 4 like 2 | 3.0 | <0.038 | regulation of cell growth |
| 9643 | Male>Female | Major histocompatibility complex, class I, A | 154.0 | <0.0048 | antigen processing and presentation of peptide antigen via MHC class I |
| 3105 | Small proline-rich protein 3 | 4.3 | <0.0316 | epidermis development |
| 6707 | Defensin β1 | 3.3 | <0.000 | chemotaxis |
| 1672 | Lipocalin 2 (oncogene 24p3) | 3.0 | <0.0193 | transport |
| 3934 | Cell adhesion molecule 1 | 2.3 | <0.0290 | T cell mediated cytotoxicity |
| 23705 | Female>Male | CDNA FLJ40891 fis, clone UTERU2001110 | 10.0 | <0.0091 | |
| * | Capping protein (actin filament), gelsolin-like | 2.9 | <0.0124 | protein complex assembly |
| 822 | Laminin α1 | 2.1 | <0.0045 | multicellular organismal development |
| 284217 | Gamma-aminobutyric acid (GABA) A receptor α6 | 2.1 | <0.0467 | ion transport |
| 2559 | Sjogren syndrome nuclear autoantigen 1 | 1.6 | <0.0148 | identical protein binding |
| 8636 | Data were analyzed with and without log transformation. The asterisk indicates gene accession number = BQ068355

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

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

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

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.
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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