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doi: [10.1074/jbc.M113.504399](https://doi.org/10.1074/jbc.M113.504399)**The Ets Transcription Factor EHF as a Regulator of Cornea Epithelial Cell Identity**^{*S}[Denise N. Stephens](#),[‡] [Rachel Herndon Klein](#),^{‡§} [Michael L. Salmans](#),^{‡§} [William Gordon](#),[‡] [Hsiang Ho](#),[¶] and [Bogi Andersen](#)^{‡§¶,1}[Author information](#) ▶ [Article notes](#) ▶ [Copyright and License information](#) ▶This article has been [cited by](#) other articles in PMC.**Abstract**Go to:

The cornea is the clear, outermost portion of the eye composed of three layers: an epithelium that provides a protective barrier while allowing transmission of light into the eye, a collagen-rich stroma, and an endothelium monolayer. How cornea development and aging is controlled is poorly understood. Here we characterize the mouse cornea transcriptome from early embryogenesis through aging and compare it with transcriptomes of other epithelial tissues, identifying cornea-enriched genes, pathways, and transcriptional regulators. Additionally, we profiled cornea epithelium and stroma, defining genes enriched in these layers. Over 10,000 genes are differentially regulated in the mouse cornea across the time course, showing dynamic expression during development and modest expression changes in fewer genes during aging. A striking transition time point for gene expression between postnatal days 14 and 28 corresponds with completion of cornea development at the transcriptional level. Clustering classifies co-expressed, and potentially co-regulated, genes into biologically informative categories, including groups that exhibit epithelial or stromal enriched expression. Based on these findings,

Related citations in PubMed[Postnatal gene expression in the normal mouse cornea](#) [Invest Ophthalmol Vis Sci. 2004][Conditional deletion of the mouse Klf4 gene results in cornea](#) [Mol Cell Biol. 2007][Critical role of Klf5 in regulating gene expression during post-embryonic development](#) [PLoS One. 2012][The Ets family contains transcriptional activators](#) [Int J Biochem Cell Biol. 2001][The Ets-transcription factor family in embryonic development](#): [Oncogene. 2000][See reviews...](#)[See all...](#)**Cited by other articles in PMC**[Ets homologous factor regulates pathways controlling cell fate](#) [Nucleic Acids Research. 2014][See all...](#)**Links**[BioProject](#)[EST](#)[Gene](#)[GEO DataSets](#)[GEO Profiles](#)[HomoloGene](#)[Nucleotide](#)[Protein](#)[PubMed](#)[Taxonomy](#)[Taxonomy Tree](#)

and through loss of function studies and ChIP-seq, we show that the Ets transcription factor EHF promotes cornea epithelial fate through complementary gene activating and repressing activities. Furthermore, we identify potential interactions between EHF, KLF4, and KLF5 in promoting cornea epithelial differentiation. These data provide insights into the mechanisms underlying epithelial development and aging, identifying EHF as a regulator of cornea epithelial identity and pointing to interactions between Ets and KLF factors in promoting epithelial fate. Furthermore, this comprehensive gene expression data set for the cornea is a powerful tool for discovery of novel cornea regulators and pathways.

Keywords: Aging, Chromatin Immunoprecipitation (ChIP), Cornea, Development, DNA Transcription, Epithelium, Eye, Gene Expression, Transcription/Developmental Factors

Introduction

Go to:

The cornea is the transparent tissue at the front of the eye, responsible for transmission and focusing of light through the iris while also protecting the eye from infections and other external damage. The cornea consists of three cellular layers: the outermost non-keratinized, stratified epithelium; the intermediate stroma, which in mammals comprises up to 90% of the cornea's thickness; and the innermost single layered endothelium (1). In the mouse, cornea development (Fig. 1A) begins around embryonic day 12. The epithelium differentiates from the surface ectoderm with KRT14 (keratin 14) expression marking the transition from ectoderm to cornea epithelium. Around the same time, mesenchymal cells from the neural crest migrate into the space between the ectoderm and the lens (2), ultimately differentiating into stromal fibroblasts (keratocytes) and the endothelium (3). The stroma and endothelium undergo organization during the late embryonic and early postnatal period; keratocytes differentiate and secrete extracellular matrix proteins, including collagen, and the endothelium proliferates, forming a monolayer across the posterior cornea. Meanwhile, the epithelium undergoes progressive stratification and differentiation in late embryogenesis and during the early postnatal period. By 2 weeks of age, the epithelium reaches nearly full thickness, and terminal differentiation markers, such as KRT12 and GJA1(Cx43), are visible. The stroma has become thicker and largely acellular, and the endothelial cells cease proliferation, having formed a barrier to maintain the fluid balance in the stroma (4).

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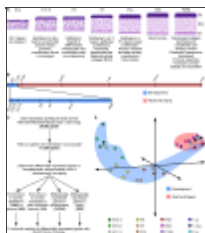


FIGURE 1.

Cornea gene expression over the lifetime of the

mouse. *A*, schematic representation of the main features of cornea development and aging in the mouse. *B*, time line depicting the ages of mouse cornea samples used in whole genome expression time course analysis. ...

In cornea aging, morphological changes are subtle ([Fig. 1A](#)) ([5](#)). Expression of cellular adhesion molecules in the epithelium decreases, and the epithelium loses repair capacity, becoming more vulnerable to infections. Collagen synthesis in the stroma is reduced, and the cornea, one of the most highly innervated tissues in the body, loses sensitivity. Endothelial cell density also decreases ([6](#)). Although the morphology of the mouse cornea during development and in the adult has been well characterized ([2](#), [7](#), [8](#)), there have only been a few studies characterizing the global gene expression of the normal cornea ([1](#), [9–11](#)), none of them profiling gene expression over stages of development and adulthood.

