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Identification of novel molecular markers through transcriptomic analysis in human fetal and adult corneal endothelial cells

Yinyin Chen[1,2,3], Kevin Huang[3], Martin N. Nakatsu[4], Zhigang Xue[1,*], Sophie X. Deng[4,*] and Guoping Fan[1,3,*]

[1]Translational Center for Stem Cell Research, Tongji Hospital, Department of Regenerative Medicine, Tongji University School of Medicine, Shanghai, China [2]School of Life Science and Technology, Tongji University, Shanghai 200092, China, [3]Department of Human Genetics, David Geffen School of Medicine and [4]Cornea Division, Jules Stein Eye Institute, University of California Los Angeles, Los Angeles, CA 90095, USA

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The corneal endothelium is composed of a monolayer of corneal endothelial cells (CECs), which is essential for maintaining corneal transparency. To better characterize CECs in different developmental stages, we profiled mRNA transcriptomes in human fetal and adult corneal endothelium with the goal to identify novel molecular markers in these cells. By comparing CECs with 12 other tissue types, we identified 245 and 284 signature genes that are highly expressed in fetal and adult CECs, respectively. Functionally, these genes are enriched in pathways characteristic of CECs, including inorganic anion transmembrane transporter, extracellular matrix structural constituent and cyclin-dependent protein kinase inhibitor activity. Importantly, several of these genes are disease target genes in hereditary corneal dystrophies, consistent with their functional significance in CEC physiology. We also identified stage-specific markers associated with CEC development, such as specific members in the transforming growth factor beta and Wnt signaling pathways only expressed in fetal, but not in adult CECs. Lastly, by the immunohistochemistry of ocular tissues, we demonstrated the unique protein localization for Wnt5a, S100A4, S100A6 and IER3, the four novel markers for fetal and adult CECs. The identification of a new panel of stage-specific markers for CECs would be very useful for characterizing CECs derived from stem cells or *ex vivo* expansion for cell replacement therapy.
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INTRODUCTION

Corneal endothelial cells (CECs) are a monolayer of endothelial cells lining the Descemet's membrane of the cornea. CECs exhibit a typical hexagonal shape and form tight junctions with the neighboring cells. The main function of CECs is to serve as a water pump via Na^{+}–K^{+}-ATPase, which actively transports bicarbonate ion from the stroma into the anterior chamber. The active bicarbonate ion flux provides the driving force for pumping the corneal fluid out of the stroma, thus keeping the stroma in a constant state of hydration to achieve corneal transparency. Adult CECs have limited capacity for cell proliferation *in vivo* as the cell cycle is arrested in the G1 phase (1), and CEC deficiency due to genetic diseases, trauma and aging would lead to irreversible corneal edema, opacity and eventual blindness (2).

CEC genetic disorders include Fuchs endothelial corneal dystrophy (FECD), posterior polymorphous corneal dystrophy (PPCD), congenital hereditary endothelial dystrophy type 1 and 2 (CHED1 and 2) and X-linked endothelial corneal dystrophy (3). The genetic defects underlying some of these hereditary diseases have been recently reported. For example, mutations in Collagen type VIII A2 (*COL8A2*), a major component of the Descemet's membrane secreted by CECs, are

*To whom correspondence should be addressed. Email: gfan@mednet.ucla.edu (G.F.); deng@jsei.ucla.edu (S.X.D.), and xuezhigang75@gmail.com (Z.X.)

associated with either early-onset FECD or PPCD, whereas mutations in the solute carrier family 4 member A11 (*SLC4A11*) gene are related to late-onset FECD and CHED2 (4). Nevertheless, many disease target genes remain to be discovered.

Clinically, besides entire corneal transplantation, surgical procedures such as endokeratoplasty or Descemet's stripping endothelial keratoplasty is developed to replace dysfunctional CECs with healthy corneal endothelium (5). However, due to the global shortage of tissue donors and limited transplant-grade cornea tissues, new methods to generate functional CECs need to be established. Recent advancements in stem cell biology have opened new avenues to expand adult or fetal CECs *ex vivo* (6) or derive CECs from neural crest cells (7), or even with human somatic stem cells such as corneal stroma stem cells, mesenchymal stem cells and bone marrow-derived endothelial progenitor cells (8–10). Theoretically, *in vitro* differentiation of CECs from pluripotent stem cells such as human embryonic stem cells (hESCs) or induced pluripotent stem cells would be a very feasible approach to provide unlimited source of CECs for cell replacement therapy.

Several protein markers have been used to identify adult CECs, most related to cell adhesion, gap junction, tight junctions and pump function such as N-cadherin, connexin-43 (11), ZO-1 (12), Na^+–K^+-ATPase (13) and Occludin (14). However, molecular markers unique to fetal and adult CECs and molecular pathways associated with maturation of CECs are not well studied. We therefore took a comprehensive approach to profile mRNA transcriptomes in both fetal and adult human CECs (HCECs) by RNA-sequencing (RNA-seq) and examine unique molecular markers in fetal and adult CECs. Bioinformatics analysis identified major gene expression changes between fetal and adult CECs in cell metabolism, cell adhesion and the transforming growth factor beta (TGF-β) signaling pathway. By further comparing CEC gene expression profiles with 12 other cell types, we identified 245 and 284 tissue-specific genes in fetal and adult CECs, respectively. In fact, several endothelial dystrophy genes are highly expressed in adult CECs, consistent with their implicated role in CEC physiology. Significantly, we validated a portion of the fetal and adult-specific markers using immunohistochemistry, demonstrating that our data analysis is a valuable resource for identifying protein biomarkers unique to fetal and adult CECs.

