• Basic Research •

Transcriptome profiling of ocular surface ectoderm derived from embryonic stem cells, and identification of CACNG6 and AQP3 as its surface markers

Lu Sun[1,2], Yu-Ming Li[2,3], Yu-Wen Song[1,2], Yi-Chen Yang[1,2], Lian Duan[1], Yang Gao[1], Jian-Xin Li[1], Yan-Kun Yu[4], Kun-Peng Pang[5], Guang-Fu Dang[1], Can-Wei Zhang[1,4]

[1]Department of Ophthalmology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Jinan 250014, Shandong Province, China
[2]Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan 250014, Shandong Province, China
[3]Department of Neurosurgery, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Shandong Medicine and Health Key Laboratory of Neurosurgery, Jinan 250014, Shandong Province, China
[4]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangdong Provincial Key Laboratory of Ophthalmology and Visual Science, Guangzhou 510060, Guangdong Province, China
[5]Department of Ophthalmology, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China
Co-first Authors: Lu Sun and Yu-Ming Li
Correspondence to: Guang-Fu Dang and Can-Wei Zhang. The First Affiliated Hospital of Shandong First Medical University, No. 16766, Jingshi Road, Jinan 250014, Shandong Province, China. dangguangfu@qq.com; yankezcw@163.com
Received: 2025-07-17 Accepted: 2026-02-06

Abstract

● **AIM:** To investigate the transcriptional profiling of ocular surface ectoderm (OSE) derived from human embryonic stem cells (hESC), and identified CACNG6 and AQP3 as the surface markers of OSE.

● **METHODS:** hESCs were differentiated into OSE, neuroectoderm (NE), surface ectoderm (SE), and other surface ectoderm (OE) cells *in vitro*. RNA-seq was performed to analyze transcriptomic profiling of hESC-derived OSE, NE, OE, and SE. The differential expressed genes (DEGs) were identified, and Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, and protein-protein interaction (PPI) network analyses were performed to screen the signals and hub genes associated to OSE commitment. Also, the highly expressed transcription factors (TFs) and membrane proteins (MPs) in OSE cells were identified.

● **RESULTS:** Transcriptome analysis revealed that OSE development is dually regulated by signals associated with both SE and NE development. The signaling pathways such as Hippo, encoding extracellular matrix (ECM)-receptor interaction, and transforming growth factor-β (TGF-β) might delineate the surface ectodermal phenotype of OSE, with *FN1*, *COL1A1*, and *TGFB1* identified as hub genes. Additionally, pathways such as axon guidance, might elucidate the influence of NE in OSE commitment, and with *PAX6*, *LHX2*, *FOXG1*, *SOX2*, *MSI1*, and *DCLK1* recognized as the hub genes. Genes implicated in retinoic acid (RA) synthesis (*ALDH1A1*, *ALDH1A3*, and *RDH10*) exhibited high expression in OSE, indicating the significant role of the RA signaling pathway in OSE development. Furthermore, OSE-specific transcription factors and surface markers (*CACNG6* and *AQP3*) were identified.

● **CONCLUSION:** This study reveals the transcriptome profiling of OSE, which could provide insights into the characteristics of OSE and the underlying molecular mechanisms involved in its derivation.

● **KEYWORDS:** ocular surface ectoderm; transcriptome; human embryonic stem cells; corneal epithelial development; surface markers

DOI:10.18240/ijo.2026.08.02

Citation: Sun L, Li YM, Song YW, Yang YC, Duan L, Gao Y, Li JX, Yu YK, Pang KP, Dang GF, Zhang CW. Transcriptome profiling of ocular surface ectoderm derived from embryonic stem cells, and identification of CACNG6 and AQP3 as its surface markers. *Int J Ophthalmol* 2026;19(8):1440-1450

INTRODUCTION

The ocular surface comprises corneal, limbal, and conjunctival epithelial cells, which, along with a stable preocular tear film, maintain its integrity[1]. The corneal and conjunctival epithelium originate from a small cohort of paired box 6 positive (PAX6+) surface ectodermal (SE) cells induced

by the optic vesicle, referred to as ocular surface ectoderm (OSE) cells by Hayashi *et al*[2]. Certain signals, such as bone morphogenetic protein 4 (BMP4) and Wnt signaling pathways, have been reported to be involved in OSE commitment[3]. Additionally, activation of Wnt, Notch, Hippo, and Hedgehog signaling pathways were detected during the differentiation of corneal epithelial cells from human embryonic stem cells (hESC)[4]. Nevertheless, due to the limited number of cells and isolation difficulties, the transcriptomic characteristics and the regulatory network associated with OSE identity remain undefined.

During gastrulation, retinal precursor cells from the anterior neural plate extends toward SE, where overlying SE cells exhibit the OSE phenotype, giving rise to the presumptive corneal epithelium and lens vesicle[5]. OSE cells express PAX6, an essential transcription factor of human neuroectoderm (NE) tissues, and p63, the master regulator during SE commitment[6-7]. Based on these, we hypothesize that the co-upregulated signaling pathways and genes between OSE and SE or NE may provide clues for investigating the regulatory network that specifies OSE fate.

In this study, we generated OSE, NE, SE, and other surface ectoderm (OE) cells from hESC, and their transcriptome profiling was analyzed through RNA-seq. Then, the differentially expressed genes (DEGs) co-upregulated in OSE, SE, and OE compared with NE was screened for investigating the signals associated with surface ectoderm phenotype, and that co-upregulated in OSE and NE compared with SE and OE were used to analyze the regulatory role of NE in OSE commitment were identified. The differently expressed transcription factors (TFs) were screened, and the biological processes and signaling pathways were enriched through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. We found expression of AQP3 and CNCAG6 in an *in vitro* model and in embryonic mice, which are considered surface markers (SMs) of OSE cells and can be used to isolate OSE cells by flow cytometry or immunomagnetic beads.

MATERIALS AND METHODS

Ethical Approval All animal experiments were approved by the Medical Ethics Committee of the First Affiliated Hospital of Shandong First Medical University, China. The Ethics Committee of the First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital approved this study (2023112801).

hESC Culture and Differentiation The hESC line H1 (WA-01) was cultured on Matrigel-coated plates using mTeSRTM1 medium (Stem Cell Technologies)[8]. Subsequently, hESCs were dissociated with GCDR (Stem Cell Technologies) and inoculated into 6-well plates of LN511E for 3–7d. The medium was changed to differentiation medium, which consisted of GMEM (Life Technologies), 10% knockout serum replacer (KSR; Life Technologies), 1 mmol/L sodium pyruvate, 0.1 mmol/L non-essential amino acids, 2 mmol/L L-glutamine, 1% penicillin-streptomycin solution, and 55 μmol/L 2-mercaptoethanol. After 3wk, cultures exhibited OSE, NE, and OE cells with distinct morphologies. For corneal epithelial cell differentiation, the cells were cultured in corneal differentiation medium (CDM; differentiation medium and Cnt-PR; 1:1, CELLnTEC Advanced Cell Systems) containing 20 ng/mL keratinocyte growth factor (KGF) and 10 μmol Y-27632 for 6wk, followed by corneal epithelium culture medium (CECM; DMEM/F12 and DMEM; 1:1) supplemented with 10% fetal bovine serum, 10 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin, 0.4 μg/mL hydrocortisone, 0.1 nmol/L cholera toxin, 2 nmol/L 3,3',5-triiodo-L-thyronine, and 1% penicillin/streptomycin for 2wk[9]. Directed differentiation of hESC into SE cells was performed as previously reported[7]. Essential 6 (E6, Life Technologies) supplemented with 1 μmol/L retinoic acid (RA) and 5 ng/mL recombinant human BMP-4 was used as the differentiation medium.

