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A Transcriptomic Analysis of Differential Gene Expression during Chick Periocular Neural Crest Differentiation into Corneal Cells

Lian Bi, **Peter Lwigale**

BioSciences, Rice University, 6100 Main Street, Houston, Texas.

Abstract

Background: Multipotent neural crest cells (NCC) contribute to the corneal endothelium and keratocytes during ocular development, but the molecular mechanisms that underlie this process remain poorly understood. We performed RNA-Seq analysis on periocular neural crest (pNC), corneal endothelium, and keratocytes and validated expression of candidate genes by in situ hybridization.

Results: RNA-Seq profiling revealed enrichment of genes between pNC and neural crest-derived corneal cells, which correspond to pathways involved in focal adhesion, ECM-receptor interaction, cell adhesion, melanogenesis, and MAPK signaling. Comparisons of candidate NCC genes to ocular gene expression revealed that majority of the NCC genes are expressed in the pNC, but they are either differentially expressed or maintained during corneal development. Several genes involved in RA, TGFβ, and Wnt signaling pathways and their modulators are also differentially expressed. We identified differentially expressed transcription factors as potential downstream candidates that may instruct expression of genes involved in establishing corneal endothelium and keratocyte identities.

Conclusion: Combined, our data reveal novel changes in gene expression profiles as pNC differentiate into highly specialized corneal endothelial cells and keratocytes. These data serve as platform for further analyses of the molecular networks involved in NCC differentiation into corneal cells, and provide insights into genes involved in corneal dysgenesis and adult diseases.

Keywords

RNA-Seq; neural crest cells; cornea; corneal endothelium; keratocytes

INTRODUCTION

The cornea is a multilayered transparent tissue comprised predominantly of cells derived from a multipotent embryonic cell population, the neural crest cells (NCC). Cranial NCC progenitors of the cornea originate from the neural tube region encompassing the caudal diencephalon and the rostral metencephalon (Lwigale et al., 2004; Creuzet et al., 2005b). This population of NCC forms different streams that migrate into the frontal-nasal,

*Corresponding author: Peter Y. Lwigale, BioSciences, Rice University, 6100 Main Street, Houston, Texas. lwigale@rice.edu.

periocular, and maxilla-mandibular regions (Noden, 1975; Johnston et al., 1979; Serbedzija et al., 1992) where they respond to environmental cues and differentiate into region-specific tissues. Periocular neural crest cells (pNC) contribute to various ocular structures including the cornea, eyelids, and connective tissues (Lwigale et al., 2004; Creuzet et al., 2005b). Defects in pNC migration, proliferation, and differentiation are associated with a condition known as anterior segment dysgenesis (ASD) (Cook, 1989). ASD is comprised of spectrum of ocular disorders characterized by malformation of the cornea, iris, lens, and eyelids (Churchill and Booth, 1996; Sowden, 2007). Despite the significance of NCC to eye development, very little is known about the molecular underpinnings of their differentiation into ocular tissues.

During chick corneal development, pNC occupy the mesenchyme surrounding the rudimentary eye for approximately two days prior to their initial migration into the presumptive corneal region at about embryonic day (E)4.5. Migration of pNC into the presumptive corneal region occurs in two waves (Hay and Revel, 1969; Lwigale et al., 2005). The first wave forms the corneal endothelium, an interior monolayer of cells that establishes a barrier and regulates fluid movement between the anterior chamber and the cornea (Waring et al., 1982). The second wave of migration occurs at about E6, when pNC invade the acellular primary stroma and differentiate into keratocytes, which synthesize the extracellular matrix of the corneal stroma (Linsenmayer et al., 1984; Quantock and Young, 2008). The intricate behavior of pNC during chick corneal development is in part regulated by the lens vesicle, given that its ablation results in precocious migration and malformation of the cornea (Beebe and Coats, 2000; Lwigale and Bronner-Fraser, 2009). The molecular signals involved in regulating pNC migration were pinpointed to lens-derived Semaphorin3A and the spatiotemporal downregulation of its receptor, *NRP1*, by a subset of pNC, which permitted their migration into the presumptive corneal region (Lwigale and Bronner-Fraser, 2009). Studies in mice have identified that retinoic acid (RA) signaling regulates transcription factors expressed by pNC including *Pitx2* and *Foxc1* that inhibit canonical Wnt signaling via upregulation of *Dkk2* (Lehmann et al., 2003; Evans and Gage, 2005; Gage et al., 2008). Knockout of any one of these genes in mice phenocopy the corneal and iridial defects observed in humans with ASD (Gage et al., 1999; Kitamura et al., 1999; Kume and Seo, 2010). Together, these studies have advanced our understanding of early corneal development, but the molecular mechanisms that transform pNC into the diverse progeny of ocular cells remain unclear.

In this study, we take advantage of the stepwise contribution of avian pNC to the nascent cornea and survey their gene expression profile during differentiation into corneal endothelium and keratocytes by RNA-Seq analysis. We evaluated changes in expression profile of candidate NCC markers following aggregation in the periocular region. We studied changes in gene expression profiles of components of the major signaling pathways (RA, TGFβ, and Wnt) associated with ocular development. We identified genes that are likely to be involved in pNC differentiation into corneal endothelium and keratocytes. Altogether, these data serve as a foundation to advance our understanding of the molecular mechanisms underlying pNC migration, proliferation and differentiation.

RESULTS AND DISCUSSION

Generation of a comprehensive transcriptome during pNC differentiation into corneal cells

During early ocular development in chick, the status of pNC differentiation can be categorized into three phases: (1) aggregation of NCC into the periocular region, which occurs between E2-E3; (2) migration of pNC into the presumptive corneal region to form the endothelial layer by E5; and (3) migration of pNC into the primary corneal extracellular matrix to form the stromal keratocytes by E7. At each of the above stages, NCC are subjected to different environmental cues that play important roles in their migration, proliferation, and differentiation (Brugmann et al., 2006; Lwigale and Bronner-Fraser, 2009). Our analysis of NCC contribution to early ocular development using the quail/chick chimera technique indicates that the periocular mesenchyme at E3 is mostly comprised of quail-derived QCPN-positive pNC that give rise to the corneal endothelium at E5, and to the keratocytes and endothelium at E7 (Fig. 1A).

To identify changes in gene expression during pNC formation corneal cells in chick, we isolated periocular mesenchyme from E3 (pNC), the monolayer of corneal endothelium at E5 (En), and the combined stroma and endothelium at E7 (KEn) (Fig. 1B). Three biological triplicates for each time point were prepared for RNA-Seq analysis as described in the methods section. Principal-components analysis (PCA) was applied to all mapped genes from the 9 samples to determine the reproducibility of biological repeats by Noiseq. The Scatter plots indicate good separation between pNC, En, and KEn, as well as good clustering of the three biological repeats of each sample, with system variance of 89% (Fig. 1C).

Identification of differentially expressed genes between pNC and corneal cells

To identify differentially expressed genes, we performed further analysis using a cutoff of abs(log2)≥1 and FDR<0.05 on genes that overlapped between NoiseqBio and EdgeR analyses (Supplementary Table 1). Based on these analyses, 3160 genes were differentially expressed between pNC and En (Fig. 1D). The 2101 upregulated genes represent En and the 1059 downregulated genes represent pNC. Likewise, 3800 genes were differentially expressed between pNC and KEn (Fig. 1D), of which 2307 upregulated genes represent KEn and the 1493 downregulated genes represent pNC. Given that both En and KEn contain corneal endothelial cells, we analyzed the difference between these two groups. Differentially expressed genes between En and KEn (2096) may either be involved in keratocyte differentiation or associated with further development of the corneal endothelium between E5 and E7. In this group, 846 genes are upregulated and 1250 genes are downregulated. KEGG pathway analysis indicated enrichment of similar pathways between pNC/En and pNC/KEn, with the top five corresponding to focal adhesion, ECM-receptor interaction, cell adhesion, melanogenesis, and MAPK signaling (Fig. 1E). A complete KEGG pathway list that includes the major signaling pathways during ocular development (RA metabolism, Wnt, and TGFβ signaling) is provided in Supplementary Table 2.

Changes in NCC gene expression in the periocular region and cornea

Given that NCC reside in the periocular region for approximately two days before their initial migration into the presumptive cornea (Hay and Revel, 1969), we investigated

whether they change their molecular signature prior to, and during their differentiation into corneal cells. A list of 47 genes was generated from candidate chick NCC genes (Simoes-Costa et al., 2014; Simoes-Costa and Bronner, 2015), and we examined their expression in pNC, En, and KEn. From this list, 43 NCC genes were expressed in pNC, of which 20 were downregulated, 7 were upregulated, and 10 were constitutively expressed in both En and KEn. Interestingly, some genes (6) were either downregulated or maintained in only En or KEn (Fig. 2A and Supplementary Table. 3). Of the 4 NCC genes that were not expressed in the pNC (*ADAM11, DLX6, ZIC3/4*), *ADAM11* was upregulated during corneal development. These results indicate that the molecular signature of NCC is maintained in the periocular region, and partially during early corneal development. This is supported by our previous study, showing that keratocytes isolated from E10 quail corneas were capable of differentiating into other NCC-derived tissues when grafted into the migratory stream of stage 9 chick embryos (Lwigale et al., 2005). To validate the expression of NCC genes, we chose one gene from each category based on whether they were downregulated (*MSX2*), maintained (*SNAI2*), or upregulated (*RHOB*) during corneal development and analyzed their spatiotemporal expression by in situ hybridization. Our results revealed that *MSX2* and *SNAI2* are expressed in the periocular region (Fig. 2B and 2C). Unlike *MSX2,* which is restricted to the periocular region, expression of *SNAI2* is maintained in the corneal endothelium (Fig. 4C, arrowhead) and stroma. In mouse, *Msx1* and *Msx2* are involved in NCC survival, proliferation, and differentiation (Liu et al., 1999; Ishii et al., 2005), and it is possible that some of the early functions are maintained only in the chick pNC. *SNAI2* is involved in the epithelial-mesenchymal transition required for NCC delamination from the neural tube (Taneyhill et al., 2007). We show that expression of *SNAI2* is maintained when pNC undergo mesenchyme-endothelial transition to form the corneal endothelium, which strongly expresses N-cadherin (Reneker et al., 2000; Lwigale et al., 2005). Inhibition of E-cadherin by *SNAI2* requires *LMO4* (Ochoa et al., 2012), which is downregulated in our RNA-Seq data (Fig 2A and Supplementary Table. 3), suggesting that *SNAI2* may play a different role that does not affect cell adhesion molecules in the corneal endothelium.