Here we have created a comprehensive gene expression profile of whole mouse cornea at key points throughout cornea development and every 6 months thereafter, from age E14.5² to 2 years postnatally. This data set will be useful for the discovery of new genes, gene groups, transcriptional regulators, and biologically important pathways in the cornea and will also assist in the characterization of transgenic mouse models and human cornea diseases. Using these data, we identified the Ets factor EHF (Ets homologous factor) as a potential regulator of cornea epithelial cell differentiation, and through a combination of ChIP-seq and RNAi, we demonstrate for the first time that EHF promotes cornea epithelial cell identity.

EXPERIMENTAL PROCEDURES

Go to:

Isolation of Total RNA and Preparation for Whole Genome Expression Arrays

Whole corneas were dissected from wild type CB6 mouse eye globes. Cornea epithelium was isolated from P28 mice by

digestion in 50% EMEM, dispase II with 50 mM sorbitol and 10 μ l/ml β -mercaptoethanol for 2 h at 37 °C (modified from Ref. [12](#)). The corneal stroma was isolated from P7 mice by embedding whole eye globes in OCT, cryosectioning at 10 μ m, and microdissecting out the stroma. Total RNA from cornea and other tissues was isolated as described previously ([13](#)). Sample purity was validated by quantitative PCR expression of tissue-specific markers and the absence of markers for adjacent tissues. Cornea samples were prepared for microarray with the NuGEN Ovation RNA Amplification System V2 and NuGEN FL-Ovation cDNA Biotin Module V2 (NuGEN Technologies, San Carlos CA). All other samples were prepared with the Ambion WT expression kit (Invitrogen). Whole genome expression was assessed with Affymetrix Mouse Gene 1.0 ST arrays in triplicate (E14.5–P60 cornea; lung, trachea) or duplicate (P180 to 2-year cornea; separated epithelium and stroma) and Mouse Genome 430 arrays in triplicate (esophagus, small intestine, stomach, and skin). Microarray data for bladder and E18.5 skin was published previously ([13](#)).

Microarray Data Analysis Array data were quantified with Expression Console version 1.1 software (Affymetrix, Inc.) using the PLIER Algorithm default values. Expression values were then filtered as present/absent at expression 150. Only known coding transcripts (24,582) representing 21,676 genes (referred to here as the transcriptome) were included in the analysis. MultiExperiment Viewer software ([14](#), [15](#)) was used to perform analysis of variance, principal component analysis (PCA), gene distance matrix, hierarchical clustering, and k -means analyses. Probe sets were called differentially expressed if they had a p value of <0.01 and a 2-fold expression change over the developmental time course. The Cyber-T web server ([16](#), [17](#)) was utilized to compare P28–P60 and 2-year whole cornea samples; genes passing a p value of <0.05 and a change in expression greater than 1.3-fold were clustered with hierarchical clustering. The Cyber-T method was also used to determine differential expression between epithelium and stroma; epithelium-enriched, stroma-enriched, or both (expressed in both samples) probe sets were determined by a -fold expression cut-off using known markers in each tissue. We used AmiGO ([18](#)) to compile a list of mouse transcriptional regulators, including DNA-binding transcription factors, chromatin modifiers, and transcription co-factors. For comparison across epithelial tissues, each array was \log_2 -transformed and mean-centered with the S.D. set to 1. Only common probe sets from Affymetrix Mouse Gene 430 and Affymetrix MoGene 1.0 ST

arrays were used for downstream analyses. For the gene distance matrix, gene chip biases were removed using distance-weighted discrimination (19). For identification of genes unique to the cornea, sample replicates were averaged, and -fold changes were calculated by comparing each tissue at E18.5 with the cornea P28 sample. Genes with -fold change ± 2.0 in all other tissues were selected for hierarchical clustering and *k*-means clustering ($k = 7$). Clustering and heat maps were generated using the pheatmap package in R (available from the R Project for Statistical Computing Web site). For comparing aging gene expression changes across tissues, we obtained data from previously published expression data sets and from GEO (Gene Expression Omnibus) (20–36). Gene ontology was performed using DAVID (37, 38).

ChIP-PCR and ChIP Sequencing ChIP assays were performed as described previously (13, 39), using IgG (Sigma; for ChIP-PCR only) or EHF antibody (Santa Cruz Biotechnology, Inc.; for ChIP-PCR and ChIP-seq). Sequencing libraries were generated for the EHF ChIP and input samples using the Illumina Tru-Seq kit, according to the Illumina protocol for ChIP-seq library preparation with some modification; after adaptor ligation, 14 cycles of PCR amplification were performed prior to size selection of the library (40). Clustering and 50-cycle single end sequencing were performed on the Illumina Hi-Seq 2000 genome analyzer. Reads were aligned to the mouse mm9 genome using Bowtie (version 0.12.7) (41), with only uniquely aligning reads retained; in total, 6.5 million mapped reads were obtained. MACS (version 1.4.2) (42) was used to call peaks, with the input sample used as the control. Galaxy was used to align peaks to gene regions and compare ChIP-seq and siRNA data (43–45). MEME and Cistrome were used for motif analysis (46, 47).