Animals All pregnant mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The pregnant mice at gestational day 10.5 and 12.5 were euthanized, and the embryos were dissected out of the uterus and used for the following studies.

Immunofluorescence Staining The cells were fixed using 4% paraformaldehyde. Following permeabilization and antigen blocking, they underwent overnight incubation at 4℃ with primary antibodies. Subsequently, they were exposed to corresponding fluorescein-conjugated secondary antibodies. Nuclei were stained with Hoechst 33342 (1:1000; Thermo Fisher Scientific). Examination was then conducted using a fluorescence microscope. Mouse embryos were fixed with 4% paraformaldehyde. After paraffin embedding, tissue sections, and de-paraffinization with xylene, the sections were autoclaved for antigen repair. The remaining steps were performed as immunofluorescence staining of the cultured cells.

RNA Extraction and Quantitative Real-Time Reverse Transcription PCR OSE, NE, and OE cells derived from hESC were isolated using an inverted phase contrast microscope (10×). RNA was extracted from each cell type, with approximately 10 clones pooled into one sample. For SE and hESC, RNA was extracted from approximately 1×10^6 cells. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was then performed using the iTaq Universal SYBR Green Supermix Kit (Bio-Rad). The data were analyzed using the $2^{-\Delta\Delta CT}$ method and visualized with Prism software.

RNA-seq The extracted RNA was also utilized for RNA-seq. RNA-seq libraries were prepared using the TruSeq

Stranded mRNA Library Prep Kit (Illumina), and sequencing was performed on a NextSeq 500 system (Illumina) to obtain transcripts per kilobase million (TPM) values for each gene.

RNA-Seq Data Analysis Gene expression analysis was performed using R version 4.1.2, and DEGs with a fold change of no less than 2 and a false discovery rate (FDR) of more than 0.05 were determined using DESeq2. The genes with TPM below 5 in all compared groups were excluded. Then, differentially expressed TFs were identified from hTFtarget database (http://bioinfo.life.hust.edu.cn/hTFtarget). Moreover, the SMs were predicted through Uniport database (https://www.uniprot.org/). GO and KEGG analysis were conducted to identify the biological functions of the DEGs and related pathways.

The STRING database (http://string-db.org/) was used to construct PPI networks. The minimum interaction score of above 0.4 was significant. Cytoscape software was used to present PPI network, and the key functional module was analyzed with Cytoscape plug-in (MCODE, http://apps.cytoscape.org/apps/mcode)[10]. Hub genes were screened by cytoHubba plug-in (http://hub.iis.sinica.edu.tw/cytohubba/). Seven algorithms were applied, and the intersection of top 20 genes obtained from each algorithm was considered as the hub genes. Then, we constructed co-expression networks *via* GeneMANIA10 (http://www.genemania.org/).

Construction of TF-Gene and TF-miRNA Regulatory Network TF-gene and TF-miRNA regulatory networks were constructed by the Networkanalyst platform. TF-gene regulatory network was constructed with the JASPARA (https://jaspar.genereg.net) database, and TF-miRNA regulatory network was acquired from the RegNetwork (http://www.regnetworkweb.org) database.

Statistical Analysis All experimental data were treated as mean±standard deviation (SD). The analysis of difference was conducted by one-way analysis of variance (ANOVA) and *t*-test, and $P<0.05$ was considered statistically significant.

RESULTS

Generation of OSE, NE, OE, and SE cells from hESCs The hESCs exhibited characteristic undifferentiated morphology (Figure 1A) and expressed high levels of pluripotency markers octamer-binding transcription factor 4 (OCT4) and Nanog homeobox (NANOG; Figure 1B). Upon induction of hESC differentiation, distinct concentric cell clusters with visible delineation were observed (Figure 1C). The differentiated cells in the SE group displayed a uniform epidermal cell phenotype (Figure 1D). The phenotypes of NE, OSE, OE, and SE cells were verified by immunofluorescence staining, qRT-PCR and RNA-seq analysis (Figures 1E–1G, 2A). The OSE cells could be further differentiated into corneal epithelial cells (CK3+). Principal component analysis (PCA) and cluster analysis validated the consistency of biological replicates (Figure 2B, 2C).

Analysis of Transcriptomic Differences Between OSE and hESC During the process of differentiation, OSE, along with NE and OE, were concurrently generated from hESC. Thus, hESC served as a control for investigating transcriptome characteristics of OSE. Totally 2116 up-regulated and 2333 down-regulated DEGs were identified. The heatmap showcasing the top 60 ranked genes (30 up-regulated and 30 down-regulated) is presented in Figure 2D. GO analysis showed that the mostly enriched term of the up-regulated DEGs was regulation of nervous system development, and the biological processes, such as epidermis development and skin development were also enriched (Figure 2E). KEGG analysis showed that the most enriched pathway among the up-regulated DEGs was axon guidance, and Hippo and transforming growth factor β (TGF-β) signaling pathways, which were reported to play crucial roles in embryonic development, were also found in the top 10 ranked pathways (Figure 2F).

Identification of Signaling Pathways Associated with Surface Ectodermal Phenotype of OSE Cells To comprehend the signaling pathways associated with the surface ectodermal phenotype of OSE cells, we analyzed the transcriptomic variances between surface ectoderm (OSE, SE, and OE) and NE cells. A total of 902 co-upregulated DEGs were identified in OSE, SE, and OE cells (Figure 3A). The heatmap displaying the top 50 ranked genes is presented in Figure 3B. Notably, the key gene TP63, associated with SE commitment, along with genes encoding extracellular matrix (ECM) proteins such as ITGB6, COL12A1, and COL3A1, were observed among the DEGs. BP terms included skin development, epidermis development, and cell junction assembly, while MF terms encompassed cadherin binding and integrin binding, and CC terms included focal adhesion and cell-substrate junction (Figure 3C). Additionally, KEGG analysis revealed enrichment in signaling pathways such as focal adhesion, ECM-receptor interaction, Hippo signaling pathway, and TGF-β signaling pathway (Figure 3D). Furthermore, we identified TFs co-upregulated in SE, OSE, and OE, including master genes of SE development such as TFAP2A, GRHL3, and TP63 (Figure 3E).