From our list of NCC genes, only *CDH11, CSRNP1, ERG, LIMS1, RHOB*, *TFAP2β, and MYC* are upregulated during corneal development. As an example from this group, we validated the spatiotemporal expression of *RHOB*. Our data indicate that *RHOB* is expressed by a few cells in the periocular region (Fig. 2D, arrow), then it is strongly expressed in the corneal endothelium (Fig. 2D, arrowhead), and in both the corneal endothelium and stroma at E7. During early development, *RHOB* is expressed in the dorsal neural tube and transiently in migratory NCC, and it is involved in the delamination process (Liu and Jessell, 1998). The function of *RHOB* during corneal development remains unclear, but it has been shown to play a critical role during barrier formation in vascular endothelial cells (Marcos-Ramiro et al., 2016). Our data also show upregulation of *TFAP2β,* which suggests its potential role during chick corneal development. Conditional knockout of *TfAp2β* in the NCC lineage in mice caused several defects in NCC-derived ocular tissues including malformation of the corneal endothelium and stroma (Martino et al., 2016).

Differentiation of pNC is regulated by multiple signaling pathways

Signals from the neural plate, ectoderm, and adjacent mesoderm mediate NCC formation at the neural plate border with the ectoderm. These include growth factors such as Wnt (Saint-Jeannet et al., 1997), bone morphogenetic protein (BMP; Liem et al., 1995), fibroblast growth factor (FGF; Monsoro-Burq et al., 2003), and RA signaling (Villanueva et al., 2002; Martinez-Morales et al., 2011). These signaling pathways regulate NCC formation at all embryonic axial levels, except for RA signaling, which is absent in the rostral cranial NCC streams that contribute to the eye (Maden et al., 1998). pNC experience similar signals from the adjacent ocular tissues (optic cup, lens, and ectoderm) prior to their migration into the presumptive corneal region. RA signaling is from the optic cup and ectoderm (Li et al., 2000; Matt et al., 2005; Molotkov et al., 2006), whereas *TGFβ* and *BMP* are expressed in the lens and optic cup (Trousse et al., 2001; Saika et al., 2002). Several *WNTs* are expressed in the ectoderm, optic cup, and lens (Jin et al., 2002; Fokina and Frolova, 2006). Interestingly, disruption of these major signaling pathways lead to dysgenesis of NCC-derived ocular tissues (Gage et al., 1999; Kitamura et al., 1999; Kume and Seo, 2010), but the mechanisms of how they are regulated and their action on pNC are not clearly understood. Our KEGG pathway analysis also indicates that the above signaling pathways change during corneal development (Supplementary Table 2), thus, we analyzed the expression of RA, TGFβ, and Wnt signaling components and their downstream targets.

RA signaling pathway—Genetic studies in mice showed that three retinaldehyde dehydrogenase enzymes (Raldh1/2/3) responsible for RA synthesis are all required for proper development (Dupe et al., 2003; Molotkov et al., 2006; Maden, 2007). Our data indicate high-level expression of components of the RA signaling pathway during pNC differentiation into corneal cells (Fig. 3A and Supplementary Table 4). These include transporter genes (*STRA6*, *CRABP1/2* and *RBP5, FABP5*), enzymes (*DHRS3*, *RDH5/12/14*, and *ALDH1A2*), regulator (*CYP26B1*), and nuclear receptors (*NR2F1/2*, *NR2C1*, *RXRA*, *RARA*, and *RARB, PPARD*). Of these genes, *ALDH1A2, DHRS3, and CYP26B1*, are significantly upregulated in En and KEn compared to pNC (Fig. 3A and Supplementary Table 4). Upregulation of *ALDH1A2* and *DHRS3* was confirmed by in situ hybridization to determine the specific localization of RA synthesis and inhibition, respectively, in the cornea. Although *ALDH1A2* is strongly expressed in the optic cup at E3, it is not expressed in the pNC. Consistent with our RNA-Seq data, *ALDH1A2* is vividly expressed in the corneal endothelium at E5 and E7 (Fig. 3C, arrowheads), with sparse staining in the stroma. Similarly, expression of *DHRS3* is minimal in the pNC at E3, but robust in the corneal endothelium at E5 and E7 (Fig. 3D, arrowheads), and also expressed in the stroma. Our data show for the first time that the chick corneal endothelium expresses *ALDH1A2* and thus, acts as a potential source for RA signaling, which may act in either an autocrine or paracrine fashion to regulate corneal development. Interestingly, our results also suggest that the RA inhibitors DHRS3 and CYP26B1 (Sakai et al., 2001; Feng et al., 2010; Kam et al., 2013) may act at the same time to modulate the levels of signaling in the corneal endothelium and stroma. This is in conjunction with previous observation that RA signaling intensified in various ocular tissues including the cornea of *Cyp26a1* null mice (Sakai et al., 2004).

Downregulated RA genes in En and KEn include *CRABP1, NR2F1* and *NR2F2* (Fig. 3A and Supplementary Table 4). Downregulation of *CRABP1*, which promotes RA degradation (Dong et al., 1999; Michalik and Wahli, 2007), may cause an increase in nuclear RA mediated by *CRABP2* that is constitutively expressed. *NR2F1* and *NR2F2* (also known as *COUP-TF1* and *COUP-TF2*) are transcription factors that function as RA nuclear receptors (Kruse et al., 2008; Pickens et al., 2013), but they have also been shown to inhibit RA signaling by binding to retinoic acid response elements (RAREs) of downstream genes (Kliewer et al., 1992; Tran et al., 1992). Our in situ hybridization analysis confirms that *NR2F2* is expressed in the pNC (Fig. 3E, arrow), but it is not detectable in the corneal endothelium or stroma (Fig. 3E). A similar pattern of downregulation is expected for the expression of *NR2F1*, which may indicate a potential increase in expression of RA-target genes in the corneal endothelium and stroma.

Activation of RA signaling leads to upregulation or downregulation of target genes (Balmer and Blomhoff, 2002; Matt et al., 2005). Examples of RA-target genes in the cornea include transcription factors that are either upregulated (*PITX2, DKK2, PAX6*, *TFAP2β*) or downregulated (*SOX9, MSX1, OTX2, LMO2*) (Fig. 3A and 3E, and Supplementary Table 4). *Pitx2*, *Foxc1, Pax6* play important roles during mouse corneal development (Smith et al., 2000; Kanakubo et al., 2006; Gage et al., 2014), and they are among the ASD genes linked to dysgenesis of the human cornea (Sowden, 2007).

TGFβ pathway—The TGFβ superfamily comprises of TGFβs, BMPs, and other ligands, which regulate differentiation, proliferation, migration, and apoptosis in numerous cell types including the NCC (Shah et al., 1996; Chai et al., 2003; Wurdak et al., 2005). Comparison of En and KEn to pNC indicated that *TGFβ1/2/3* and *BMP4/7* are expressed during corneal development. *TGFβ2* was upregulated and *BMP5* was the only gene downregulated in En and KEn (Fig. 4A and Supplementary Table 5). In mice TGFβ2 signaling from the lens induces expression of *Pitx2* and *Foxc1* in the NCC mesenchyme of the presumptive cornea, and its absence causes severe ocular defects including corneal thinning and absence of the corneal endothelium (Saika et al., 2001; Ittner et al., 2005). Ectopic expression of BMP5 in the chick neural retina transforms it into retinal pigment epithelium (Steinfeld et al., 2017), but its function in the pNC remains unclear.

The following regulators of TGFβ (*CRM1, THBS1, LTBP1/2, DCN, TGIF1/2, SMURF1/2, RBX1, SKP1* AND *CUL1*) are expressed during corneal development. Of these, *THBS1, LTBP1/2,* and *DCN* are upregulated in En and KEn (Fig. 4A and Supplementary Table 5), indicating their potential function during corneal development. Thbs1 activates TGFβ and inhibits angiogenesis (Good et al., 1990; Schultz-Cherry et al., 1994; Tolsma et al., 1997). In the adult cornea, *THBS1* is expressed in the corneal endothelium and stroma (Hiscott et al., 2006), where it plays a role as an antiangiogenic agent during wound healing (Hiscott et al., 1999; Matsuba et al., 2011; Blanco-Mezquita et al., 2013). *LTBP1* is expressed in a few cells in the periocular region, but it is robust in the corneal endothelium and stroma (Fig. 4B). LTBP1 plays an important role during secretion, deposition, and activation of TGFβ in glioma cells (Tritschler et al., 2009) and it is possible that it plays similar roles during corneal development. DCN inhibits TGFβ signaling by binding directly to its active form (Yamaguchi et al., 1990; Border et al., 1992), and it has also been shown to prevent

fibrillogenesis of corneal collagen and to cause fibrosis during wound healing (Rada et al., 1993; Mohan et al., 2010). Therefore, upregulation of *DCN* in En and KEn indicates potential modulation of TGFβ signaling, which might be required for activation of specific downstream genes during corneal development.

Our data indicate that *BAMBI* is the only TGFβ regulator that is downregulated in En and KEn (Fig. 4A). BAMBI is a pseudo-receptor with similar structure to TGFβR1 that inhibits TGFβ signaling and activates canonical Wnt signaling (Onichtchouk et al., 1999; Lin et al., 2008). We confirmed that *BAMBI* is expressed in the pNC (Fig. 4C, arrowhead) where it may play a role in Wnt signaling, but it is not detectable in the corneal endothelium and stroma, which correspond with the upregulation of TGFβ signaling in the cornea.