RESULTS

Tissue-specific gene expression in fetal and adult CECs

Because markers for either fetal or adult CEC are not well studied, we performed RNA-Seq in three adult and two fetal CEC tissues and produced ~179 million uniquely mapped reads. To identify genes that were specific to either fetal or adult CEC, we procured 97 samples from the public domain representing 12 different types of cells and tissues (Supplementary Material, Table S2). For analytical reasons, we only selected RNA-Seq datasets generated by the Illumina system to prevent potential platform biases.

Hierarchal clustering revealed that fetal CECs cluster closer to relatively immature cell types, such as hESCs, neural progenitor cells (NPCs) and human umbilical vein endothelial cells (HUVECs; Fig. 1A). In contrast, adult CECs clustered away from these early stage cells and grouped together with other terminally differentiated somatic cell types. By principal component analysis (Fig. 1B), we found that adult CECs are positioned closer to adult brain samples, consistent with the fact that CECs are derived from neural crest cells that share the same cell origin of the neural tube. Overall, identical cell types clustered tightly together, suggesting that each cell type exhibits well-defined transcriptional patterns. In particular, adult and fetal CEC clustered closely together compared with other cell types, indicating that CECs also have a unique transcriptional profile.

To gain insight into the exact molecular differences between adult and fetal CECs from other cell types, we performed statistical analysis (one-way analysis of variance, or ANOVA) and identified genes that were specifically overexpressed in either adult or fetal CECs. Using the criteria of ANOVA Tukey–Kramer multiple comparison corrected P-values <0.01 and an additional cutoff of at least 2-fold greater expression compared with other 12 tissues, we identified a total of 284 (Fig. 2A) and 245 genes (Fig. 2B) that are specifically enriched in adult and fetal CECs, respectively. Gene ontology (GO) analysis revealed that the 284 up-regulated genes in adult CECs mainly participate in inorganic anion transmembrane transporter activity, transcription factor activity and cyclin-dependent protein kinase inhibitor activity (Fig. 3A), reflecting the unique features of mature CECs. Genes unique to fetal CECs are involved in extracellular matrix structural constituent, platelet-derived growth factor binding and growth factor activity (Fig. 3B). These results probably reflect the specific functional traits of fetal CECs for growth factor contribution during fetal CEC development.

We next took a systems approach and asked whether adult or fetal CECs express a unique transcriptional network (or module) of genes. By weighted gene co-expression network analysis (WGCNA), we identified single modules that represent adult ($n = 300$) and fetal ($n = 343$) CECs transcriptional networks (Supplementary Material, Figs S1 and S2). When we overlapped this result with our single-gene statistical analysis, we found fairly good concordance with 70% overlap with adult ($n = 209$) and 44% in fetal ($n = 160$) CECs (Supplementary Material, Table S1). Taken together, our multi-approach analysis suggests that the unique gene expression profiles in fetal and adult CECs are highly robust.

WGCNA can also lead to a natural measure of gene similarity (or membership) to a particular module. Genes with high membership in a module are generally regarded as hub genes, and tend to play central roles in the network. Interestingly, in adult CECs, we identified *SLC4A11* as one of the top-ranking hub genes. *SLC4A11* has been linked with hereditary corneal dystrophies (15,16) and knockdown of *SLC4A11* in cultured CECs has been shown to induce apoptosis (17). Thus, our network analysis helped to refine and rank the set of key players in the adult or fetal CEC networks. We provided a ranked list of adult and fetal CEC stage-specific hub genes (Supplementary Material, Table S1). Together, our analysis has uncovered a clear transcriptional signature in adult