The PPI network comprised 776 nodes and 10 826 interaction pairs (Figure 4A), and identified a key functional module consisting of 29 DEGs (Figure 4B). This module included key genes in the TGF-β signaling pathway (*TGFB1*, *TGFB2*, *TGFBR2*, and *TAGLN*) and genes involved in extracellular matrix formation (*COL5A1*, *COL5A2*, and *FBLN1*). Three common up-regulated hub genes (*FN1*, *COL1A1*, and *TGFB1*) were identified (Figure 4C), and their gene expression was

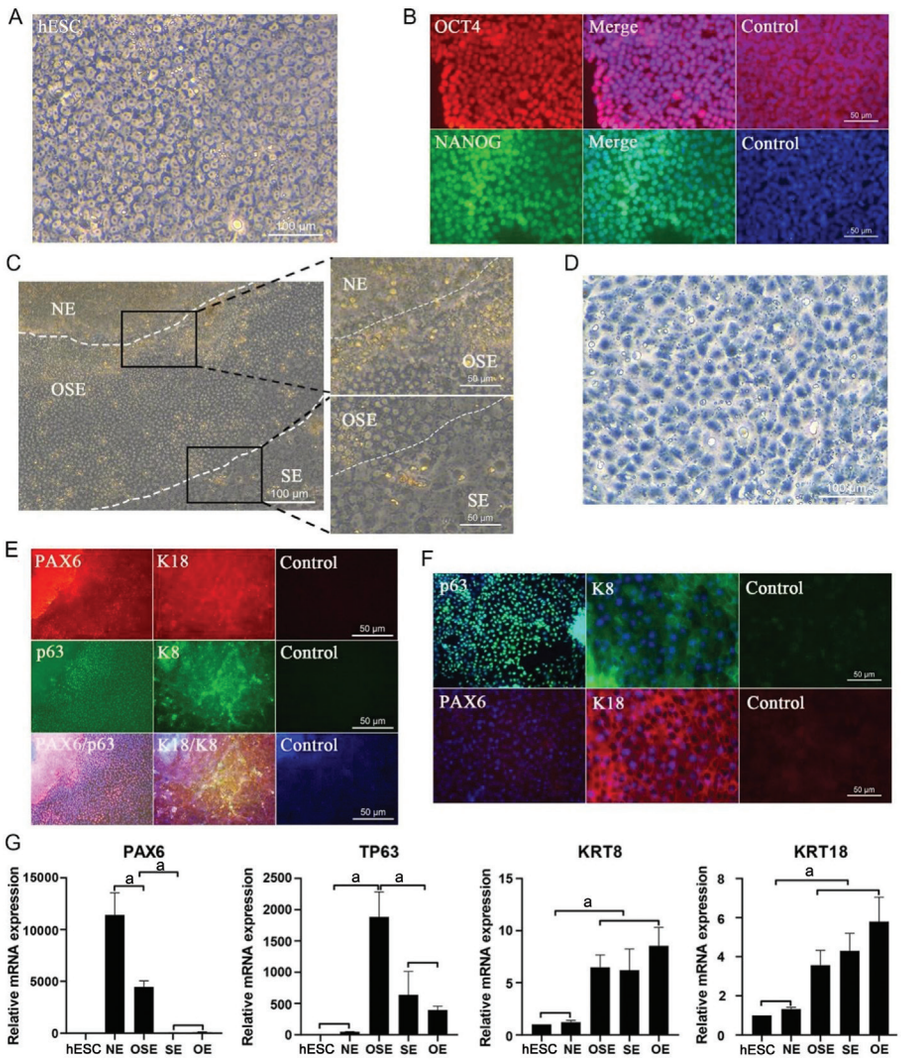

Figure 1 Generation of OSE, NE, OE, and SE cells from hESC A: hESC photographed microscopically. Scale bar, 100 μm; B: hESCs highly expressed OCT4 (red) and NANOG (green). Nuclei, blue. Scale bar, 50 μm; C: Microscopic observation of NE, OSE, and OE cells. Scale bar, 100 μm; D: Microscopic observation of SE cells. Scale bar, 100 μm; E: Expression of PAX6 (red), p63 (green), K18 (red), and K8 (green) in the cultures derived from hESCs. Nuclei, blue. Scale bar, 50 μm; F: Expression of PAX6 (red), p63 (green), K18 (red), and K8 (green) in SE cells. Nuclei, blue. Scale bar, 50 μm; G: Gene expression of *PAX6*, *TP63*, *KRT8*, and *KRT18* in NE, OSE, SE, and OE cells. The hESCs were used as the control. [a]P<0.05. OSE: Ocular surface ectoderm; NE: Neuroectoderm; OE: Other surface ectoderm; SE: Surface ectoderm; hESC: Human embryonic stem cells; OCT4: Octamer-binding transcription factor 4; NANOG: Nanog homeobox; PAX6: Paired box 6; p63/*TP63*: Tumor protein p63; K18/*KRT18*: Keratin 18; K8/*KRT8*: Keratin 8.

validated by qPCR (Figure 4D). Additionally, we constructed TF-gene and TF-miRNA co-regulatory networks. The TF-gene network detected 18 TFs interacting with hub genes (Figure 4E), while the TF-miRNA network identified 8 miRNAs and 50 TFs (Figure 4F).

Co-upregulated Genes and Signaling Pathways in NE and OSE Cells We postulated that signals originating from NE cells may contribute to OSE development. DEGs analysis revealed 181 co-upregulated DEGs in OSE and NE compared to OE and SE (Figure 5A). The top 50 up-regulated genes are depicted in Figure 5B, with genes associated with nervous system and eye development, such as *RAX*, *PAX6*, *CNTFR*, *SIX3*, and *TH*, among others. GO analysis showed enrichment of co-upregulated DEGs in BP terms related to forebrain development, axonogenesis, and axon guidance, while CC terms included neuronal cell body, distal axon, and growth cone (Figure 5C). KEGG analysis revealed enrichment in signaling pathways such as axon guidance and motor proteins (Figure 5D). Additionally, TFs associated with neuronal development, such as SOX6, SOX11, PAX6, and RAX, were also expressed in OSE cells (Figure 5E).

The PPI network comprised 81 nodes and 392 interaction pairs (Figure 6A), with a key functional module containing 10 DEGs, including PAX6, SOX11, SOX2, SIX3, and FOXG1 (Figure 6B). PAX6, SOX2, LHX2, FOXG1, MSI1, and DCLK1 were identified as hub genes (Figure 6C), and their