The TGFβ superfamily receptors *TGFβR1/2*, *BMPR1A/2*, and downstream components including *SMAD1/2/4/5/6* are all constitutively expressed in pNC, En, and KEn (Fig. 4A and Supplementary Table 5). Several transcription factors downstream of TGFβ signaling are either upregulated (*MYC, TWIST2, FOS*) or downregulated (*SOX9, LIN28A*). Surprisingly, some TGFβ activated transcription factors such as *SNAI1, TBX3, SOX9,* and *LIN28A* are downregulated, whereas some that are inhibited by TGFβ (*IRX3* and *MYC*) are upregulated (Fig. 4E and Supplementary Table 5). Our data also indicate that majority of the transcription factors activated by BMP signaling such as *ID1/2/3*, *MSX1*, and *HEY1* are downregulated during corneal development (Fig. 4A and 4E and Supplementary Table 5). An example of a TGFβ downstream target that is upregulated in En and KEn is *TGFβ-induced* (*TGFβI*, also known as *BIGH3*), which was confirmed to be expressed in the corneal endothelium (Fig. 4D, arrowhead) and stroma. *TGFβI* encodes an ECM protein induced by TGFβ that interacts with collagen (Skonier et al., 1992; Hashimoto et al., 1997). Recent studies have shown that mutations in *TGFβI* in humans cause a condition known as granular corneal dystrophy, which is characterized by opaque deposits in the corneal stroma (Kattan et al., 2017; Nielsen et al., 2017; Chao-Shern et al., 2018). Therefore its upregulation in our data suggests that TGFβI may play a role in organizing collagen fibrils synthesized by the corneal endothelium and keratocytes during early development.

Wnt signaling—Defects in Wnt signaling pathway cause dysgenesis of the anterior ocular tissues in humans and mice (Ittner et al., 2005; Reis and Semina, 2011; Bankhead et al., 2015). Analysis of the components of the Wnt signaling pathway indicates that despite the expression of several ligands in chick ocular tissues (Fokina and Frolova, 2006), only *WNT2B, WNT4, WNT5A, WNT6, WNT9A, and WNT9B* are highly expressed in the pNC and its corneal derivatives during development (Fig. 5A and Supplementary Table 6). Genes representing regulators of Wnt signaling at multiple levels including *FRZB, DACT1/2, DKK3, SFRP1/2, BAMBI, AXIN1/2, NLK, GROUCHO, WLS, AES*, and *CTBP1* are also constitutively expressed in pNC, En and KEn. In this group, only *WLS* and *BAMBI* mediate Wnt signaling and the rest are inhibitors. We confirmed by in situ hybridization that both *WLS* and *AES* are constitutively expressed in the pNC, corneal endothelium, and stroma (Fig. 5B and 5C). Wnt receptors and co-receptors including *FRZ1/2/6/7/9* and *LRP5*/6 are also constitutively expressed. *FRZ8/10* are upregulated in En but downregulated in KEn, and *FRZ4* is downregulated during corneal development (Fig. 5A and Supplementary Table 6). Downstream components of Wnt signaling such as *DVL1/3*, *GSK3B*, and *CTNNB1* are

either upregulated or constitutively expressed, whereas *TCF7, TCF7L1* and *LEF1* are downregulated. Combined, our data suggest that the pNC, corneal endothelium and keratocytes have the potential for canonical Wnt signaling. However, the expression of multiple inhibitors including the upregulation of *DKK2* suggest that Wnt signaling is regulated at multiple levels during corneal development. We therefore examined the expression of Wnt target genes in En and KEn and observed that some Wnt-upregulated genes are downregulated (*SOX17, FST, JAG1, STRA6, SALL4, RET, TBX3*), whereas others are upregulated (*NOS2, MYC, AXIN2, FN1, CYR61, PITX2, MMP2, LBH, WISP1*) in our data (Fig. 5E and Supplementary Table 6). However, *CLDN1* is upregulated in En but downregulated in KEn, and *SP5* is downregulated in En but upregulated KEn. Our data also show that some Wnt-downregulated genes (*TNFRSF1, CDH1*) are upregulated during corneal development. As an example of a Wnt target gene, we confirmed the expression of *PITX2* and observed vivid expression in the pNC, En, and KEn (Fig. 5D). Mouse studies have shown that Pitx2 is activated by Wnt signaling (Kioussi et al., 2002; Briata et al., 2003), but also indirectly acts as a Wnt inhibitor via upregulation of Dkk2 (Gage et al., 2008).

WNT11, the main ligand for the Wnt/PCP pathway, is upregulated in KEn, and *WNT5A*, which functions in both the Wnt/calcium and Wnt/PCP pathways (Yamanaka et al., 2002; Sato et al., 2010), is maintained at all stages of corneal development. Components of the Wnt/PCP pathway (*DAMM2, MAPK10, ROCK2, PRICKLE2, RHOA, RAC1, CDC42)* and Wnt/calcium pathway (*NFACTC1, PRKCA, CAMK2D, RYK, CAMK2B*) are either upregulated or constitutively expressed. These results raise the possibility that non-canonical Wnt signaling occurs during corneal development and it may be involved in inducing cell migration and polarity.

Differential expression of transcription factors during early corneal development

Our data indicate that differential expression of components of the major signaling pathways (RA, Wnt, TGFβ) correspond with the transformation of pNC into corneal endothelium and keratocytes. To identify the genes involved in the process of pNC differentiation into corneal cells, we examined the changes in expression of transcription factors (Table 1 and Supplementary Table 7). Upregulated genes with known ocular functions include *PAX6 and ZNF469. PAX6*, which is induced by RA signaling, is expressed in various ocular tissues and it has been shown to play a major role in eye development (Hill et al., 1991; Fujiwara et al., 1994; Baulmann et al., 2002). Analysis of NCC-derived cells in $Pax6^{sey/+}$ mice showed abnormal migration into the eye (Kanakubo et al., 2006). *ZNF469* is involved in ECM synthesis and defects in this gene cause connective tissue disorders in humans including an ocular defect known as brittle cornea syndrome (Zlotogora et al., 1990; Al-Hussain et al., 2004; Vincent et al., 2014). Based on these examples, the upregulated genes may represent transcription factors involved in key processes of corneal development including cell migration, proliferation, and differentiation.

The downregulated genes represent transcription factors that are only expressed in the pNC (Table 1 and Supplementary Table 7). Among these are some of the *bona fide* NCC genes such as *FOXD3*, *SOX9*, and *SOX10* (Fig. 2A). *FOXD3* is required for self-renewal and

maintenance of NCC multipotency (Labosky and Kaestner, 1998; Dottori et al., 2001; Mundell and Labosky, 2011), thus its downregulation may be required during pNC differentiation into corneal cells. *SOX9* plays an early role during NCC induction (McKeown et al., 2005), but it is later required for chondrogenesis (Zhao et al., 1997; Sahar et al., 2005). Therefore, it is possible that the expression of *SOX9* in the pNC may promote the formation of orbital bones, whereas its downregulation in En and KEn might be involved in preventing ossification of the cornea. Similarly, *SOX10* is required during NCC induction, but it is later involved in their differentiation into non-mesenchymal derivatives including melanocytes and peripheral neurons (Southard-Smith et al., 1998; Dutton et al., 2001). Downregulation of *SOX10* in En and KEn raises the possibility that both the melanocyte and neural lineages are prevented in the cornea.

Also, among the downregulated genes are representatives of transcription factors not expressed by cranial NCC, therefore we considered them specific for the periocular mesenchyme. pNC that do not form the cornea give rise to various skeletomuscular derivatives and also contribute pericytes to the ocular vasculature (Creuzet et al., 2005a). This is reflected in the pNC transcription factors that play important roles in osteogenesis, myogenesis, and vasculogenesis. For example, *HEY1* is a downstream target of Notch signaling that is involved in vasculogenesis and osteoblastic differentiation (Fischer et al., 2004; Salie et al., 2010). *DACH2* is required during eye and limb development in drosophila (Mardon et al., 1994), and it synergizes with *EYA2* during myogenesis in chick (Heanue et al., 1999). *CITED2* is a negative regulator of *Hif-1* that is required for normal lens development and regression of the hyaloid vasculature (Chen et al., 2009).

Identification of genes involved in pNC differentiation into corneal endothelium

The corneal endothelium is comprised of polarized cells that form tight junctions and transport fluids between the cornea and anterior chamber, which is critical for maintaining stromal deturgescence and transparency (Waring et al., 1982). Our data indicate that most genes involved in membrane transport, cell polarity, and cell junctions are upregulated in both En and KEn (Table 2 and Supplementary Table 8). Examples of membrane transporter genes that are upregulated in the corneal endothelium include *AQP1* and *ABCB5*. AQP1 is a bi-directional water transporter (Sui et al., 2001), which indicates potential increase in fluid absorption during pNC differentiation into corneal cells. ABCB5 is an ATP-dependent transporter that is also a well-known marker for limbal stem cells (Chen et al., 2005; Ksander et al., 2014).

The characteristic hexagonal shape of the corneal endothelium is due to forces that straighten the actomyosin fibers on the apical surface that faces the anterior chamber. The apical surface is also marked by ZO-1, whereas the basal surface that is in contact with the Descemet's membrane is labeled by α3β1 (He et al., 2016). Interestingly, although *ZO-1* (*TJP1*) is a major component of the adult corneal endothelium (Petroll et al., 1999; Ramachandran and Srinivas, 2010), it was not upregulated in our data sets (Supplementary Table 8). Suggesting that its expression and function may arise at a later time point than the stages of corneal development used for this study. Our data indicate that *THY1*, which is involved in cell polarity, is upregulated (Table 2). THY1 is predominantly expressed in

neurons, where it is involved in cell attachment, migration and polarization (Tiveron et al., 1994; Leyton et al., 2001; Kong et al., 2013). THY1 is also localized on apical surfaces of epithelial cells (Powell et al., 1991), but it has not been studied in the corneal endothelium.