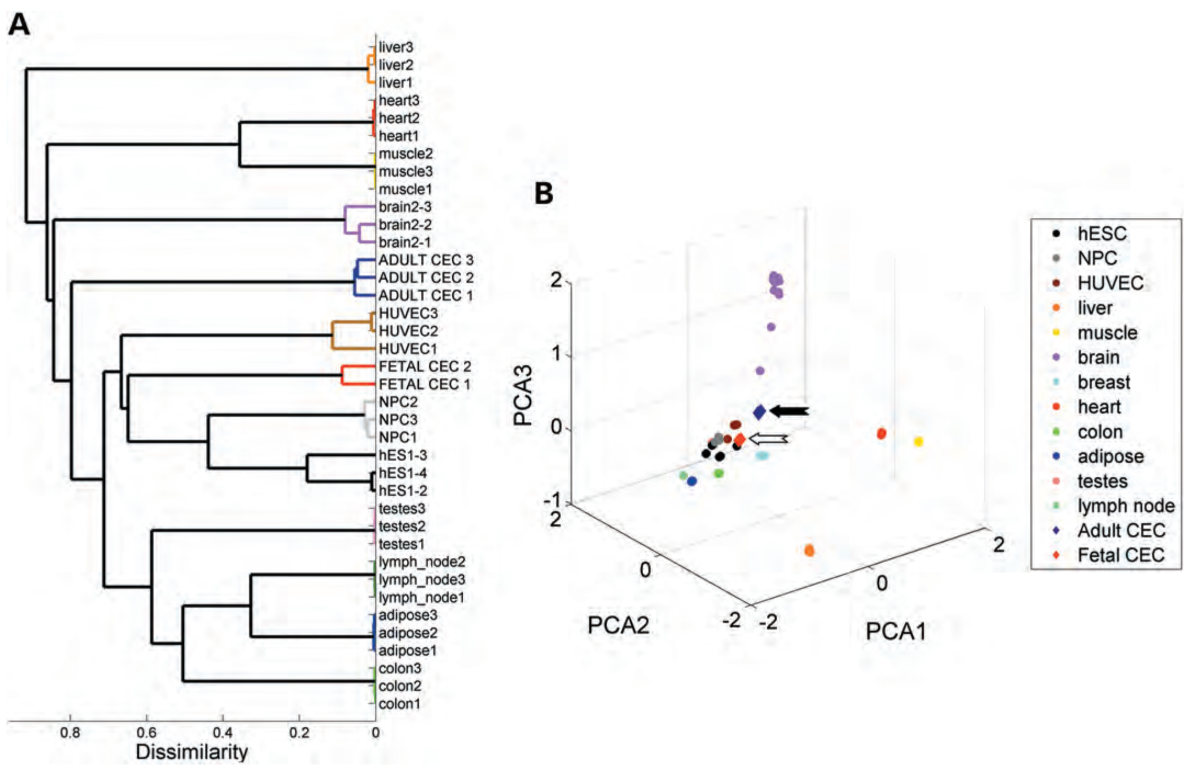

Figure 1. Adult and fetal CECs exhibit distinct global mRNA expression patterns. (**A**) Unbiased hierarchical clustering shows fetal and adult CEC relationship with representative samples from other tissues and cell types. (**B**) Three-dimensional scatterplot of the first three principal components (PCA1, PCA2 and PCA3) demonstrate that fetal samples cluster to hESCs, NPCs and HUVECs, and adult CEC cluster closer to brain samples. Ninety-seven samples from 12 different tissue and cell types were downloaded from the GEO database. Solid and open arrows point out adult and fetal CECs.

and fetal CECs that are not present in other cell and tissue types examined, including other types of endothelial cells.

Differential expression pattern between adult and fetal CECs

We next directly compared adult and fetal CECs and identified 688 and 1763 genes that are expressed by more than 2-fold [false discovery rate (FDR) $< 5\%$] in adult and fetal CECs, respectively. To increase the stringency and identify the most differentially expressed genes between adult and fetal CECs, we used a 5-fold change cutoff, and by this criterion, we identified 518 and 668 genes that are highly expressed in adult and fetal CECs, respectively. Remarkably, functional annotation of the differentially expressed genes suggests distinct functional differences between adult and fetal CECs. For example, GO and kyoto encyclopedia of genes and genomes (KEGG) pathway analysis indicated that many genes involved in metabolic processes are up-regulated in adult CECs (Fig. 4A and Table 1), whereas fetal CECs showed GO terms enriched in metallopeptidase activity, extracellular structure and TGF-β signaling (Fig. 4B and Table 2). Interestingly, calcium ion binding proteins such as S100 family members (S100A4, S100A5 and S100A6) are expressed higher in adult CECs. Collectively, our analysis revealed clear differences between adult and fetal CECs, indicating that CECs acquire transcriptional changes in select pathways during developmental maturation.

Stage-specific marker genes in adult and fetal CECs are confirmed at the protein level

Although we have identified many putative molecular markers to distinguish adult and fetal CECs on the RNA level, it is still unclear whether these markers can be confirmed at the protein level. We therefore collected fetal and adult corneal tissues for immunohistochemistry analysis. As expected, immunostaining analysis showed expression of tight junction protein ZO-1 in both fetal (17 weeks in gestation) and adult CECs ($>$37 years old; Supplementary Material, Fig. S3A and B). Na^+–K^+-ATPase, which is one of the most important markers for the pump function in CECs, is highly expressed in adult CECs but not in fetal CECs (Supplementary Material, Fig. S3C and D), consistent with the observations in our RNA-Seq analysis (Supplementary Material, Table S3). This result suggests that pump function is not yet fully established in 17-week-old fetal CECs.

We next examined components of the Wnt signaling pathways since it is known to be an important regulator of ocular tissue development (18,19). Indeed, genes involved in Wnt signaling are highly enriched in fetal CECs (Supplementary Material, Table S1). Our immunocytochemistry experiment showed that Wnt intracellular signaling molecules such

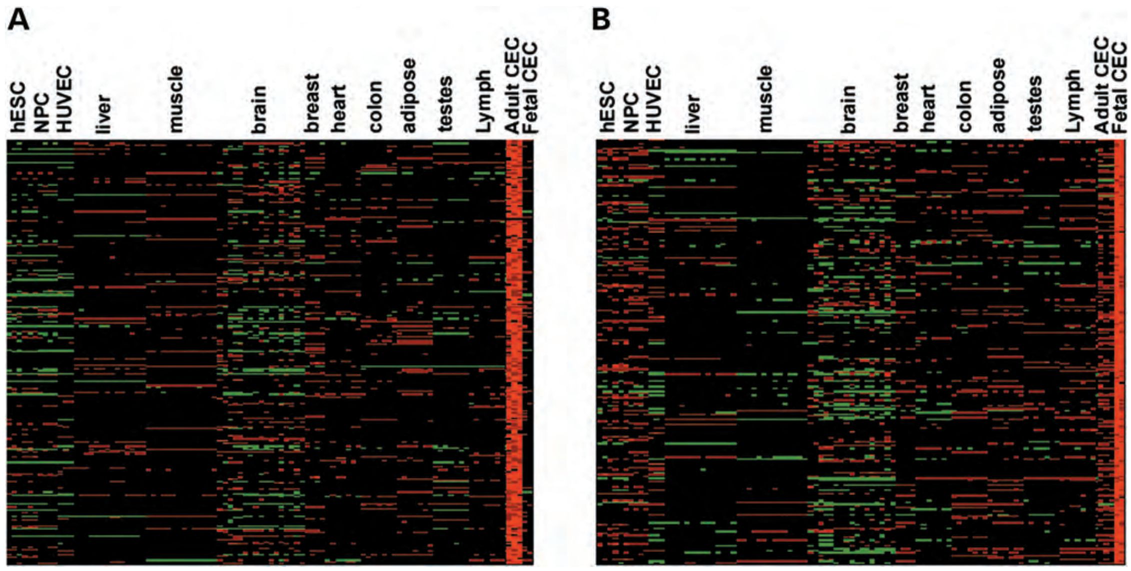

Figure 2. Heatmap analysis of the up-regulated gene in adult and fetal CECs. Heatmap of relative expression levels for genes that are highly expressed in (**A**) adult or (**B**) fetal CECs.