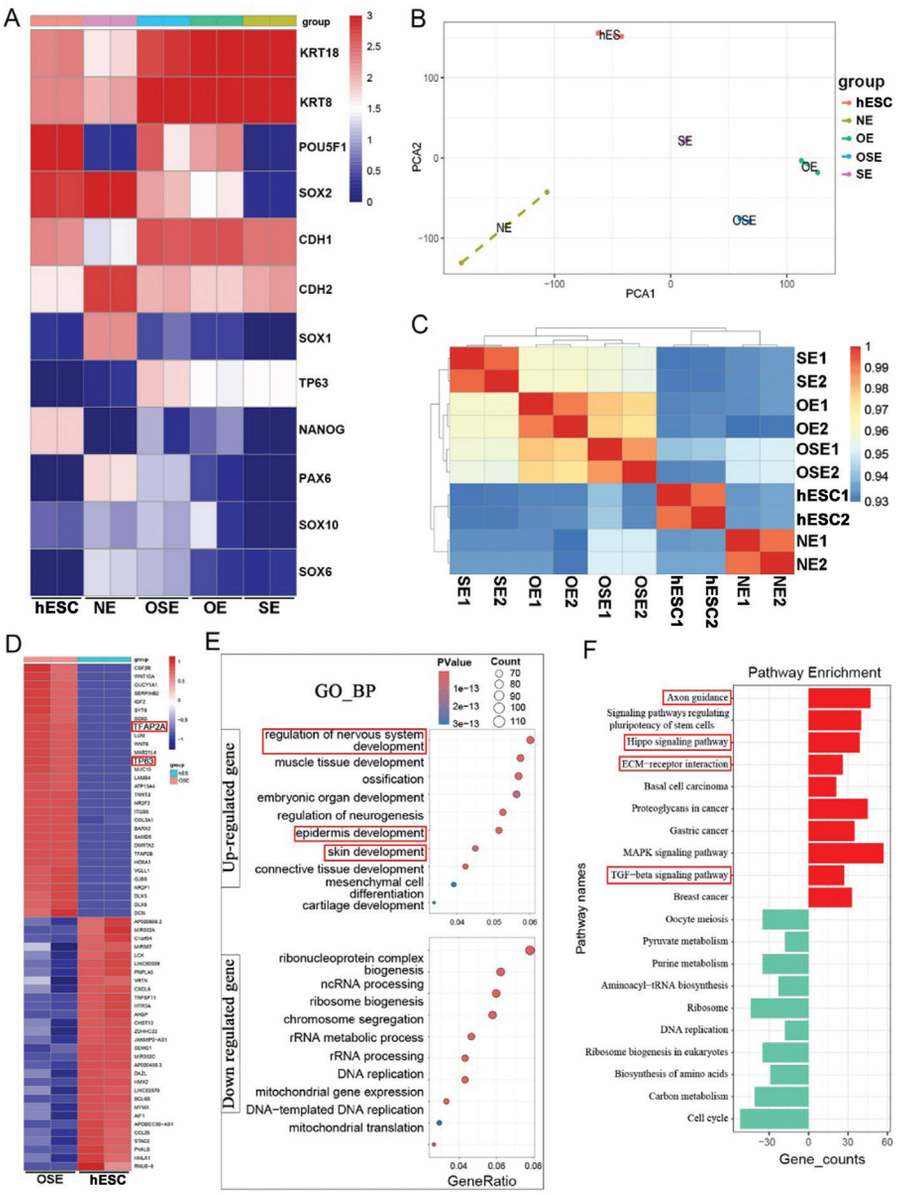

Figure 2 Transcriptome analysis of NE, OSE, OE, and SE cells derived from hESCs A: Heatmap showing the expression of the marker genes of NE, OSE, OE, SE, and hESCs; B: PCA of RNA-seq data; C: Cluster analysis verified the consistency of the biological replicates; D: Heatmap showing top 60 DEGs (30 up-regulated and 30 down-regulated) between OSE and hESC; E: GO_BP enrichment analysis of DEGs; F: KEGG pathway analysis of DEGs. OSE: Ocular surface ectoderm; NE: Neuroectoderm; OE: Other surface ectoderm; SE: Surface ectoderm; hESC: Human embryonic stem cells; PCA: Principal component analysis; GO_BP: Gene Ontology_ Biological Process; DEGs: Differential expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; KRT18: Keratin 18; KRT8: Keratin 8; POU5F1: POU class 5 homeobox 1; SOX: SRY-box transcription factor; CDH: Cadherin; TP63: Tumor protein p63; NANOG: Nanog homeobox; PAX6: Paired box 6.

gene expression was validated by qPCR (Figure 6D). The TF-gene network comprised 35 TFs, with some genes such as *FOXC1*, *YY1*, *STAT1*, and *JUN* interacting with the hub genes involved in the specification of SE phenotype (Figure 6E). Additionally, 14 miRNAs co-expressed in NE and OSE cells were screened (Figure 6F).

OSE-Specific Transcriptome Characterization The construction of OSE-specific transcriptome patterns may facilitate an in-depth understanding of the developmental processes and functional characteristics of OSE. Therefore, we scrutinized OSE-specific high-expression transcriptome features. 92 specifically highly expressed DEGs were identified in OSE, in comparison to hESC, NE, OE, and SE (Figure 7A). Figure 7B illustrated the heatmap of the top 50 genes. In GO analysis, the DEGs mainly enriched in the biological processes related to transcriptional regulation, cell differentiation, and camera-type eye development (Figure 7C). KEGG analysis revealed 2 enriched pathways, retinol metabolism and mineral absorption (Figure 7D). Two functional modules were obtained, DLX5-DLX6-TP63 and ALDH1A1-ALDH1A3-RDH10 modules (Figure 7E). The 13 genes were identified as the hub genes, including *TP63*, *DLX6*, and *DLX5* (Figure 7F).