Whole Genome Expression Arrays for Primary Human Cornea Epithelial Cells and siRNA Experiments

Primary human cornea epithelial cells from CELLnTEC Inc. were grown in CnT-20 medium as directed by the manufacturer. Cells were trypsinized and plated, 100,000 cells/well, in a 12-well dish containing 1 μ l of Lipofectamine RNAiMAX and either 30 nM *EHF* siRNA or scrambled siRNA. The *EHF* siRNA was pooled from three individual siRNAs targeting *EHF* (Ambion, ID s25397, s25398, s25399) in the same concentration. *EHF* mRNA knockdown was verified by quantitative PCR, and reduction in EHF protein level was demonstrated in immortalized human cornea epithelial cells by Western blot. Total RNA was extracted from the plated cells with the Purelink RNA miniprep kit

well with previous studies

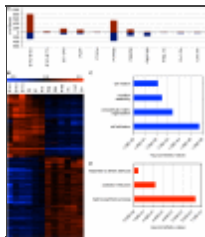


FIGURE 2.

Identification of a gene expression transition point between P14 and P28 points to the importance of distinct biological functions in cornea during development and adulthood. A, number of differentially regulated genes ($p < 0.01$, >1.5 -fold ...

To search for gene expression patterns matching the phases of development and adulthood as defined by PCA, we identified genes showing greater than 2-fold average change in expression between the developmental (E14.5–P14) and adult (P28 to 2 year) time points. Remarkably, 1106 genes feature a striking expression switch at the P14–P28 development-adulthood transition point ([Fig. 2B](#)). Gene ontology (GO) analysis of these genes identified overrepresentation of genes associated with specific functions in the two phases, indicating the activity of distinct biological processes during cornea development and adult homeostasis. During development, the biological terms *cell adhesion*, *extracellular matrix organization*, *myofibril assembly*, and *cell motion* most likely represent active migration of cornea keratocytes and keratinocytes characteristic for morphogenesis of the cornea ([Fig. 2C](#)). During adulthood, the biological terms *lipid biosynthetic process*, *oxidation reduction*, and *response to abiotic stimulus* most likely represent the cornea's role as a barrier to environmental exposure, which becomes more important as the cornea fully differentiates ([Fig. 2D](#)). Together, these data point to an important gene expression transition point between P14 and P28 that reflects distinct functions of the developing and adult cornea.

Distinct Spatio-temporal Gene Expression Profiles Underlie Cornea Development

The PCA analysis ([Fig. 1D](#)), and the identification of a transcriptional switch at the development-adult transition point ([Fig. 2B](#)), suggested that gene expression could be analyzed separately for the development and adulthood phases, using P28 as the end point for development and the starting point for adulthood.

We first investigated gene expression patterns during development from E14.5 to P28 by analysis of variance, identifying 10,209 genes (12,000 probe sets; 43% of the mouse transcriptome) as significantly differentially regulated over the developmental time course. *k*-Means clustering grouped the developmentally regulated genes by their temporal expression pattern into 40 distinct clusters (Fig. 3 and supplemental Table S1). To facilitate classification of the temporal gene expression clusters vis á vis epithelial and stromal expression, we evaluated the ratio of probe set expression in isolated adult cornea epithelium and stroma (Fig. 4A). By setting cut-offs based on the -fold enrichment of numerous known epithelial and stromal genes, we generated a list of epithelial (1832 probe sets, 1455 genes) and stromal (1147 probe sets, 902 genes) enriched genes, representing 6 and 4% of the mouse transcriptome, respectively. We then overlapped the lists of “epithelial” and “stromal” genes with the developmental clusters, discovering that a number of clusters contained a significant overrepresentation of epithelial or stromal enriched genes, whereas other clusters were composed of genes expressed relatively equally in both cell layers (Fig. 4B).

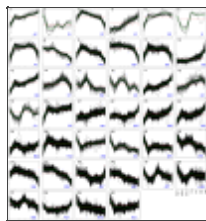


FIGURE 3.

***k*-Means clustering of developmental cornea expression data.** Differentially regulated genes were grouped into 40 temporal expression clusters. *x* axis, time point (labels shown on graph for cluster 36); *y* axis, \log_2 expression. Each tick mark represents ...

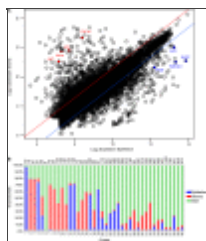


FIGURE 4.

Evaluation of epithelial and stromal enrichment of genes expressed in the mouse cornea. A, gene expression data for separated cornea epithelium and

stroma samples at P28. Expression of each gene in the epithelium (*x axis*) was plotted against its expression

...

We observed that the shapes of the temporal expression patterns in some clusters were similar and that they differed mostly in the magnitude of gene expression change (Fig. 3); many of these clusters also contained similar GO terms (Table 2). Using these temporal pattern similarities, and considering membership of known marker genes as well as the representation of epithelium- and stroma-enriched genes, we combined 31 of the 40 *k*-means clusters into 10 superclusters (Fig. 5). Grouping the clusters in this manner resulted in three “epithelial” (A, C, and G) and four “stromal” (B, E, F, and H) superclusters. Of the remaining three superclusters, one supercluster (D) contains both epithelial and stromal genes, although it is slightly more stromal than epithelial in nature. The last two superclusters (I and J) are primarily composed of genes expressed in both epithelium and stroma, containing GO categories that represent housekeeping-type genes, and showing modest changes in expression.

Cluster sets	Example genes	Selected GO set
1	ADAMTS1, ADAMTS1-AS1, COL1A1	Cellular development, EMT, Differentiation
2	COL1A1, COL1A2, COL3A1	Extracellular matrix organization, Collagen
3	COL1A1, COL1A2, COL3A1	Extracellular matrix organization, Collagen

TABLE 2

Characteristics of the developmental *k*-means clusters

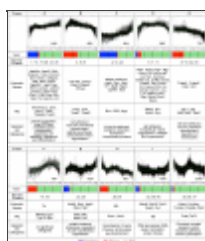


FIGURE 5.