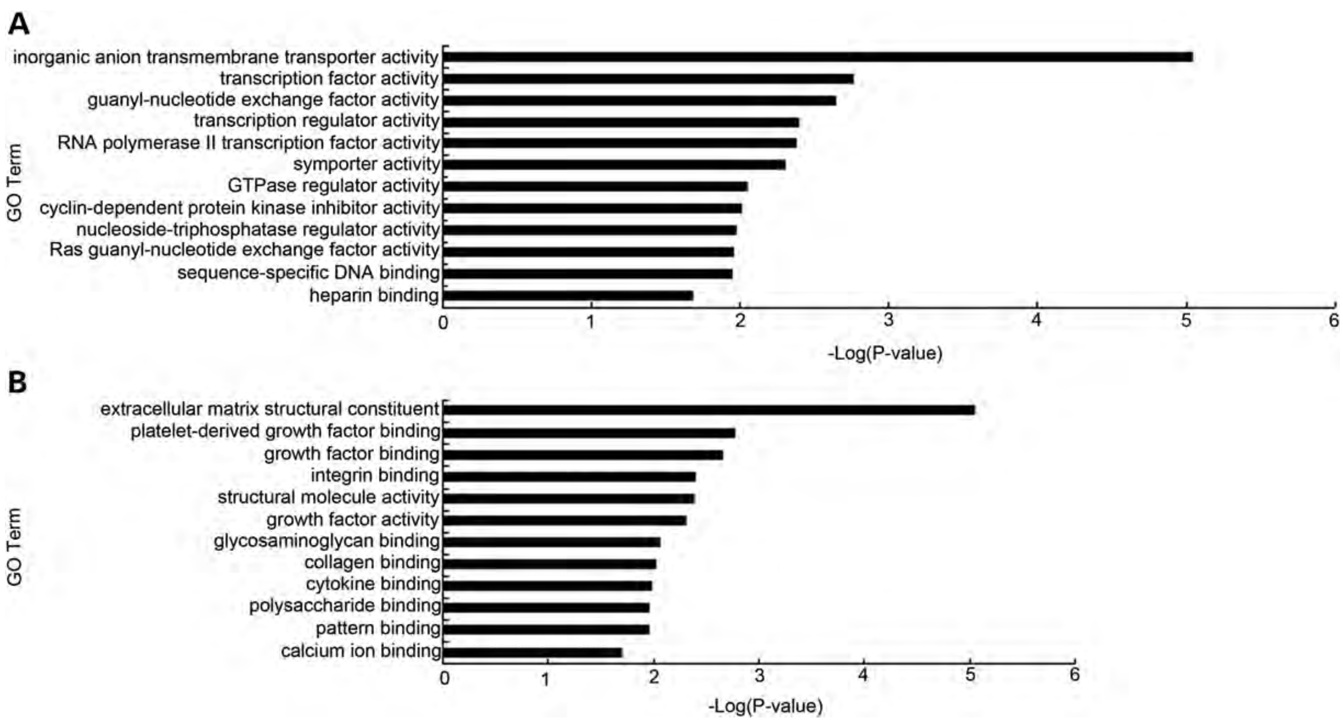

Figure 3. GO analysis of CEC tissue-specific signature genes. Bar graphs showing the significance of enrichment terms for a set of uniquely expressed genes in (**A**) adult CECs and (**B**) fetal CECs compared with 12 tissue and cell types. *P*-values <0.05.

as axin2 (Supplementary Material, Fig. S3E and F) and beta-catenin (Supplementary Material, Fig. S3G and H) are readily detected in both fetal and adult CECs. However, we found that Wnt5a is only expressed in fetal, but not adult CECs (Fig. 5A and B), suggesting that, at the protein level, the Wnt5a pathway is only functional in fetal CECs.

The S100 protein family consists of several calcium-binding proteins and have been shown to regulate a variety of cellular processes (20). We found that mRNAs in sub-members of the S100A protein family (S100A4, S100A5 and S100A6) are highly expressed in adult, but not fetal CECs (Supplementary Material, Table S1). Our immunohistochemistry confirmed that S100A4 and S100A6 proteins were readily detected in adult CECs (Fig. 5C and E) or fetal cornea epithelium (Supplementary Material, Fig. S4), but not in fetal cornea endothelium (Fig. 5D and F). Therefore, S100A4 and S100A6