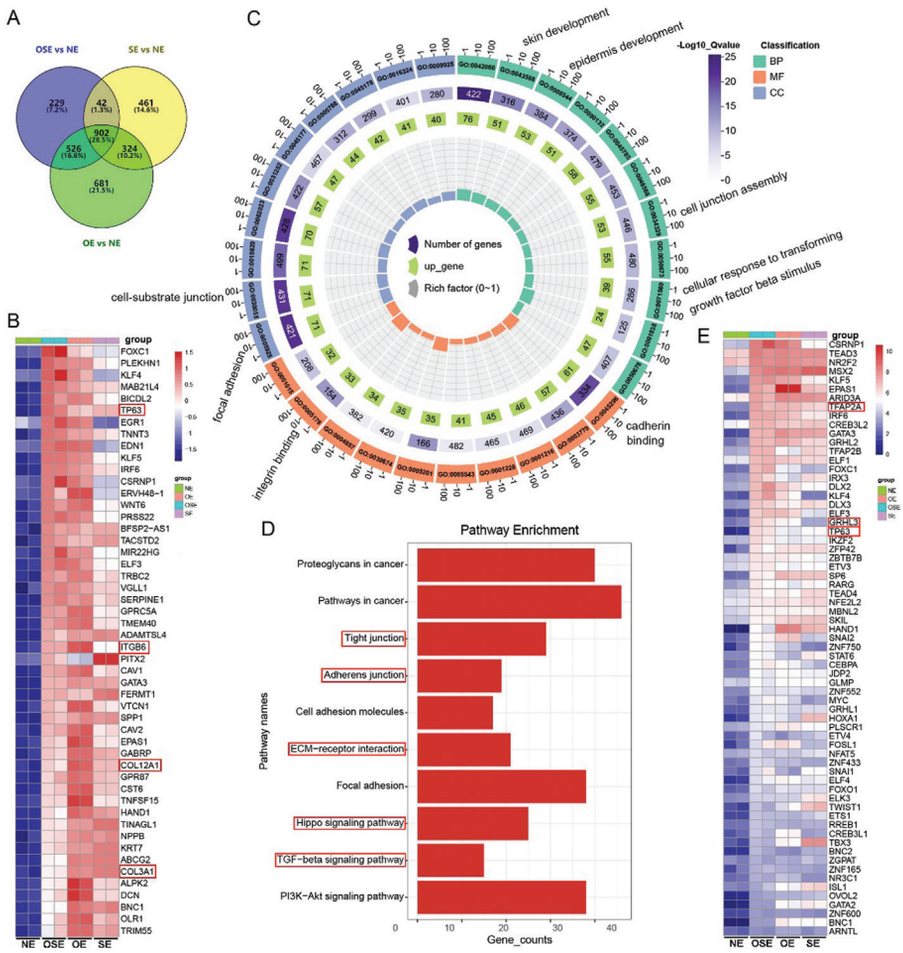

Figure 3 Identification of the signals associated with surface ectodermal phenotype A: Venn diagram showing the DEGs that are up-regulated in OSE, SE, and OE compared to NE; B: Heatmap showing top 50 up-regulated DEGs; C: Results of GO enrichment analysis; D: KEGG pathway analysis of DEGs upregulated in OSE, SE, and OE, P<0.05 were considered significant; E: Heatmap showing up-regulated TFs. OSE: Ocular surface ectoderm; NE: Neuroectoderm; OE: Other surface ectoderm; SE: Surface ectoderm; DEGs: Differential expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; TFs: The highly expressed transcription factors.

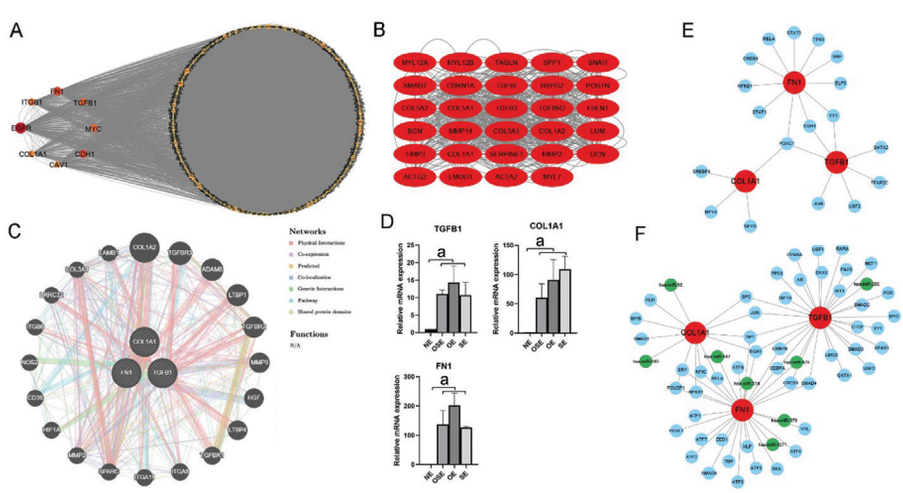

Figure 4 Analysis of the regulatory network in SE lineage-determination A: PPI network of upregulated DEGs in OSE, SE, and OE compared to NE. The redder the color of the gene in the network, the higher its connectivity with other genes; B: A key functional module of DEGs; C: Analysis of hub genes and their co-expressed genes by GeneMANIA; D: Detection of the hub gene expression in OSE, SE, and OE through qPCR, [a]P<0.05 were considered significant; E: Screening of the TFs interacting with the hub genes through TF-gene network. Highlighted red nodes represent hub genes, and blue nodes represent TFs; F: The TF-miRNA co-regulatory network interacting with the hub genes. The red nodes are hub genes, the blue nodes represent TF-genes, and the green nodes represent miRNAs. OSE: Ocular surface ectoderm; NE: Neuroectoderm; OE: Other surface ectoderm; SE: Surface ectoderm; DEGs: Differential expressed genes; PPI: Protein-protein interaction; TGFB1: Transforming growth factor β1; COL1A1: Collagen type I alpha 1 chain; FN1: Fibronectin 1; qPCR: Quantitative real-time polymerase chain reaction; TFs: The highly expressed transcription factors.

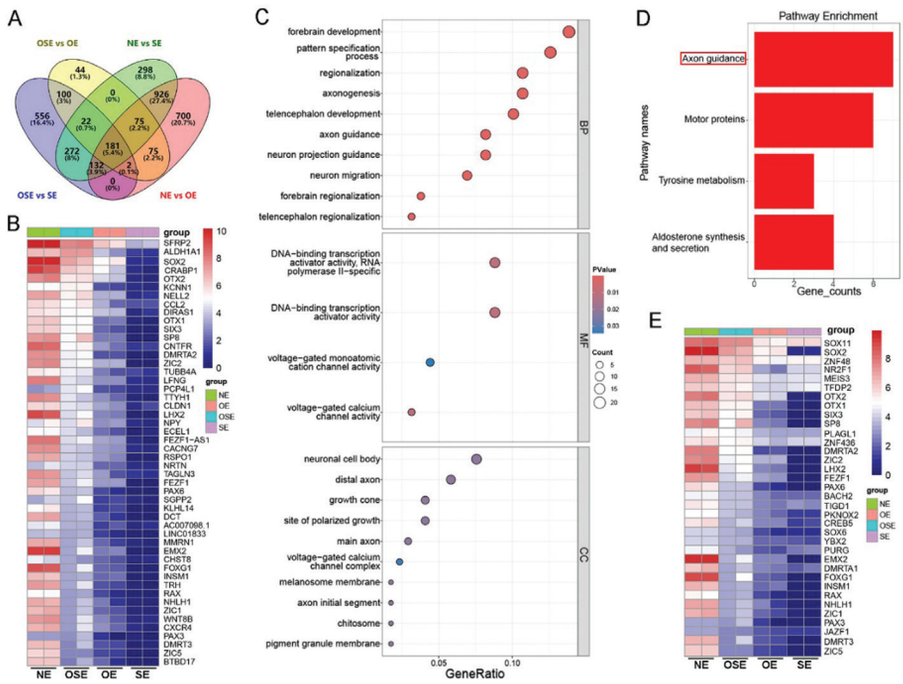

Figure 5 Differential gene analysis of NE and OSE versus SE and OE by RNA-seq A: Venn diagram showing the DEGs co-upregulated by NE and OSE relative to SE and OE; B: Heatmap displaying the co-upregulated genes in NE and OSE; C: GO enrichment analysis of the DEGs co-upregulated in NE and OSE; D: KEGG pathway analysis of DEGs upregulated in NE and OSE, $P<0.05$ were considered significant; E: Heatmap showing up-regulated TFs. OSE: Ocular surface ectoderm; NE: Neuroectoderm; OE: Other surface ectoderm; SE: Surface ectoderm; DEGs: Differential expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; TFs: The highly expressed transcription factors.

TFs play a crucial role in determining cell fate decisions. Moreover, monitoring cell SMs recognized by antibodies is essential for isolating viable target cell types for further studies. Thus, OSE-specific TFs and SMs were screened, resulting in the identification of 18 TFs and 2 SMs (AQP3, CACNG6) in OSE cells (Figure 7G). Immunofluorescence staining confirmed the expression of AQP3 and CACNG6 (Figure 7H). However, in mouse embryonic eyes, AQP3 and CACNG6 also expressed in retinal progenitor cells (RPCs), though the expression of CACNG6 was very weak (Figure 7I).