Time course clustering of cornea developmental genes reveals distinct gene expression patterns associated with epithelium and stroma. Shown are the expression profiles of superclusters (A–J) that were formed by combining similar *k*-means clusters ...

Comparing the epithelial and stromal superclusters, the most striking difference is evident between P14 and P28 when

expression in the epithelial clusters is increasing while expression in stromal clusters is decreasing (Fig. 5). This observation suggests that during this time period, increased expression of many epithelial genes is required for the formation of an effective epithelial barrier that protects the adult eye from injury and infection. Meanwhile, it is likely that the expression of many stromal components decreases during the same period to allow the cornea to develop a transparent state. Another difference between epithelial and stromal clusters is the respective up- and downward trend in gene expression over the whole time course. In particular, two of the three epithelial clusters (A and G) exhibit a robust up-regulation in gene expression between E14.5 and E15.5, corresponding to the formation of a two-layered epithelium.

Developmental Co-regulation of Cornea Genes Suggests New Transcriptional Regulators for Cornea

In addition to pointing to new biological functions through GO categories, the clusters suggest linkage between transcriptional regulators (*TRs*) and target genes (Fig. 5). For example, cluster A contains the well characterized epithelial differentiation genes *Krt12*, *Aldh3a1*, and *Upk1b* (55–57) and known transcriptional regulators for epithelial differentiation, including *Klf4* and *Klf5* (58, 59). In fact, the KLF5 target genes *Dsp* and *Dsg1b* (59) are both found in this cluster. We overlapped the gene expression data for *Klf4* and *Klf5* conditional knock-out corneas with the superclusters and observed differences in cluster membership between genes up-regulated and down-regulated after *Klf4* and *Klf5* deletion. For instance, genes that appear to be positively regulated by KLF4 and KLF5 (down-regulated after gene deletion) are enriched in epithelial-type superclusters like A and C. In contrast, genes that are up-regulated after deletion of these two factors tend to fall into stromal-type superclusters like B and E (Fig. 6).

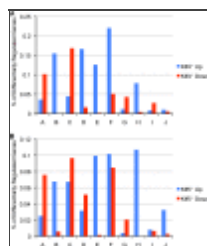


FIGURE 6.
Differentially regulated genes in *Klf4*^{-/-} and *Klf5*^{-/-} corneas overlapped with the developmental superclusters (see Fig. 5). A, percentage of genes in each supercluster affected by the cornea deletion of

Klf4. B, percentage ...

Supercluster A also contains transcriptional regulators not previously known to have a function in the cornea epithelium, including the Ets factor EHF, a candidate new regulator for the cornea epithelial differentiation program. Cluster C contains Ets factor *Elf3* (*Ese1*), previously implicated in cornea epithelial differentiation (50), along with epithelial markers *Aqp5* and *Itga6* (60, 61). Interestingly, epithelial gene repressor *Zeb1* (62) and its uncharacterized family member *Zeb2* were located in cluster E, a stromal cluster. Known stromal transcription factors *Pitx2* and *Twist2* (63, 64) were grouped in cluster B, which contained stromal markers *Fbln2*, *P4ha2* (65, 66), and genes enriched for positive regulation of cell-substrate adhesion. This cluster also contained *Twist1*, suggesting it as a potential regulator of stromal gene expression. We also examined where endothelial genes were located across the clusters and found them to be evenly distributed among the clusters (data not shown). We conclude that membership in the superclusters can be mined to develop new hypotheses about regulatory relationships in the cornea epithelium and stroma.

To identify genes uniquely expressed in the cornea compared with other epithelial tissues, we utilized previously published (13) and new microarray expression data from seven other mouse epithelial tissues, comparing these to gene expression during cornea development (Fig. 7). At the transcript level, the developing cornea most resembles E18.5 bladder and E14.5 skin and is most distinct from E18.5 lung (Fig. 7A). Using data from the most differentiated time points (where multiple time points were available), we compared the expression of genes across these eight epithelial tissues, finding that 124 genes were expressed 2-fold or higher in the cornea compared with all of the other tissues (Fig. 7B and supplemental Table S2). Of these cornea-enriched genes, about half are members of epithelium-enriched time course clusters, and two-thirds are cornea epithelium-enriched (data not shown). Among these genes were, as expected, previously described genes, such as *Krt12*, *Upk1b* (57), and *Slurp1* (67), but also the transcription factor *Ehf* with unknown functions in the cornea. We conclude that relatively few genes are highly enriched in cornea compared with other epithelia and that these genes are likely to carry out cornea-specific functions.

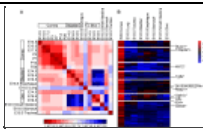


FIGURE 7.

Comparison of the whole genome expression profiles of various mouse epithelial tissues. A, gene distance matrix comparing correlation of global expression profiles of developmental cornea time points with the indicated epithelial tissues. The correlation ...

Ageing-associated Gene Expression Changes in the Cornea; Enrichment of Epithelium- and Stroma-enriched Genes

To identify genes with significant changes in expression during aging, we utilized Cyber-T (16, 17) to compare cornea mRNA expression between young adult (average of P28 and P60) and old (2-year) mice. At $p < 0.01$, 608 genes (664 probe sets, 2.5% of the transcriptome) are differentially expressed (Table 3 and supplemental Table S3). This compares with 10,209 genes (45% of the transcriptome) differentially expressed during cornea development, indicating that a much smaller portion of the genome exhibits dynamic expression changes in aging than in development. Furthermore, as found in other aging studies, most expression changes were less than 2-fold, whereas dramatic expression changes were common in development. Analyzing 43 aging gene expression data sets from 32 mouse tissues available in GEO2R (20, 21), we found that the portion of the transcriptome changing in aging varies from less than 1% up to 24% of transcripts, with the majority of tissues showing changes in less than 10% of the transcriptome (Table 4). With 2.8% of probe sets changing in cornea aging, the cornea falls into a group of tissues with a relatively modest change in aging-related gene expression.