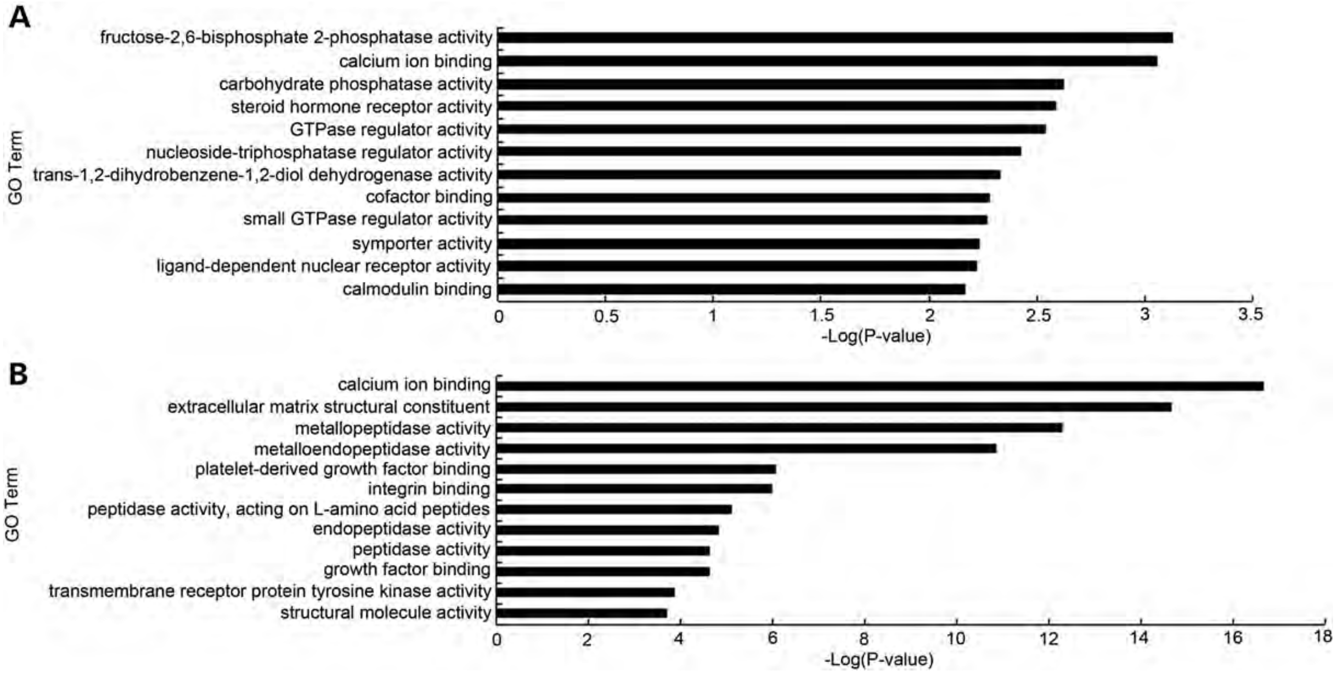

Figure 4. GO analysis of CEC stage-specific signature genes. Bar graphs showing the significance of enrichment terms for a set of higher expressed genes in (**A**) adult over fetal CECs and (**B**) fetal over adult CECs. *P*-values <0.05.

Table 1. Analysis of KEGG pathway via DAVID software for 518 adult stage-specific CECs compared with fetal CECs

Term	Count	Genes	*P*-value
Glycolysis/ Gluconeogenesis	10	ALDOA, GPI, LDHA, ALDOC, PKM2, ENO2, FBP1, PGK1, ALDH3B1, ENO1	1.96E−04
Arginine and proline metabolism	8	GLS2, SAT1, GLUL, GOT1, ACY1, GATM, NAGS, NOS3	0.002188
Fructose and mannose metabolism	6	ALDOA, PFKFB4, AKR1B10, PFKFB2, ALDOC, FBP1	0.005918
Pentose phosphate pathway	5	ALDOA, GPI, ALDOC, FBP1, RBKS	0.010124
p53 signaling pathway	7	STEAP3,TNFRSF10B, CCND3,ZMAT3,CYCS, GADD45G, RRM2B	0.030056
Nitrogen metabolism	4	GLS2, CTH, GLUL, CA3	0.044035

P-value <0.05.

calcium-binding proteins are two additional novel markers for distinguishing mature adult versus immature fetal CECs.

We also examined cellular localization for proteins that are highly expressed in both adult and fetal CECs. Significantly, we found novel localizations of intracellular proteins such as a stress-induced protein Immediate Early Response 3 (IER3) during CEC maturation. Our immunostaining experiment demonstrated that IER3 can be readily detected in both fetal and adult CECs, but with a stark contrast in subcellular localization. IER3 localizes in the cytoplasm of adult CECs, whereas it is found in the nucleus of fetal CECs. Thus, IER3 is another useful protein marker to characterize the maturation of CECs in development (Fig. 5G and H).

DISCUSSION

CECs are critical for maintaining corneal transparency through regulating stromal hydration. Although previous studies have examined select CEC gene expression via RT–PCR, no information is available concerning the entire transcriptome of either fetal or adult CECs. In this study, we conducted a comprehensive mRNA-Seq analysis of fetal and adult CECs, uncovering both tissue- and stage-specific gene transcripts. These molecular signatures of fetal and adult CECs would be very useful to develop biomarkers to characterize different stages of CECs both *in vivo* and *in vitro*.

Here, we have found that genes highly expressed in adult CECs are tightly associated with special CEC functions. For example, the barrier function of CECs is set up by tight junction, which is well correlated with a high level of ZO-1 and N-cadherin mRNAs in adult CECs. Similarly, AQP1, Na^+–K^+-ATPase, $Na^+/HCO3^-$ and other genes relevant to pump function of mature CECs are detected at a higher level in adult CECs than in fetal CECs. Adult CECs are in a quiescent state with very limited proliferation ability. Consistently, in our analysis, we found that Ki67 and cyclinD2 are dramatically down-regulated in adult CECs compared with fetal CECs.