Genes involved in cell junctions include *CLDN4, CLDN3, AND CDH2 (N-CADHERIN).* Of these genes, *CLDN3* is involved in tight junction barrier function in the submandibular glands (Mei et al., 2015; Yokoyama et al., 2017), and it is possible that it plays a similar role in the corneal endothelium. *CDH2* mediates cell adhesion in neural cells and it is a well-known marker for corneal endothelial cells (Reneker et al., 2000). Its upregulation indicates the potential for formation of intercellular bonds at the onset of endothelial differentiation.

We also detected genes that are highly expressed in the adult corneal endothelium (Chng et al., 2013). These were either upregulated (*MGARP, MYOC, COL8A2*, *LDHA*) or constitutively expressed at all three time points (*VIM, TSPAN6, TPJ2, ATP1B1, TPT1, PRDX6*) (Table 2 and Supplementary Table 8).

Identification of genes involved in pNC differentiation into keratocytes

Keratocytes are characterized by their ability to synthesize the stromal ECM, which is comprised of collagens and proteoglycans that constitute more than 90% of the cornea (Knupp et al., 2009; Hassell and Birk, 2010). Our data show that genes for several ECM-related proteins are upregulated during corneal development (Table 3 and Supplementary Table 9). A majority of the upregulated corneal ECM genes belong to collagen (Supplementary Table 9). They represent fibrillar collagens (*COL1/2/5/6/11/27*) required for the corneal structure, and nonfibrillar collagens, which are either associated with the collagen fibrils (*COL9/14/16*) or expressed on cell surfaces (*COL4/8/17*) (Shaw and Olsen, 1991; Kadler et al., 1996; Knupp et al., 2009). Collagens form the bulk of the corneal stroma and associate with ECM proteins and regulatory enzymes that align and space the structural fibrils in configurations that elicit transparency (Rada et al., 1993; Michelacci, 2003; Hassell and Birk, 2010). Our data also show that genes for several proteins expressed in the adult cornea are upregulated in En and KEn. They include glycoproteins (*NTN3, VIT, SPON2, OLFML2B,* and *THSB2*), proteoglycans (*OGN[Mimecan], KERA,* and *DCN*), other ECM proteins (*EGFL6, NPNT, PTN,* and *LTBP1/2*), and regulatory enzymes (*MMP27*, *ADAMTS2/5/8,* and *COLGALT2*). We also identified some ECM genes that are significantly downregulated in the En and KEn (*VWF*, *VTN, HAPLN1, LEPREL1, HPSE2* and *CSGALNACT1*). Given the functional differences between the corneal endothelium and keratocytes (Waring et al., 1982; Linsenmayer et al., 1984; Quantock and Young, 2008), we were surprised that many of the ECM related genes were expressed by both cell-types. One possibility is that some of these genes are transiently expressed in the corneal endothelium, and this could be resolved by comparing their localization at later stages of development. It is also possible that most of the ECM proteins are intracellularly degraded upon synthesis in the corneal endothelium (Ko and Kay, 2001; Lee et al., 2012), and do not contribute to the corneal matrix.

CONCLUSIONS

We have used RNA-Seq analysis to provide an unbiased depiction of gene expression profile of NCC in the periocular region and during their differentiation into corneal endothelium and stromal keratocytes. We confirmed the expression patterns of candidate genes at in the periocular region and during corneal development by in situ hybridization. We highlighted minor deviations in gene expression following aggregation of cranial NCC in the periocular region, suggesting that pNC maintain a level of multipotency that enables their contribution to various ocular structures later in development. We focused on three major pathways (RA, Wnt and TGFβ) involved in corneal development and identified for the first time that several modulators are upregulated during pNC differentiation into corneal cells, which could be critical in directing the expression of downstream transcription factors with potential roles in establishing endothelial and keratocyte identities. Previous studies have shown that there is crosstalk between these pathways during corneal development. The RNA-Seq data provides a useful tool for generating gene regulatory networks for pNC migration, proliferation, and differentiation. These data not only provide a foundation for identifying genes with novel function during corneal development, they also provide a valuable resource for future studies of corneal diseases, stem cell biology, and bioengineering of corneal tissues.

EXPERIMENTAL PROCEDURES

Animals

Fertilized White Leghorn chicken (Gallus gallus domesticus) and Japanese quail eggs (Coturnix coturnix japonica) were obtained from commercial sources and incubated at 38°C under humidified conditions. Quail-chick chimeras were generated to track cells of neural crest origin in ocular tissues by grafting dorsal neural tube explants from HH stage 9 (Hamburger and Hamilton, 1951) quail donors to stage-matched chick embryos as previously described (Lwigale et al., 2005). Chick and chimeric embryos were incubated until E3, E5, or E7. Embryos were manipulated according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Rice University.

Tissue collection from chick embryos

Periocular neural crest (pNC) mesenchyme was obtained by dissecting the anterior half of the eyes from E3 embryos. Corneal endothelium (En) tissues were obtained by trimming E5 corneas at boundary with the periocular region. Combined keratocytes and corneal endothelium (KEn) tissues were obtained by trimming E7 corneas at the boundary with the limbus region. All tissues were incubated in dispase (1.5 mg/ml; Worthington) at 38°C for 5 minutes, then rinsed twice in Ringer's solution. For the pNC, the ectoderm, lens vesicles, and optic cups were removed and discarded, and the periocular mesenchyme from 26 eyes was pooled into each sample. For the En, the corneal epithelium was removed and discarded, and the endothelial layers from 120 eyes were pooled into each sample. For the KEn, the corneal epithelium was removed and discarded, and the stroma and endothelium from 10 corneas were pooled into each sample. The KEn samples included the corneal endothelium to capture any changes in gene expression at this time point. Three biological repeats were made for each group for a total of 9 samples. All samples were immediately immersed in

Trizol (Life Technologies), flash frozen in liquid nitrogen, and shipped in dry ice to BGI.US® for library preparation and sequencing.

RNA Sequencing and Mapping

Sequencing library of each sample was prepared according to Illumina Standard Protocols. High-throughput sequencing was performed by Illumina HiSeq™4000 (Single Read, 50bp) and generated an average of 47,074,369 raw sequencing reads from each sample. Raw reads were filtered by removing reads with adaptors, removing reads with more than 10% unknown bases, and removing low quality reads (if more than 50% of base has Phred quality score (Q score) <5). An average of 46,928,426 clean reads with Q30 >95% (Richterich, 1998) were generated for all nine samples for further analysis. Clean reads were mapped to Gallus gallus reference assembly Galgal5 and annotated gene model (GenBank: GCA_000002315.3) using Bowtie 2 (Langmead and Salzberg, 2012) and HISAT (Kim et al., 2015). The average mapping ratio with reference gene was 76.26% and average genome mapping ratio was 93.10%. The mapped reads were then counted by RSEM (Li and Dewey, 2011). Transcripts Per Kilobase Million (TPM) values were generated by normalization of counts with library size and gene length (Wagner et al., 2012).

Data access

RNA-Seq data sets have been deposited in the in the NCBI's GEO database (https://www.ncbi.nlm.nih.gov/geo/browse/). Accession number (GSE120434).

DEGs and Pathway Analyses

Differentially expressed genes (DEGs) were screened out by EdgeR (Robinson et al., 2010) and NoiseqBio (Tarazona et al., 2011; Tarazona et al., 2015). Each pair (En/pNC, KEn/pNC, or KEn/En) were compared and DEGs chosen based on the following standards: (1) CPM (count per million) greater than 1 in at least three samples; (2) Log2 fold change greater than 1 in both EdgeR and NoiseqBIO; (3) FDR (corrected p-value, q-value) less than 0.05 in EdgeR and Probability (equal to 1-FDR) more than 95% in NoiseqBio. To reduce false positive rate, overlapped (common) genes from EdgeR and NoiseqBio were defined as DEGs for further analysis (Supplementary Table 1). KEGG pathway analysis (Kanehisa et al., 2012) was performed by DAVID Bioinformatics Resources (Huang da et al., 2009). Top significant pathways were ordered by q-value, which is a corrected p-value by multiple hypothesis testing using the Benjamini-Hochberg, with 0.01 as a cutoff.

Neural crest genes with TPM greater than 1 in three pNC samples are considered to be expressed. Genes associated with RA, Wnt and TGF-beta pathways were analyzed with KEGG pathway, Map Database, and published data from Pubmed. Transcription factors, corneal endothelium, and ECM genes were analyzed with various tools including KEGG Orthology database, KEGG pathway database, InterPro database, and published data from Pubmed.

In situ hybridization

Freshly isolated E3 chick heads and either E5 or E7 eyes were fixed overnight at 4°C in modified Carnoy's fixative (60% ethanol, 30% formaldehyde, and 10% glacial acetic acid).

Samples were embedded in paraffin and sectioned between 10–12 μm. Probes were designed by Primer-blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) or adapted from Gallus Expression in Situ Hybridization Analysis (Geisha; http://geisha.arizona.edu/geisha/) and cloned into pCR®II-TOPO® vector or pCR®4-TOPO® with dual promoters (Invitrogen). A list of primer sequences used for generating in situ probes is provided in Table 4. Digoxigenin-labeled riboprobes were generated by in vitro transcription with Superscript III®. Section in situ hybridization was performed as previously described (Etchevers et al., 2001). Brightfield images were captured using a Zeiss Axiocam mounted on AxioImager2 microscope (Zeiss).

Immunostaining

Paraffin sections of E3, E5, and E7 quail-chick chimeras were rehydrated and prepared for immunostaining following standard protocols. Mouse anti-QCPN monoclonal antibody (1:1, IgG1, DHSB) was used to identify quail neural crest-derived cells during corneal development. Secondary antibody (Alexa 594 Goat anti-mouse IgG1, Invitrogen) was used at a concentration of 1:200. Imaging was performed with AxioImager2 fluorescent microscope with ApoTome (Zeiss).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key findings:

- RNA-Seq profiling revealed enrichment of genes between periocular neural crest corneal endothelium, and keratocytes.
- The top KEGG pathways of the differentially express genes include focal adhesion, ECM-receptor interaction, cell adhesion, melanogenesis, and MAPK signaling.
- Candidate neural crest genes are expressed in the periocular neural crest, but they are either differentially expressed or maintained during cornea development.
- Modulators of major signaling pathways associated with ocular development (retinoic acid, TGFβ, and Wnt), transcription factors, and differentiation genes were differentially expressed.