DISCUSSION

OSE cells, belonging to SE, serve as precursor cells for corneal and conjunctival epithelia, crucial for maintaining ocular surface integrity and normal visual function[11-12]. The signals regulating SE development should be involved in OSE commitment. Moreover, due to the adjacent location, the signals from RPCs or neuro-retina derived from NE also play important roles in ocular surface morphogenesis during embryonic development[5,13]. Direct analysis of OSE cells from living organisms is limited by ethical concerns and technical challenges, such as the invasiveness of tissue collection and the difficulty of obtaining adequate cell quantities for experimental studies. As a result, hESC-derived OSE cells provide a valuable alternative, offering a human-specific *in vitro* model that effectively simulates OSE development. This model facilitates detailed investigations into early human OSE development, which are challenging to achieve with animal models. Herein, we generated OSE, NE, SE, and OE cells from hESCs and elucidated the gene expression characteristics of OSE, along with the hub genes and regulatory networks involved in its commitment through RNA-seq. We believe that these findings will expand our understanding of ocular surface biology and its associated diseases.

Key TFs associated with NE and SE, such as PAX6, TP63, and TFAP2A, were identified in OSE cells through DEGs analysis between OSE and hESC. GO analysis revealed that up-regulated DEGs in OSE cells were enriched in neural system development and epidermis development, indicating the regulation of OSE development by signals from both NE and SE. While both surface ectoderm and neuroectoderm arise from ectoderm, they differentiate into distinct cell types. Surface ectoderm gives rise to external body structures like skin, sweat glands, and corneal epithelial cells, whereas neuroectoderm primarily forms the nervous system, including the brain, spinal cord, and retina[14]. In addition to OSE, SE and OE represent two other lineages of the surface ectoderm. To identify signals associated with the surface ectodermal phenotype, genes upregulated in OSE, SE, and OE relative to NE were analyzed, focusing on ECM components and hub genes such as *FN1*, *COL1A1*, and *TGFB1*. The analysis

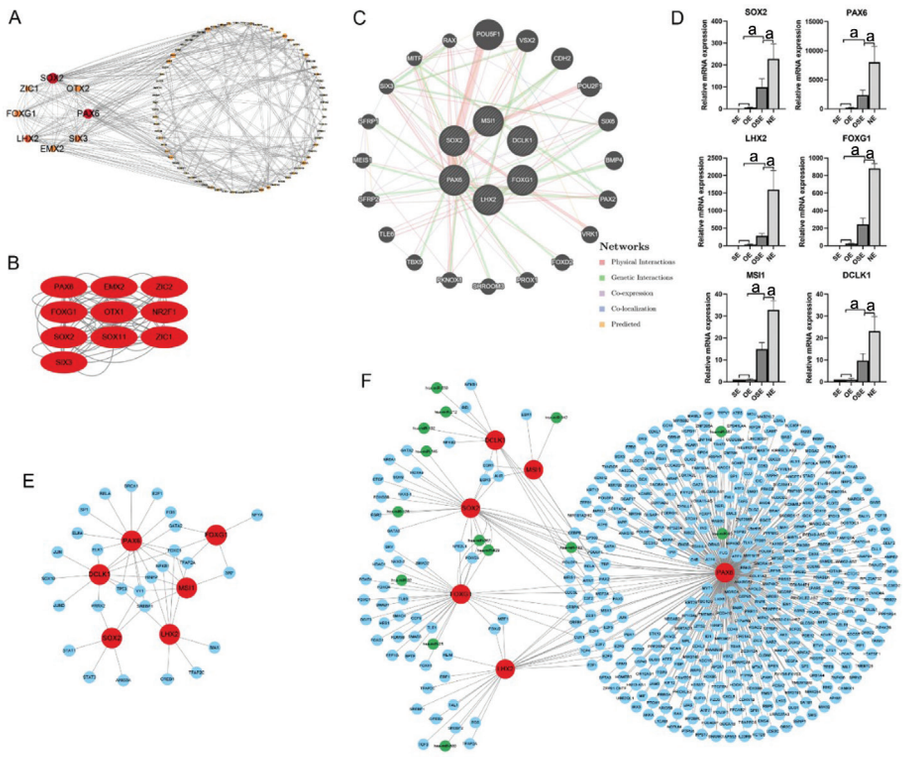

Figure 6 Analysis of the up-regulate regulatory network in NE and OSE versus SE and OE A: PPI network diagram of the DEGs co-upregulated in NE and OSE. The redder the color of a gene in the network, the higher its connectivity with other genes; B: A key functional module of DEGs; C: Analysis of hub genes and their co-expressed genes by GeneMANIA; D: Detection of the hub gene expression in NE and OSE through qPCR, [a]*P*<0.05 were considered significant; E: Screening of the TFs interacting with the hub genes through TF-gene network. Highlighted red nodes represent hub genes, and blue nodes represent TFs; F: The TF-miRNA co-regulatory network interacting with the hub genes. The red nodes are hub genes, the blue nodes represent TF-genes, and the green nodes represent miRNAs. OSE: Ocular surface ectoderm; NE: Neuroectoderm; OE: Other surface ectoderm; SE: Surface ectoderm; DEGs: Differential expressed genes; PPI: Protein-protein interaction; TFs: The highly expressed transcription factors; qPCR: Quantitative real-time polymerase chain reaction; *SOX2*: SRY-box transcription factor 2; *PAX6*: Paired box 6; *LHX2*: LIM homeobox 2; *FOXG1*: Forkhead box G1; *MSI1*: Musashi RNA-binding protein 1; *DCLK1*: Doublecortin-like kinase 1.

highlighted the enrichment of pathways including ECM-receptor interaction, Hippo signaling, and TGF-β signaling. Moreover, single-cell sequencing of corneal-like organoids revealed that the transcription levels of FN1, COL1A1, COL1A2, COL5A2, and COL5A1 increased by over 20-fold. This heightened expression may correspond to the intense transcriptional activity of these collagens observed during corneal development in organoid formation[15].

TGF-β signaling has been identified as a critical regulator of skin cell induction and fate decision, with TGF-β inhibitors leading to the depletion of OSE cells in cultures derived from induced pluripotent stem cells (iPS)[2,16]. These findings underscore the pivotal role of the TGF-β signaling pathway in specifying the SE phenotype and OSE development. The Hippo-YAP signaling pathway, essential in various stages of embryonic development, interacts with other developmental and regulatory signals including TGF-β, Wnt, and Notch[17-18]. Additionally, compared to SE and OE, the upregulated DEGs in OSE and NE showed enrichment in forebrain development and axon guidance. Notably, hub genes such as *PAX6*, *LHX2*, and *FOXG1*, known regulators of axon guidance and neurodevelopment, were among the identified genes[19-21].