Gene symbol	Gene description
Ap2b1	Apoptosis-inducing factor 2
Capn1	Calpain domain 1, family 1, subfamily 1, orthologous to 1
Chchd10	Cholesterol efflux factor 10
Chchd11	Cholesterol efflux factor 11
Chchd12	Cholesterol efflux factor 12
Chchd13	Cholesterol efflux factor 13
Chchd14	Cholesterol efflux factor 14
Chchd15	Cholesterol efflux factor 15
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Chchd94	Cholesterol efflux factor 94
Chchd95	Cholesterol efflux factor 95
Chchd96	Cholesterol efflux factor 96
Chchd97	Cholesterol efflux factor 97
Chchd98	Cholesterol efflux factor 98
Chchd99	Cholesterol efflux factor 99
Chchd100	Cholesterol efflux factor 100

TABLE 3

List of the genes most up-regulated in cornea during aging

aging; these genes fall into nine distinct clusters based

...

Comparing the differentially expressed genes in aging with those differentially expressed in development, we found 382 genes in common, whereas 226 genes were uniquely changing in aging ([Fig. 8B](#)). The genes commonly altered in development and aging are associated with cell adhesion and regulation of cell morphogenesis ([Fig. 8C](#)), processes that are important in both development and aging. The genes changing uniquely in aging are enriched for translation-related genes and were not epithelium- or stroma-enriched; most of these translation-related genes are down-regulated. Interestingly, recent work in mice has demonstrated that down-regulation of translation pathways results in increased longevity ([69](#)). This reduced translation in the cornea may contribute to the functional maintenance of the cornea in aged mammals.

Next, we asked whether previously defined aging-regulated genes from other tissues also change in the cornea. We compared differentially regulated genes in the aging cornea with those in 42 other aging data sets from a variety of tissues ([Table 4](#)). To a large extent, unique genes were expressed in each one of these tissues. However, the aging transcriptomes of tail skin, cochlea, and lungs were more similar to cornea than predicted by random chance ($p < 0.01$), whereas several aging transcriptomes were less similar to cornea than predicted, including those from myoblasts, heart ventricle, muscle, bone marrow, and brain. We found that 479 genes were differentially expressed in the cornea and in at least one other aging gene expression data set, with relatively few altered across numerous tissues ([Fig. 8D](#)). The top common genes represent several known age-regulated pathways, such as cell adhesion, ion transport, and cell death, suggesting that these particular genes may be important aging regulators in their respective pathways, regardless of the tissue in which they are expressed.

ChIP-seq and Functional Studies Identify EHF as a Regulator of Cornea Epithelial Cell Identity

To identify potential new regulators of cornea epithelial differentiation, we decided to focus on the Ets transcription factor EHF. The *Ehf* transcripts are progressively up-regulated during development, remaining highly expressed during adulthood with a limited (1.3-fold) but significant increase in expression as aging progresses ([Fig. 9A](#)). The EHF protein is selectively expressed within nuclei of the mouse cornea epithelium ([Fig. 9B](#)), and in the

cornea, *Ehf* transcripts are expressed at a greater than 2-fold higher level than in the other epithelial tissue we profiled ([Fig. 9C](#)).

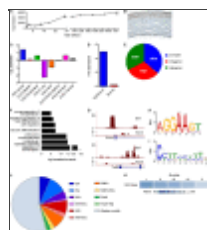


FIGURE 9.

ChIP-seq on mouse cornea epithelium identifies the Ets factor EHF as a potential regulator of cornea epithelial gene expression. *A*, expression of *Ehf* in mouse cornea over the lifetime of the mouse. *Ehf* belongs to supercluster A, which contains known cornea ...

The localization of *Ehf* in supercluster A with numerous cornea epithelial differentiation genes ([Fig. 5](#)) and its preferential expression in the epithelium ([Fig. 9D](#)) suggested that it is a potential regulator of cornea epithelial cell differentiation. To identify direct EHF targets, we performed ChIP-seq on cornea epithelium isolated from 3-month-old mouse corneas. At a false discovery rate of 5%, 15,384 EHF ChIP-seq peaks were called against the input control. Of these, 5570 peaks were found in the proximal promoter regions of genes, defined as a 3-kb window from 2 kb upstream to 1 kb downstream of transcription start sites ([Fig. 9E](#)). GO analysis of genes bound by EHF revealed a highly significant enrichment for transcriptional regulation ([Fig. 9F](#)); in particular, EHF bound the promoters of other Ets genes like *Ets1*, *Ets2*, and Ets2 repressor factor *Erf* (data not shown). Additional significantly enriched gene ontology categories included signaling, cell migration, and epithelium morphogenesis ([Fig. 9F](#)). The top enriched peaks include genes such as wound healing-related *Plaur* ([70](#)) and *Tcf4*, recently implicated in limbal stem cell maintenance ([71](#)) ([Fig. 9G](#)).

De novo motif searches on the 1000 most highly enriched EHF peaks identified, as expected, an enrichment of a motif similar to the core Ets motif ([Fig. 9H](#)). Intriguingly, a motif matching the binding site for KLF4, a transcription factor involved in cornea epithelial differentiation, was also highly enriched in these EHF ChIP-seq peaks ([Fig. 9I](#)), suggesting that there may be co-regulation of target genes and overlap in biological functions between these two transcription factors. This finding is further

supported by the observation that ~30% of genes affected by knock-out of *Klf4* in the cornea (72) have an EHF peak within 20 kb of their transcription start site (data not shown).