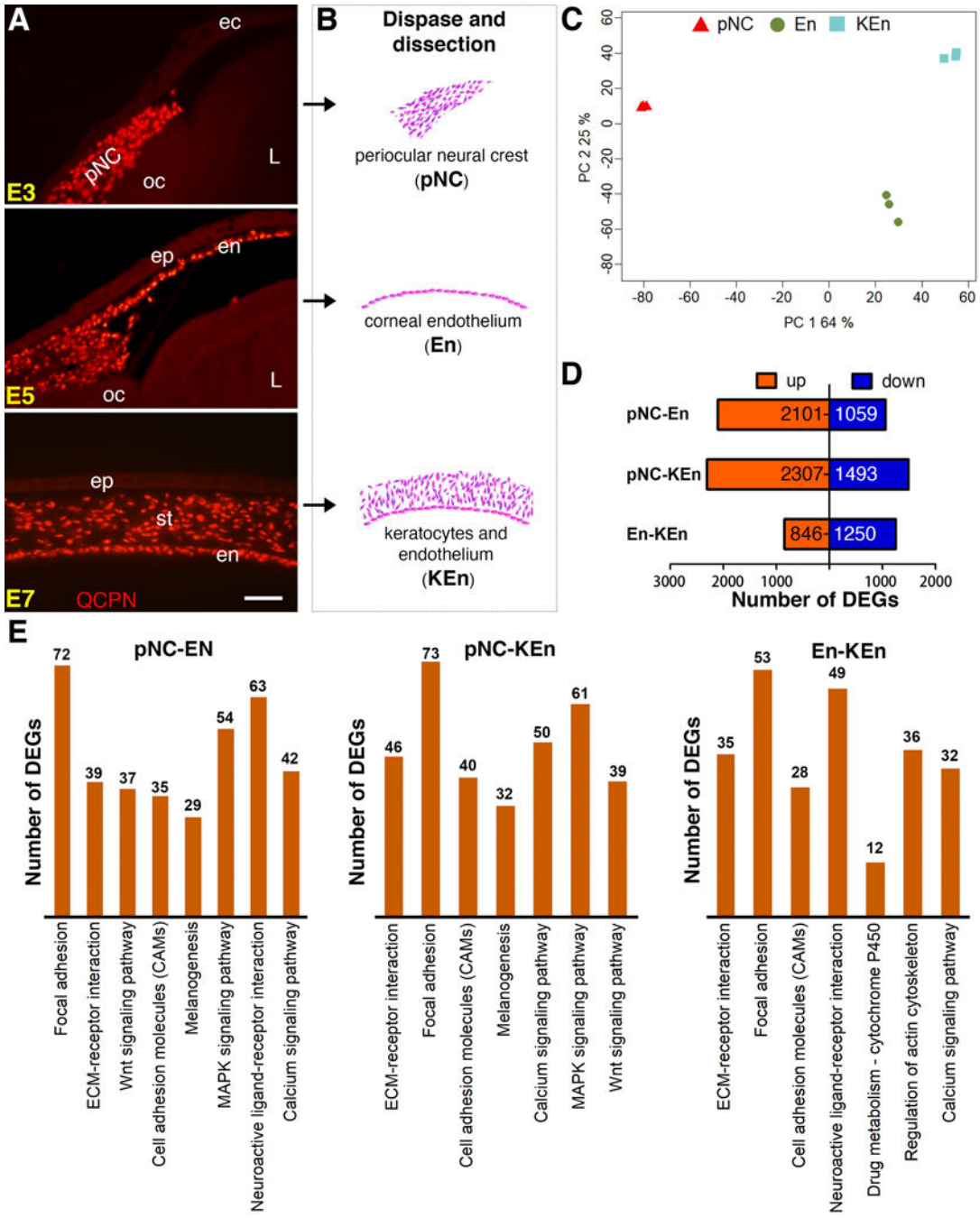

Figure 1. Experimental design for tissue isolation, RNA-Seq analysis, and comparison of genes identified in pNC, En, and KEn.

(A) Localization of neural crest cells in the periocular region and their corneal derivatives revealed by immunostaining cross sections of quail-chick chimera eyes for QCPN (red) at various stages of ocular development. (B) Periocular neural crest cells (pNC), corneal endothelium (En), or the combined keratocytes and corneal endothelium (KEn) were isolated at E3, E5, and E7, respectively. (C) PCA plot showing that the triplicate samples from pNC (Red), En (Green), and KEn (Blue) cluster together. Principal components 1 and 2 summarize 89% of the system variance. (D) Bar graph showing numbers of differentially

expressed genes (red indicates upregulated and blue indicates downregulated genes). (E) Histogram representation indicating the number of DEGs between pNC-En, pNC-KEn, and En-KEn belonging to significant KEGG pathways. Abbreviations: ec, ectoderm; oc, optic cup; L, lens; ep, epithelium; en, endothelium; st, stroma. Scale bar: 100 μm.

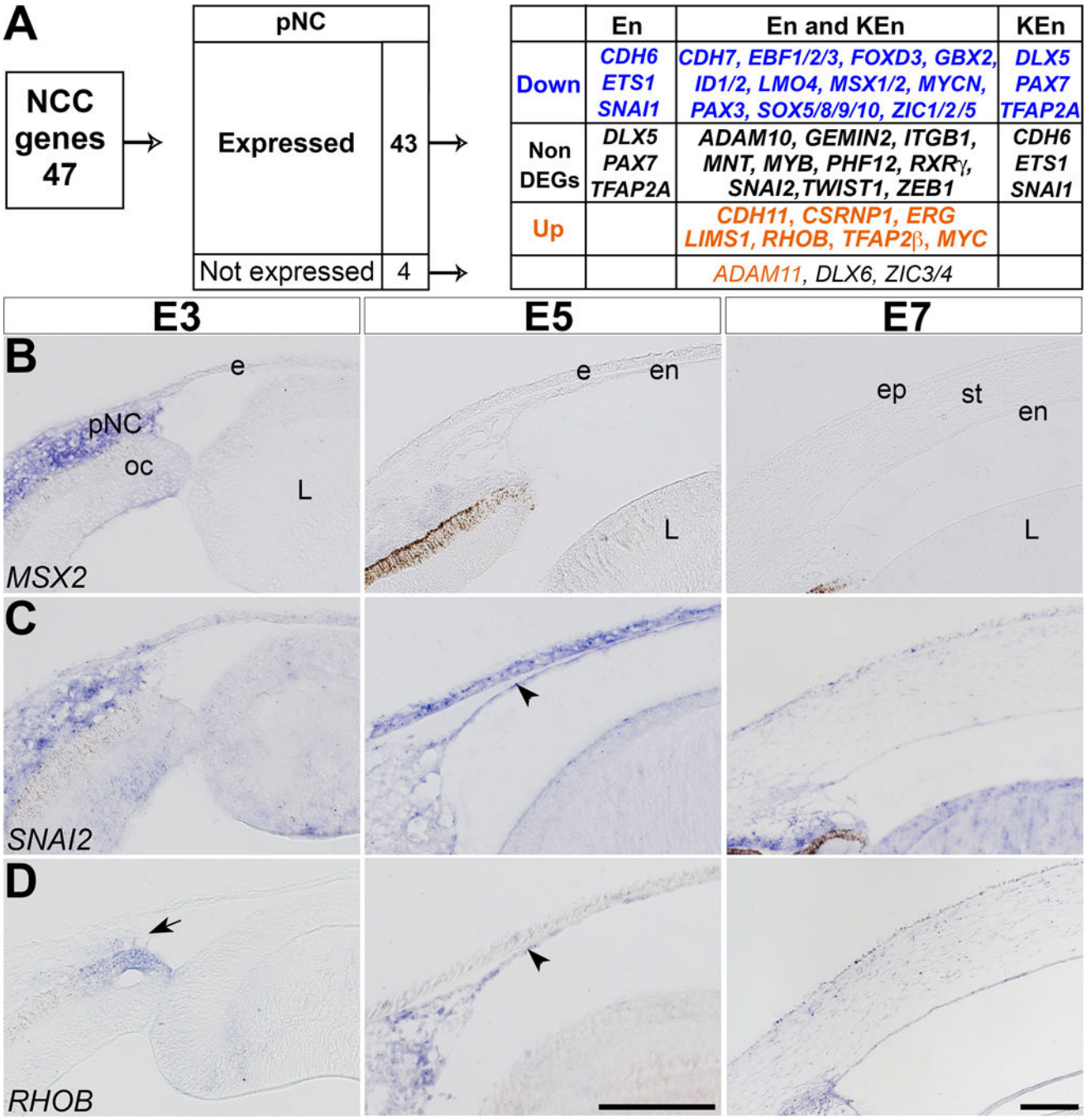

Figure 2. Changes in the molecular identity of NCC during their localization in the periocular region and differentiation into corneal cells.

(A) Analysis based on 47 candidate NCC genes indicates that 43 genes were expressed and 4 were not expressed in the periocular region. Of the 43 expressed genes, 20 were downregulated, 10 were constitutively expressed, and 7 were upregulated during corneal development. (B-D) Section in situ hybridization of E3, E5, and E7 anterior eyes indicating that: (B) *MSX2* is strongly expressed in the periocular region but undetectable in the corneal endothelium and stroma; (C) *SNAI2* is maintained at all three time points; and (D) *RHOB* is minimal in the periocular region, but it is strongly expressed in the corneal endothelium and

stroma. Arrow indicates periocular region and arrowheads indicate corneal endothelium. Abbreviations: pNC, periocular neural crest; ec, ectoderm; oc, optic cup; L, lens; ep, epithelium; en, endothelium; st, stroma. Scale bars: 100 μm.