It has been postulated that both Wnt and TGF-β pathways play a role in CEC development. The Wnt pathways can be separated into two different pathways: canonical frizzle receptor/beta-catenin-dependent T cell factor pathway and non-canonical Ryk receptor/Jun NH2-terminal kinase and calcium/calmodulin-dependent kinase pathways. These two

Table 2. Analysis of KEGG pathway via DAVID software for fetal CEC stage-specific gene expression

Term	Count	Genes	*P*-value
ECM-receptor interaction	22	COL4A2, COL4A1, COL3A1, COL2A1, COL5A, COL5A1, SDC3, LAMA2, GP5, LAMA4, ITGA5, TGA8, ITGA7, COL6A3, COL1A2, COL6A2, COL6A1, TNN, COL1A1, THBS2, COL11A1, FN1	1.63E−12
Axon guidance	22	PLXNC1, PLXNA1, GNAI1, EFNB1, EFNB2, EPHB3, CXCL12, EPHA3, SLIT3, EPHB2, SEMA5B, EPHB6, SEMA6B, EPHA6, CXCR4, ROBO1, FYN, SEMA3F, SEMA3D, ROBO2, SEMA3A, SRGAP1	8.34E − 09
Focal adhesion	26	COL3A1, COL2A1, BCL2, COL6A3, COL6A2, COL6A1, TNN, PIK3R3, THBS2, COL11A1, FN, COL4A2, COL4A1, IGF1, FLNC, COL5A2, COL5A1, LAMA2, LAMA4, ITGA5, FYN, CCND2 ITGA8, ITGA7, COL1A2, COL1A1	8.22E−08
Arrhythmogenic right ventricular cardiomyopathy	10	LAMA2, SLC8A1, CACNA2D1, PKP2, ITGA5, ITGA8, ITGA7, LEF1, CACNG4, SGCD	0.001988
Hypertrophic cardiomyopathy	10	LAMA2, SLC8A1, CACNA2D1, ITGA5, ITGA8, ITGA7, IGF1, CACNG4, SGCD, TGFB2	0.004299
Pathways in cancer	23	FGF18, RET, AR, COL4A2, BMP2, COL4A1, LEF1, KITLG, IGF1, CDK6, FGF12, GLI2, MMP2, FZD7, TGFB2, LAMA2, SMO, FZD10, CBLB, LAMA4, BCL2, PIK3R3, FN1	0.005296
Dilated cardiomyopathy	10	LAMA2, SLC8A1, CACNA2D1, ITGA5, ITGA8, ITGA7, IGF1, CACNG4, SGCD, TGFB2	0.007243
Cell adhesion molecules	12	NCAM2, CADM3, CLDN19, CD34, ITGA8, CD276, NLGN1, NLGN3, CDH3, JAM2, SDC3, CLDN15	0.010309
Small cell lung cancer	8	LAMA2, LAMA4, COL4A2, COL4A1, BCL2, CDK6, PIK3R3, FN1	0.037463
TGF-beta signaling pathway	8	BMP2, LTBP1, ACVRL1, LEFTY2, DCN, THBS2, CHRD, TGFB2	0.044046
Gap junction	8	TUBB2B, GNAI1, TUBB6, LPAR1, PLCB1, ITPR3, TUBA1A, TUBB4	0.048826

P-value <0.05.

pathways are targeted by different Wnt molecules such as Wnt1, Wnt3a and Wnt8 for the canonical pathway, whereas Wnt5a, Wnt4 and Wnt11 for the non-canonical pathway (21). Of note, we found that 27 genes in the Wnt pathway are highly expressed in fetal CECs (Supplementary Material, Tables S1 and S4), particularly for the non-canonical Wnt pathway genes such as Wnt5a and Wnt4, raising the possibility that the non-canonical Wnt pathway may be important for CEC development. Similarly, eight TGF-β signaling pathway members (TUBB2B, GNAI1, TUBB6, LPAR1, PLCB1, ITPR3, TUBA1A and TUBB4) are detected at a much higher level in fetal CECs than in adult CECs. TGF-β family genes are involved in regulating cell differentiation, proliferation and extracellular matrix production in ocular tissue (22). In fact, TGF-β2 null mice exhibit an absence of endothelium and fusion of the lens with the cornea (23). In coupling with high levels of TGF-β signaling molecules, we also observed enrichment of genes in the extracellular matrix structural constituent in fetal CECs. Overall, our stage-specific gene expression analysis supports the notion that both Wnt and TGF-β pathways are important for the differentiation and maturation of fetal CECs into adult CECs.