To determine what additional cofactor motifs might be present within the full data set of EHF ChIP-seq peaks, we performed directed motif searches using the Cistrome motif-based interval screener with position-specific scoring matrix. This search revealed significant enrichment of the EHF, ETS2, PAX6, KLF4, and KLF5 motifs within the EHF peaks when compared with negative control regions of identical size (Fig. 9J). The KLF5 motif was most highly enriched and was found in ~30% of EHF peaks. Interestingly, the Ets motif associated with ETS2 was more highly enriched within the EHF peaks than the published EHF motif. These two motifs are similar, sharing the core Ets sequence within their respective motifs, and both EHF and ETS2 fall into the same class in an analysis where Ets factors were grouped by the sequence similarity of their motifs (73). It is possible that EHF binding specificity may vary depending on the context of the binding and the cofactors with which it interacts. PAX6 was also enriched in a small but significant fraction of EHF peaks, suggesting that PAX6 and EHF may co-regulate a subset of their target genes. Furthermore, we found that the PAX6 and Ets motifs co-occurred within peaks, as did the KLF and Ets motifs. In contrast, there was no overlap between PAX6 and KLF motifs, supporting the idea that EHF interacts separately with PAX6 and KLF factors to regulate different subsets of gene targets. Similar to recent studies on other transcription factors (74, 75), we found a significant fraction of EHF peaks without the canonical EHF or Ets motifs. In these instances, EHF may be recruited through protein-protein interactions rather than binding the DNA directly, or under *in vivo* conditions, like in our experiment, EHF may bind to several different motifs in a context-dependent manner.

To assess the relationship between gene expression and EHF binding, we ranked the gene expression data from the isolated mouse cornea epithelium at P28 and divided it into quintiles based on expression levels. EHF peaks were most enriched in the most highly expressed quintile and least enriched in the quintile with the lowest expressed genes, consistent with a role for EHF in regulating cornea epithelial identity by increasing the expression of differentiation genes (Fig. 9K).

To identify genes regulated by EHF, we knocked down *EHF* (Fig. 10A) in primary human cornea epithelial cells and assessed global gene expression with microarrays. We found nearly 500

differentially regulated genes, 365 down-regulated and 132 up-regulated, suggesting that in the cornea epithelium, EHF acts predominantly as a transcriptional activator. Overlapping the EHF-regulated genes with the mouse superclusters ([Fig. 10B](#)), we observed that the genes belonging to the epithelial superclusters (A, C, and G) were predominantly down-regulated upon *EHF* knockdown, providing further evidence that EHF is primarily a transcriptional activator of cornea epithelium differentiation genes. Interestingly, genes up-regulated after *EHF* knockdown were overrepresented in certain stromal superclusters (B, E, and H), suggesting that EHF also suppresses expression of stromal genes in cornea epithelial cells. Consistent with these findings, GO analysis of down-regulated genes in response to *EHF* knockdown revealed an enrichment of ectoderm development/epithelial differentiation and response to wounding, including genes in the epidermal differentiation complex (cytoband 1q21–1q22) ([76](#)) ([Fig. 10C](#)). In contrast, GO analysis of up-regulated genes in response to *EHF* knockdown, identified angiogenesis and blood vessel morphogenesis, further supporting the notion that EHF represses genes that could compromise corneal clarity.



FIGURE 10.

EHF ChIP-seq, in combination with gene expression data from cornea epithelial cells lacking EHF, indicates that EHF is a regulator of cornea epithelial cell identity. A, mRNA levels of *EHF* in human cornea epithelial cells after siRNA knockdown (si*EHF* ...

To study the functional effects of EHF binding, we overlapped the genes affected by *EHF* knockdown with genes that have a linked EHF peak, defined as 20 kb upstream and throughout the gene's body. About 50% of genes affected by *EHF* knockdown have a linked EHF peak ([Fig. 10D](#)). These genes are enriched for epithelial differentiation-associated GO categories, including tissue morphogenesis, epithelial development, regulation of phosphorylation, and negative regulation of proliferation ([Fig. 10](#)

E). With respect to the quintiles of ranked epithelial gene expression, genes with an EHF peak that were down-regulated by the knockdown tended to fall into the highly expressed epithelial quintiles, whereas genes with an EHF peak up-regulated by the knockdown tended to fall into the lower expressed quintiles (Fig. 10F). This suggests a role for EHF in promoting epithelial identity, primarily through the activation of genes in cornea epithelium but also through the repression of non-epithelial genes.

To identify potential regulatory patterns, we determined where the ChIP-seq and siRNA data fell across the temporal expression clusters (Fig. 10G). Whereas the genes with ChIP-seq peaks in their proximity distributed fairly evenly across the clusters, when overlapped with the siRNA data, there was a significant difference in the number of peaks overlapping up-regulated *versus* down-regulated genes within the individual clusters. All of the strong epithelial superclusters (A, C, and F), have EHF peaks near genes down-regulated by *EHF* knockdown, suggesting that at these loci, EHF binding is strongly associated with gene activation. Stromal superclusters E and H and weak stromal supercluster D had EHF peaks near genes that are up-regulated by knockdown of *EHF*, suggesting repression of stromal or non-epithelial genes. The equally enriched superclusters I and J were mostly composed of genes not bound by EHF. These data further support the idea that EHF acts in the cornea epithelium to activate epithelial genes and repress non-epithelial genes, like those found in stroma-enriched clusters.