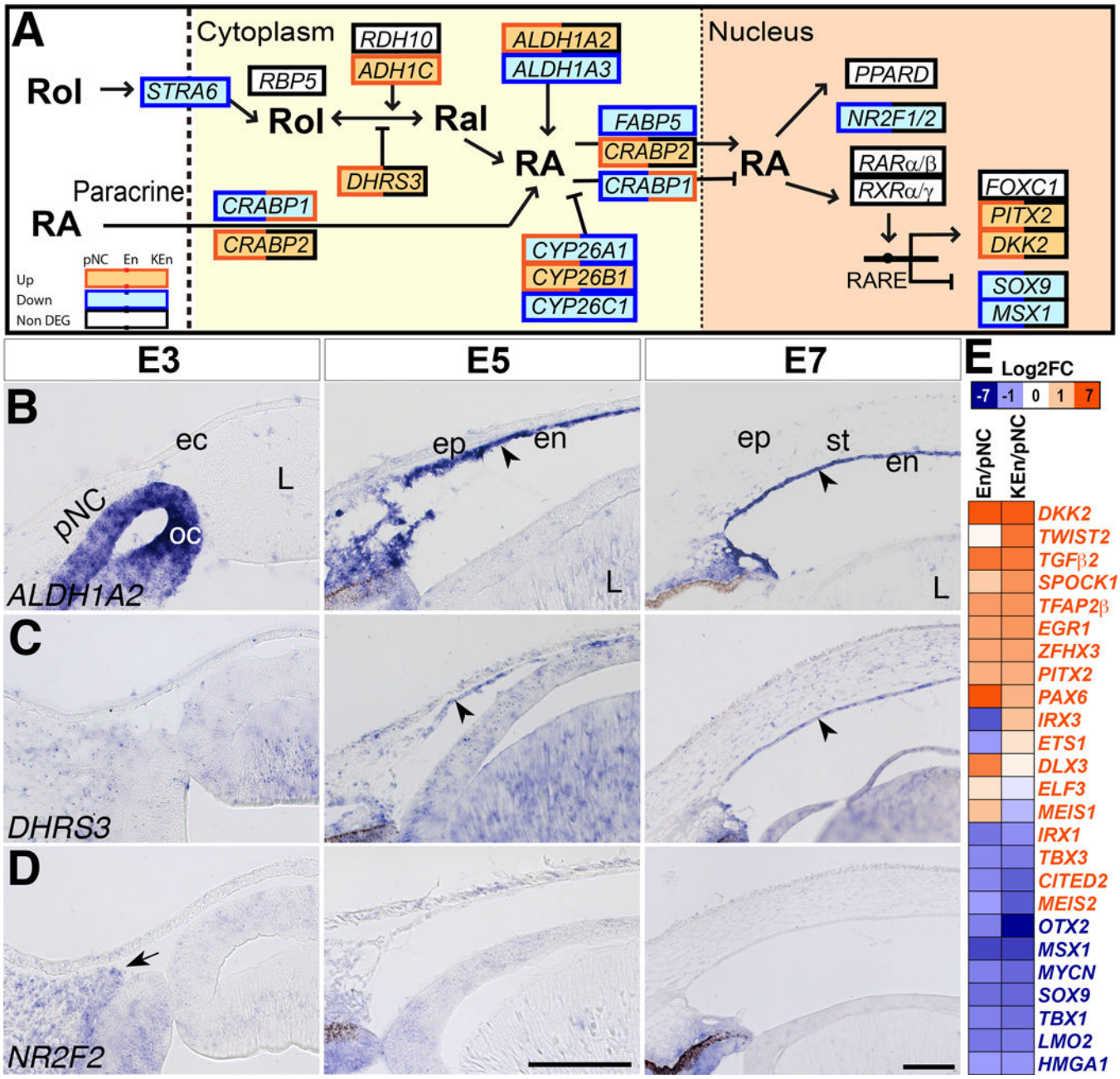

Figure 3. RA signaling pathway.

(A) Black boxes indicate constitutively expressed genes, red boxes indicate upregulated genes, and blue boxes indicate downregulated genes. Double colored boxes indicate partial upregulation, downregulation, or maintenance during corneal development. (B-D) Section in situ hybridization of E3, E5, and E7 anterior eyes showing that: (B) *ALDH1A2* is expressed in the optic cup but not the periocular mesenchyme at E3, but it is vividly expressed in the corneal endothelium (arrowhead), and sparsely expressed in the stroma at E7. (C) Expression of *DHRS3* is ubiquitous but low at E3, but becomes strong in the corneal endothelium (arrowhead) and stroma. (D) *NR2F2* is expressed in the periocular mesenchyme at E3 (arrow), but it is not detectable in the corneal endothelium and stroma at

E5 and E7. (E) Heatmap showing the relative upregulation and downregulation of RA target genes between En/pNC and KEn/pNC. Font color of genes indicates RA-upregulated (red) or RA-downregulated (blue) genes. Abbreviations: pNC, periocular neural crest; ec, ectoderm; oc, optic cup; L, lens; ep, epithelium; en, endothelium; st, stroma. Dotted lines represent the cell and nuclear membranes. Scale bars: 100 μm.

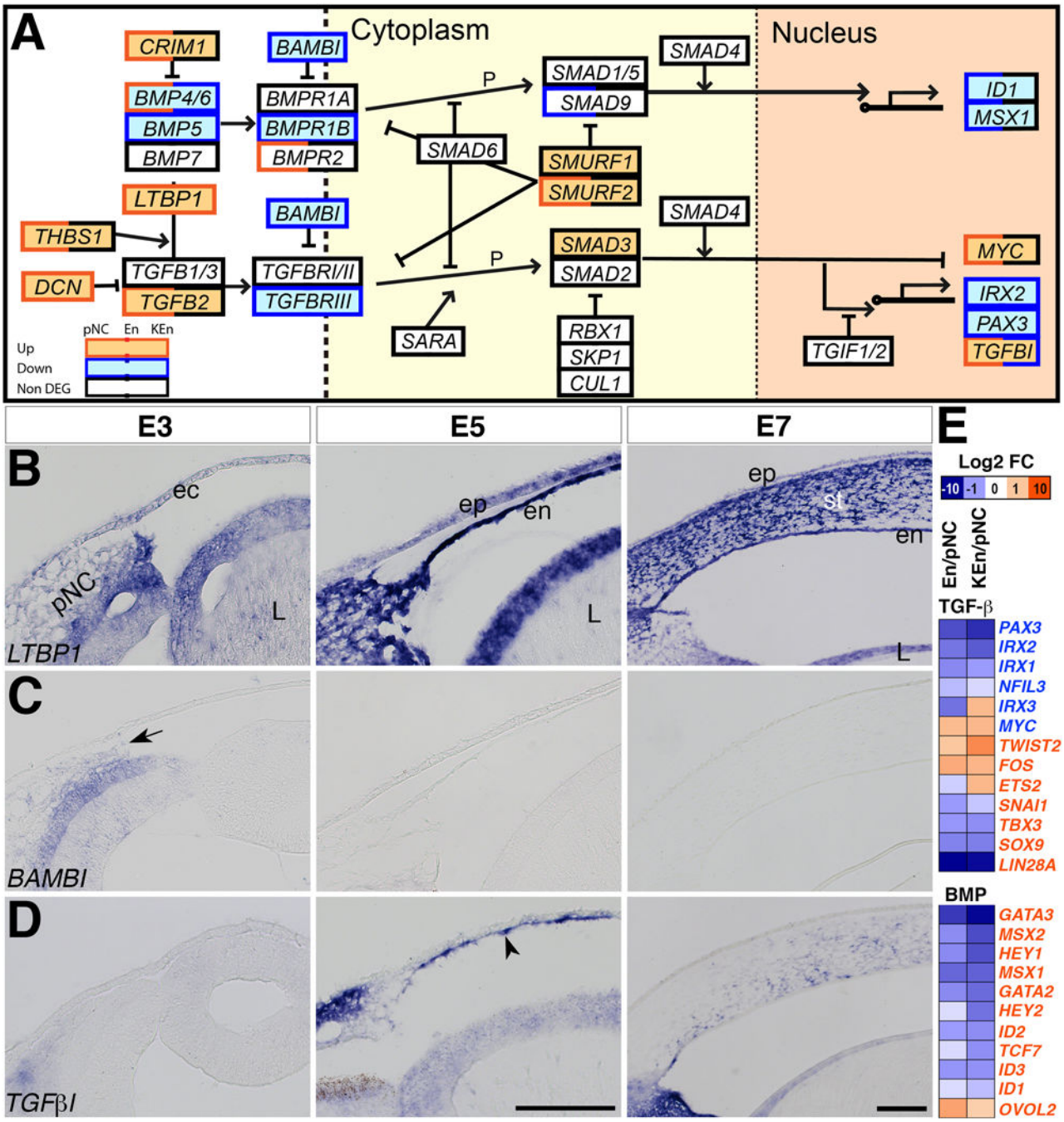

Figure 4. TGFβ Signaling pathway.

(A) Black boxes indicate constitutively expressed genes, red boxes indicate upregulated genes, and blue boxes indicate downregulated genes. Double colored boxes indicate partial upregulation, downregulation, or maintenance during corneal development. (B-D) Section in situ hybridization of E3, E5, and E7 anterior eyes showing that: (B) *LTBP1* is lightly expressed in the periocular mesenchyme at E3 and it is strongly expressed in the corneal endothelium and stroma. (C) *BAMBI* is expressed in the optic cup and low in the periocular mesenchyme and it is not detectable in the corneal endothelium and stroma. (D) Expression

of *TGFβi* is also low in the periocular mesenchyme at E3, but it is vividly expressed in the corneal endothelium at E5 (arrowhead) and stroma at E7. (E) Heatmap showing the relative upregulation and downregulation of TGFβ and BMP target genes between En/pNC and KEn/pNC. Font color of genes indicates TGFβ- or BMP-upregulated (red) or TGFβ-downregulated (blue) genes. Abbreviations: pNC, periocular neural crest; ec, ectoderm; oc, optic cup; L, lens; ep, epithelium; en, endothelium; st, stroma. Dotted lines represent the cell and nuclear membranes. Scale bars: 100 μm.