Recently, derivation of CEC-like cells *in vitro* from progenitor cells or somatic stem cells has been investigated for transplantation treatment of CEC deficiency. Hatou *et al.* (8) isolated cornea-derived precursors from the adult corneal stroma to make tissue-engineered corneal endothelium. Joyce *et al.* (9) investigate the potential of umbilical cord blood mesenchymal stem cells to become HCECs. Recently, Ju *et al.* (7) tested the feasibility of differentiating neural crest cells into functional corneal endothelial cells. In all these studies, the markers used for characterizing CEC-like cells were limited to ZO-1, Na^+–K^+-ATPase and N-cadherin. Our demonstration of four novel protein markers including S100A4, S100A6, Wnt5a and IER3 in this study will certainly aid the characterization of CEC-like cells from the above studies. S00A4 is one of S100 family genes encoding calcium-binding proteins involved in cell morphogenesis in multiple cell lineages (24). S100A6 was previously shown in the plasma membrane of corneal and limbal epithelial cells (25). It has been debated whether S100 expression is specific in human corneal endothelium (26,27). In the present study, we found that S100A4 and S100A6 are detectable in adult CECs as well as in adult and fetal corneal epithelium, but not in fetal CECs. Thus, S100A4 or S100A6 should be considered as a mature marker for adult CECs. IER3 is a stress-induced protein (28) encoded by radiation-inducible immediate-early gene IEX-1, previously detected as a potential target gene in human cancer, inflammatory diseases or hypertension (29,30). In keratinocytes, it is observed that IER3 can translocate from nucleus to cytoplasm in response to 1alpha,25-dihydroxyvitamin D3 for reduced cell growth (31). Consistently, our observed IER3 translocation from the nucleus in fetal CECs to the cytoplasm in adult CECs may also be associated with reduced cell proliferation and growth in adult CECs. Taken together, our newly identified CEC protein markers as well as stage-specific CEC mRNAs would be very useful to characterize and quality-control a variety of CEC-like cells derived from either *ex vivo* expansion of corneal progenitors or *in vitro* differentiation of somatic and pluripotent stem cells.

Lastly, corneal endothelial dystrophies are a set of hereditary corneal diseases characterized by slowly progressive edema of the cornea in different modes, each associated with different target genes. We noted that several of our newly identified CEC-tissue-specific genes are also corneal disease target genes. For example, *COL8A2* and *SLC4A11*, two major PPCD-related genes (32), are expressed at an extremely high level in both fetal and adult CECs. Similarly, *CDKN1A* exhibits a much higher expression level in adult CECs than fetal CECs. *CDKN1A* is an important marker of cellular senescence (33) and mediates part of the cellular stress response in the pathogenesis of both early and late-onset FECD (34). Our current study identifies over two hundred

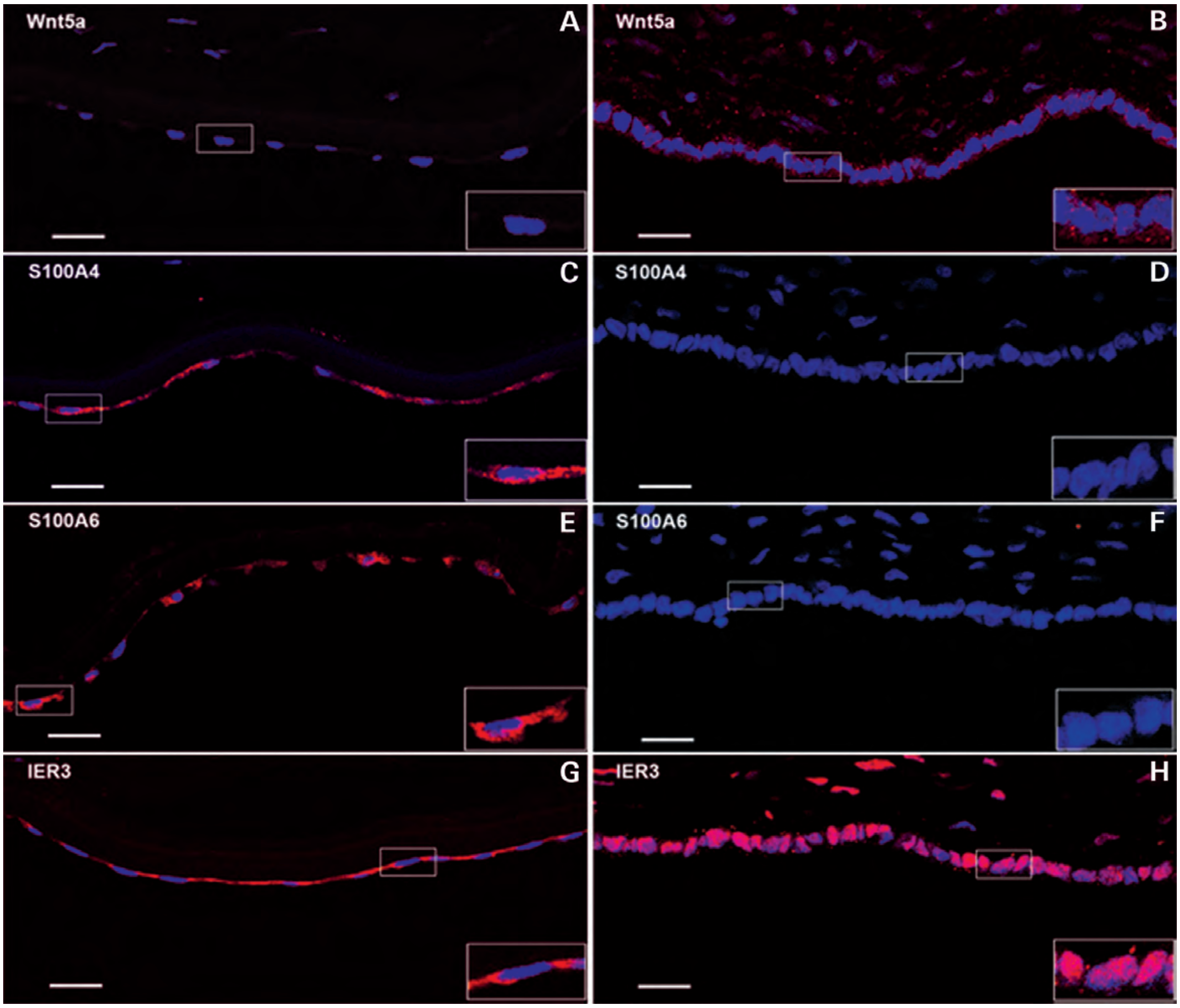

Figure 5. Immunostaining of novel marker proteins expressed in adult and fetal CECs. The bottom monolayer of the samples depicts corneal endothelium. A red signal shows the expression of each antigen as detected by a specific antibody. The blue signal is Hoesch dye. Left panels show adult CEC samples and right panels display fetal CEC samples. (**A**) and (**B**) are immunofluorescent labeling of Wnt5a in adult and fetal CECs; (**C**) and (**D**) detect S100A4 expression in adult and fetal CECs; (**E**) and (**F**) for S100A6; (**G**) and (**H**) showing IER3 protein localization in adult and fetal CECs. Scale bar, 20 µm.