The specific genes that are down-regulated after *EHF* knockdown point to multiple possible roles of EHF in the epithelium. Several genes encoding keratin-cross-linking SPRR proteins are down-regulated, suggesting a role in barrier function (77). Alternatively, the down-regulation of anti-angiogenic SERPIN genes (78, 79) and the up-regulation of angiogenic genes in the epithelium points to a role for EHF in maintenance of corneal transparency through suppression of angiogenic factors. Another possible role of EHF is in immune response of the cornea. Genes in the TNF superfamily and the SAA family and *IL19* are all down-regulated. TNF genes are regulators of inflammation and apoptosis (79), whereas SAA proteins and *IL19* are both responsive to bacterial lipopolysaccharides (80, 81). In sum, our data suggest that EHF regulates cornea epithelial cell identity by activating differentiation genes and repressing non-epithelial genes.

DISCUSSION

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Our genome-wide cornea mRNA expression study is the first performed over the lifetime of the mouse, providing a detailed, continuous profile of whole mouse cornea transcript expression through embryonic and early postnatal development into aging. Previous work compared whole cornea mRNA expression in P9 and 6-week-old mice, identifying the most abundantly expressed transcripts, transcriptional regulators, and enriched gene groups in the developing and adult mouse cornea (11). Another study compared cornea stroma mRNA expression from P10 and adult mice with that of cultured keratocytes, fibroblasts, and myofibroblasts, revealing a role for the stroma in maintenance of transparency as well as in barrier protection during times of infection or injury (10). A third study compared whole cornea mRNA expression in P10 and 7-week-old mice with that of lens and tendon, defining cornea-enriched genes compared with the lens and tendon (9). The comprehensive picture of cornea gene expression in our study provides new insights into both cornea development and aging, helps define the transition point between development and adulthood, and identifies new pathways and genes involved in development and aging. In particular, we have identified the Ets factor EHF as a promoter of cornea epithelial cell identity.

A Cornea Gene Expression Switch at the Juncture of Development and Adulthood

The timing for the completion of cornea development remains controversial. Based on morphology, early electron microscopic studies argued that development is completed at eye opening (P14) (8, 82). In contrast, an *in vivo* confocal microscopy study reported that epithelial and stromal development is concluded by P30 (83), whereas another study reported that the cornea did not fully mature until 2 months of age (84). A global comparison of the gene expression profiles in our study through PCA argues that cornea development ends between P14 and P28 (Fig. 1D). This conclusion is also supported by the underlying structure in our data set, where over 1000 genes show a prominent switch in expression between P14 and P28 (Fig. 2B). Among these genes, we identified a group of myofibril assembly transcripts that are expressed in development and not aging. Although actins and myosins have been studied in the cornea, where they were found to play roles in cell migration, regulation of cell shape, and wound healing (85–87), we have identified several transcripts in this group with unknown roles in the cornea, including actins $\alpha 1$ (*Acta1*) and $\gamma 1$ (*Actg1*) and myosins heavy chain 10 (*Myh10*) and light chain 2 (*Myl2*) (Fig. 2C), which were previously linked to cardiac and/or

skeletal muscle; MYH10 was recently shown to coordinate collagen translation in mouse embryonic fibroblasts (88). These findings suggest that these genes associated with striated muscle may play additional roles in the developing cornea, potentially in collagen fibril organization and/or epithelial cell migration.

Gene Expression in Corneal Development Previous studies have demonstrated that co-expression of genes is often a characteristic of co-regulated genes (89, 90), particularly among genes that are highly expressed and/or co-regulated by multiple transcription factors. Thus, the identification of a set of temporal gene expression clusters in development (Fig. 3) allows us to make inferences about potential transcriptional relationships in the cornea that could not have been discovered with previous cornea microarray studies. The inclusion of expression data from separated epithelium, separated stroma, and other epithelial tissues further refined the data. Validating this approach, we observed that known target genes of the transcription factors KLF4 and KLF5 (58, 59, 72) were located within the same supercluster as *Klf4* and *Klf5*. Our clustering approach allowed us to gain insights into the previously published gene expression data for mice deleted for *Klf4* and *Klf5* in the cornea. Incorporating information about cluster membership into the list of genes affected by the *Klf4* and *Klf5* deletions demonstrates that these factors primarily activate genes in epithelial enriched clusters, whereas many of the genes up-regulated in response to gene deletion reflect the previously described secondary changes in the stroma.

The data are also useful for gaining insights into cornea stroma development. Thus, we found that *Twist2*, which has a known role in stroma development (64), belongs to stroma-enriched supercluster B. Interestingly, the family member *Twist1*, which has an unknown function in cornea, also falls into supercluster B, raising the possibility that it also is involved in stroma development, perhaps jointly with *Twist2* (91).

Aging in the Cornea Our analysis of aging in the cornea identified 608 differentially regulated transcripts representing 2.8% of the transcriptome. The gene expression changes during cornea aging are modest compared with development, consistent with reports on other aging tissues (22). Aging genes grouped into broad clusters by overall trends of down-regulation or up-regulation (Fig. 4A). The enrichment of genes within each cluster captured several different pathways commonly regulated in aging, such as lipid biosynthetic process, cell adhesion and TGF β signaling, blood vessel development, electron transport and oxidative

phosphorylation, and protein translation. The GO analysis also identified categories that correspond to the morphological changes observed in the cornea, such as an increase in epithelial cell differentiation and a decrease in cell adhesion and cell motion. We also discovered that of these aging-regulated genes, a disproportionate number are stroma-enriched. This may relate to the quiescent state of keratocytes (4) as opposed to the constant cell renewal in the epithelium.