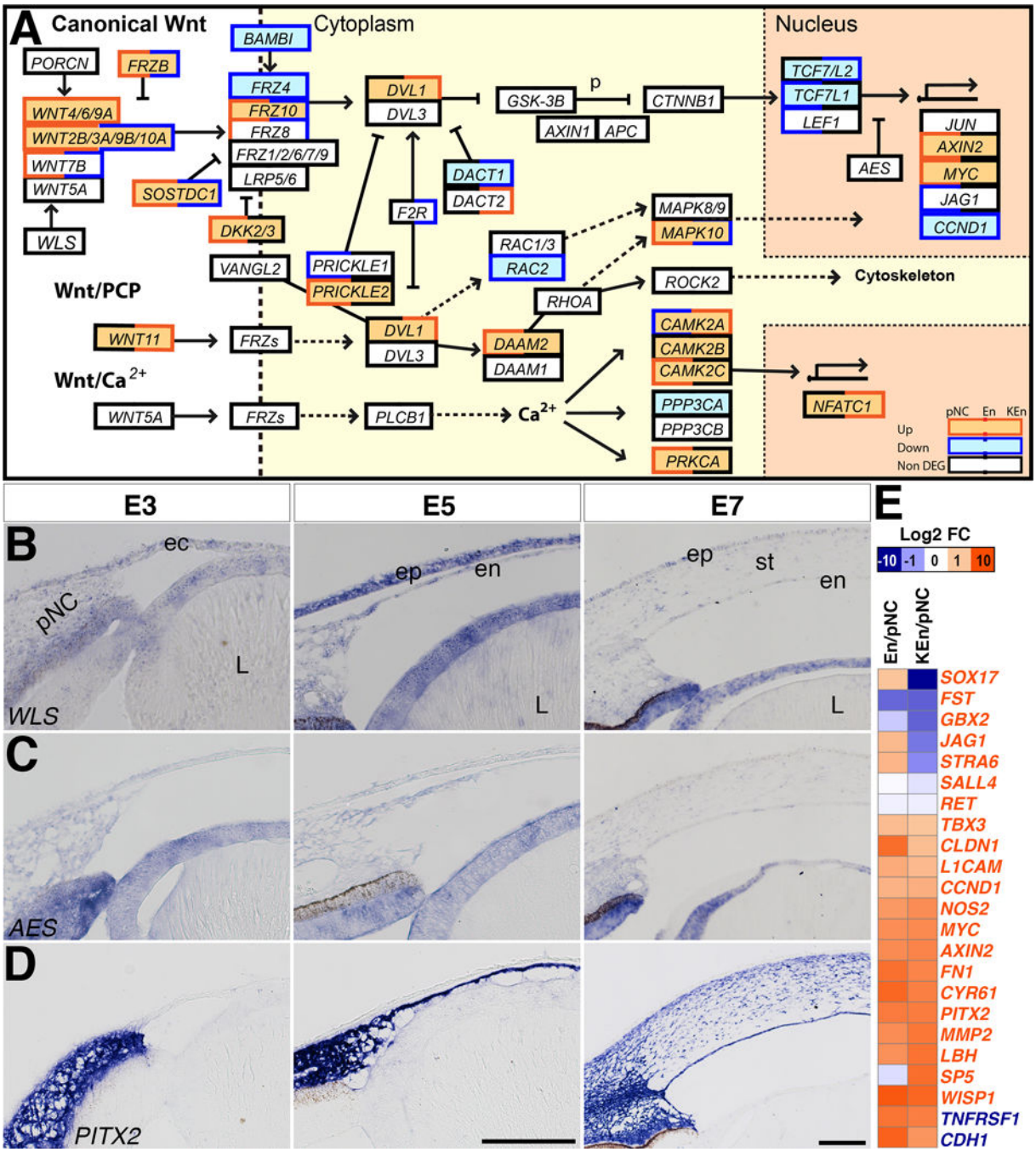

Figure 5. Wnt Signaling pathway.

(A) Black boxes indicate constitutively expressed genes, red boxes indicate upregulated genes, and blue boxes indicate downregulated genes. Double colored boxes indicate partial upregulation, downregulation, or maintenance during corneal development. (B-D) Section in situ hybridization of E3, E5, and E7 anterior eyes showing that: (B) *WLS* and (C) *AES* are both expressed in the periocular mesenchyme at E3, and in the corneal endothelium and stroma. (D) *PITX2* is strongly expressed in the periocular mesenchyme, corneal endothelium, and stroma. (E) Heatmap showing the relative upregulation and

downregulation of Wnt target genes between En/pNC and KEn/pNC. Font color of genes indicates Wnt-upregulated (red) or Wnt-downregulated (blue) genes. Abbreviations: pNC, periocular neural crest; ec, ectoderm; oc, optic cup; L, lens; ep, epithelium; en, endothelium; st, stroma. Dotted arrows indicate implied connection in the literature. Scale bars: 100 μm.

Table 1.

Top 20 differentially expressed transcription factors in En/pNC and KEn/pNC

Gene ID	Symbol	Log2FC (En/pNC)	q-value	Gene ID	Name	Log2FC (KEn/pNC)	q-value
Upregulated				**Upregulated**			
101750712	***ZNF750***	9.86	2E-18	100857422	***POU3F4***	8.77	1E-121
418669	***SHOX***	8.22	2E-224	100858556	***DMRT2***	7.09	2E-135
396546	***HMX1***	8.03	2E-19	426886	***TWIST3***	6.69	5E-142
396346	***SOHO-1***	7.85	5E-20	418669	***SHOX***	6.67	5E-128
100858556	***DMRT2***	7.59	3E-160	101748749	***ZNF469***	6.12	6E-108
419863	***IRF6***	6.99	2E-30	101750712	***ZNF750***	5.46	7E-05
395943	***PAX6***	6.69	4E-14	107051987	***POU3F3***	5.29	3E-36
100857422	***POU3F4***	5.65	2E-58	396158	***MYBL1***	5.28	2E-226
424652	***GLIS1***	5.34	1E-167	395405	***TWIST2***	5.12	0
777244	***SHOX2***	5.30	1E-91	396346	***SOHO-1***	4.86	1E-08
395714	***BARX2***	4.69	9E-19	423315	***NPAS3***	4.56	2E-156
101748749	***ZNF469***	4.66	3E-71	777244	***SHOX2***	4.34	2E-58
395590	***DLX3***	4.53	6E-19	373990	***TBX2***	4.10	1E-240
421416	***FOSL2***	4.45	6E-61	768789	***FOXE1***	4.08	1E-43
374101	***SCX***	4.05	2E-194	100859610	***CREB3L1***	3.86	2E-120
429506	***GRHL2***	3.98	3E-22	396210	***NFIA***	3.79	3E-159
373990	***TBX2***	3.97	8E-228	419631	***TFAP2E***	3.61	3E-82
428651	***GRHL1***	3.71	3E-41	395713	***TFAP2B***	3.50	2E-240
423923	***EMX2***	3.64	9E-114	427171	***PIK3R1***	3.44	3E-113
419631	***TFAP2E***	3.42	1E-74	374101	***SCX***	3.41	1E-139
Downregulated				**Downregulated**			
418313	***TBX15***	−10.53	7E-38	418313	***TBX15***	−10.53	2E-41
373932	***NKX3–2***	−8.69	6E-65	107053847	***DLX2***	−10.44	5E-65
396383	***ISL1***	−7.84	2E-15	428534	***SOX17***	−10.25	4E-37
396391	***GATA5***	−7.35	8E-12	419106	***GATA3***	−9.68	6E-123
396129	***SIM1***	−7.33	2E-30	724086	***SIX2***	−9.55	3E-137
396109	***FOXD1***	−7.00	2E-119	428021	***ZIC2***	−9.30	4E-94
419106	***GATA3***	−6.89	2E-100	395573	***SOX10***	−8.93	5E-147
503512	***FOXL2***	−6.75	1E-90	396109	***FOXD1***	−8.18	1E-138
395752	***NKX6–1***	−6.60	9E-50	395191	***OTX2***	−7.86	2E-19
395176	***EBF2***	−6.12	5E-72	395633	***MYF5***	−7.81	4E-16
395794	***FOXD3***	−5.98	3E-49	395794	***FOXD3***	−7.69	1E-61
374137	***MEOX2***	−5.93	2E-36	421786	***LIN28B***	−7.65	0
374127	***PAX3***	−5.74	2E-81	374127	***PAX3***	−7.46	7E-103
107053847	***DLX2***	−5.68	3E-47	503512	***FOXL2***	−7.46	4E-102
395795	***FOXD2***	−5.57	5E-133	396391	***GATA5***	−7.35	1E-13
419388	***PRDM16***	−5.56	7E-155	395752	***NKX6–1***	−7.19	5E-57

Gene ID	Symbol	Log2FC (En/pNC)	q-value	Gene ID	Name	Log2FC (KEn/pNC)	q-value
100857760	***FOXF2***	−5.54	5E-100	395596	***EPAS1***	−7.05	8E-174
395961	***NR5A2***	−5.42	5E-25	373932	***NKX3–2***	−6.98	8E-67
724086	***SIX2***	−5.35	4E-94	395977	***GFI1B***	−6.97	1E-25
428021	***ZIC2***	−4.68	4E-57	374137	***MEOX2***	−6.93	8E-44

Bold values indicate differentially expressed genes between the two groups (abs(log2)≥1 and q < 0.05). Minus signs indicate downregulation. The individual TPM and standard deviations for the RNAseq replicates are listed in Supplementary Table 7.

Table 2.

Differential expression of corneal endothelium related genes in En/pNC and KEn/pNC.