RA plays a pivotal role in regulating the transcription of key developmental genes, influencing the development of various organs and tissues, including the spinal cord, forelimbs, and eyes[22]. Our analysis revealed significantly higher expression of genes involved in retinol metabolism, such as ALDH1A1-ALDH1A3-RDH10, in OSE cells compared to other cell types. Moreover, the expression levels of RA receptors RARB and RARG were also notably elevated in OSE compared to NE cells. In human embryos at 10–21wk post-conception, developing corneal epithelial cells exhibit high expression of key module genes identified in this study, including ALDH1A1, ALDH3A1, CLU, and KRT12[9]. These lend support to the notion that the RA signaling pathway may play a regulatory role in OSE development. Additionally, we identified a functional module consisting of hub genes DLX5, DLX6, and TP63, which have been implicated in craniofacial abnormalities and ectrodactyly[7,23], and exhibited substantial upregulation in OSE cells. Notably, TP63, a master regulator

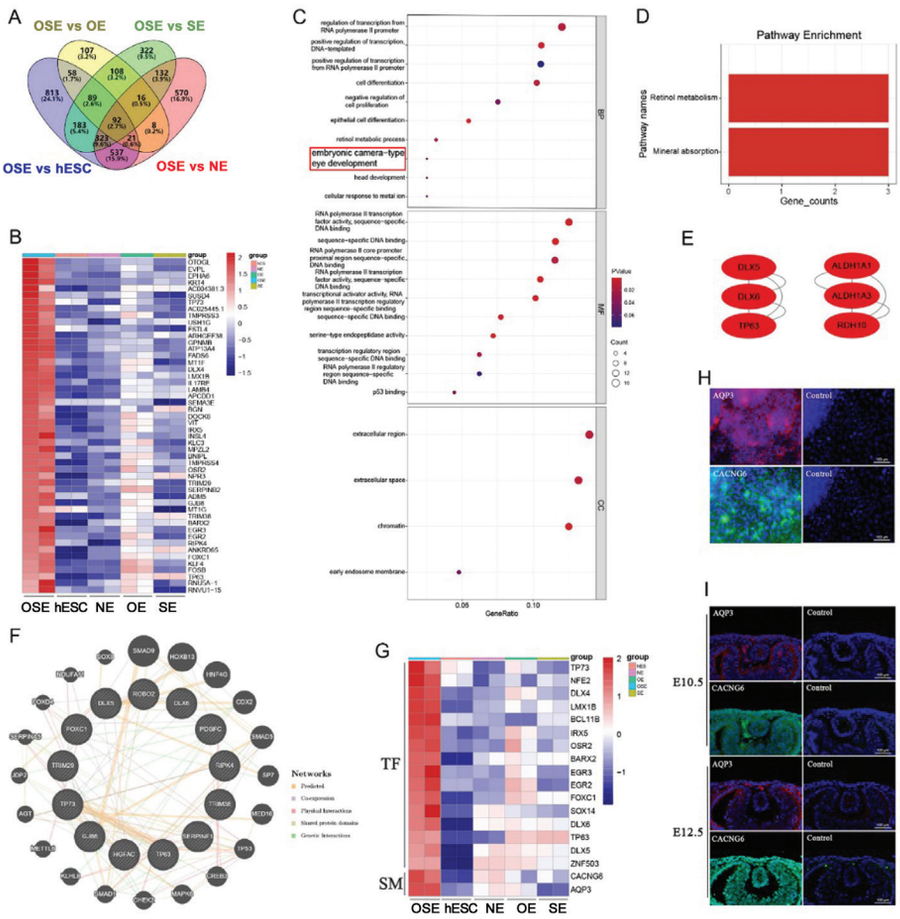

Figure 7 OSE-specific transcriptome characteristics compared to hESC, NE, SE, and OE A: Venn diagram showing the DEGs upregulated in OSE compared to hESC, NE, SE, and OE; B: Heatmap showing the OSE-specific highly expressed genes; C: GO enrichment analysis results of the upregulated DEGs in OSE; D: KEGG analysis results of the upregulated DEGs in OSE; E: The functional modules of the upregulated DEGs in OSE; F: The hub genes screening from the upregulated DEGs in OSE and their co-expressed genes constructed by GeneMANIA; G: Heatmap showing highly expressed TFs and SMs of OSE; H: Immunofluorescence staining of AQP3, CACNG6 in OSE cells derived from hESCs; I: Immunofluorescence staining of AQP3, CACNG6 in OSE cells in mouse embryonic eyes. OSE: Ocular surface ectoderm; NE: Neuroectoderm; OE: Other surface ectoderm; SE: Surface ectoderm; hESC: Human embryonic stem cells; DEGs: Differential expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; TFs: The highly expressed transcription factors; SMs: Surface markerss; GO: Gene Ontology; AQP3: Aquaporin 3; CACNG6: Calcium voltage-gated channel auxiliary subunit gamma 6.

of SE identity, showed significantly higher expression levels in OSE compared to SE and OE cells, suggesting a pivotal role of this functional module in OSE development. Additionally, FOXC1, previously reported to govern corneal epithelium fate[24], exhibited high expression level in OSE cells. This underscores the potential significance of FOXC1 as a regulator of OSE development far from the role in determining corneal epithelium fate. Furthermore, we screened and identified 18 TFs and 2 surface markers (CACNG6 and AQP3) that were highly expressed specifically in OSE cells. These markers hold promise for identifying or isolating OSE cells in future studies. AQP3, a member of the AQP family, was reported to be responsible for transmembrane transport of small molecules, including water, glycerol, and urea in corneal and conjunctival epithelium, contributing to ocular surface lubrication[25-27]. CACNG6, an integral membrane protein was associated with Ca^{2+} channel regulation[28]. Here we showed that CACNG6 and AQP3 expressed in OSE cells, although their roles were still not clear. In mouse embryonic eye, CACNG6 and AQP3 were also detected in RPCs. This also reflects the similarity in some gene expression between OSE and RPCs. In the meantime, these two surface markers can be used as surface markers for fetal OSE and RPCs. It should be noted that our *in vivo* temporal analysis is focused on the E10.5-E12.5 window. Although this establishes expression stability during a key morphogenetic period, profiling the precise onset (*e.g.*, at E8.5 or earlier) remains technically demanding due to the limited tissue size and fragility of earlier-stage embryos. Future methodological refinements will enable a more exhaustive developmental timeline analysis. In addition, due to the technical and ethical limitations of human embryo research, the *in vivo* expression of CACNG6 and AQP3 were not assessed

in human. Although our findings identify them as candidate markers, their utility as specific surface markers for human OSE and RPCs still need further confirm by the validation of their precise expression patterns, cellular specificity, and biological roles in human developmental contexts. Future studies will incorporate complementary approaches, such as proteomics, live-cell imaging, and *in vivo* validation, to address these limitations and improve the robustness of the conclusions.

In summary, our findings suggest that OSE development may be influenced by signals associated with both nervous system and epidermis development. Key signaling pathways such as TGF-β and Hippo, along with ECM composition and hub genes like *FN1*, *COL1A1*, and *TGFB1*, may contribute to defining the surface ectodermal phenotype of OSE. Additionally, the axon guidance signaling pathway and hub genes such as *PAX6*, *SOX2*, *LHX2*, *FOXG1*, *MSI1*, and *DCLK1* may play roles in mediating the effects of NE on OSE commitment. Furthermore, the significant upregulation of genes involved in retinol metabolism highlights the importance of RA in OSE development. These findings deepen our understanding of OSE cells and provide valuable insights into the molecular mechanisms underlying their derivation.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. Nan Cao for his guidance and assistance in this study.