genes that are highly expressed in adult and fetal CECs when compared with 12 other tissue types (Supplementary Material, Table S2). Because these CEC signature genes are enriched for the genes critical for CEC functions, we speculate that some of these highly expressed signature genes in adult and fetal CECs could be good candidates of disease target genes underlying many other hereditary CEC dystrophies.

MATERIALS AND METHODS

Adult and fetal CEC samples

Adult cornea samples (age 31, 56 and 64 years old) were obtained from normal subjects who donated to the eye bank in UCLA Jules Stein Eye Institute. Fetal CEC samples were dissected from fetal ocular samples (16–18 weeks of gestation) provided by UCLA Department of Pathology & Laboratory as approved by UCLA IRB. The whole corneal endothelial layers were carefully peeled off under a dissecting microscope and were immediately lysed in reagents for RNA purification.

RNA isolation

RNA were extracted from those adult and fetal tissues using the RNeasy Micro Kit (Qiagen, USA). RNA concentration was detected by nanodrop spectrophotometer.

Library construction and high-throughput sequencing

RNA-Seq library construction followed the protocol described in Illumina TruSeq™ RNA Sample Preparation Guide. Library construction was started with 100 ng of total RNA, via poly-T oligo-attached magnetic beads to purify the poly-A containing mRNA molecules. RNA fragments were reverse-transcribed into first cDNA strand, followed by synthesis second cDNA strand. After end repaired with a single 'A' base, cNDAs were ligated with different adapters for PCR amplification. After being amplified for 15 cycles, the concentration of the product was tested by the Qubit Fluorometer (Invitrogen, USA). We subjected 10 nmol of each sample to sequencing according to the manufacturer's instruction with Illumin Hi-seq 2000. Reads were mapped to the hg19 genome using burrows-wheeler alignment (35).

Statistical and bioinformatics analysis

For all datasets analyzed, we first transformed mapped transcript read counts to the reads per kilobase per million mapped reads (RPKM) metric, and genes with an average RPKM < 1 were filtered out, followed by quantile normalization. We performed Student's *t*-test with Storey's multiple testing correction to produce the FDR. Genes that had greater than a mean fold-change of 2 and FDR <5% were considered differentially expressed. All statistical analysis was performed using built-in Matlab functions. GO and KEGG pathway analyses were performed via David bioinformatics on-line software.

Tissue-specific expression

We curated multiple samples from the public domain, restricting ourselves to datasets generated by the Illumina Solexa platform to reduce platform biases. We carried out one-way ANOVA, and then selected for genes that were specifically overexpressed in either adult or fetal CEC samples. Two cutoffs were used: (1) Tukey–Kramer multiple comparison corrected *P*-values <0.01, which is a relatively conservative method for one-way ANOVA with different sample sizes and (2) mean 2-fold expression over all tissue types. These analyses were performed in Matlab using built-in functions (anova1, and multcomp).

Weighted gene co-expression network analysis

We constructed a signed weighted correlation network as previously described (36). Briefly, a matrix of pairwise correlations was generated between all pairs of genes across all samples. This matrix was raised to the power 12 to produce the adjacency matrix, which is used to calculate the Topological Overlap (TO), a robust and biologically meaningful measure of network interconnectedness. Genes with highly similar co-expression relationships were grouped together by performing average linkage hierarchical clustering on the TO. Module membership was calculated for each gene i in module q, as MMiq = cor(xi, Eq), where xi is the expression profile of gene i and Eq is the eigengene of module q. Genes with high module memberships are generally regarded as hub genes. WGCNA is a publically available R package that has been described in detail in the reference (36).

Immunostaining

Adult and fetal cornea tissues were fixed with 4% PFA for 30 min at room temperature, and cryo-protected in 30% sucrose/PBS overnight. After embedded in OCT, frozen section were cut using a microtome-cryostat (Leica, USA) in 10 μm. The sections were washed three times in phosphate-buffered saline (PBS) and then permeabilized in 0.4% Triton-X 100 for 1 h. Blocking was carried out using 4.5% normal donkey serum, then sections were incubated overnight at 4°C in primary antibodies diluted in blocking solution. The primary antibodies used in the experiment were anti-Wnt5a (Santa Cruz), S100A4 (Abnova), S100A6 (Abnova), IER3 (Abgent), ZO-1 (Invitrogen), Na^+–K^+-ATPase (Millipore), Axin2 (abcam) and Beta-catenin (BD). After being washed three times in PBS, sections were incubated in fluorochrome-conjugated secondary antibodies for 1 h at room temperature, cell nuclei staining was done by Hoechst, and the slides were mounted with 5% *n*-propylgallate (Sigma) in 50% glycerol. Immunostaining results were taken using a 40× objective under fluorescent light with a confocal microscope (Leica TCS-SP; Leica Microsystems).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement: None declared.

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