Gene ID	Symbol	Log2FC (En/pNC)	q-value	Log2FC (KEn/pNC)	q-value
Membrane transporters					
420384	*AQP1*	**9.24**	0	**10.34**	0
420606	*ABCB5*	**8.04**	8E-114	**7.12**	5E-89
417849	*BEST3*	**7.29**	4E-34	**6.78**	1E-27
776258	*SLC35F4*	**6.21**	4E-60	**7.21**	1E-91
769904	*FAM26E*	**5.65**	3E-167	**7.19**	2E-268
395443	*SLC1A3*	**−5.16**	7E-135	**−4.38**	7E-121
428653	*KCNS3*	**−4.43**	2E-11	**−4.76**	7E-14
396532	*SLC4A1*	**−4.34**	1E-19	**−7.32**	5E-35
770954	*KCNK2*	**−4.26**	1E-70	**−5.07**	1E-90
414871	*KCNH6*	**−3.27**	4E-29	−0.78	0.0012
Cell polarity					
378897	*THY1*	**3.70**	6E-180	**4.87**	1E-293
Tight junction					
395144	*CLDN4*	**7.16**	6E-20	**2.57**	0.0308
374029	*CLDN3*	**5.76**	1E-18	0.72	0.3202
429506	*GRHL2*	**3.98**	3E-22	−0.13	0.8131
424910	*CLDN1*	**3.00**	1E-05	**−1.79**	0.0112
769245	*CLDN19*	**2.93**	1E-07	**2.80**	2E-07
420022	*RAMP2*	**−3.27**	2E-32	**−5.47**	3E-59
374028	*CLDN5*	**−2.73**	2E-29	**−3.35**	3E-41
396047	*SNAI1*	**−1.61**	9E-10	−0.85	0.0011
422858	*PPP2R2C*	**−1.38**	6E-05	**3.56**	2E-54
420481	*MPP7*	**−1.23**	7E-18	**−1.49**	7E-26
Gap junction					
374196	*PDGFA*	**4.78**	7E-35	**3.18**	5E-17
395771	*GJB6*	**3.61**	9E-11	0.53	0.4574
374128	*PDGFB*	**1.93**	9E-12	0.86	0.0035
395581	*HTR2B*	**1.81**	0.001	**3.40**	6E-11
408035	*EGF*	**1.75**	5E-04	**−2.32**	0.0024
430991	*ADRB3*	**−3.71**	3E-11	**−3.08**	1E-09
771678	*GUCY1A2*	**−3.47**	8E-26	**−4.19**	6E-35
396502	*GJA5*	**−3.04**	2E-11	**−7.27**	2E-35
404529	*GJA4*	**−2.11**	1E-12	**−5.78**	2E-43
422407	*GUCY1A3*	**−2.08**	1E-14	**−5.46**	2E-50
Adherens junction					
414745	*CDH2*	**3.31**	1E-100	**1.59**	4E-27
414849	*CDH13*	**3.14**	0.444	**3.61**	2E-15

Gene ID	Symbol	Log2FC (En/pNC)	q-value	Log2FC (KEn/pNC)	q-value
423718	*CDH23*	**2.37**	0.107	**1.68**	3E-10
419222	*CDH4*	**1.90**	9E-13	−0.45	5E-09
415797	*CDH11*	**1.49**	5E-36	0.01	1E-36
374068	*CDH5*	**−8.37**	4E-32	**−4.73**	3E-97
374007	*CDH7*	**−6.90**	9E-61	**−1.72**	1E-79
Expressed in adult corneal endothelium *					
770869	*MGARP*	**6.35**	0	**5.75**	0
424391	*MYOC*	**5.00**	5E-23	**1.07**	0.0282
428221	*COL8A2*	**3.12**	2E-69	**3.69**	1E-92
396221	*LDHA*	**1.88**	2E-24	**3.26**	1E-65
418425	*CD200*	**−3.08**	3E-42	**−4.47**	3E-71
396092	*ALCAM*	**−2.00**	1E-41	**−1.57**	5E-28
374110	*PTGDS*	**−1.44**	0.009	−0.41	0.4664

Bold values indicate differentially expressed genes between the two groups (abs(log2)≥1 and q < 0.05). Minus signs indicate downregulation. The individual TPM and standard deviations for the RNAseq replicates are listed in Supplementary Table 8.

* Chng et al., 2013.

Table 3.

Differential expression of ECM related genes in En/pNC and KEn/pNC

Gene ID	Symbol	Log2FC (En/pNC)	q-value	Log2FC (KEn/pNC)	q-value
Collagens					
428448	*COL6A6*	**2.72**	2E-17	**11.03**	0
396292	*COL6A2*	**6.41**	0	**10.60**	0
396000	*COL6A1*	**4.51**	8E-137	**7.71**	1E-306
416696	*LOC416696*	**8.85**	9E-29	**7.14**	4E-21
101747382	*LOC101747382*	**4.86**	6E-120	**7.04**	2E-218
107056318	*LOC107056318*	0.19	0.763	**-4.18**	3E-11
421873	*COL19A1*	0.16	0.7209	**−3.16**	9E-14
101751793	*LOC101751793*	−1.79	5E-13	**−2.93**	4E-29
422350	*COL4A6*	−0.11	0.5729	**−2.03**	2E-32
422348	*COL4A5*	0.00	0.9913	**−1.60**	2E-21
Glycoproteins					
396387	*NTN3*	**1.75**	4E-07	**6.03**	7E-127
421471	*VIT*	**10.87**	5E-30	**5.67**	2E-11
422905	*SPON2*	**3.62**	4E-44	**5.61**	5E-96
424366	*OLFML2B*	**5.59**	0	**5.41**	0
414837	*THBS2*	**2.97**	7E-99	**4.69**	3E-215
419031	*VWF*	**−2.75**	8E-25	**−10.87**	6E-102
395935	*VTN*	**−5.57**	3E-35	**−5.70**	4E-39
417180	*LAMC3*	**−3.01**	4E-67	**−5.70**	2E-161
427850	*RELN*	**−2.02**	5E-14	**−5.26**	4E-53
395531	*NID1*	**−1.49**	1E-48	**−3.49**	2E-212
Proteoglycans					
374039	*OGN*	**6.04**	2E-295	**8.13**	0
373995	*KERA*	**4.86**	1E-160	**6.42**	1E-242
107056545	*LOC107056545*	**4.55**	1E-05	**6.20**	1E-08
417892	*DCN*	**2.58**	3E-33	**4.25**	4E-77
395798	*ACAN*	0.23	0.6479	**3.46**	5E-20
414143	*LEPREL1*	**−3.90**	7E-71	**−6.99**	1E-128
396475	*HAPLN1*	**−2.51**	8E-18	**−6.31**	1E-70
770863	*GPC3*	**−1.29**	3E-08	**−4.51**	8E-64
419184	*SDC4*	0.01	0.9846	**−2.37**	2E-16
Other ECM proteins					
418637	*EGFL6*	**4.31**	8E-96	**8.18**	3E-307
422535	*NPNT*	0.65	0.0021	**6.25**	1E-151
418125	*PTN*	**4.32**	2E-290	**5.52**	0
421461	*LTBP1*	**2.17**	2E-76	**3.21**	3E-153
428888	*LTBP2*	**2.23**	1E-66	**2.82**	7E-104

Gene ID	Symbol	Log2FC (En/pNC)	q-value	Log2FC (KEn/pNC)	q-value
395912	*MGP*	0.69	0.0939	**−3.23**	4E-10
Enzymes and Regulators					
395850	*MMP27*	**8.15**	3E-158	**9.07**	8E-209
416291	*ADAMTS2*	0.82	0.0511	**7.57**	1E-120
769222	*ADAMTS8*	**5.08**	8E-271	**4.54**	8E-219
424447	*COLGALT2*	**2.36**	3E-44	**4.28**	3E-131
427971	*ADAMTS5*	**5.14**	1E-174	**4.09**	3E-112
422488	*CSGALNACT1*	**−1.49**	2E-08	**−3.95**	1E-34
423834	*HPSE2*	**−3.12**	2E-19	**−1.42**	2E-05

Data is arranged to show the top 5 upregulated and downregulated genes in each category. Bold values indicate differentially expressed genes between the two groups (abs(log2)≥1 and $q < 0.05$). Minus signs indicate downregulation. The individual TPM and standard deviations for the RNAseq replicates are listed in Table 12.

Table 4.

List of primers used for in situ hybridization.

Gene ID	Symbol	Forward primer	Reverse primer
NCC genes			
395245	*MSX2*[1]	5’-CGAGGAGCACCACAAAGTCAAG-3’	5’-GACAGGAGTAGCATAGAGTCCAACG-3’
432368	*SNAI2*	5’-CTGTGTGGACTACAACCGGG-3’	5’-CTTCACATCCGAGTGGGTCT-3’
395734	*RHOB*	5’-TCGTCTTCAGCAAGGACGAG-3’	5’-GCAATTGATGCAGCCGTTCT-3’
RA genes			
416880	*ALDH2*	5’-ATCCCTTGGAACTTCCCCCT-3’	5’-ACTGATGCCCAACAGCAACT-3’
419480	*DHRS3*	5’-CCTTCCCTTCCTTCGCTTTAT-3’	5’-GTGCTTCCAAACTCCACATTTC-3’
386585	*NR2F2*	5’-CAAAGTTGGCATGAGACGGG-3’	5’-AGCTTCCCGAATCGTGTTGG-3’
TGF-β			
421461	*LTBP1*[2]	5’-TGCATCAAACCTAACTGTGCA-3’	5’-TCGGAAGTTAGTGGCTGTCA-3’
428413	*BAMBI*[3]	5’-GATCGCCATTCCAGCTACAT-3’	5’-TTTGCTGTCGTTGATCTTGC-3’
395897	*TGFBI*	5’-CACACAGCTCTACTCCGACC-3’	5’-GGCCAACTCAAACAGGGTCT-3’
Wnt genes			
100858542	*AES*	5’-TGTTTCCACAAAGCCGACAC-3’	5’-TTCTCCCCATCGTCGTCTTG-3’
424707	*WLS*	5’-AGTGATCGCCTTTCTGGTGG-3’	5’-GCTATGGGTCCAACCTGCTT-3’
395862	*PITX2*	5’-AGCGGACGCACTTCACCAGC-3’	5’-CGCAGCTCAGTCCGTGGCAA-3’

[1]Adapted from Geisha (http://geisha.arizona.edu/geisha/search.jsp?entrez_gene=461423)

[2]Lorda-Diez et al., 2010.

[3]Adapted from Geisha (http://geisha.arizona.edu/geisha/search.jsp?entrez_gene=461645)