Authors' Contributions: Conceptualization, Sun L, Li YM, and Dang GF, Zhang CW; methodology, Sun L, Dang GF, and Zhang CW; software, Song YW, Li JX, and Yang YC; validation, Gao Y, Yu YK, and Duan L; formal analysis, Sun L and Zhang CW; writing—original draft preparation, Sun L and Li YM; writing—review and editing, Pang KP, Dang GF, and Zhang CW; funding acquisition, Zhang CW. All authors have read and agreed to the published version of the manuscript.

Foundations: Supported by the National Natural Science Foundation of China (No.82000853); the Natural Science Foundation of Shandong Province (No.ZR2022MH154).

Conflicts of Interest: Sun L, None; **Li YM,** None; **Song YW,** None; **Yang YC,** None; **Duan L,** None; **Gao Y,** None; **Li JX,** None; **Yu YK,** None; **Pang KP,** None; **Dang GF,** None; **Zhang CW,** None.

REFERENCES

1 Jurkunas U, Johns L, Armant M. Cultivated autologous limbal epithelial cell transplantation: new frontier in the treatment of limbal stem cell deficiency. *Am J Ophthalmol* 2022;239:244-268.

2 Hayashi R, Ishikawa Y, Sasamoto Y, *et al*. Co-ordinated ocular development from human iPS cells and recovery of corneal function. *Nature* 2016;531(7594):376-380.

3 Kobayashi Y, Hayashi R, Shibata S, *et al*. Ocular surface ectoderm instigated by WNT inhibition and BMP4. *Stem Cell Res* 2020;46:101868.

4 He J, Ou S, Ren J, *et al*. Tissue engineered corneal epithelium derived from clinical-grade human embryonic stem cells. *Ocul Surf* 2020;18(4):672-680.

5 Fujimura N. WNT/β-catenin signaling in vertebrate eye development. *Front Cell Dev Biol* 2016;4:138.

6 Kamuro R, Yoshihara M, Hara S, *et al*. PAX6-dependent differentiation landscape of ocular surface epithelium *via* single-cell RNA sequencing in hiPSC-derived ocular developmental organoid. *Commun Biol* 2025;8(1):1220.

7 Pattison JM, Melo SP, Piekos SN, *et al*. Retinoic acid and BMP4 cooperate with p63 to alter chromatin dynamics during surface epithelial commitment. *Nat Genet* 2018;50(12):1658-1665.

8 Zhang C, Du L, Sun P, *et al*. Construction of tissue-engineered full-thickness cornea substitute using limbal epithelial cell-like and corneal endothelial cell-like cells derived from human embryonic stem cells. *Biomaterials* 2017;124:180-194.

9 Li M, Guo H, Wang B, *et al*. The single-cell transcriptomic atlas and RORA-mediated 3D epigenomic remodeling in driving corneal epithelial differentiation. *Nat Commun* 2024;15:256.

10 Ye H, Sun A, Xiao W, *et al*. Differential circular RNA expression profiling of orbital connective tissue from patients with type I and type II thyroid-associated ophthalmopathy. *Invest Ophthalmol Vis Sci* 2022;63(12):27.

11 Roshandel D, Semnani F, Rayati Damavandi A, *et al*. Genetic predisposition to ocular surface disorders and opportunities for gene-based therapies. *Ocul Surf* 2023;29:150-165.

12 Song Y, Xu T, Li H, *et al*. Delivery of Itgb1-siRNA by triptolide-modified and anti-Flt1 peptide-guided ionizable cationic LNPs for targeted therapy of corneal neovascularization. *J Control Release* 2025;383:113811.

13 Li J. Role of Smad4 from ocular surface ectoderm in retinal vasculature development. *Int J Ophthalmol* 2020;13(2):231-238.

14 Guo Y, Wu W, Ma X, *et al*. Comparative gene expression profiling reveals key pathways and genes different in skin epidermal stem cells and corneal epithelial cells. *Genes Genom* 2019;41(6):679-688.

15 Maiti G, Monteiro de Barros MR, Hu N, *et al*. Single cell RNA-seq of human cornea organoids identifies cell fates of a developing immature cornea. *PNAS Nexus* 2022;1(5):pgac246.

16 Li X, Xie R, Luo Y, *et al*. Cooperation of TGF-β and FGF signalling pathways in skin development. *Cell Prolif* 2023;56(11):e13489.

17 Piccolo FM, Kastan NR, *et al*. Role of YAP in early ectodermal specification and a Huntington's Disease model of human neurulation. *ELife* 2022;11:e73075.

18 Yang CC, Yang XX, Zhao XJ, *et al*. Semaphorin 7A impairs barrier function in cultured human corneal epithelial cells in a manner dependent on nuclear factor-kappa B. *Int J Ophthalmol* 2024;17(3):444-453.

19 Diacou R, Nandigrami P, Fiser A, *et al*. Cell fate decisions, transcription factors and signaling during early retinal development. *Prog Retin Eye Res* 2022;91:101093.

20 Hettige NC, Peng H, Wu H, *et al*. FOXG1 dose tunes cell proliferation dynamics in human forebrain progenitor cells. *Stem Cell Reports* 2022;17(3):475-488.

21 Manuel M, Pratt T, Liu M, *et al*. Overexpression of Pax6 results in microphthalmia, retinal dysplasia and defective retinal ganglion cell axon guidance. *BMC Dev Biol* 2008;8(1):59.

22 Berenguer M, Duester G. Retinoic acid, RARs and early development. *J Mol Endocrinol* 2022;69(4):T59-T67.

23 Vera-Carbonell A, Moya-Quiles MR, Ballesta-Martínez M, *et al*. Rapp-Hodgkin syndrome and SHFM1 patients: delineating the p63-Dlx5/Dlx6 pathway. *Gene* 2012;497(2):292-297.

24 Yang W, Lee SK, Lehmann OJ, *et al*. FoxC1 activates limbal epithelial stem cells following corneal epithelial debridement. *Exp Eye Res* 2023;234:109599.

25 King LS, Kozono D, Agre P. From structure to disease: the evolving tale of aquaporin biology. *Nat Rev Mol Cell Biol* 2004;5(9):687-698.

26 Melnyk S, Bollag WB. Aquaporins in the cornea. *Int J Mol Sci* 2024;25(7):3748.

27 Levin MH, Verkman AS. Aquaporin-dependent water permeation at the mouse ocular surface: *in vivo* microfluorimetric measurements in cornea and conjunctiva. *Invest Ophthalmol Vis Sci* 2004;45(12):4423-4432.

28 Lee JS, Kim JH, Bae JS, *et al*. Association of CACNG6 polymorphisms with aspirin-intolerance asthmatics in a Korean population. *BMC Med Genet* 2010;11(1):138.