We also identified three cornea- and cornea epithelium-enriched genes in the aging data set, *Slurp1*, *1600014C10Rik*, and *Ehf*. SLURP1, which plays an immunomodulatory role in the cornea epithelium, suppressing neutrophil infiltration, is down-regulated in proinflammatory conditions of the cornea (67). The down-regulation of *Slurp1* in aging may suggest decreased immune privilege in aging but also suggests potentially a quicker response to injury. The human ortholog of *1600014C10Rik*, *C19orf12*, encodes a small mitochondrially localized protein that may play a role in lipid homeostasis (92). Mutations in this gene are associated with some cases of neurodegeneration with iron accumulation in the brain (92). In the adult cornea, *1600014C10Rik* is moderately expressed and down-regulated to an unusual extent (−7.41-fold) in aging, suggesting that it may play a role in decreased mitochondrial function in the cornea, potentially leading to increased oxidative stress and increased cellular damage. The *Ehf* gene, which is up-regulated during aging, promotes cornea epithelial differentiation (see below); EHF may contribute to the increased epithelial differentiation in the aging cornea.

Our findings on gene expression during cornea aging support some of the general principles derived by previous meta-analyses of aging data sets (22, 93). First, relatively few genes change in aging compared with development. Second, the changes across different tissues are relatively unique, although functionally similar tissues do show more similarity than functionally distinct tissues. Finally, although there are relatively few specific genes that change across many tissues, there are several pathways that are commonly age-regulated. However, although we did find individual genes belonging to the following categories, we did not find enrichment for immune response-, inflammation-, or cell cycle-related genes, processes shown to be common across numerous aging organs (93). The lack of differential regulation of genes in these specific categories is informative because it highlights ways that the cornea may be distinct from other tissues. In particular, because one of the primary functions of the cornea is to maintain transparency for

vision, the regulation of immune response- and inflammation-related genes must be tightly controlled to prevent changes that disrupt vision.

EHF as a Regulator of Epithelial Cell Fate in the Cornea EHF (Ets homologous factor), a member of the ESE (epithelial specific expression) Ets transcription factor family (94), caught our interest because 1) it is expressed relatively highly in cornea compared with several other epithelial tissues; 2) it is selectively expressed in the epithelium of the cornea; 3) its temporal expression pattern during cornea development correlates highly with the expression of known cornea differentiation genes in supercluster A, including that of *Aldh3a1* and *Krt12*; and 4) its expression increases during aging, correlating with aging-associated differentiation. Previous studies found that EHF expression is enriched in epithelial tissues (95), particularly glandular epithelia, where it binds targets related to late stage differentiation (96). It has been shown to be highly expressed in cornea (97), conjunctiva (53), and prostate endocrine cells (98), although the roles of EHF in these tissues are yet to be described.

Through siRNA knockdown of *EHF* in cornea epithelial cells, we discovered that epithelium-related genes and genes in the epidermal differentiation complex were down-regulated. These data support our hypothesis that EHF is involved in the epithelial differentiation program of the cornea and suggest that it is primarily a transcriptional activator of these genes. We also found that non-epithelial genes were up-regulated and mapped to stromal superclusters, suggesting that EHF is also a repressor of non-epithelial/stromal genes within cornea epithelial cells.

We performed *in vivo* ChIP-seq of separated cornea epithelium and compared it with the siRNA data to identify genes both bound and regulated by EHF, thus further elucidating the role of EHF in the cornea epithelium. Multiple genes involved in immunity-related functions are differentially regulated, such as *CXCL14* (2.65-fold), *SGPP2* (-2.12-fold), and *PLAUR* (-1.38-fold). *CXCL14*, a chemokine shown to be up-regulated in epidermal cell differentiation (99), may play a role in immune function of differentiated cornea epithelium. However, it is also known to play a role in cell migration and angiogenesis, and its up-regulation after *EHF* knockdown points to a role of EHF in suppressing this function. *SGPP2* is involved in lipid metabolism and may be induced during the inflammatory response (100). The regulation of these genes suggests a role for EHF in regulating the immune response of the differentiated cornea epithelium. The cornea is

both highly innervated and populated by antigen-presenting cells (101), and EHF may be involved in regulating signaling to these cells. Other interesting EHF-regulated genes that we identified with the combined ChIP-Seq/siRNA experiment include *CCNA1* (2.24-fold), *FGFR3* (−1.9-fold), and *TCF4* (1.36-fold). *FGFR3* plays a role in negative regulation of epithelial proliferation (102) and positive regulation of epithelial cell commitment (103, 104), whereas *CCNA1* plays a role in regulation of the cell cycle (104). *TCF4* is associated with proliferation in limbal epithelial stem cells and is not expressed in more differentiated cornea epithelial cells (71). Together, our data suggest that EHF modulates epithelial cell fate through repression of proliferation and non-epithelial genes and activation of epithelial differentiation and immunity-related genes.

The discovery of a role for EHF in the cornea epithelium provides an example of the utility of the cornea transcriptome profile. Through the time course data, we gained insights into the potential role of EHF and its target genes, which we verified through ChIP and knockdown studies. Interestingly, we found predicted *KLF4* and *KLF5* binding sites within a significant portion of the EHF ChIP-seq peaks. Furthermore, we found that 30% of the genes deregulated after either *Klf4* or *Klf5* gene deletion in the cornea contain EHF peaks in their close proximity. Together, these data suggest the possibility that EHF collaborates with *KLF4* and *KLF5* to specify cornea epithelial cell identity.

Acknowledgments

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This article contains [supplemental Tables S1–S3](#).

²The abbreviations used are:

En embryonic day *n*
Pn postnatal day *n*
GO gene ontology
PCA principal component analysis
ChIP-seq ChIP sequencing.

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